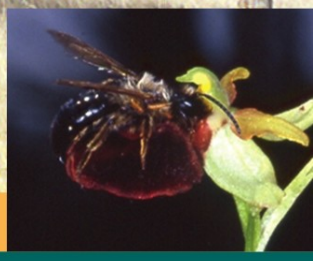


Matthias Glaubrecht
Editor

Evolution in Action

Springer

Evolution in Action

Matthias Glaubrecht
Editor
In cooperation with Harald Schneider

Evolution in Action

Case studies in Adaptive Radiation, Speciation and the Origin of Biodiversity

Special volume originating from contributions to the Priority Programme SPP 1127 "*Radiations: Origins of Biological Diversity*" of the Deutsche Forschungsgemeinschaft

Springer

Dr. Matthias Glaubrecht
Museum für Naturkunde Berlin
Leibnitz Institute for Research in
Evolution and Biodiversity at the
Humboldt University
Invalidenstraße 43
10115 Berlin
Germany
matthias.glaubrecht@mfn-berlin.de

In cooperation with

Dr. Harald Schneider
Department of Botany
Natural History Museum
London

ISBN 978-3-642-12424-2 e-ISBN 978-3-642-12425-9
DOI 10.1007/978-3-642-12425-9
Springer Heidelberg Dordrecht London New York

Library of Congress Control Number: 2010930166

Cover illustration:

Top: Typical mound of termites species (*Macrotermes michaelseni*) at Kajiado, Kenya (from Marten et al., this volume; photography: Manfred Kaib).

Left: Wild tomatoes (*Solanum* sect. *Lycopersicon*): *S. chilense* from the Moquegua population, Peru (from Stephan & Städler, this volume; photography: Gabriel Clostre).

Middle: New and so far undescribed freshwater gastropod species of *Tylomelania* from Lake Poso on Sulawesi, Indonesia (from Rintelen et al., this volume; photography: Chris Lukhaup).

Right: Orchid *Ophrys sphegodes* with pseudocopulating males of the pollinator *Andrena nigroaenea* (from Ayasse et al., this volume; photography: Manfred Ayasse).

Cover design: WMXDesign GmbH, Heidelberg, Germany

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

Radiations, or Evolution in Action

We have just celebrated the "Darwin Year" with the double anniversary of his 200th birthday and 150th year of his masterpiece, "*On the Origin of Species by means of Natural Selection*". In this work, Darwin established the factual evidence of biological evolution, that species change over time, and that new organisms arise by the splitting of ancestral forms into two or more descendant species. However, above all, Darwin provided the mechanisms by arguing convincingly that it is by natural selection – as well as by sexual selection (as he later added) – that organisms adapt to their environment. The many discoveries since then have essentially confirmed and strengthened Darwin's central theses, with latest evidence, for example, from molecular genetics, revealing the evolutionary relationships of all life forms through one shared history of descent from a common ancestor. We have also come a long way to progressively understand more on how new species actually originate, i.e. on speciation which remained Darwin's "*mystery of mysteries*", as noted in one of his earliest transmutation notebooks. Since speciation is the underlying mechanism for radiations, it is the ultimate causation for the biological diversity of life that surrounds us.

As we have learned, at the latest during last year's celebration of Charles Darwin and his discovery of evolution, it was not only the immediate natural objects from the "*Beagle's*" circumnavigation of the globe 1831–1836 that Darwin observed, collected and reported on that provided him with basal evidence for organic evolution. Even more important was his second, longer journey to discovery after his return to England, when Darwin developed his "*theory to work with*", as he once wrote, after thinking about the Malthusian paradigm of the enormous fertility of organisms that surpassed the capacity of available resources. It was only through this second voyage during more than two decades, from early 1837 to his epochal publication in 1859 when Darwin patiently and dedicatedly substantiated his theory, that he truly completed the "Copernican Revolution" in biology. That way he finally brought "the origin and adaptations of organisms in their profusion and wondrous variation into the realm of science", as Ayala and Avise recently pointed out (Proc Natl Acad Sci USA, 106 (2009): 2475–2476).

Around the globe, we have in 2009 commemorated two centuries of Darwin with numerous colloquia, conferences, cloud-gathering festivals, and museum exhibitions, and with new books and research articles in journals. The research papers compiled in the present volume also reveal some of the many aspects among the wide spectrum of current approaches in evolutionary research, following largely in Darwin's footsteps. In many ways, we today still relate to the plethora of observations and notes Darwin made more than a century ago. On the other hand, we have the privilege to use modern techniques, for example, from molecular biology and from systematic phylogeny, to allow the reconstructing of the relationships of organisms and the course of evolution – an accumulation of knowledge Darwin could not have imagined but that he certainly would have loved to know about.

Beyond doubt, Charles Darwin's contribution to our understanding of the origin of biodiversity cannot be overestimated, as a very natural transition will lead from the Darwin Year to 2010 as the Year of Biodiversity and Conservation. This book is a contribution to both celebrations, with the studies and model cases presented showing the progress and dynamic of research based on Darwinian theories as well as shedding light on the implications in context with the current biodiversity crises. The great importance of adaptive (and non-adaptive) radiations for biodiversity is widely accepted, but our understanding of the processes and mechanisms involved is still limited, and generalizations need to be based on the accumulation of more evidence from additional case studies. Our model cases are, more often than not, in need of being conserved, with their immediate habitats where we find and study them being better protected.

The studies presented in this volume are those urgently needed case studies focusing on a variety of organisms and different aspects of radiations. As case studies in evolution, they are also taking advantage of the progress in molecular biology and bioinformatics, two areas that have revolutionized modern biology. The scientific results presented herein are excellent examples not only of evolution in action, but also of active research on evolutionary processes and their most apparent outcome, viz. the biodiversity that we want to conserve for future generations to enjoy.

This volume aims at bringing together the immediate results of studies and projects conducted within a priority programme funded by the Deutsche Forschungsgemeinschaft (DFG) from 2002 to 2008 (see more on this in the following introductory chapter by Bill Martin). Here, the insights of 25 research groups with a total of 109 contributors are arranged in three parts: The first part (1) is concerned with approaches in botany (8 papers), the second part (2) with host-plant interactions (4 papers), and the third part (3) with approaches in zoology (13 papers); all summarizing the advances we have made so far.

The authors were asked to present their research with scientific rigor, albeit not necessarily presenting it in the usual form of a research paper, but if possible as a more readable review. That way, we have hoped to not so much write only for the few other experts in our immediate field of expertise (be it *Solanum* genetics, *Crematogaster* ants on Malaysian *Macaranga* plants, or *Tylomelania* snails in lakes on Sulawesi), but for a wider audience. At the same time, we hope to present

here a colorfully illustrated survey of current evolutionary biology research in Germany. These papers or chapters, although they were all independently written, are here grouped according to their main subjects, their hypotheses tested, and their major findings and implications. Of course, other arrangements are also possible; however, the present compilation follows an inherent design suggested by their contents that I will briefly outline here.

Starting with model cases for radiations in ferns on Madagascar (Schneider et al.), in plants on Macaronesian islands (Thiv et al.) and *Hordeum* in the Americas (Blattner et al.), a main underlying theme in this book will be the question of the driving forces responsible for species evolution. This is discussed, for example, for key innovations for ferns (Schneider et al.), the mating system in *Capsella* (Paetsch et al.) and, in particular, for ecological factors – the latter actually being the major recurrent factor in focus in many of the papers compiled here – such as pollinator-driven speciation in orchids (Ayasse et al.). The botany section concludes with two papers looking into population genetics and genetic diversity in plants used for human food, such as tomatoes (Stephan et al.) and wheat and barley (Kilian et al.).

In part II, four papers look at case studies in host–plant interactions as a special case of biotic evolution, searching for general principles that apply to those animals that directly live on, in or with plants and vice versa. The paper by Weising et al. discusses *Macaranga* speciation, the paper by Feldhaar et al. on speciation in *Crematogaster* adds the ant perspective to the story. Another intriguing case study on plants comes from the plethora of forms in wild roses interacting with fungi and insects (Kohnen et al.), while Johanneson et al. follow the traces of speciation in plant-dwelling tephritid flies.

Part III on the zoological approaches starts with examining radiations again and some of the proposed key factors responsible for diversification and speciation, exemplified by the incorporation of photosynthetic units in seaslugs (Wägele et al.), by the role of cuticular properties in fungus-growing termites (Marten et al.), and by the differential properties of the electric organ in African fishes promoting ecological speciation (Tiedemann et al.). With that, one of the major subjects of modern speciation studies is once more emerging, viz. testing the contribution of ecological versus geographical factors, as then also investigated in the paper by Schubart et al. on the adaptive nature of a radiation of freshwater crabs on Jamaica. The three subsequent papers look more specifically into the spatial component of speciation, using as exemplars the formerly assumed "ring species" of the *Larus* gull complex (Liebers-Helbig et al.), water frogs in the eastern Mediterranean (Plötner et al.), and hitherto cryptic species in Corsican *Limax* slugs (Nitz et al.). In addition to the latter paper that also deals predominantly with reproductive characters and properties, two more chapters examine the role of sexual selection in speciation, as illustrated for Cretan land snails by Sauer and Hausdorf, and by Mayer et al. for acoustically communicating grasshoppers, both in their way testing or providing evidence for non-ecological radiations. The possibility of sympatric speciation is further examined by Herder and Schliewen for lacustrine fishes in lakes on the Indonesian islands of Sulawesi. I am convinced that these central highland lakes provide us with a highly suitable "natural laboratory" for speciation studies, potentially even

better suited than other ancient lake systems, in order to test the differential role of allopatry versus sympatry, with a suite of geographical and ecological factors discernable, as shown for example in our own study of the endemic *Tylomelania* gastropods (Rintelen et al.). In the subsequent paper by Köhler et al., we further examine these themes for another closely related limnic snail group; however, this time not for a lacustrine but instead a riverine setting. Finally, the zoological section is complemented by another study on limnic snails (a group of invertebrates obviously on its way of being recognized as an emerging model system in evolutionary biology), with Wilke et al. investigating, this time explicitly, the possibility of non-adaptive radiations.

As is evident from the present compilation in this book, we are still far away from being able to provide a balanced view on radiation and speciation, as we are not even close to looking comprehensively at the major organisms, regions, or factors involved. While some taxa are examined herein very thoroughly, others are completely missing. Nevertheless, we discuss some of the most prominent factors and highlight future avenues of research. In any case, I am convinced that these papers presented here all show, in a variety of ways, evolution in action.

As authors of these papers as well as participants of the DFG priority programme, we are in great debt to the organisers, Klaus Bachmann and William Martin, who provided a major trigger for synthesizing our work. We are grateful for the financial support by the DFG and the continuous support by its representative, Roswitha Schönwitz, as well as grateful to the members of the review board for the many stimulating suggestions and discussions during these six exciting and successful research years, and to the reviewers of the 25 papers published here for their comments and constructive criticisms.

I would like to thank Harald Schneider who has helped during the review process with handling the botanical and host–plant interaction papers, Bill Martin for establishing contact with Springer, Heidelberg, and Sabine Schwarz and Anette Lindqvist of its Life Sciences Editorial Office for their encouragement and help throughout the process of editing this volume.

Berlin Matthias Glaubrecht
February 2010

Introduction to the Priority Programme "*Radiations: Origins of Biological Diversity*"

This book results from a focused research programme, a priority programme, that was funded by the German Research Foundation (DFG) during the period 2002–2007 entitled "*Radiations: Origins of Biological Diversity*" (priority programme SPP 1127). The programme was a landmark boost for studies in ecology and evolution at the species level in Central Europe, and it brought together a broad spectrum of evolutionary biologists, united by a single pressing question about the driving forces behind species diversification.

This is a good opportunity to answer a question that I have often been asked: how did the programme SPP 1127 come to be? Adjunct to the annual meeting of the German Botanical Society for 2000 in Jena, officers and representatives of the DFG invited a group of about 20 scientists from botany, zoology, microbiology, and ecology to a one-day brainstorming session on evolution and systematics, with the goal of identifying some of the big outstanding questions in the field, efforts towards whose solution would generate substantial progress in our understanding of biodiversity. It also had to be something harboring interest and research potential for those in the plant, animal, and microbial fields. Our brains astorm, and with the flames of discussion raging, we searched our gray matter hour upon hour for issues genuinely original, scientifically of outstanding value, and at the same time of broad enough appeal to attract the interests of chemists and physicists, for example, so as to be of obvious significance beyond a specialist audience. Good ideas are cheap. We needed a *great* idea. By mid-afternoon, things started to look pretty bleak, as did many faces around the table, and we had got to the point where we were discussing with mostly genuine enthusiasm things like "The postglacial recolonization of Europe", which is no doubt of great interest, especially for those of us living in Central Europe, but maybe not the biggest evolutionary topic ever identified.

Just when it seemed that there were no big questions left or otherwise identifiable in our storm-swept minds about species-level evolution, the waters parted. One of the world's leading botanists, Friedrich Ehrendorfer, opened up the afternoon session with the memorably booming words "Radiations! If we want to understand

the origin of species, we need to look where evolution is striding at its greatest pace." We all immediately recognized that a great idea had just been born, and from there on, the brainstorming was all downhill towards the goal of formulating a research proposal to the DFG with the aim of looking into the evolutionary impact of radiations, or evolution in action, as we have called it here in this book. That procedure entailed inviting several dozen potentially interested scientists to a colloquium in order to have short presentations and discuss the issue of radiations further, which we did; we being Konrad Bachmann, director of the Institut of Plant Genetics and Crop Plant Research in Gatersleben, Germany, and myself. That meeting brought the already considerable level of momentum to a point where people were suddenly expecting Konrad and I to write a proposal, as if we had signed some contract stating that we didn't have enough work on our hands and were desperately looking for more. It was at that time that I kept hearing things like "you write really well" from everyone in that circle, which I now understand to be a special kind of democracy known in most countries under the name of "mobbing". Only now do I notice that neither before that time nor thereafter had anyone ever directly commented on my writing skills.

Because the topic of radiations is rather broad, the priority programme (SPP) proposal that Konrad and I cobbled together contained some subtopics to help keep research efforts focussed. These subtopics were (1) the role of the reproductive system in speciation, (2) spatial separation and allopatric speciation, (3) the role of key characters in adaptive radiations, and (4) coupled radiations, where two or more groups seem to be cospeciating. It turns out that the SPP proposal was very popular with the decision-making bodies of the DFG, who earmarked rather generous sums for the study of species-level biodiversity on the basis of our document. Subsequently, the SPP proposal was also popular with the field in general, as an avalanche of outstanding individual research proposals came in for the first round. Over the six years that the priority programe SPP 1127 ran, the DFG allocated over eight million euros to fund 102 two-year research proposals aimed at species-level biodiversity. That substantial investment in basic science will return long-term benefits in our understanding of biological diversity and the role of radiative speciation processes in its origins. This volume summarizes some of the advances that emerged from projects that were funded during the priority programme. I hope that readers find it informative and attractive.

Over the course of SPP 1127, we held three successful colloquia, organized by Konrad and myself, and a very successful workshop on population genetic methods, kindly organized and run by Wolfgang Stephan and Thomas Städler from the Institute of Evolutionary Biology at the Ludwig Maximillian University in Munich. Various national and international activities in the field of biodiversity research emerged around SPP 1127 (a very notable one being the New Zealand Plant Species Radiations Network organized by Pete Lockhart of Massey University, with which several levels of interaction and exchange materialized), for which we are grateful.

Everyone associated with SPP 1127 owes special thanks to the programme's steering committee, who gave us good advice and assistance along the way. The steering committee was, in alphabetical order, Alfried Vogel (Natural History

Museum, London), Ian Baldwin (Max Planck Institute for Chemical Ecology, Jena), Spencer Barrett (University of Toronto), David Penny (Massey University, New Zealand), Edmund Gittenberger (National Museum of Natural History, Leiden, The Netherlands), Scott Hodges (University of California at Santa Barbara), Johannes Vogel (Natural History Museum, London), Dick Olmstead (University of Washington), and Wolfgang Wägele (Zoological Museum König, Bonn). Their time and their efforts in helping us to make the most of our research opportunities are very deeply appreciated. Thanks also go to Harald Schneider and Matthias Glaubrecht, who took it upon themselves to edit this volume.

All the participants in SPP 1127 owe the German Research Foundation and in particular Roswitha Schönwitz, our responsible programme officer who kept us on track from start to finish, an enormous round of special thanks for helping to make the program materialize. We also owe an equally special round of thanks to Friedrich Ehrendorfer from the University of Vienna for maintaining clarity of mind while the rest of our brains were storming away, and for coming up with the topic of radiations.

Düsseldorf
June 2009

William Martin

Contents

Contributors

Christian Albrecht Department of Animal Ecology & Systematics, Justus Liebig University, Giessen, Germany

Çiğdem Akın Biodiversity and Conservation Laboratory, Department of Biology, Middle East Technical University, 06531 Ankara, Turkey

Manfred Ayasse Institute for Experimental Ecology, University of Ulm, Albert-Einstein-Allee 11, 89069 Ulm, Germany, manfred.ay.asse@uni-ulm.de

Gudrun Bänfer Plant Molecular Systematics, University of Kassel, 34109 Kassel, Germany

Christina Baier Leibniz Institute of Plant Genetics and Crop Science (IPK), 06466 Gatersleben, Germany

Peter Beerli Department of Scientific Computing, Florida State University, Tallahassee, FL 32306-4120, USA

Mandy Benke Department of Animal Ecology & Systematics, Justus Liebig University, Giessen, Germany

Dirk Berger Senckenberg Natural History Collections Dresden, Museum of Zoology, Königsbrücker Landstraße 159, 01109 Dresden, Germany, dirk.berger@senckenberg.de

Jean-Michel Bichain Muséum National d'Histoire Naturelle, Paris, France

C. Can Bilgin Biodiversity and Conservation Laboratory, Department of Biology, Middle East Technical University, 06531 Ankara, Turkey

Frank R. Blattner Leibniz Institute of Plant Genetics and Crop Research (IPK), Corrensstraße 3, 06466 Gatersleben, Germany, blattner@ipk-gatersleben.de

Martin Brändle Department of Ecology, Philipps University Marburg, Marburg, Germany

Roland Brandl Animal Ecology, Department of Ecology, Faculty of Biology, University of Marburg, Karl-von-Frisch Str. 8, 35032 Marburg, Germany, brandlr@staff.uni-marburg.de

Ingo Burghardt Australian Institute of Marine Science, PMB No. 3 Townsville QLD 4810 Australia and University of Technology Sydney, Climate Change Cluster, PO Box 123 Sydney, NSW 2007, Australia

Nadia Byrstiakova Department of Plant Science, University of Cambridge, Cambridge, CB2 3EA, UK and Department of Botany, Natural History Museum, London, SW7 5BD, UK

Keith A. Crandall Department of Biology, Brigham Young University, Provo, UT 84602-5255, USA

Thorsten Diegisser Institut für Zoologie, Abteilung Ökologie, Universität Mainz, Saarstrasse 21, 55099 Mainz, Germany

Wiebke Dorstewitz Plant Molecular Systematics, University of Kassel, 34109 Kassel, Germany

Korinna Esfeld Staatliches Museum für Naturkunde Stuttgart, Rosenstein 1, 70191 Stuttgart, Germany and University of Bern, Altenbergrain 21, CH-3013 Bern, Switzerland, korinna.esfeld@ips.unibe.ch

Gerhard Falkner Staatliches Museum für Naturkunde Stuttgart, Rosenstein 1, 70191 Stuttgart, Germany and Département Systématique et Evolution. UMS Taxonomie et collections, Muséum National d'Histoire Naturelle Paris, 55 rue de Buffon, F-75005 Paris, France, Falkner@malaco.de

Heike Feldhaar Behavioural Physiology and Sociobiology (Zoology II), Biocenter, Am Hubland, University of Würzburg, 97074 Würzburg, Germany and Behavioural Biology, University of Osnabrück, Barbarastr. 11, 49076 Osnabrück, Germany, feldhaar@biologie.uni.osnabrueck.de

Christina Fey-Wagner Plant Molecular Systematics, University of Kassel, 34109 Kassel, Germany

Philine G.D. Feulner Unit of Evolutionary Biology/Systematic Zoology, Institute of Biochemistry and Biology, University of Potsdam, 14476 Potsdam, Germany and Evolutionary Bioinformatics Group, Westfälische-Wilhelms-Universität Münster, Institute for Evolution and Biodiversity, Hüfferstrasse 1, 48149 Münster, Germany, p.feulner@uni-muenster.de

Brigitte Fiala Animal Ecology and Tropical Biology (Zoology III), Biocenter, Am Hubland, University of Würzburg, 97074 Würzburg, Germany

Roman Fricke Philipps-Universität Marburg, Marburg, Germany

Jürgen Gadau Behavioural Physiology and Sociobiology (Zoology II), Biocenter, Am Hubland, University of Würzburg, 97074 Würzburg, Germany and School of Life Sciences, Arizona State University, PO Box 874501, Tempe, AZ 85287-4501 USA

Friederike Gallenmüller Abert-Ludwigs-Universität-Freiburg, Freiburg, Germany

Matthias Glaubrecht Museum für Naturkunde Berlin, Leibniz Institute for Research in Evolution and Biodiversity at the Humboldt University, Invalidenstraße 43, 10115 Berlin, Germany, matthias.glaubrecht@mfn-berlin.de

Julia Gögler Institute for Experimental Ecology, University of Ulm, Albert-Einstein-Allee 11, 89069 Ulm, Germany, julia.goegler@uni-ulm.de

Brigitte Gottsberger Department of Population Ecology, University of Vienna, Rennweg 14, A-1030 Vienna, Austria, brigitte.gottsberger@univie.ac.at

Yvonne Grzymbowski Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany

Gaston-Denis Guex Zoologisches Institut, Universität Zürich-Irchel, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

Daniela Guicking Plant Molecular Systematics, University of Kassel, 34109 Kassel, Germany

Cornelia Haefeli Zoologisches Institut, Universität Zürich-Irchel, Winterthurerstrasse 190, 8057 Zurich, Switzerland

Katharina Händeler Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany

Bernhard Hausdorf Zoological Museum, University of Hamburg, Martin-Luther-King-Platz 3, 20146 Hamburg, Germany, hausdorf@zoologie.uni-hamburg.de

Gerhard Haszprunar Zoologische Staatssammlung München, Münchhausenstr. 21, 81247 München, Germany, haszi@zsm.mwn.de

Jochen Heinrichs Albrecht-von-Haller Institut für Pflanzenwissenschaften, Georg-August Universität Goettingen, Göttingen, Germany and FU Berlin, Institut fuer Biologie, Angewandte Genetik, 14195 Berlin, Germany

Andreas J. Helbig (†) Vogelwarte Hiddensee, University of Greifswald, Germany

Sabine Hennequin Department of Botany, Natural History Museum, London SW7 5BD, UK

Fabian Herder Sektion Ichthyologie, Zoologisches Forschungsmuseum Alexander Koenig, Adenauerallee 160, 53113, Bonn, Germany and Department of lchthyology, Bavarian State Collection of Zoology (ZSM), Münchhausenstr 21, 81247 München, Germany, f.herder.zfmk@uni-bonn.de

Hansjürg Hotz Zoologisches Institut, Universität Zürich-Irchel, Winterthurer-strasse 190, CH-8057 Zurich, Switzerland

Herbert Hurka Department of Botany, University of Osnabrueck, Barbarastraße 11, 49076 Osnabrueck, Germany

Sabine S. Jakob Leibniz Institute of Plant Genetics and Crop Research (IPK), Corrensstraße 3, 06466 Gatersleben, Germany

Thomas Janssen Albrecht-von-Haller Institute of Plant Sciences, Georg-August University, 37073 Göttingen, Germany and National Botanic Garden of Belgium, Bouchout Domain, Nieuwelaan 38, 1860 Meise, Belgium

Jes Johannesen Institut für Zoologie, Abteilung Ökologie, Universität Mainz, Saarstrasse 21, 55099 Mainz, Germany, jesjo@uni-mainz.de

Manfred Kaib Department of Animal Physiology, Faculty of Biology, University of Bayreuth, Universitätsstr. 30, 95440 Bayreuth, Germany, manfred.kaib@uni-bayreuth.de

Benjamin Kilian Institute of Botany III, Heinrich-Heine-Universität Düsseldorf, Universitätsstrasse 1, 40225 Düsseldorf, Germany and Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Genebank/ Genome Diversity, Correns-strasse 3, 06466 Gatersleben, Germany, kilian@ipk-gatersleben.de

Frank Kirschbaum Institute of Animal Sciences, Humboldt-University, Berlin, Germany and Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany

Katrin Klinge Georg-August Universität Göttingen, Göttingen, Germany

Peter de Knijff Department of Human Genetics, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, The Netherlands, knijff@lumc.nl

Marcus Koch Heidelberg Institute of Plant Sciences, University of Heidelberg, Im Neuenheimer Feld 345m, 69120 Heidelberg, Germany, mkoch@hip.uni-heidelberg.de

Frank Köhler Museum für Naturkunde Berlin, Invalidenstraße 43, 10115 Berlin, Germany and Australian Museum, 6 College Street, Sydney, NSW 2010 Australia, frank.koehler@austmus.gov.au

Ines Köhnen Friedrich Schiller Universität Jena, Jena, Germany

Annette Kohnen Philipps-Universität Marburg, Marburg, Germany

Hans-Peter Kreier Albrecht-von-Haller Institut für Pflanzenwissenschaften, Georg-August Universität Goettingen, Göttingen, Germany, and FU Berlin, Institut fuer Biologie, Angewandte Genetik, 14195 Berlin, Germany

Tim Kröger-Kilian Plant Molecular Systematics, University of Kassel, 34109 Kassel, Germany

Dorit Liebers-Helbig Deutsches Meeresmuseum, Katharinenberg 14-20, 18439 Stralsund, Germany, dorit.liebers@meeresmuseum.de

Spartak N. Litvinchuk Institute of Cytology, Russian Academy of Sciences, Tikhoretsky prospekt 4, 194064 St. Petersburg, Russia

Wolfgang Maier Ruhr-Universität Bochum, Bochum, Germany

Andreas Marten Animal Ecology, Department of Ecology, Faculty of Biology, University of Marburg, Karl-von-Frisch Str. 8, 35032 Marburg, Germany

William Martin Institute of Botany III, Heinrich-Heine-Universität Düsseldorf, Universitätsstrasse 1, 40225 Düsseldorf, Germany, W.Martin@uni-duesseldorf.de

Frieder Mayer Museum für Naturkunde Berlin, Leibniz Institute for Research on Evolution and Biodiversity at the Humboldt University Berlin, Invalidenstraße 43, 10115 Berlin, Germany, frieder.mayer@mfn-berlin.de

Sara Mayland-Quellhorst Department of Botany, University of Osnabrueck, Barbarastraße 11, 49076 Osnabrueck, Germany

Ute Moog Plant Molecular Systematics, University of Kassel, 34109 Kassel, Germany

Haiko Muth Albrecht-von-Haller Institut für Pflanzenwissenschaften, Georg-August Universität Goettingen, Göttingen, Germany and FU Berlin, Institut fuer Biologe, Angewandte Genetik, 14195 Berlin, Germany

Barbara Neuffer Department of Botany, University of Osnabrueck, Barbarastraße 11, 49076 Osnabrueck, Germany

Barbara Nitz Zoologische Staatssammlung München, Münchhausenstr. 21, D-81247 München, Germany and Institute of Epidemiology, Helmholtz Center München, German Research Center for Environmental Health, München/Neuherberg, Germany, Barbara.Nitz@zsm.mwn.de

Franz Oberwinkler Eberhard Karls Universität Tübingen, Tübingen, Germany

Torsten Ohst Museum für Naturkunde Berlin, Leibniz Institute for Research on Evolution and Biodiversity at the Humboldt University Berlin, Invalidenstraße 43, 10115 Berlin, Germany

Elisabeth Otto Albrecht-von-Haller Institut für Pflanzenwissenschaften, Georg-August Universität Goettingen, Göttingen, Germany and FU Berlin, Institut fuer Biologie, Angewandte Genetik, 14195 Berlin, Germany

Melanie Paetsch Department of Botany, University of Osnabrueck, Barbarastraße 11, 49076 Osnabrueck, Germany, Melanie.Paetsch@biologie.uni-osnabrueck.de

Somsak Panha Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok, 10330 Thailand, somsak_panha@yahoo.com

Thekla Pleines Leibniz Institute of Plant Genetics and Crop Research (IPK), Corrensstraße 3, 06466 Gatersleben, Germany

Jörg Plötner Museum für Naturkunde Berlin, Leibniz Institute for Research on Evolution and Biodiversity at the Humboldt University Berlin, Invalidenstraße 43, 10115 Berlin, Germany, joerg.plotner@mfn-berlin.de

Nicolas Pruvost Institut für Evolutionsbiologie und Umweltwissenschaften, Universität Zürich-Irchel, Winterthurerstrasse 190, 8057 Zurich, Switzerland

France Rakotondrainibe Museum national d'Histoire naturelle, Dpt. Systematique et Evolution, UMR-CNRS 5202, CP 29, 75231 Paris, Cedex 05, France

Michael J. Raupach Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany

Heinz-Ulrich Reyer Zoologisches Institut, Universität Zürich-Irchel, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

Kristina von Rintelen Museum für Naturkunde, Leibniz-Institut für Evolution- und Biodiversitätsforschung an der Humboldt-Universität zu Berlin, Invalidenstraße 43, 10115 Berlin, Germany

Thomas von Rintelen Museum für Naturkunde, Leibniz-Institut für Evolution- und Biodiversitätsforschung an der Humboldt-Universität zu Berlin, Invalidenstraße 43, 10115 Berlin, Germany

Christiane Ritz Justus-Liebig-Universität Giessen, Giessen, Germany

Francesco Salamini Department of Plant Breeding and Genetics, Max Planck Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, 50829 Köln, Germany and Fondazione Parco Tecnologico Padano, Via Einstein – Localita Cascina Codazza, 26900 Lodi, Italy

Tobias Santl Biologie 1, Institut für Zoologie, Universität Regensburg, 93040 Regensburg, Germany

Jan Sauer Zoological Museum, University of Hamburg, Martin-Luther-King-Platz 3, 20146 Hamburg, Germany, ja_sa@web.de

Ulrich K. Schliewen Department of Ichthyology, Bavarian State Collection of Zoology (ZSM), Münchhausenstr. 21, 81247 München, Germany, schliewen@zsm.mwn.de

Harald Schneider Albrecht-von-Haller Institute of Plant Sciences, Georg-August University, 37073 Göttingen, Germany and Department of Botany, Natural History Museum, London, SW7 5BD, UK, h.schneider@nhm.ac.uk

Robert Schreiber Museum für Naturkunde Berlin, Leibniz Institute for Research on Evolution and Biodiversity at the Humboldt University Berlin, Invalidenstraße 43, 10115 Berlin, Germany

Christoph D. Schubart Biologie 1, Institut für Zoologie, Universität Regensburg, 93040 Regensburg, Germany, christoph.schubart@biologie.uni-regensburg.de

Wolfram Schulze Animal physiology, University of Bayreuth, Universitätsstraße 30, 95440 Bayreuth, Germany, wschulze@biologie.uni-erlangen.de

Alfred Seitz (†) Institut für Zoologie, Abteilung Ökologie, Universität Mainz, Saarstrasse 21, 55099 Mainz, Germany

Thomas Speck Abert-Ludwigs-Universität-Freiburg, Freiburg, Germany

Thomas Städler Plant Ecological Genetics, ETH Zurich, Institute of Integrative Biology, Universitätstrasse 16, 8092 Zurich, Switzerland, thomas.staedler@env.ethz.ch

Jesper T. Stenderup Biologie 1, Institut für Zoologie, Universität Regensburg, 93040 Regensburg, Germany and Natural History Museum of Denmark, University of Copenhagen, 1350 København, Denmark

Wolfgang Stephan Department of Biology II, Section of Evolutionary Biology, University of Munich (LMU), Grosshaderner Strasse 2, 82152 Planegg-Martinsried, Germany, stephan@bio.lmu.de

Viviane Sternkopf Deutsches Meeresmuseum, Katharinenberg 14-20, 18439 Stralsund, Germany

Johannes Stökl Institute for Experimental Ecology, University of Ulm, Albert-Einstein-Allee 11, 89069 Ulm, Germany and Institut für Zoologie, AG Chemische Ökologie, Universität Regensburg, Universitätsstraße 31, 93053 Regensburg, Germany, johannes.stoekl@biologie.uni-regensburg.de

Günter Theissen Friedrich Schiller Universität Jena, Jena, Germany

Mike Thiv Staatliches Museum für Naturkunde Stuttgart, Rosenstein 1, 70191 Stuttgart, Germany, mike.thiv@smns-bw.de

Ralph Tiedemann Unit of Evolutionary Biology/Systematic Zoology, Institute of Biochemistry and Biology, University of Potsdam, Karl-Liebknecht-Strasse 24-25, Haus 26, 14476 Potsdam, Germany, tiedeman@uni-potsdam.de

Teja Tscharntke Georg-August Universität Göttingen, Göttingen, Germany

Thomas Uzzell Laboratory for Molecular Systematics and Ecology, Academy of Natural Sciences, 1900 B. F. Parkway, Philadelphia, PA 19103, USA

Andrea Vaupel Philipps-Universität Marburg, Marburg, Germany

Miriam Vogel Plant Molecular Systematics University of Kassel, 34109 Kassel, Germany

Heike Wägele Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany, hwaegele@evolution.uni-bonn.de

Tobias Weil Biologie 1, Institut für Zoologie, Universität Regensburg, 93040 Regensburg, Germany

Kurt Weising Plant Molecular Systematics, University of Kassel, 34109 Kassel, Germany, weising@uni-kassel.de

Rob Westaway Faculty of Mathematics, Computing and Technology, The Open University, Abbots Hill, Gateshead NE 83DF, UK and IRES, Newcastle University, Newcastle-upon-Tyne, NE1 7RU, UK

Thomas Wilke Department of Animal Ecology & Systematics, Arbeitsgruppe Tierökologie, Justus Liebig University, Heinrich-Buff-Ring 26-32, 35392 Gießen, Germany, tom.wilke@allzool.bio.uni-giessen.de

Volker Wissemann Justus-Liebig-Universität Giessen, Institut für Allgemeine Botanik & Pflanzenphysiologie, AG Spezielle Botanik, Heinrich-Buff-Ring 38, 35390 Giessen, Germany, volker.wissemann@bot1.bio.uni-giessen.de

Tina Wöhrmann Plant Molecular Systematics, University of Kassel, 34109 Kassel, Germany

Part I
Approaches in Botany

Rapid Radiations and Neoendemism in the Madagascan Biodiversity Hotspot

Harald Schneider, Thomas Janssen, Nadia Byrstiakova, Jochen Heinrichs, Sabine Hennequin, and France Rakotondrainibe

Abstract This study recorded evidence for three independent rapid radiations of scaly tree ferns in Madagascar. These three radiations happened in the late Cenozoic and were likely triggered by the fluctuations of the global climate. It is the first report for this kind of relationships of ferns diversity in the Madagascan biodiversity centre and climate change, but it is consistent with similar reports for other species-rich lineages in the megadiverse Madagascan rainforest habitats. Finally, we generated a biogeographic scenario describing the origin of the three Malagasy clades of scaly tree ferns. We also found evidence for range loss as a result of deforestation in historical times. The presented system is an excellent example of the contribution of rapid radiations to the highly threatened biodiversity of Madagascar and adjacent islands.

H. Schneider (✉)
Department of Botany, Natural History Museum, London SW7 5BD, UK
Albrecht-von-Haller Institute of Plant Sciences, Georg-August University, 37073 Göttingen, Germany
e-mail: h.schneider@nhm.ac.uk

T. Janssen
Albrecht-von-Haller Institute of Plant Sciences, Georg-August University, 37073 Göttingen, Germany
National Botanic Garden of Belgium, Bouchout Domain, Nieuwelaan 38, 1860 Meise, Belgium

N. Byrstiakova
Department of Botany, Natural History Museum, London SW7 5BD, UK
Department of Plant Science, University of Cambridge, Cambridge CB2 3EA, UK

J. Heinrichs
Albrecht-von-Haller Institute of Plant Sciences, Georg-August University, 37073 Göttingen, Germany

S. Hennequin
Department of Botany, Natural History Museum, London SW7 5BD, UK

F. Rakotondrainibe
Dpt. Systematique et Evolution, Museum national d'Histoire naturelle, UMR-CNRS 5202, CP 29, 75231 Paris, Cedex 05, France

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_1,

1 Introduction

Adaptive radiations are widely accepted as one of the major processes in the accumulation of biodiversity on oceanic islands as illustrated by classical examples such as Darwin's finches on the Galagagos islands and Hawaiian silverswords (Schluter 2000; Gavrilets and Losos 2009; Losos and Ricklefs 2009). Geographic isolation, restricting the colonisation of these islands by new taxa or genotypes, increases the opportunities for successful colonisers not only to occupy their preferred niche but also to adapt to currently unoccupied niches by transformation of their niche preferences. Isolated islands, e.g., oceanic islands such as Hawaii and Galapagos, provide new ecological opportunities for the few successful colonisers. They are therefore of special interest to evolutionary biologists because they allow the study of evolutionary processes in a well-defined macroecological/macroevolutionary framework. The few successful colonisers may be the founders of new lineages by rapid diversification and/or by evolving traits that their ancestors at the mainland did not exploit as the result of competition by close relatives. A classical example is island woodiness. Although adaptive radiations are well studied for several island archipelagos such as Hawaii, Galapagos, and the Canary islands, their importance for the Madagascan biodiversity centre is less well understood. The Madagascan region is composed of the continental fragments Madagascar and the Seychelles plus the volcanic island chains of the Comoros and the Mascarenes (Vences et al. 2009). The region, but especially Madagascar itself, is famed for its unique biodiversity and ranked as one of the most important hotspots of biodiversity in the world based on species richness and number of endemic species (Myers et al. 2000; Kier et al. 2009; Lagabrielle et al. 2009). Recent evolutionary studies established Madagascar as one of the major model regions for the understanding of the accumulation of high rates of endemism by the combination of rarity of colonisation events, adaptive radiations, and climatic fluctuations (Vences et al. 2009).

The oceanic Mascarene Islands, located in the western Indian Ocean, are of similar importance in respect to our understanding of evolutionary processes and the conservation of their unique biodiversity (Kier et al. 2009; Lagabrielle et al. 2009). The two major islands, Mauritius and Reunion, provide a unique natural laboratory for the study of evolutionary processes. They share many features such as general climate, average distance to the next larger landmass, and their origin as volcanic islands, but they differ profoundly from each other in their age and current habitat diversity (Table 1).

Unfortunately, this unique diversity of the Malagasy biodiversity centre is highly threatened and the establishment of effective conservation strategies is imperative (Allnut et al. 2008; Kremen et al. 2008; Lagabrielle et al. 2009; Vences et al. 2009). To do so, we urgently need a much more comprehensive assessment of the biodiversity of the region especially by considering the potential impact of global climate warming. Species-rich groups are a particular challenge for conservation because they may be formed by adaptive and/or rapid radiations, and the

Table 1 Overview of the taxonomic diversity of the scaly tree ferns in the Madagascan biodiversity hotspot (*TS* total number of species, *TT* total number of taxa; *EN* percentage of endemic taxa) and physical diversity such as classification of the islands, main substrate, age of the islands and size of the island(s). These islands are classified either as continental fragments (CF) or oceanic islands (OC). The proximal maximum age of the island is given based on the oldest evidence of the island formation as a result of volcanic activity in the case of oceanic islands or evidence for the separation from other continents in the case of continental fragments. AFR indicates the age of the separation from Africa, IND the age of the separation from India. The Comoros plus Mayotte include several islands of different ages with the maximum age of 15 million years for the oldest existing island of the group

	TS	TT	EN (%)	Origin	Main substrate	Age	Size Km^2
Comoros incl. Mayotte	3	4	25	OI	Volcanic	<15	2,236
Madagascar	47	63	96	CF	Granitic	~130 AFR ~88 IND	587,040
Mauritius	4	5	60	OI	Volanic	~7.8	1,860
Reunion	4	4	50	OI	Volcanic	~2.1	2,512
Seychelles	1	1	100	CF	Granitic	~75 AFR	455

morphological differentiation may be often cryptic. Assessment of this megadiversity using traditional taxonomic approaches may therefore have vastly underestimated the number of species and thus misled conservation activities (Andreone et al., 2008; Vieites et al. 2009).

Recent studies on lineages including many Malagasy endemics have found evidence for repeated colonisation from Africa in the Tertiary with subsequent radiation events (Douady et al. 2002; Yoder et al. 1996, 2003; Yoder and Yang 2004; Raselimananana et al. 2009; Tsy et al. 2009). The opposite mechanisms, out of Madagascar migrations, have been reported for a few lineages such as chameleons (Raxworthy et al. 2002). Other studies found evidence for long-term survival and recent speciation events along ecological gradients and/or in response to Tertiary and Quaternary climatic fluctuations (Raxworthy et al. 2003; Townsend et al. 2009; Vences et al. 2009). Thus, the current diversity of Madagascar and adjacent islands is likely to be the result of a unique combination of factors such as isolation since the break-up with India allowing the slow accumulation of biodiversity, colonisation by African taxa with subsequent adaptive radiations, and species turnover as the result of climatic fluctuations in the last 10 million years.

Evolutionary sciences rely mainly on inductive research (Losos and Ricklefs 2009). General conclusions such as the importance of adaptive radiation require the accumulation of case studies exploring evidence using alternative analytical approaches and a wide range of taxa. So far, ferns have been widely ignored in studies on adaptive radiation especially in Madagascar. Until 2005, not a single study focused on the origin of the fern diversity of the Madagascan region. This present study was designed to overcome this shortcoming in our current knowledge of the origin of fern diversity of the Malagasy region. Malagasy ferns comprise more than 500 species of which more than 20% are likely endemic to the region (Table 1; Janssen et al. 2008).

2 Research Approach

2.1 Taxonomy

In assessing the scaly tree fern species diversity in the Madagascan region, the taxonomic account was generated by extensive fieldwork and exhaustive study of herbarium specimens deposited at the major herbaria holding collections from the Madagascan region. The fieldwork included extensive collecting visit in Madagascar but also visits to the Comoros, Mascarenes, Seychelles and Tanzania by T.J. and/or F.R. T.J. also visited Sri Lanka to study the scaly tree ferns of that island because relationships between Malagasy and Ceylonese scaly tree ferns were suggested by Holttum (1981).

2.2 Phylogeny

In reconstructing the phylogenetic relationships of scaly tree ferns occurring in the Madagascan region, two key questions required to be addressed. First, we needed to establish evidence for clades that diversified in the Madagascan region, and second, we wanted to determine the relationships of the Madagascan scaly tree ferns to relatives occurring in other parts of the world. We took advantage of the study of Korall et al. (2007) who established a phylogenetic framework for these ferns using a representative worldwide sampling. The study of Korall et al. (2007) is based on DNA sequences of five regions of the plastid genome, e.g., *rbcL*, *rbcL-accD* intergenic spacer, *rbcL-atpB* intergenic spacer, *trnG-trnR* region, and the *trnL-trnF* region. The same regions were sequences for 51 samples of scaly tree ferns occurring in the Madagascan area including the Seychelles and the Mascarene (Janssen et al. 2008). The sampling covers more than 70% of the taxonomic diversity of the area. Three species endemic to Sri Lanka were also included to be able to test Holttum's hypothesis mentioned above. The sampling includes at least a single specimen for nearly all taxa recognised in the morphology-based taxonomic revision. These new sequences were combined with the sequence data previously used by Korall et al. (2007) and the alignment adjusted manually. The sequence alignment was than employed to reconstruct the phylogeny using standard phylogenetic analytical procedures including maximum parsimony, maximum likelihood, and Bayesian inference of phylogeny (see Janssen et al. 2008).

2.3 Nuclear Markers

Sequences of the nuclear genome region were used to explore the relationships revealed by plastid genome sequences. Plastid genomes are usually inherited

maternally in ferns (Gastony and Yatskievych 1992; Vogel et al. 1998) and they do not allow us to recognise introgression and hybridisation without considering other evidence. Both hybridisation and introgression can be identified by incongruence between the phylogenetic hypotheses obtained by markers of potentially biparental inheritance such as nuclear genome sequences. Several nuclear markers, e.g., *gapCp*, *LFY*, and *pgiC*, were originally tested for consistency and quality of sequence products. The initially tested regions have been studied previously for other fern lineages (Schuettpelz et al. 2008; Shepherd et al. 2008). We did not obtain consistently good PCR products of *gapCp* and *pgiC* but did of *LFY*. The *LFY* primers used were designed by A. Driskoll (http://www.uvm.edu/%7Ebarring/barrprotos.html) and employed for the first time for scaly tree ferns (S.H., personal communication). The phylogeny of the intron 2 of the single copy *LFY* was reconstructed using the same phylogenetic analyses as for the plastid phylogeny. Both phylogenies were compared visually for incongruence.

2.4 Establishing Biogeographic Hypothesis

The ancestral distribution ranges were estimated using different approaches that take advantage of the generated phylogenetic framework by maximising the ancestral area reconstruction using either maximum parsimony or maximum likelihood. In addition, DIVA and Bayesian-DIVA analyses were performed (Ronquist 1997; Nylander et al. 2008). DIVA analyses are based on an explicit model of biogeographic history by considering dispersal and vicariance as the two main processes, whereas Bayesian DIVA analyses do not rely on a single optimal tree but take phylogenetic uncertainty into account. Species ranges were classified in five classes, because we are only interested in the global origin of the scaly tree ferns occurring in the Madagascan region. The classes are "Neotropical" for species occurring in South America, Central America and the Caribbean, "Asia to Australasia" for species occurring in continental Asia, Sri Lanka, throughout Malesia to Australia and New Zealand, and "Africa" for species occurring in continental Africa. Species occurring in Comoros, Madagascar, Mascarenes, and Seychelles were divided into two classes. One group includes only the Malagasy species, whereas the other group includes species occurring on the smaller islands.

2.5 Divergence Time Estimates

To reconstruct the time frame of the divergence of Malagasy scaly tree ferns, divergence times were estimated using DNA sequence variation constraint by fossil evidence. We used different approaches to estimate the divergence times using the software packages r8s (Sanderson 2002) and BEAST (Drummond and Rambaut 2007). Analyses assuming a relaxed molecular clock provided us with the most convincing results (Janssen et al. 2008).

2.6 Niche Modelling

To explore climatic niche evolution using distribution range modelling, distribution data are used to estimate the climatic niche preferences of each species and their respective pools of related species, e.g., all Malagasy scaly tree ferns (see Janssen et al. 2008). This approach was used to reconstruct the fundamental climatic niche and subsequently to estimate the fundamental distribution range of these taxa under the current climatic conditions but also with different moisture and temperature regimes such as scenarios predicting the Malagasy climate in the last glacial minimum (LGM, 18 BYA).

3 Results

3.1 Taxonomic Revision

The revision of the scaly tree ferns of the Madagascan region resulted in the description of 12 new species for Madagascar and 1 new species for Mauritius (Janssen and Rakotondrainibe 2006, 2007, 2008). In addition, several new variaties are now recognised (Fig. 1). Combined the number of new species and new variaties resulted in a substantial increase (>25%) of the known number of scaly tree ferns occurring in the Madagascan region: 24 for Madagascar and 2 for Mauritius (Table 1).

3.2 Scaly Tree Fern Phylogeny

Scaly tree fern species of the Madagascan region belong to three clades that correspond to morphologically distinct groups. The first clade includes species without indusia and is nested within the *Gymnosphaera* clade. The second clade includes indusiate species with tripinnate leaves, whereas the third clade consists of indusiate species with bipinnate leaves (Fig. 1). These two clades are nested within the *Alsophila* clade but not closely related to each other (Fig. 2). These three clades include either exclusively species occurring in the Madagascan region or also African species in addition to the Madagascan species. The sister clade of two of the three clades occur in Africa, whereas the sister taxon of one clade occurs in the Neotropics, especially in the Caribbean. The Ceylonese species form a clade nested within *Alsophila* but not closely related to any of the Madagascan species (Fig. 2).

3.3 Radiations of Scaly Tree Ferns in Madagascar

Divergence time estimation recovered evidence for three rapid radiations of scaly tree ferns in the Malagasy region, one for each clade. Our analyses did not suggest

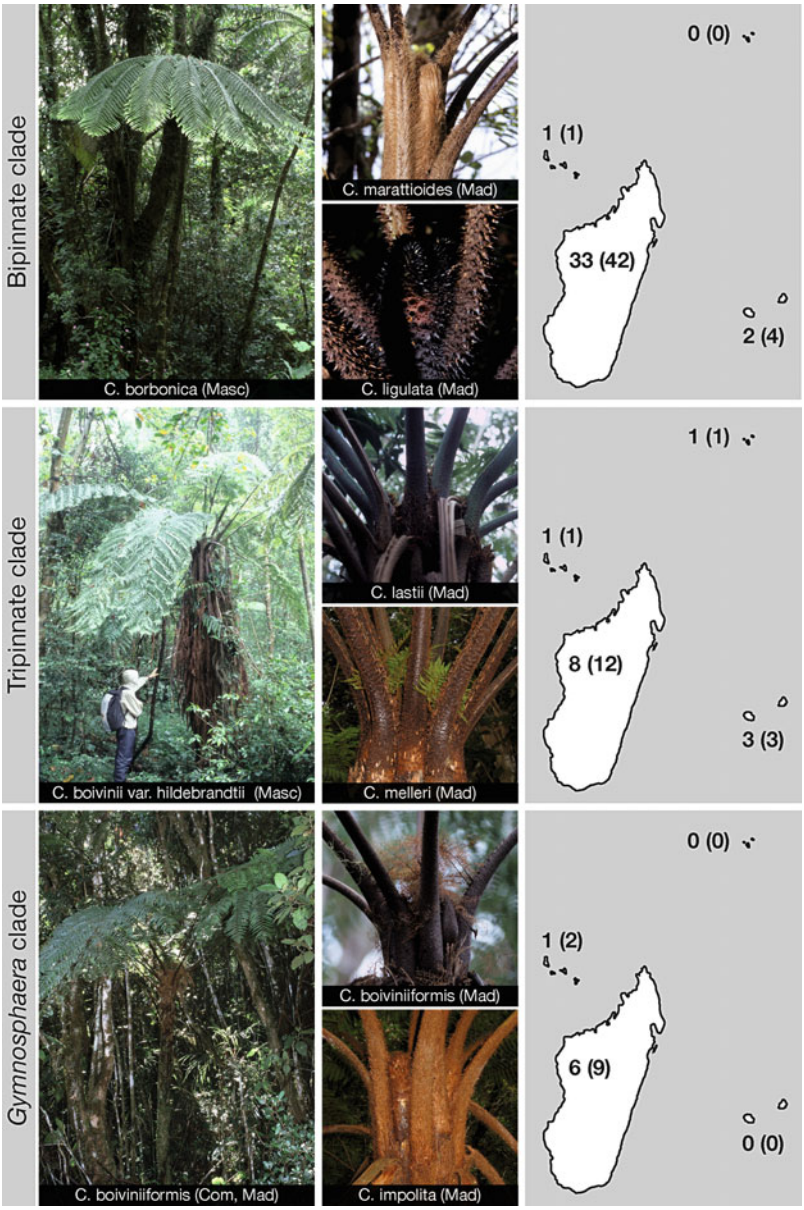


Fig. 1 Introduction to the three clades of scaly tree ferns occurring in Madagascar and adjacent region. The habit of a typical representative is given for each clade plus images of the shoot apex of two additional representatives. The *maps* outline the distribution of each clade including the number of taxa on the Seychelles, Comoros, Mascarenes, and Madagascar. The first *number* corresponds to the total number of species whereas the *number in parentheses* corresponds to the total number of taxa (species and variations). *Mad* Madagascar, *Masc* Mascarenes

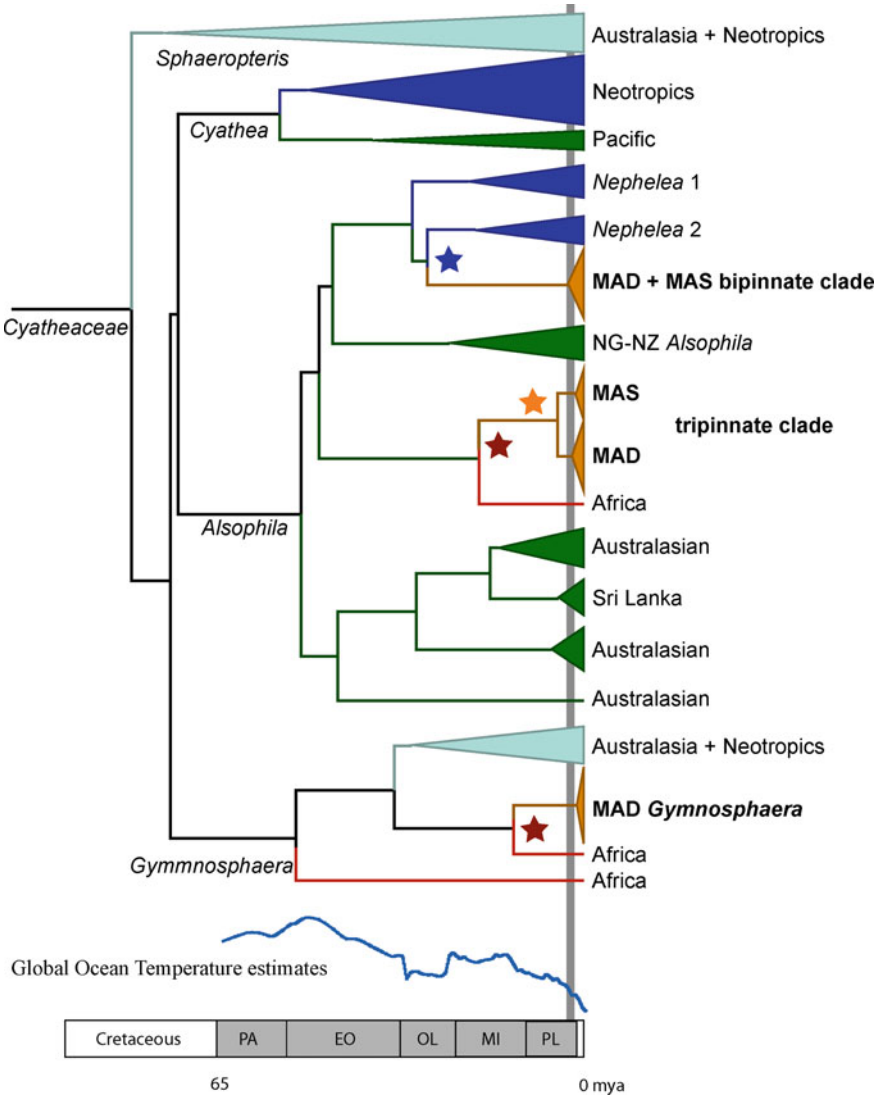


Fig. 2 Simplified chronogram of the tree ferns with the distribution ranges indicated based on Janssen et al. (2008). A simplified time scale is given at the *bottom* of the figure. *PA* Paleocene, *EO* Eocene, *OL* Oligocene, *MI* Miocene, *PL* Pliocene. The *colour* of the clades corresponds to the distribution ranges given in the *right column*. *MAD* Madagascar, *MAS* Mascarenes. *Stars* indicate a sequence of dispersal followed by vicariance events. The *colour* indicates the occurrence of each clade: *bright blue* Asia to Australasia plus a few species in the Neotropics, *dark blue* Neotropics, *green* Asia to Austalasia, *orange* Madagascar and adjacent regions, *red* Africa. The global temperature estimate graph is based on Haq et al. (1987) and indicates the fluctuations. The *grey bar* indicates the period in which the three Malagasy scaly tree fern radiations are onset

the successful colonisation of Madagascar as the trigger of the radiations because of the recovered time period of the potential earliest colonisation and the observed radiations onset. The temporal onset of the three radiations fall into the Pliocene or the earliest Pleistocene (Janssen et al. 2008). This time period is characterised by strong climatic fluctuations with a general trend to cooler global climates. Several other studies have found evidence for a similar coincidence of rapid radiations or similar divergence events in lineages that are species rich in Madagascar. In agreement with other authors, we suspect late Tertiary climatic fluctuation as the main cause of the unique Malagasy rainforest diversity (Fig. 2).

3.4 Comparing Current Distribution and Fundamental Distribution

The current distribution was estimated by plotting the occurrences of herbarium specimens and our own field observation on map of Madagascar (Fig. 3a). The current distribution is closely linked to the preservation of rainforests. The species diversity is especially high in areas known to be well collected, and thus the lack of records may to some extent reflect limited collecting activities in many areas of Madagascar. The estimation of the fundamental distribution of tree ferns in Madagascar, however, indicated a much wider distribution range than the one indicated by the observed distribution (Fig. 3b). The strong difference between predicted and observed distribution indicates a significant loss of habitats in historic times. The likely reason is the deforestation of large areas of Madagascar since the first human settlers arrived. The close correlation of scaly tree fern distribution and the occurrence of rainforests suggests that these species will be excellent indicators in conservation surveys focusing on rainforest habitats. Tree ferns are easily spotted but the species identification is difficult for nonexperts. An interesting aspect of the Malagasy scaly tree ferns is their adaptation to more seasonal climates in comparison to other scaly tree ferns (Fig. 3c) (Janssen et al. 2008; Bystriakova et al., in preparation.). The three Malagasy lineages of scaly tree ferns show the same notable shift in their climatic niche preferences. However, we currently lack sufficient observations to establish a hypothesis describing ecological differentiation among the Malagasy species with the exception of species occurring on the Mascarene islands.

3.5 Scaly Tree Ferns on the Mascarene Islands

The two major islands of the Mascarene island, Reunion and Mauritius, house similar species numbers (see Table 1), but the pattern gets considerably more complex if the phylogenetic relationships are taken into account. One out of the four species on each island is the invasive *Cyathea* (*Sphaeropteris*) *cooperi* that is

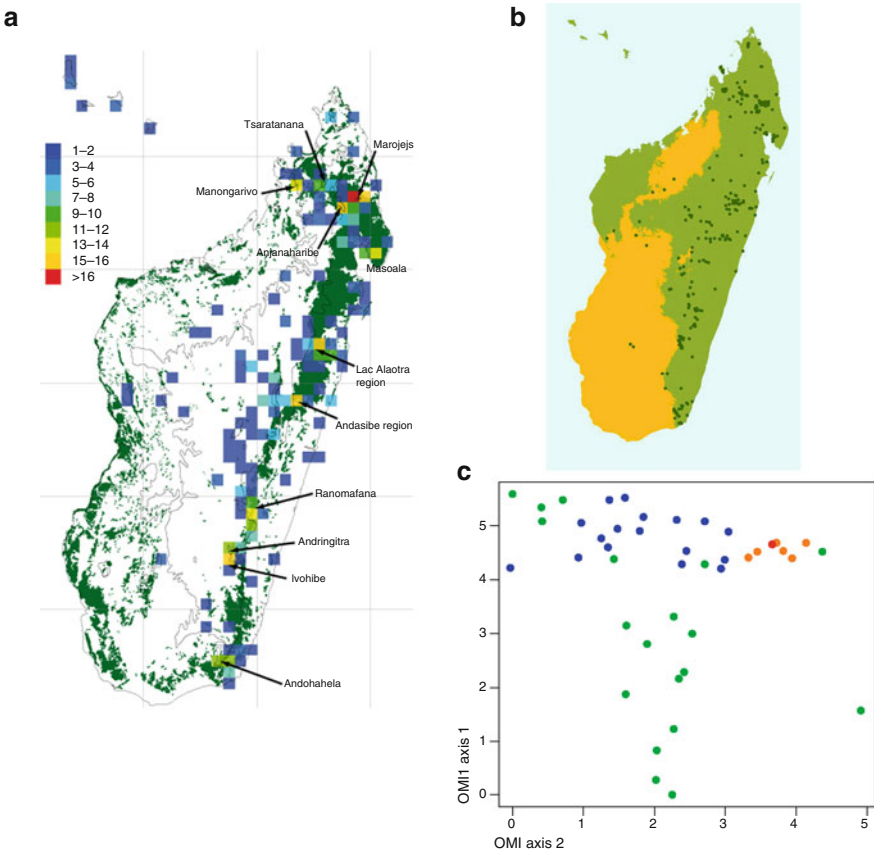


Fig. 3 Geographic distribution of scaly tree ferns in Madagascar. (**a**) Current distribution in Madagascar based on herbarium collections and fieldwork observations. The *green shaded* areas correspond to well-preserved forest areas. The colour code indicates the number of records for each grid (see, for additional information, Janssen and Rakotondrainibe 2008). (**b**) Fundamental distribution range of scaly tree ferns in Madagascar and Comoros as predicted in our niche modelling analyses (Bystriakova, unpublished). (**c**) Ordination of climatic niche preferences of tree ferns (Janssen et al. 2008; Bystriakova et al., in preparation.). *Orange squares* indicate Madagascan species, whereas the other colours indicate: African (*red*), Neotropical (*blue*), and Asian/Austalasian (*green*) species. The Madagascan species show very similar climatic preferences and are distinct from non-Madagascan species with the exception of African species (Bystriakova et al., in preparation.)

a potential threat to the native tree fern diversity in lowland habitats. This taxon is usually found in habitats heavily influenced by human activities. The other species are endemic to the Mascarenes. In Reunion, one out of the three native species, *Cyathea* (*Alsophila*) *borbonica* var. *borbonica*, belongs to the bipinnate clade, the other two to the tripinnate clade. These two sister species, *C*. (*Alsophila*) *excelsa*

and *C*. (*Alsophila*) *glauca,* show ecological differentiation. The first one occurs from 200 to 1,700 m, whereas the second one occurs from 1,300 to 2,000 m. *Cyathea excelsa* is also found in Mauritius whereas *C*. *glauca* is not known from there, which is to be expected given the highest point of Mauritius is below 1,000 m. However, our current data do not allow us to explore the hypothesis that *C*. *glauca* originated in Reunion. *C*. *excelsa* and *C*. *glauca* may have been separated in Mauritius and independently colonised Mauritius. *C*. *glauca* may have gone extinct in Mauritius as a result of habit. Mauritius was once more like Reunion today as a result of active volcanism, as observed in Reunion. Today's landscape of Mauritius is mainly the result of erosion since the end of the active phase of volcanism. A very different pattern was found in *C*. (*Alsophila*) *borbonica,* which includes only a single variaty in the lowland rainforests of Reunion but two distinctive variaties in Mauritius. The diversity of rare local endemic Mauritian taxa is further increased by *Cyathea* (*Alsophila*) *grandgaudiana* which combines the bipinnate leaves with the occurrence of aborted sporangia. This character is also known from the tripinnate *C*. *excels*, but it is not known if the character combination indicates hybridisation between the bipinnate and tripinnate scaly tree ferns in Mauritius. Current evidence is insufficient to explore fragmentation as the cause of the establishment of the Mauritian endemics.

4 Perspectives

Our study reports the first comprehensive example for a fern radiation in Madagascar. Recently, a second example has also been suggested for the genus *Elaphoglossum* (Vasco et al. 2009), but this example still needs a more rigid biogeographic analysis. Similar studies on other fern lineages will be of major interest because not all lineages may show a radiation in Madagascar but instead may provide better insights into the exchange of plant diversity between Madagascar and other areas such as Africa and Asia. So far, we do not have any indication for a fern species occurring in Madagascar that traces back their separation from their relatives to Gondwana times. In contrast, ferns join the lineages that contain signals for the important impact of the late Cenozoic radiations to the diversification of rainforest taxa in Madagascar.

Tree ferns occur mainly in the wet tropical parts of Madagascar and are easily spotted and registered. They are therefore extremely well suited to be used to assess the existence of relatively undisturbed rainforest patches, but the identification of the species requires expertise. It is increasingly clear that the biodiversity of Madagascar can only be protected for some selected areas (Kremen et al. 2008), and the occurrence of scaly tree ferns may be a good criteria for rainforest habitats.

The low level of genetic differentiation in the plastid genome limits the study of the evolutionary processes, especially the ecological differentiation among scaly tree fern species. Future studies will need to focus on the establishment of more

variable markers such as single nucleotide polypmorphisms (SNPs). The investment into these kind of markers will be well repaid because the Malagasy scaly tree ferns offer unique opportunities to study ecological and genetic aspects of fern/plant speciation in the tropics but also to understand the processes of rapid radiations.

Acknowledgements This project was supported by the Deutsche Forschungsgemeinschaft (SCHN758/2-1) as part of the Schwerpunkt Radiationen and by the National Geographic Society (7702-4). We are greatly in dept to collaborators, who supported our fieldwork or provided material for sequencing, and the curators of various herbaria, who allowed us to access their collections.

References

Allnut TF, Ferrier S, Manion G, Powell GVN, Ricketts TH, Fisher BL, Harper GJ, Irwin ME, Kremen C, Labat J-N, Lees DC, Pearce TA, Rakotondrainibe F (2008) A method for quantifying biodiversity loss and its application to a 50-year record of deforestation across Madagascar. Conserv Lett 1:173–181

Andreone F, Carpenter AI, Cox N, du Preez L, Freeman K, Furrer S, Garcia SG, Glaw F, Glos J, Knox D, Köhler J, Mendelson JR, Mercurio V, Mitermeier RA, Moore RD, Rabibisoa NH, Randriamahazo H, Randrianasolo H, Rasoamampiona Raniosoa N, Ravoahangimlala Ramilijaona O, Raxworthy CJ, Vallan D, Vences M, Vieites DR, Weldon C (2008) The challenge of conserving amphibian megadiversity in Madagascar. PLoS Biol 6:e118

Douady CJ, Catzdelis F, Kao DJ, Springer MS, Stanhope MJ (2002) Molecular evidence for the monophyly of Tenrecidae (mammalia) and the timing of the colonization of Madagascar by Malagasy Tenrecs. Mol Phylogenet Evol 22:357–363

Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 7:214

Gavrilets S, Losos JB (2009) Adaptive radiation: contrasting theory with data. Science 323:732–737

Gastony GJ, Yatskievych G (1992) Maternal inheritance of the chloroplast and mitochondrial genomes in cheilanthoid ferns. Am J Bot 79:716–722

Haq BU, Hardenbol J, Vail PR (1987) Chronology of fluctuating sea levels since the Triassic. Science 235:1156–1167

Holttum RE (1981) The tree ferns of Africa. Kew Bull 36:463–482

Janssen T, Bystriakova N, Rakotondrainibe F, Coomes D, Labat J-N, Schneider H (2008) Neoendemism in Madagascan scaly tree ferns results from recent, coincident diversification bursts. Evolution 62:1876–1889

Janssen T, Rakotondrainibe F (2006) A revision of the fern family Cyatheaceae in the Mascarene Islands. Adansonia 28:213–241

Janssen T, Rakotondrainibe F (2007) An update of the revision of *Cyathea* subgen. *Alsophila* sect. *Gymnosphaera* (Cyatheaceae) in Madagascar and the Comoros including a discussion of putative hybridization events. Adansonia 29:195–213

Janssen T, Rakotondrainibe F (2008) A revision of the indusiate scaly tree ferns (Cyatheaceae: *Cyathea* subgen. *Alsophila* sect. *Alsophila*) in Madagascar, the Comoros and the Seychelles. Adansonia 30:221–376

Kier G, Kreft H, Lee TM, Jetz W, Ibisch PL, Nowicki C, Mutke J, Barthlott W (2009) A global assessment of endemism and species richness across island and mainland regions. Proc Natl Acad Sci USA 106:9322–9327

Korall P, Metzgar JS, Conant DS, Schneider H, Pryer KM (2007) Phylogeny of scaly tree ferns (Cyatheaceae) as revealed by five plastid loci. Am J Bot 94:873–886

Kremen C, Cameron A, Moilanen A, Phillips SJ, Thomas CD, Beentje H, Dransfield J, Fisher BL, Glaw F, Good TC, Harper GJ, Hijsmans RJ, Lees DC, Louis E Jr, Nussbaum RA, Raxworthy CJ, Razafimpahanana A, Schatz GE, Vences M, Vieites DR, Wright PC, Zjhra ML (2008) Aligning conservation priorities across taxa in Madagascar with high-resolution planning tools. Science 320:222–226

Lagabrielle E, Rouget M, Payet K, Wistebaar N, Durieux L, Baret S, Lombard A, Strasberg D (2009) Identifying and mapping biodiversity processes for conservation planning in islands: a case study in Reunion Island (Western Indian Ocean). Biol Conserv 142:1523–1535

Losos JB, Ricklefs RE (2009) Adaptation and diversification on islands. Nature 457:830–836

Myers N, Mittermeier RA, Mittermeier CG, da Fonsega GAB, Kent J (2000) Biodiversity hotspots for conservation priorities. Nature 403:853–858

Nylander JAA, Olsson U, Alstrom O, Sanmartin I (2008) Accounting for phylogenetic uncertainty in biogeography: a Bayesian approach to dispersal-vicariance analysis of the thrushes (Aves: Turdus). Syst Biol 57:257–268

Raselimananana AP, Noonan B, Praveen Karanth K, Gauthier J, Yoder AD (2009) Phylogeny and evolution of Malagasy plated lizards. Mol Phylogenet Evol 50:336–344

Raxworthy CJ, Forstner MR, Nussbaum RA (2002) Chameleon radiation by oceanic dispersal. Nature 415:784–787

Raxworthy CJ, Martinez-Meyer E, Horning N, Nussbaum RA, Schneider GE, Ortega-Huerta MA, Townsend Peterson A (2003) Predicting distributions of known and unknown reptile species in Madagascar. Nature 426:837–841

Ronquist F (1997) Dispersal-vicariance analysis: a new approach to the quantification of historical biogeography. Syst Biol 46:195–203

Schluter D (2000) The ecology of adaptive radiation. Oxford University Press, Oxford

Schuettpelz E, Grusz AL, Windham MD, Pryer KM (2008) The utility of nuclear *gapCp* in resolving polyploidy fern origins. Syst Bot 33:621–629

Shepherd LD, Perrie LR, Brownsey PJ (2008) Low-copy nuclear DNA sequences reveal a predominance of allopolyploids in a New Zealand *Asplenium* fern complex. Mol Phylogenet Evol 49:240–248

Townsend TM, Vieites DR, Glaw F, Vences M (2009) Testing species-level diversification hypotheses in Madgascar: the case of microendemic *Brookesia* leaf chemaleons. Syst Biol 58:641–646

Tsy JMLP, Lumaret R, Mayne D, Vall AOM, Abutoba YIM, Sagna M, Roseta SOR, Danthu P (2009) Chloroplast DNA phylogeography suggests a West African centre of origin for the baobab, *Adansonia digitata* L. (Bombacoideae, Malvaceae). Mol Ecol 18:1707–1715

Vasco A, Moran RC, Rouhan G (2009) Circumscription and phylogeny of the *Elaphoglossum ciliatum* group (*E.* sect, *Lepidoglossa*, Dryopteridaceae) based on cpDNA sequences. Taxaon 58:825–834

Vences M, Wollenberg KC, Vieites DR, Less DC (2009) Madagascar as a model region of species diversification. Trends Ecol Evol 24:456–465

Vieites DR, Wollenberg KC, Andreone F, Koehler J, Glaw F, Vences M (2009) Vast underestimation of Madagascar's biodiversity evidenced by an integrative amphibian inventory. Proc Natl Acad Sci USA 106:8267–8272

Vogel JC, Russell SJ, Rumsey FJ, Barrett JA, Gibby M (1998) Evidence for maternal transmission of chloroplast DNA in the genus *Asplenium*. Bot Acta 111:247–249

Yoder AD, Burns MM, Zehr S, Delefosse T, Vernon G, Goodmann SM, Flynn JJ (2003) Single origin of Malagasy carnivora from an African ancestor. Nature 421:734–737

Yoder AD, Cartmill M, Ruvolo M, Smith K, Vilgalys R (1996) Ancient single origin of Malagasy primates. Proc Natl Acad Sci USA 93:5122–5126

Yoder AD, Yang Z (2004) Divergence dates for Malagasy lemurs estimated from multiple gene loci: geological and evolutionary context. Mol Ecol 13:757–773

Rapid Radiation in the Barley Genus *Hordeum* (Poaceae) During the Pleistocene in the Americas

Frank R. Blattner, Thekla Pleines, and Sabine S. Jakob

Abstract Evidence was found for a rapid radiation of the grass genus *Hordeum* in the Americas during the last 2 million years, accumulating 23 species in South and North America, while only 10 *Hordeum* species occur in other regions of the world. The differences in species richness are caused by distinct evolutionary mechanisms in the Americas and Eurasia, as recovered by the integration of phylogenetic and phylogeographic analyses with modeling of ecological niches. The Eurasian region is mainly characterized by a loss of biodiversity during the Pleistocene glaciations, while vivid speciation took place in the Americas during this time period. Thus, speciation in Eurasia was mainly affected by severe genetic bottlenecks probably due to small populations surviving in ice-age refugia, while such restrictions in New World species groups seem less pronounced. Particularly in southern Patagonia, speciation was due to multiple geographical subdivisions of relatively large populations during the last million years, without measurable reduction of genetic diversity or population sizes. This together with long-distance colonization of remote areas was the main cause of species diversity in the New World.

1 Introduction

Hordeum comprises about 33 species and belongs with other cereals, such as wheat and rye, and several important forage grasses, to the grass tribe Triticeae. *Hordeum* is characterized by three single-flowered spikelets (triplets) at each rachis node of the inflorescence, the lateral ones often sterile or only rudimentarily present. The economically most important species of the genus is barley, *H. vulgare*, which is used to feed livestock, as flour in human consumption, and malted for beer and

F.R. Blattner (✉), T. Pleines, and S.S. Jakob
Leibniz Institute of Plant Genetics and Crop Research (IPK), Corrensstraße 3, 06466 Gatersleben, Germany
e-mail: blattner@ipk-gatersleben.de

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_2,

Fig. 1 Examples of *Hordeum* species. From *left* to *right*: *H. marinum* from southwestern Europe, *H. brachyantherum* from western North America, flowering inflorescences of *H. patagonicum*, and *H. comosum* from southern South America

whisky production. *Hordeum* species are naturally distributed all over the northern hemisphere, in South Africa and in southern South America. With 16 native species, this latter region is also the main center of species diversity (von Bothmer et al. 1995). The species nearly always occur in open habitats (Fig. 1), often in steppe or meadow vegetation, along streams and ditches, many on salt-influenced soils, and nowadays also in disturbed habitats along streets and irrigation channels within their natural distribution areas. Some species like wall barley (*H. murinum*) and sea barley (*H. marinum* and *H. gussoneanum*) have been introduced nearly worldwide in temperate and dry regions, and occur now as weeds in disturbed and agricultural habitats. Several taxonomic treatments of *Hordeum* exist. The most extreme views are those of Dewey (1984) and Löve (1984) who divided the group into *Hordeum* s.str., consisting either of *H. vulgare* or *H. vulgare* and *H. bulbosum*, while all other species were included in the genus *Critesion*. This concept of generic delimitation was not generally agreed upon, and von Bothmer et al. (1995) in their last revision of the genus maintained the traditional circumscription as a single genus.

The infrageneric delimitation has also changed several times during the recent decades, involving criteria such as life cycle or awn length (Nevski 1941). von Bothmer and Jacobsen (1985) recognized four sections, *Hordeum*, *Anisolepis*, *Stenostachys*, and *Critesion*. These sections were maintained in the 1995 revision of the genus, although cytogenetic data did not entirely support this classification and the authors stated that it might be artificial (von Bothmer et al., 1995). Petersen and Seberg (2003) proposed a new sectional treatment of the genus based on phylogentic analyses of single-copy nuclear and chloroplast DNA data. They delimited four different sections from von Bothmer et al. (1995): *Hordeum*, *Sibirica*, *Stenostachys*, and *Critesion*. Although based on a phylogenetic interpretation of DNA sequence data, this new classification also contradicted the earlier cytogenetic results, exemplified by the definition of so-called *genomes* in *Hordeum* and other Triticeae taxa. Within *Hordeum*, four genomes have been described,

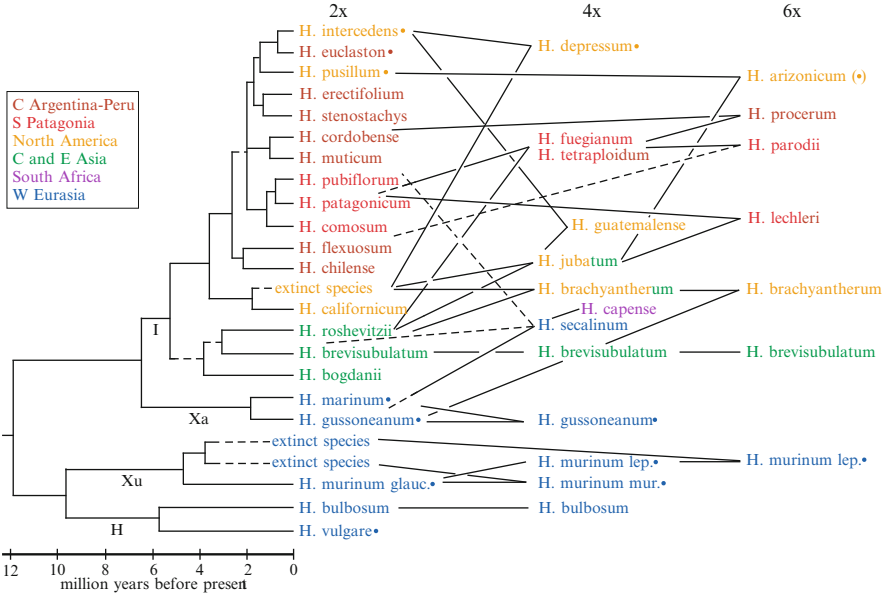


Fig. 2 Scheme representing our current understanding of phylogenetic relationships among *Hordeum* species (modified from Blattner 2009). It is derived from sequence analyses of nuclear rDNA ITS data for all species (Blattner 2004), ITS, DMC1 and EF-G for diploid taxa (Blattner 2006), AFLP fingerprints for the closely related diploid American species (Pleines and Blattner 2008), and for some species groups also chloroplast *trn*L-F sequences (Jakob and Blattner 2006). Diploids (2×) are drawn directly to the branches of the phylogenetic tree, while polyploids (4×, 6×) are mapped on the *right*. *Lines* connect the taxon names with their respective (partly putative) parental species. *Dashed lines* indicate topological uncertainties. *Bullets* behind taxon names mark annual taxa. *Letters* along the branches of the tree denominate the distribution of Triticeae genomes within the genus. While for the diploid species, estimations of clade ages were conducted, no such analyses were done for the polyploids. The existence of extinct species could be inferred from sequences, which are present in polyploid taxa, but do not occur in any extant diploid species

named **H**, **I**, **Xa**, and **Xu** (Linde-Laursen et al. 1992; Wang et al. 1996; Blattner, 2009). The distribution of the genomes among *Hordeum* species is given in Fig. 2. Although the concept of genomes is widely used in cultivated plants and their wild relatives, the systematic value of chromosomal similarity is debated (e.g., Petersen and Seberg 2003), as it is unclear how the amount of meiotic chromosome pairing in interspecific hybrids, that is used to define genomes, is connected to phylogenetic relationships.

Several attempts have been undertaken to clarify the phylogeny of *Hordeum* species, since barley is a major crop on the world scale and its wild relatives bear potential as genetic resources for crop improvement. However, most analyses suffered from often arbitrary taxon sampling or methodological shortcomings. Thus, surprisingly few phylogenetic studies with a complete or at least representative taxon sample have been conducted. Studies with higher species numbers or

including at least all diploid species have been published by Doebley et al. (1992), Komatsuda et al. (1999), Nishikawa et al. (2002), and Petersen and Seberg (2003, 2004). The results were contradictory, partly due to inconsistencies between chloroplast and nuclear phylogenies, and partly caused by differences in taxon sampling. Furthermore, all studies revealed poor resolution of the closely related New World taxa of the genus.

Speciation, the separation of a phylogenetically coherent group of individuals into two independently evolving lineages, is the major driving force of biodiversity. From a geographical perspective three major settings can result in speciation. These are: (1) speciation in allopatry, which means individuals of a species become geographically separated and evolve afterwards into two different lineages, as gene flow between sister populations is absent due to the geographical barrier; (2) parapatry, which means that a small peripheral population evolves into a different lineage, often involving restricted gene flow between the main population in the center and the peripheral isolate; and while these processes are probably involved in the majority of speciation events, the occurrence of (3) sympatric speciation is still under debate, as in this case no geographical barrier reduces gene flow between the differentiating lineages. As gene flow generally counteracts diversification and genetic recombination melts down diversifying allele combinations, strong selection against interbreeding has to maintain separated gene pools from a very early stage of diversification to arrive at separate species in a sympatric setting. Sympatric speciation can therefore be mainly postulated in situations such as those found in lakes or on small islands where geographic barriers are less likely (Coyne and Orr 2004). Otherwise, allopatric speciation involving geographical barriers is the preferred null hypothesis, particularly when taking into account the climate changes during the last 2 million years (my), when Pleistocene temperature oscillations resulted in range shifts and often forced species into isolated ice-age refugia. The major exception to the general rarity of sympatric speciation occurs nearly only in plants, where polyploidization, i.e., the doubling of chromosome numbers, is quite frequent while it seems comparatively rare in animals. Changes in chromosome number result in immediate reproductive isolation and therefore allow differentiation of two lineages in the same area without gene flow.

1.1 Framework of the Studies, Questions to Answer

Initially, the high number of *Hordeum* species in South America in comparison to other areas of the world allowed two hypotheses explaining this observation: (1) either the genus occurred for a longer time in South America and therefore had more time to evolve species diversity in this region, or (2) speciation rates increased in comparison to other areas after *Hordeum* arrived in South America. This second hypothesis was much more likely given that the majority of closely related genera of Triticeae occur in the northern hemisphere, particularly in western Asia, thus making a South American origin of the genus highly improbable. Also, the slight

morphological differences among several of the South American *Hordeum* species might indicate a relatively young age of this group. Thus, we assumed an ongoing rapid radiation of South American *Hordeum* taxa in comparison to species groups from other areas of the world. Putative causes for a rapid radiation could be (1) ecological differentiation of the taxa filling different open niches after an initial arrival in this area, or (2) several allopatric divisions of populations resulting in a multitude of geographically separated species. Clear ecological differences among species regarding soil and climate conditions together with often sympatric occurrence of species within a certain area could support an ecological speciation scenario in South America (Coyne and Orr 2004; Jakob et al. 2009). As seemingly increased speciation in South America could also be caused by higher extinction rates in areas of the northern hemisphere, we compared Old and New World *Hordeum* groups in a phylogenetic context to estimate speciation rates in different areas of the world. To understand possible reasons for shifts in speciation or extinction rates and to correlate them with climatic or geological changes it seemed also advisable to assign proximate ages to species groups within the genus. Moreover, the occurrence of *Hordeum* species on all continents apart from Australasia also demands the understanding of the historical biogeography, i.e., when and from where different regions of the earth were colonized.

The study of speciation processes first needs a sound phylogenetic framework. Therefore, several analysis approaches were conducted involving sequencing of nuclear loci and an analysis of amplified fragment length polymorphisms (AFLP; Vos et al. 1995) for the closely related species of the New World. In addition, analyses of chloroplast diversity were conducted to understand the nature of inconsistencies between nuclear and plastid phylogenies and to reconstruct the evolutionary history (phylogeography) of populations, species and species groups within the genus. To understand ecological differences among species, we also conducted cultivation experiments in greenhouses, and used modeling of the species' potential climatic niches to detect niche shifts among closely related taxa and probable Pleistocene refugia of species.

2 Materials and Employed Methods

In our molecular analyses of the evolution of species within the genus *Hordeum*, we generally tried to include a multitude of individuals per species in phylogenetic analyses to account for intraspecific variation. Thus, we transferred sampling schemes normally used in population genetic and phylgeographic analyses into phylogenetics. Wherever possible, we included material collected directly from wild populations. As we have up to now not been able to cover the entire distribution area of the genus with field trips, we complemented our *Hordeum* collection by materials from germplasm repositories (gene banks) and partly also herbaria. Seed material was germinated, grown in greenhouses, taxonomically determined, and ploidy levels were determined by flow cytometric analysis of genome sizes.

This was necessary, as *Hordeum* species are not always easily discernable, and materials from gene banks or botanic gardens often proved to be wrongly named. Moreover, in several species, cytotypes with different ploidy levels occur. Genome sizes are mostly reliable identifiers of species affiliation in *Hordeum* (Jakob et al. 2004), thus knowledge of the geographic origin of materials together with genome size provides in most cases enough information to verify species determinations.

To arrive at a robust species phylogeny, the ITS region of the nuclear ribosomal DNA was used to reconstruct phylogenetic relationships among all *Hordeum* species plus several outgroups from Triticeae, *Bromus*, and *Brachypodium* (Blattner 2004). As in most diploid *Hordeum* species two clusters of 45S rDNA located on two different chromosomes are present (Taketa et al. 1999, 2001) and most polyploid taxa originated from allopolyploidization, extensive sequencing of cloned ITS amplicons was used to determine the nature of the ITS regions present in individuals of most species. For all diploid and a few polyploid *Hordeum* species, ITS data were also combined with sequence data from two nuclear single copy genes (Blattner 2006): disrupted meiotic cDNA (DMC1; Petersen and Seberg 2004) and elongation factor gamma (EF-G; Komatsuda et al. 1999) which improved statistical support of basal branches in comparison to the analysis of only the ITS region. It did not, however, provide a better resolution of relationships among the American taxa. Therefore, we conducted in addition a phylogenetic analysis based on AFLP data (Vos et al. 1995) of the diploids from the Americas (Pleines and Blattner 2008) to define species groups particularly within the South American members of the genus. Nuclear rDNA ITS and the dataset combining three nuclear loci were also used to date branching points within the phylogeny of the genus. As calibration point, the split between barley and wheat about 13 my ago (mya) was used (Gaut 2002) in a penalized likelihood approach with R8S (Sanderson 2002) to estimate ages within *Hordeum*.

The historical biogeography of *Hordeum* was inferred from the phylogenetic tree derived from the combination of the nuclear loci, estimates of clade ages, and geographical distribution of the species. The age of the crown group of *Hordeum* (12 my) and the relevant subgroups (4–6 my) allowed for the assumption that the continents were in their present-day positions and that the relevant land bridges as Beringia and Central America were nearly or already in place at the time of origin of the taxa (Blattner 2006).

Chloroplasts, which are assumed to be maternally inherited in grasses, provide tools to analyze species histories. They are distributed by seeds only and have an effectively haploid genome and therefore a smaller effective population size than nuclear genes. This prevents problems with allelic recombination and results mostly in clearer geographically structured data in comparison to nuclear markers (Pleines et al. 2009). We used chloroplast data to analyze species or species groups to arrive at phylogeographic hypotheses of these taxa. To understand the general pattern of chloroplast allele distribution among species, we initially analyzed the chloroplast *trn*L-*trn*F region (*trn*L-F) in 875 individuals covering all taxa of *Hordeum* (Jakob and Blattner 2006). *Trn*L-F consists of two exons and the intron in the *trn*L gene, the intergenic spacer between this gene and *trn*F, and the *trn*F gene

itself. The transfer RNA (tRNA) genes are highly conserved among plants and are therefore suited as PCR primer binding sites, while intron and spacer sequences consist of variable DNA stretches, providing sequence differences useful in phylogenetic and population genetic analyses (Shaw et al. 2007).

For phylogenetic analyses of nuclear data, we used phenetic, cladistic, and model-based analysis algorithms implemented in PAUP* 4 (Swofford 2002) and MRBAYES 3.1 (Ronquist and Huelsenbeck 2003), which resulted in single or multiple trees. Statistical support of taxon groups was evaluated by bootstrap analyses (in neighbor-joining and parsimony analysis) and posterior probabilities from Bayesian inference. For chloroplast data, we also used a network approach (Posada and Crandall 2001), as data structure of the *trn*L-F region proved not to be tree-like. Thus, the reconstruction of an allele or haplotype genealogy provided a better representation of relationships among these haplotypes than all tree-based algorithms (Jakob and Blattner 2006). Haplotype genealogies were also used in studies of single species or species groups. In these cases, the number of included individuals was increased to get a good representation of species geographical distributions. In some of these more detailed studies, we included not only the *trn*L-F region but also sequenced highly variable parts of the chloroplast genome (Jakob et al. 2007; Jakob and Blattner 2010). These consisted either of AT-rich repetitive structures or mononucleotide repeat microsatellites, mostly poly-A/T stretches. Inclusion of microsatellites resulted in better-resolved relationships among chloroplast haplotypes within narrow taxonomic groups, but in more distantly related taxa, homoplasy was quite high. To overcome this problem, we invented a two-step procedure of network construction, where a backbone network was built on sequence variation at the slowly evolving parts of the analyzed loci, and subhaplotypes of these backbone haplotypes were created taking into account variation at microsatellite loci (Bänfer et al. 2006; Jakob et al. 2007).

To understand the geographic settings of speciation processes, we used phylogeographic analyses. Phylogeography as a distinct discipline arose in the late 1980s and combines microevolutionary, i.e., population genetics, and macroevolutionary concepts, i.e., phylogenetics and systematics, with the distribution of genetic variation in space and time (Avise et al. 1987; Avise 2000). In contrast to population genetics, which explains allele distribution mainly by gene flow, phylogeography explicitly seeks to determine historical processes that shaped the extant distribution of genetic variation. Thus, the genetic variation within a species is organized into a genealogy and overlaid by the geographical distribution of the alleles of the marker region under study (Avise 1989). The analysis then interprets patterns of congruence or incongruence between the extant geographic distribution of alleles and their genealogical relationships on the background of different recent and historical processes influencing the structuring of genetic diversity within and among populations, i.e. geographic barriers, dispersal events, population size changes, and gene flow. As chloroplast alleles may persist through multiple speciation events, i.e. not reaching reciprocal monophyly for quite a long time, identical alleles might be found in several closely related taxa (Jakob and Blattner 2006). This can result in wrong interpretations of time axis and geographical patterns if only single

species are analyzed. To prevent such errors, we partly analyzed entire species groups together, if they are characterized by shared chloroplast haplotypes (Jakob et al. 2007, 2009).

To illustrate general differences in species evolution in *Hordeum* between Eurasia and the Americas, we here review three studies, dealing with (1) the *H. marinum* species group from the Mediterranean and adjacent regions, (2) the North American relatives of *H. californicum*, and (3) a group of southern Patagonian *Hordeum* species. In all cases, we conducted detailed phylogeographic analyses, partly in conjunction with modeling of climate niches of the species. Finally, we will discuss our current understanding of speciation processes in the genus and its general relevance for evolutionary biology.

3 Results and Discussion

3.1 Phylogeny of Hordeum

Based on several analysis approaches, our current understanding of species relationships within *Hordeum* is given in Fig. 2. In accordance with earlier cytogenetic work and in contrast to some other phylogenetic studies, the four genome groups in *Hordeum* proved monophyletic. The basal split in *Hordeum* was estimated to date back 12 my and separated the **H** and **Xu**-genome species from taxa belonging to the **I** and **Xa**-genome groups. Within the **H**, **Xa**, and **Xu**-genome groups only five extant species occur, while the majority of *Hordeum* species belong to the **I**-genome group (26 species). The latter segregated about 5 mya into two geographically defined lineages, of which one consists of all diploid Asian taxa, the other comprises all American species. Within this latter group, species are mostly not older than 1–2 my. While this separation is quite clear for diploid taxa, several allopolyploids exist combining **I** genomes from Asian and American taxa (e.g., tetraploid *H. brachyantherum*, *H. jubatum*, and *H. guatemalense*, or hexaploid *H. arizonicum* and *H. lechleri*). Allopolyloids involving two different genomes (**I** and **Xa**) are less frequent and restricted to tetraploid *H. secalinum* and its daughter taxon *H. capense*, as well as hexaploid *H. brachyantherum* that originated in historic times in a restricted area of California via hybridization of native tetraploid *H. brachyantherum* with introduced diploid *H. gussoneanum*. No naturally occurring taxa are known which combine the **H** or **Xu** genome or one of these with other Triticeae genomes, while the **I** genome can also be found in allopolyploid genera such as *Elymus* and *Elytrigia* (Dewey 1984, Mason-Gamer 2001).

The phylogenetic hypothesis (Fig. 2) shows that none of the up to now proposed infrageneric classifications of *Hordeum* (e.g., Nevski 1941; von Bothmer and Jacobsen 1985; Petersen and Seberg 2003) is sustained by the discovered monophyletic units, and also the split of the genus in *Hordeum* and *Critesion*, as proposed by Dewey (1984) and Löve (1984), would make *Critesion* paraphyletic. Thus, our

phylogenetic results support the concept of a single genus *Hordeum* (von Bothmer et al. 1995). To arrive at a closer match of phylogenetic relationships and infrageneric taxonomic units, a new classification of the genus was proposed (Blattner 2009) subdividing *Hordeum* in subgenus *Hordeum* with sections *Hordeum* (**H**-genome taxa) and *Trichostachys* (**Xu**-genome taxa), and subgenus *Hordeastrum* consisting of sections *Marina* (**Xa**-genome taxa), *Stenostachys* (**I**-genome taxa) and *Nodosa* (allopolyploids combining **I** and **Xa** genomes).

3.2 Biogeography

The dated phylogeny of the genus (Fig. 2) provides evidence that older lineages within extant *Hordeum* are all restricted to western Eurasia, while Central Asian and American clades are of younger age. The scenario (Fig. 3) for the historical biogeography of the genus (Blattner 2006) assumes, therefore, the origin of *Hordeum* somewhere in western Eurasia, maybe in Southwest Asia. It is not, however, possible to pinpoint the exact area of origin, as climate changes during the last 12 my may have resulted in range shifts of species. This area is in accord with the distribution of most other Triticeae genera, which also mainly occur in central Eurasia. From this ancient area, *Hordeum* migrated in a western direction into the Mediterranean basin and also eastwards into Central Asia. The lineage in this latter area belongs to the **I**-genome taxa and is most probably sister to all New World

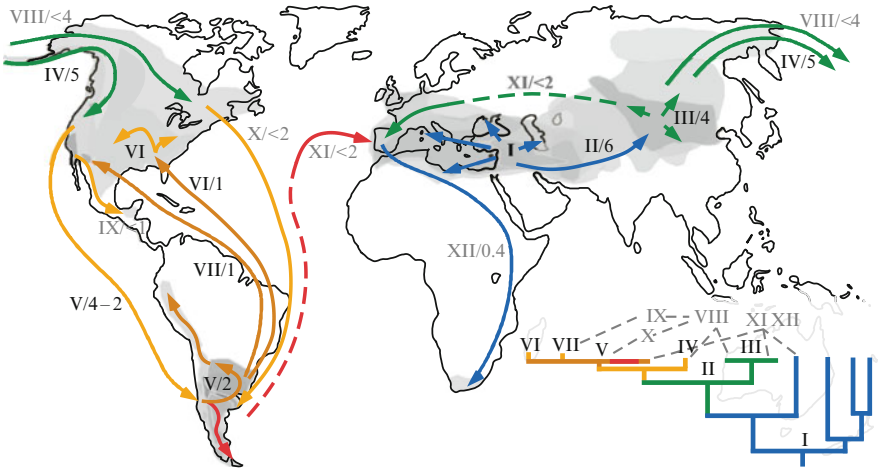


Fig. 3 Historical biogeography scenario of *Hordeum* plotted on the distribution map of extant *Hordeum* species. *Shading* of the areas reflects species numbers in the respective regions. *Black roman numbers* refer to distribution and colonization events in the diploid parts of the phylogenetic tree as shown in the *insert*, while the distribution events on the polyploid level were indicated in *gray roman numbers*. *Numbers* behind the *slashes* give approximate ages of colonization events in millions of years. Figure modified from Blattner (2006)

species of the genus. Therefore, a colonization of the Americas from Asia via Beringia is possible given the northern latitude climate during the relevant time interval about 5 mya was suitable for temperate taxa. From North America *Hordeum* reached South America between 4 and 2 mya, most probably via long-distance dispersal between western North America and today's Chile. Also, other long-distance dispersals were inferred. Thus, North America was colonized two times, independently from South America (resulting in *H. intercedens* and *H. pusillum*) and once again from East Asia (*H. jubatum*), South America one more time from North America (*H. lechleri*), Europe either from South America or Central Asia (*H. secalinum*), and South Africa from Europe (*H. capense*). Most of these dispersal routes coincide with migration routes of birds, which can often be found at ditches in *Hordeum* habitats (Blattner 2006). Thus, we guess that seeds glued to the legs or beaks of migratory birds by wet mud (Darwin 1859) were involved in long-distance colonization in *Hordeum*. Although we infer up to eight intercontinental long-distance dispersals involved in shaping the extant distribution area of *Hordeum*, species within a certain area are more closely related to each other in comparison to species occurring in other regions, apart from the two North American species nested within the South American species group. This means that colonizations of distant areas are still rare events in the genus and do not obliterate the general phylogenetic–geographic correlation.

3.3 Speciation Rates

Phylogenetic and biogeographic analyses of *Hordeum* revealed that the New World species and particularly the species in South America originated quite recently, mostly during the last 2 my. This confirmed our initial hypothesis of a young and rapid radiation on this continent leading to the high extant species number, as opposed to an ancient occurrence of the genus on that continent as assumed by Baum and Johnson (2003). In contrast to South America, we inferred in Eurasia only one speciation event at the diploid level during this time period (*H. gussoneanum*–*H. marinum*). A comparison of average net diversification rates for diploids from both areas resulted accordingly in 0.42 ($\pm$0.11) species^{-my} in the Americas compared to 0.11 ($\pm$0.03) species^{-my} in Eurasia (Jakob and Blattner 2006). This rate is quite high, particularly as it does not even include speciation at the polyploid level, and for the Americas is close to continental peak diversification rates (Baldwin and Sanderson 1998). The calculation of net diversification could not, however, finally answer our questions whether speciation rates are exceptionally high in South America or if speciation is occurring at a normal *Hordeum*-specific pace there, while Eurasia is characterized by high extinction rates resulting in much lower net diversification. To answer this question, we used an approach derived from the distribution of missing alleles in a chloroplast genealogy of the genus.

3.4 *Chloroplast Genealogy of* Hordeum

To identify reasons for inconclusive results of earlier chloroplast phylogenies in the grass genus *Hordeum* and to get insights in population demography, we established a genealogy of chloroplast haplotypes by sequencing the *trn*L-F region in 875 individuals, covering all 33 species of the genus (Jakob and Blattner 2006). Our analysis resulted in 88 chloroplast haplotypes. Their relationships are given in the genealogy shown in Fig. 4. The network reveals a clear geographical structure, with chloroplast lineages distributed mainly in the Old World or the New World all converging at the central haplotype HT1. In the New World, the major division is between North American and South American haplotypes, the latter subdivided into a northern (Chile, central Argentina, Uruguay, and northern Andes) and a southern group (southern Patagonia and Andes) with one chloroplast lineage exclusive to this southern group.

A pronounced difference between Eurasia and the Americas is the occurrence of missing intermediates (38 in the Old World vs 9 in the New World). A second conspicuous difference between these areas concerns the number of haplotypes per species and the number of shared haplotypes among different species. Most species in the Old World possess only a small number of haplotypes, in some cases even just one single haplotype was found. The coalescence of these haplotypes is shallow and they converge comparatively early into their last common ancestor. In the New World, we found up to 18 different chloroplast haplotypes within southern Patagonian species with quite deep coalescences. Furthermore, up to six species share

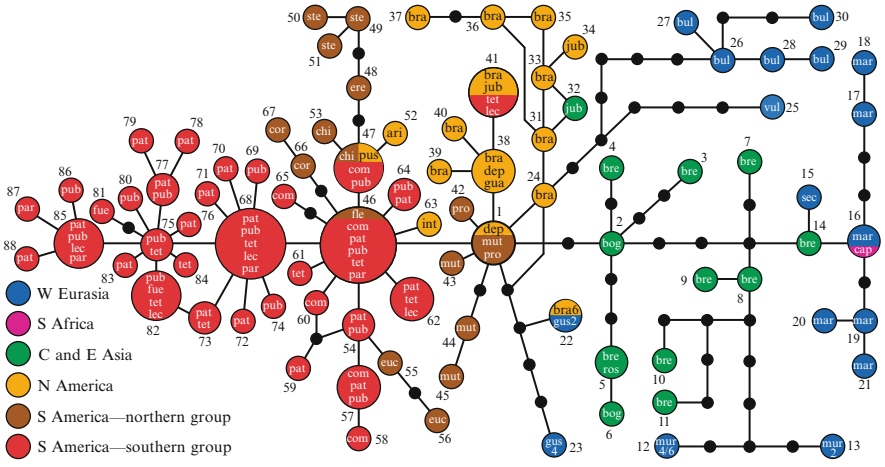


Fig. 4 Chloroplast haplotype genealogy of *Hordeum* based on a statistical parsimony network analysis of sequences of the *trn*L-F region from 875 individuals covering all *Hordeum* species. Species names are abbreviated by the first three characters of the species epithet. The network is characterized by a high number of missing intermediate haplotypes (*black dots*) in the Eurasian part, and many haplotypes shared among up to six species in the New World part. Figure modified from Jakob and Blattner (2006)

single haplotypes. In Eurasia, missing intermediates and mostly species-specific chloroplast types indicate far-reaching loss of genetic diversity, probably caused by repeated genetic bottlenecks for the populations in this area. Also, in the northern South American species group, species-specific chloroplast haplotype lineages indicate genetic bottlenecks, although probably less severe than in Eurasia (Jakob et al. 2010). The patterns found in southern Patagonia are consistent with long-term stable population sizes and large populations involved in speciation events. We explain these differences with quite different Pleistocene histories of South America and Eurasia, and also within southern South America. While east–west stretching mountain ranges and the Mediterranean Sea prevented Eurasian species from migration to southerly refugia, no such barriers were present in the Americas. Thus, species could shift with changing climate conditions to suitable habitats. This resulted in low species extinction rates in the New World, while extinction was quite high in Eurasia.

3.5 *The Eurasian* Hordeum marinum *Species Complex*

The *H. marinum* group consists of two annual grass species of western Eurasian saline meadows or marshes. The two taxa split in the Pleistocene about 2 mya (Blattner 2006). *Hordeum marinum* and the diploid cytotype of *H. gussoneanum* co-occur throughout the Mediterranean basin, while the tetraploid cytotype of *H. gussoneanum* overlaps with its diploid progenitor geographically only in the very far eastern Mediterranean, extending from there eastwards into Asia. Using chloroplast sequences of the *trn*L-F region and six chloroplast microsatellite loci, ecological predictive models based on climate data and the present geographical distribution of the two species, we analyzed differentiation processes in the *H. marinum* group (Jakob et al. 2007).

The chloroplast data indicated clear differences in the history of both species. Interestingly, both species possess highly distinct chloroplast lineages (Fig. 4), with *H. gussoneanum* revealing chloroplast haplotypes, which are otherwise extinct in Eurasia but have survived in the New World (Jakob and Blattner 2006). Phylogeographic and population genetic analyses clearly showed that the two diploid species originated allopatrically within different small and isolated ice-age refugia. For *H. marinum*, we found a subdivision between genetically quite variable populations from the Iberian Peninsula and more uniform populations from the remaining Mediterranean. As an explanation, we assume Pleistocene fragmentation of an earlier widespread population and survival in a southern Iberian and a southern Central Mediterranean refuge, where temperatures were also suitable for the species during Pleistocene cold cycles.

Chloroplast variation was completely absent within the cytotypes of *H. gussoneanum*, indicating a severe and recent genetic bottleneck. Due to this lack of chloroplast variation, only the combination of ecological habitat modeling with molecular data analyses allowed conclusions about the history of this taxon. The distribution areas of the two cytotypes of *H. gussoneanum* overlap today in parts of

Turkey, where the polyploid probably originated. Afterwards, it underwent a pronounced ecological shift, compared to its diploid progenitor, allowing it to colonize mountainous inland habitats between the Mediterranean basin and Afghanistan.

3.6 North American H. californicum *Group*

Analyses of the North American group of diploid *H. californicum* and three tetraploid taxa possessing related chloroplast haplotypes in the *Hordeum* chloroplast genealogy (Jakob and Blattner 2006) show that genetic diversity is high in comparison to *H. marinum*, particularly in the polyploid taxa. *Hordeum californicum* and *H. depressum* are both today mainly restricted to California and possess very similar haplotypes. *Hordeum brachyantherum* and *H. jubatum*, on the other hand, show a wider distribution range and also diverse chloroplast haplotypes. Many haplotypes are shared between these two taxa, even among individuals not occurring in the same area (Pleines and Blattner, unpublished). Reasons for the high haplotype diversity could either be multiple origins of the polyploids, each time introducing different maternal chloroplast types, or extended geneflow among polyploids and older diploid taxa, enriching chloroplast diversity in the polyploids. *Hordeum jubatum* probably survived the last ice-age in a northern refuge in Beringia, as can be deduced from high chloroplast diversity in Alaska. A different pattern was found in *H. brachyantherum* where we assume survival of widespread populations south of the North American ice shields in California and coastal regions of British Columbia and Newfoundland (Pleines and Blattner, unpublished).

In North America, the number of missing haplotypes is as equally low as in South America, indicating no severe genetic restrictions during the ice-age cold cycles. Contrary to the situation in Europe, *Hordeum* species in North America were probably geographically widespread during the Pleistocene. This may be because of fewer east–west oriented mountain ranges hindering migration, which is often used to explain higher extant biodiversity in North America in comparison to Europe (Thingsgaard 2001). This does not, however, hold for the only diploid of this species group, which today occurs in relatively small populations in California. This difference between diploid and polyploid taxa of this group can also indicate a wider ecological amplitude of the latter, resulting in fewer geographic restrictions from changing climate.

3.7 Southern Patagonian Species

In the huge steppe of the Patagonian plains and adjacent Andes of southern South America thrives a group of three sympatrically distributed diploid *Hordeum* species (*H. comosum, H. pubiflorum*, and *H. patagonicum*), which originated during the last 1.3 my from a common progenitor. For this group, we conducted population genetic

and phylogeographic analyses based on sequences of the chloroplast *trn*L-F region from 922 individuals (Jakob et al. 2009). We found a high number of older chloroplast haplotypes shared among these species. Furthermore, the interior haplotypes are geographically widespread whereas young tip haplotypes are mostly species specific and locally restricted (Fig. 4). Almost no missing haplotypes were detected, in great contrast to the Eurasian species. The chloroplast patterns found in Patagonian *Hordeum* species point to speciation through vicariance, where large populations became separated but did not indicate population bottlenecks typical for speciation in peripheral isolates or due to founder events. The combination of many shared haplotypes together with the low number of missing intermediate haplotypes is furthermore compatible with a constantly growing effective population size in all species, resulting in the preservation of nearly all newly arising chloroplast types (Avise 2000) and the maintenance of shared ancient polymorphisms.

Analysis of the distribution of genetic diversity within and among species inferred an origin of *H. comosum* in the central Argentine Andes, while *H. patagonicum* and *H. pubiflorum* originated in southern Patagonia. The extant occurrence of *H. comosum* in southern Patagonia and *H. pubiflorum* northward along the Argentine Andes was caused by reciprocal migration after the origin of the species.

Surprisingly, molecular data provided neither evidence for Pleistocene genetic bottlenecks nor evidence for range shifts towards the north during the last glacial maximum and recolonization of southerly habitats afterwards, as is commonly assumed for lowland species of the southern hemisphere. Rather, our molecular data indicated in situ survival of large populations of *Hordeum* species within their extant distribution ranges even in southernmost Patagonia and Tierra del Fuego. Ecoclimatic niche modeling used to reconstruct the potential Pleistocene distribution of the species about 21,000 years ago during the last glacial maximum shows that climate conditions were sufficient for the species to survive Pleistocene cold cycles in Patagonia itself without significant geographic restrictions. Molecular data together with ecological niche modeling indicate stable geographic distribution areas of the species for at least the Holocene. As the *Hordeum* species are characteristic taxa of different steppe habitats, we speculate that the Patagonian steppe might be an old vegetation unit occurring for up to 4.5 my in southern South America.

4 Conclusions

Earlier phylogenetic analyses in *Hordeum* encountered different and partly contradicting species relationships when analyzing chloroplast and nuclear loci. To explain these differences, we conducted a genealogical analysis of chloroplast alleles in 875 individuals covering all *Hordeum* species. This analysis revealed far-reaching incomplete lineage sorting in the New World taxa of the genus due to chloroplast alleles surviving several speciation events for up to 4 my. These shared alleles together with several distinct alleles occurring within single species makes the

outcome of species relationships almost arbitrary when only one or few individuals per species are included in phylogenetic analysis. The long allele survival times of haploid chloroplasts might imply that the time to reach reciprocal monophyly could even be higher in diploid nuclear alleles, rendering single copy loci also possibly problematic for phylogenetic analyses in *Hordeum*. Furthermore, in phylogeographic analyses, it seems advisable to include not only individuals from the species under study but also a representative sample from closely related taxa. From our experience with closely related species from genera of other plant families (*Allium*, *Crocus*, and *Hypericum*), we predict that the phenomenon of shared chloroplast alleles is not restricted to *Hordeum* but might be found in most other plant groups.

Our data support high speciation rates in the New World and particularly in South America, involving constantly growing effective population sizes in this area. On the other hand, *Hordeum* in Eurasia clearly suffered from far-reaching extinction, erasing populations and most probably also many species (Jakob and Blattner 2009), resulting in low extant species numbers and also relatively low chloroplast diversity. As far as this can be inferred from still existing genetic diversity, speciation mostly took place in small populations in geographically separated Pleistocene refugia. Other processes involved in speciation in *Hordeum* are multiple intercontinental long-distance dispersals, resulting in immediate reproductive isolation between the source and the newly founded population (Pleines and Blattner 2008). Even in southern South America where ecological speciation seemed possible to us, our analyses resulted up to now only in the inference of allopatric speciation through vicariance events. Thus, although many *Hordeum* species occur today in sympatry, sympatric speciation seems very rare in the genus. This supports the view that sympatric speciation might only occur in very exceptional cases, while normally, geographic barriers are involved in speciation events. In the future, we want to understand how these sympatrically occurring and still crossable *Hordeum* species prevent hybridization when occurring in close proximity, and analyze speciation processes in the polyploid taxa.

Acknowledgments We thank M. Arriaga and R. Gomez (Natural History Museum "Bernado Rivadavia, Buenos Aires) for kindly providing help in the organization of field work in Patagonia, P. Cichero (APN, Buenos Aires) for research permits in Argentine national parks, many friends, colleagues and gene banks for plant materials, and the Deutsche Forschungsgemeinschaft for financial support in the frame of SPP 1127.

References

Avise JC (1989) Gene trees and organismal histories: a phylogenetic approach to population biology. Evolution 43:1129–1208

Avise JC (2000) Phylogeography: the history and formation of species. Harvard University Press, Cambridge

Avise JC, Arnold J, Ball RM, Bermingham E, Lamb T, Neigel JE, Reeb CA, Saunders NC (1987) Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Annu Rev Ecol Syst 18:489–522

Baldwin BG, Sanderson MJ (1998) Age and rate diversification of the Hawaiian silversword alliance (Compositae). Proc Natl Acad Sci USA 95:9402–9406

Bänfer G, Moog U, Fiala B, Mohamed M, Weising K, Blattner FR (2006) A chloroplast genealogy of myrmecophytic *Macaranga* species (Euphorbiaceae) in Southeast Asia reveals hybridization, vicariance and long-distance dispersals. Mol Ecol 15:4409–4424

Baum BR, Johnson DA (2003) The South African *Hordeum capense* is more closely related to some American *Hordeum* species than to the European *Hordeum secalinum*: a perspective based on the 5S rDNA units (Triticeae: Poaceae). Can J Bot 81:1–11

Blattner FR (2004) Phylogenetic analysis of *Hordeum* (Poaceae) as inferred by nuclear rDNA ITS sequences. Mol Phylogenet Evol 33:289–299

Blattner FR (2006) Multiple intercontinental dispersals shaped the distribution area of *Hordeum* (Poaceae). New Phytol 169:603–614

Blattner FR (2009) Advances in phylogenetic analysis and new infrageneric classification of *Hordeum* (Poaceae). Breed Sci 59:471–480

von Bothmer R, Jacobsen N (1985) Origin, taxonomy and related species. In: Rasmussen DC (ed) Barley. Monographs in agronomy, vol 26. American Society of Agronomy, Madison, WI, pp 19–56

von Bothmer R, Jacobsen N, Baden C, Jørgensen RB, Linde-Laursen I (1995) An ecogeographical study of the genus *Hordeum*, 2nd edn, Systematic and ecogeographic studies on crop genepools 7. International Plant Genetic Resources Institute, Rome

Coyne JA, Orr HA (2004) Speciation. Sinauer Associates, Sunderland

Darwin C (1859) The origin of species by means of natural selection, 6th edn. John Murray, London

Dewey RD (1984) The genomic system of classification as a guide to intergeneric hybridization with the perennial Triticeae. In: Gustafson JP (ed) Gene manipulation in plant improvement. Plenum, New York, pp 209–279

Doebley J, von Bothmer R, Larson S (1992) Chloroplast DNA variation and the phylogeny of *Hordeum* (Poaceae). Am J Bot 79:576–584

Gaut BS (2002) Evolutionary dynamics of grass genomes. New Phytol 154:15–28

Jakob SS, Meister A, Blattner FR (2004) The considerable genome size variation of *Hordeum* species (Poaceae) is linked to phylogeny, life form, ecology, and speciation rates. Mol Biol Evol 21:860–869

Jakob SS, Blattner FR (2006) A chloroplast genealogy of *Hordeum* (Poaceae): long-term persisting haplotypes, incomplete lineage sorting, regional extinction, and the consequences for phylogenetic inference. Mol Biol Evol 23:1602–1612

Jakob SS, Ihlow A, Blattner FR (2007) Combined ecological niche modelling and molecular phylogeography revealed the evolutionary history of *Hordeum marinum* (Poaceae) – niche differentiation, loss of genetic diversity, and speciation in Mediterranean Quaternary refugia. Mol Ecol 16:1713–1727

Jakob SS, Blattner FR (2010) Two extinct diploid progenitors were involved in allopolyploid formation in the *Hordeum murinum* (Poaceae: Triticeae) taxon complex. Mol Phylogenet Evol 55:650–659

Jakob SS, Martinez-Meyer E, Blattner FR (2009) Phylogeographic analyses and paleodistribution modeling indicates Pleistocene in situ survival of *Hordeum* species (Poaceae) in southern Patagonia without genetic or spatial restriction. Mol Biol Evol 26:907–923

Jakob SS, Heibl C, Rödder D, Blattner FR (2010) Population demography influences climatic niche evolution: evidence from diploid South American *Hordeum* species (Poaceae). Mol Ecol 19:1423–1438

Komatsuda T, Tanno K, Salomon B, Bryngelsson T, von Bothmer R (1999) Phylogeny in the genus *Hordeum* based on nucleotide sequences closely linked to the *vrs1* locus (row number of spikelets). Genome 42:973–981

Linde-Laursen I, von Bothmer R, Jacobsen N (1992) Relationships in the genus *Hordeum*: Giemsa C-banded karyotypes. Hereditas 116:111–116

Löve A (1984) Conspectus of the Triticeae. Feddes Repert 95:425–521
Mason-Gamer RJ (2001) Origin of North American species of *Elymus* (Poaceae: Triticeae) allotetraploids based on granule-bound starch synthase gene sequences. Syst Bot 26:757–768
Nevski SA (1941) Beiträge zur Kenntnis der wild wachsenden Gersten in Zusammenhang mit der Frage über den Ursprung von *Hordeum vulgare* L. und *H. distichon* L. (Versuch einer Monographie der Gattung *Hordeum)*. Trudy Bot Inst Akad Nauk SSSR Ser 1(5):64–255
Nishikawa T, Salomon B, Komatsuda T, von Bothmer R, Kadowaki K (2002) Molecular phylogeny of the genus *Hordeum* using three chloroplast DNA sequences. Genome 45:1157–1166
Petersen G, Seberg O (2003) Phylogenetic analyses of the diploid species of *Hordeum* (Poaceae) and a revised classification of the genus. Syst Bot 28:293–306
Petersen G, Seberg O (2004) On the origin of the tetraploid species *Hordeum capense* and *H. secalinum* (Poaceae). Syst Bot 29:862–873
Pleines T, Blattner FR (2008) Phylogeographic implications of an AFLP phylogeny of the American diploid *Hordeum* species (Poaceae: Triticeae). Taxon 57:875–881
Pleines T, Jakob SS, Blattner FR (2009) Application of non-coding DNA regions in intraspecific analyses. Plant Syst Evol 282:281–294
Posada D, Crandall KA (2001) Intraspecific gene genealogies: trees grafting into networks. Trends Ecol Evol 16:37–45
Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574
Sanderson MJ (2002) Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. Mol Biol Evol 19:101–109
Shaw J, Lickey EB, Schilling EE, Small RL (2007) Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. Am J Bot 94:275–288
Swofford DL (2002) PAUP*. Phylogenetic analysis using parsimony (* and other methods), version 4. Sinauer Associates, Sunderland
Taketa S, Harrison GE, Heslop-Harrison JS (1999) Comparative physical mapping of the 5S and 18S–25S rDNA in nine wild *Hordeum* species and cytotypes. Theor Appl Genet 98:1–9
Taketa S, Ando H, Takeda K, von Bothmer R (2001) Physical location of 5S and 18S–25S rDNA in Asian and American diploid *Hordeum* species with the I genome. Heredity 86:522–530
Thingsgaard K (2001) Population structure and genetic diversity of the amphiatlantic haploid peatmoss *Sphagnum affine* (Sphagnopsida). Heredity 87:485–496
Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Freuters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP, a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414
Wang RRC, von Bothmer R, Dvorak J, Fedak G, Linde-Laursen I, Muramatsu M (1996) Genome symbols in the Triticeae. In: Wang RRC, Jensen KB, Jaussi C (eds) Proceedings of the 2nd international Triticeae symposium. Utah State University, Logan, pp 29–34

Studying Adaptive Radiation at the Molecular Level: A Case Study in the Macaronesian Crassulaceae-Sempervivoideae

Mike Thiv, Korinna Esfeld, and Marcus Koch

Abstract Oceanic islands frequently harbour prominent examples of plant radiations. Here, we investigated the Macaronesian Crassulaceae-Sempervivoideae (MCS) including the genera *Aichryson*, *Monanthes* and *Aeonium*. This species-rich clade displays a large variety in morphological and ecological features, and is, therefore, often considered as adaptive radiation. We demonstrated, however, a distinct increase in the speciation rate only in the crown group of *Aeonium*. Analysing homologues of the selected candidate genes, *APETALA1*, *APETALA3* and *PEPC* (Phosphoenolpyruvate carboxylase gene), revealed that an increase of cladogenesis is paralleled by an increased number of paralogous copies found for all these genes in *Aeonium*. These could be the result of gene duplications or early polyploidisation. The number of nonsynonymous mutations exceeds synonymous substitutions among sequences of MCS, thus indicating accelerated evolutionary rates. This was highest in *APETALA1* followed by *APETALA3* and *PEPC*. We conclude that reproductive isolation but also, to a lesser extent, physiological adaptation and hybridisation were major triggers in the evolution of this group.

M. Thiv (✉)
Staatliches Museum für Naturkunde Stuttgart, Rosenstein 1, 70191 Stuttgart, Germany
e-mail: mike.thiv@smns-bw.de

K. Esfeld
Staatliches Museum für Naturkunde Stuttgart, Rosenstein 1, 70191 Stuttgart, Germany
University of Bern, Altenbergrain 21, 3013 Bern, Switzerland

M. Koch
Heidelberg Institute of Plant Sciences, University of Heidelberg, Im Neuenheimer Feld 345m, 69120 Heidelberg, Germany

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_3,

1 Introduction

1.1 Speciation and Adaptive Radiation

The primary factors which drive evolution are still widely unknown and are of great interest in current biological research. In general, it is accepted that genetic divergence among segregating populations, polyploidy and/or hybridisation may lead to the formation of new species (Futuyma 2009). Putting this into a more specific perspective, several studies have attempted to specify the genes which may be involved in plant speciation (e.g. White and Doebley 1998; Haag and True 2001; Barrier et al. 2003). It has been conjectured that changes in regulatory genes, rather than accumulative diversification in structural genes, are responsible for the interspecific morphological variation (King and Wilson 1975). Molecular developmental studies support this theory by indicating the large impact of mutations in key regulatory genes (e.g. Purugganan 1998). For example, inflorescence transcription factors have been shown to be responsible for many morphological differences between maize and teosinte (White and Doebley 1998; Haag and True 2001). Also, a comparison between genes expressed in flower buds of *Arabidopsis thaliana* and *A. lyrata* indicated that regulatory genes show increased evolutionary rates (Barrier et al. 2003).

Highly diversified clades of organisms are typically referred to as adaptive radiations, defined as the occurrence of a burst of speciation and rapid phenotypic evolution under conditions of high ecological opportunity (sensu Schluter 1998). These types of radiating species are mostly found on islands. Several of these island groups have been studied intensively (e.g. Givnish 1998; Schluter 2000; Hawaiian archipelago: Wagner and Funk 1995; Baldwin 1997; Jordan et al. 2003; Canary Islands: Kim et al. 2008; cf. Reyes-Betancort et al. 2008). However, these studies did not distinghuish genes of regulatory function from those which are neutrally evolving, and therefore merely used as markers for phylogenetic reconstructions. In contrast, another study, dealing with nuclear coding genes, showed an acceleration in the mutation rate of regulatory genes compared to a photosynthetic structural gene in the adaptively radiated Hawaiian silverswords (Asteraceae; Barrier et al. 2001). Our study is focusing on a classic example of an adaptive radiation, the Macaronesian Crassulaceae-Sempervivoideae (MCS), and we aim to explain speciation processes in the light of the evolution of nuclear genes.

1.2 Island Radiations and Macaronesian Crassulaceae-Sempervivoideae

Oceanic island organisms may reflect an evolutionary scenario different from their continental counterparts. The Canary Islands are known for spectacular examples of

species radiations in various groups of flowering plants. Along with *Echium* (Boraginaceae; Böhle et al. 1996), *Argyranthemum* (Asteraceae; Francisco-Ortega et al. 1996, 1997), *Sonchus* (Asteraceae; Kim et al. 1996), the MCS are a classic example of adaptive radiation (Lems 1960; Voggenreiter 1974; Kull 1982; Lösch 1990). This group comprises the genera *Aeonium* (including *Greenovia*; ±44 spp.), *Monanthes* (±13 spp.) and *Aichryson* (±13 spp.; Liu 1989; Mort et al. 2002; Fairfield et al. 2004). Their taxonomy is relatively well understood – except for some taxa, e.g. *Monanthes* spp. (Lems 1960; Bramwell 1968; Liu 1989; Nyffeler 1995; Hohenester and Welss 1993) and the phylogeny has been investigated (Mes 1995; Mes and 't Hart 1996; Mes et al. 1997; Jorgensen and Frydenberg 1999; Mort et al. 2002; Fairfield et al. 2004). Accordingly, sister to the island group is *Sedum* sect. *Monanthoidea* consisting exclusively of *S. jaccardianum*, *S. surculosum* and *S. modestum* (Fig. 1) which occur on the North African mainland (Uhl 1961; Mes 1995).

Compared with more than 70 species of the MCS, the low number of taxa in sect. *Monanthoidea* – assuming equal extinction rates – suggests that this clade did not undergo a large radiation. Phylogenetic reconstructions based on the nuclear, ribosomal ITS region and several chloroplast markers (Mes 1995; Mort et al. 2002; own data) together with the high morphological and ecological variation were interpreted as further evidence for an adaptive radiation within the MCS relative to their closest relatives, *Sedum* sect. *Monanthoidea*. Although the exact interspecific relationships within *Aeonium* often remain unresolved or weakly supported, the general picture is displayed by genetic nuclear and plastid data. Accordingly, *Aichryson* is the most basal genus in the MCS and sister to *Aeonium* and the perennial *Monanthes* species (Mes 1995; Mort et al. 2002). The position of the annual *Monanthes icterica*, however, remained unclear – showing closest affinities either to *Monanthes* or *Aichryson* (Mes et al. 1997; Nyffeler 1995; Mort et al. 2002).

The MCS have their centre of diversity and distribution on the Canary Islands, however, a few taxa are also found on the Cape Verde Islands (1 sp.), Madeira (2 spp.), in Morocco (1 sp.), or E Africa/Arabia (2 spp.; Liu 1989; Hohenester and Welss 1993; Mes 1995; Jorgensen and Frydenberg 1999). These occurrences are regarded as the result of secondary, recent long-distance dispersal events (Mes et al. 1996; Mort et al. 2002). Species of *Aeonium*, *Monanthes* and *Aichryson* are usually restricted to special habitat requirements (Hohenester and Welss 1993; Jorgensen and Frydenberg 1999). According to Hohenester and Welss (1993), they mainly occur in "Macaronesian lava and rock plant communities" from sea level to 1,700 m altitude. These habitats are found throughout a large range of vegetation types (e.g. laurel and pine forests) and are distinguished by different rainfall, temperature, light and edaphic regimes (Liu 1989; Lösch 1990). Furthermore, it has been shown that the Macaronesian Sempervivoideae are specifically physiologically adapted to these habitats (Lösch and Kappen 1981; Lösch 1990; Pilon-Smits et al. 1992; Mort et al. 2007). At the same time. the Macaronesian Sempervivoideae show many variations in flower (e.g. organ merosity, petal length, colour), inflorescence (e.g. branching, shape) and growth form morphology (e.g. life history traits,

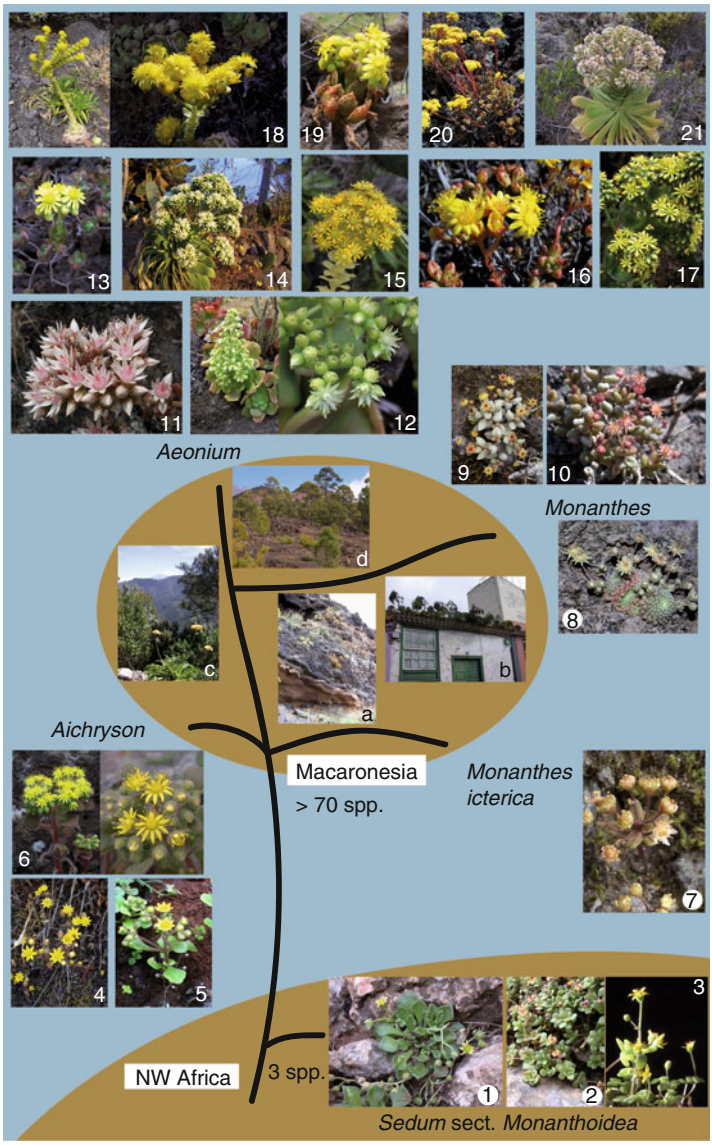


Fig. 1 The simplified phylogenetic relationships of Macaronesian Crassulaceae-Sempervivoideae (MCS), sister group to *Sedum* sect. *Monanthoidea* (1.–3.), derived from chloroplast and nrITS data (see Fig. 2, cf. Mort et al 2002) displaying the morphological and ecological diversity of representative members. (1) *Sedum jaccardianum**, (2) *S. surculosum**, (3) *S. modestum**, (4) *Aichryson parlatorei*, (5) *Ai. punctatum*, (6) *Ai. laxum**, (7) *Monanthes icterica**, (8) *M. silensis*, (9) *M. laxiflora*, (10) *M. anagensis**, (11) *Aeonium volkeri*, (12) *A. canariense**, (13) *A. saundersii**, (14) *A. urbicum*, (15) *A. holochrysum*, (16) *A. sedifolium*, (17) *A. cuneatum**, (18) *A. aureum**, (19) *A. smithii**, (20) *A. spathulatum*, (21) *A. pseudourbicum*. In all figures: *A.* = *Aeonium*, *M.* = *Monanthes* and *Ai.* = *Aichryson*. Candidate genes were sequenced for taxa with an asterisk. Habitats of MCS: (a) rocks at sea level with *A. sedifolium*, (b) roofs with *A. urbicum*, (c) laurel forests, (d) *Pinus canariensis* forest at higher altitude. All photos M. Thiv except 3. R. Harling SMNS

habits; Liu 1989; Hohenester and Welss 1993; Fig. 1). This diversity has been the subject of several considerations on the origin, correlations to ecology and evolution of these traits (Lems 1960; Pilon-Smits et al. 1992; Mes and 't Hart 1996; Jorgensen and Frydenberg 1999; Jorgensen and Olesen 2000; Jorgensen and Olesen 2001; Mort et al. 2002, 2007). Based on this evidence, up to the present, the MCS have widely been regarded as an example of an adaptive radiation (Lems 1960; Voggenreiter 1974; Mes 1995).

1.3 Candidate Genes

Neutrally evolving DNA segments, like chloroplast DNA, are often not able to trace the evolution of rapid (adaptive) radiations (e.g. reviewed by Baldwin et al. 1998). In contrast, it may be more promising to analyse genes which are assumed to be involved in a particular trait in which the species differ from each other and which could have undergone a drastic change. The big challenge is to detect those genes among the large number of genes in a plant, e.g. >25,000 genes in *Arabidopsis* (Arabidopsis Genome Initiative 2000). If genomic data are not available for a special taxon, one may deduce the function of a gene from other, closely related organisms. In our project, we analyse three nuclear genes which we suspect to be involved in the characteristics of the phenotype among MCS. Within the adaptive radiation, a high variation of floral/inflorescence morphology is found. Moreover, the colonisation of a vast range of habitats characterised by different moisture regimes is linked to different CAM activities of MCS (see Fig. 1). Therefore, homologues of genes affecting flower and inflorescence morphology (*APETALA1* = *AP1*, *APETALA3* = *AP3*) were selected, and a gene encoding for Phosphoenolpyruvate carboxylase (PEPC) which is important to the plant's water system (see Sect. 2.1).

In this study, we present an analysis on the evolution of these candidate genes and we discuss possible factors which were driving the evolution of the MCS. We address the following questions. (1) Are the MCS an adaptive radiation (sensu Schluter 1998)?. (2) Can different functional or possible orthologous and paralogous copies be detected in these genes? (3) Do candidate genes reflect the phylogenetic relationships as inferred from widely used genetic markers (nrITS; Figs. 1 and 2)? (4) What is the relative impact of these nuclear coding genes in terms of selection regimes? And (5) what might have triggered the evolution of the MCS?

2 Material and Methods

The major points of methodology are summarised here. For a detailed description, refer to Esfeld (2009) and Thiv et al. (in preparation).

2.1 Selection of the Study Group

2.1.1 nrITS

All available sequences from Mort et al. (2002) replenished by new data were used for a phylogenetic analysis using Bayesian inference (Fig. 2). Based on the resulting consensus tree, an ultrametric tree was produced using r8s (Sanderson 2004). Based on this tree, a logarithmic lineage through time (LTT) plot (Fig. 2) was created manually.

2.1.2 Candidate Genes

A total of 17 taxa were included in this study. The selection represents 12 species of the MCS (8 species of *Aeonium*, 2 of *Aichryson* and 2 of *Monanthes*), the three sister group species of *Sedum* sect. *Monanthoidea* and two outgroup taxa of *Sedum*. The species sampled represent all major clades of the MCS according to Mort et al. (2002) and, at the same time display the morphological and ecological variation of this group (Fig. 1). Voucher and sequence data are given in Esfeld (2009) and (Thiv et al., in preparation).

2.2 Selection of Candidate Genes

2.2.1 Regulatory Genes

It has been hypothesised that regulatory genes coding for transcriptional factors are strongly involved in the diversification of plants. Homeotic MADS box genes belong to this group. In the general ABCDE model (Theißen 2001; Erbar 2007), each class controls the identity of certain floral organs in eudicots. A-class genes affect the sepals and petals, B-class genes the petals and stamens, C-class genes the stamens, carpels and ovules, D-class genes only ovules, and E-class genes impact all flower organs.

APETALA1 (*AP1*) is an A-class homeotic MADS box gene. For *Arabidopsis*, it could be shown that *AP1* also has an impact on the number of inflorescence branches and the flowering time (Mandel and Yanofsky 1995).

APETALA3 (*AP3*) belongs to the B-class floral homeiotic MADS box genes which define the organ identity and position of petals and stamens (Weigel 1998). In *Arabidopsis*, a QTL site for petal and stamen size was hypothesised to be localised in a region which includes *AP3* (Juenger et al. 2000).

2.2.2 Structural Gene

PEP carboxylase is widely expressed in most plant tissues. PEPC catalyses the fixation of CO_2 to yield oxaloacetate. It plays a key role in the primary fixation of CO_2 in xerophytic CAM and C_4 plants and has an anaplerotic function in many cells

(Chollet et al. 1996). It could also be shown that PEPC is expressed at a higher level in water-stress situations of *Kalanchoe blossfeldiana*, a close relative of MCS (Gehrig et al. 1995). More evidence for a link between PEPC and ecological adaptation is provided by Lösch and Kappen (1981), Lösch (1990), Pilon-Smits et al. (1992) and Mort et al. (2007), who found divergent CAM activities for various species of MCS.

2.3 Laboratory Work

Fresh floral tissue from early to mature stages of selected MCS was collected and used for RNA extraction and cDNA amplification. PCRs using different degenerated *PEPC*, *AP1*, *AP3* primers were conducted (Kramer et al. 1998; Barrier et al. 2003; Litt and Irish 2003; Gehrig et al. 1995; Esfeld 2009; Thiv et al., in preparation). Based on these sequences, specific primers were created which yielded amplification based on genomic DNA, followed by cloning and sequencing of at least five copies per clone (Mort and Crawford 2004). Exons and introns were determined by comparison with cDNA fragments. Only unique exon regions without any stop codons were considered for analyses. For more details, see Esfeld (2009).

We aimed at completing sequencing of all three candidate genes for our taxon selection. Despite several attempts, some accessions have not been successful, e.g. *Aichryson* could not be sequenced for *AP1*.

2.4 Data Analysis

2.4.1 Phylogenetic Analyses

Bayesian inference of phylogeny using all datasets was explored using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2006). Models were selected by AIC in Modeltest 3.7. (Posada and Crandall 1998). Detailed descriptions of options used for analyses are given in Esfeld (2009).

Ka/Ks Values

In order to test the selection regime at the molecular level, rates of nonsynonymous and synonymous substitutions are compared (Nei and Kumar 2000). While nonsynonymous mutations yield a change in amino acids, a synonymous mutation at the exchangeable third position in a codon leaves the amino acid unaltered. The relative rate of nonsynonymous and synonymous substitution is calculated as the number of synonymous substitutions per synonymous site (Ks) and the number of nonsynonymous substitutions per nonsynonymous site (Ka) for a pair of sequences (Nei and Gojobori 1986; Nei and Kumar 2000; Hurst 2002). Thus, the quotient

Ka/Ks per site, also called ω value, is an indicator for the selection regime at the molecular level. In the case of neutral evolution, an equated value of Ka/Ks ∼1 is expected, purifying selection would yield a Ka/Ks value <1 and positive selection (diversifying selection) would result in Ka/Ks >1 (Nei and Kumar 2000). Ka/Ks values of the above-mentioned genes were calculated according to the Nei and Gojobori method (1986) with Jukes-Cantor correction and complete deletion of gaps implemented in MEGA version 4 (Tamura et al. 2007). Standard errors were obtained by a bootstrap procedure (1,000 replicates). We calculated Ka/Ks values for the clusters we found in all three candidate genes. The mean values and standard deviations for the corresponding sequences are given in Figs. 3–5. Comparison of significant differences of Ka/Ks values were done using a *t* test.

2.4.2 Dating

Molecular Dating

To achieve a rough estimate when cladogenesis events in MCS happened, molecular divergence of nrITS was used. Clock-like evolution was tested using a likelihood ratio (LR) test (Felsenstein 1981; Sanderson 1998; Nei and Kumar 2000). Like several other recent studies (Richardson et al. 2001; Bossuyt and Milinkovitch 2001; Cooper et al. 2001), a geological calibration date was used for molecular dating estimates. Except for terminal taxa which are likely the result of recent dispersal, the complex biogeographic structure of MCS did not allow us to attribute a single island to an appropriate endemic clade. Instead, we fixed the age of Fuerteventura to 20.7 mya (Carracedo 1994) as the oldest Canarian island, and as the upper bound for the possible age of the stem node of the island radiation. Although we are aware that this single calibration point is not precise and, moreover, the taxon's real age may be underestimated under specific conditions (Heads 2005), the age estimates of many other Canarian plants (Böhle et al. 1996; Kim et al. 1996) are much younger than the geological age of the archipelago and are in accordance with our results. We applied the Bayesian dating method (Thorne et al. 1998; Thorne and Kishino 2002) following Rutschmann (2004) and Thiv et al. (2006) using baseml (PAML, Yang 1997), estbranches (Thorne et al. 1998) and multidivtime (Kishino et al. 2001; Thorne and Kishino 2002).

3 Results and Discussion

3.1 Are the MCS the Result of an Adaptive Radiation?

Defining "adaptive radiations" raises several problems. First, it is difficult to quantify species-rich clades versus species-poor sister groups. Secondly, the measurement and the evaluation of morphological and ecological diversity

require the collection of large amounts of data. These points make it unfeasible to test this phenomenon (Schluter 1998). At the same time, the degree of "adaptation" of certain features is difficult to test, as previously illustrated in the MCS (Jorgensen and Frydenberg 1999; Jorgensen and Olesen 2001). When comparing the phenotypic variation between MCS and their continental sister from the genus *Sedum* one finds a high diversification in the island group (Fig. 1). Firstly, concerning vegetative characters such as habits and life forms, the insular taxa comprise annual (*Aichryson*, *Monanthes icterica*), perennial woody (*Aeonium*, *Aichryson tortuosum*, *Ai. bethencourtianum*) and perennial or short-lived monocarpic herbaceous (*Monanthes*, *Aeonium*) plants with different levels of branching systems (e.g. Liu 1989; Jorgensen and Olesen 2000; Mort et al. 2002; Fairfield et al. 2004). Secondly, this plasticity is found in floral characters affecting reproductive traits. Their flowers are choripetalous and actinomorphic showing typical Crassulaceae features. They mainly vary in flower merosity (5- to polymerous), size, colour and nectariferous glands born at the base of the carpels. This large floral variety, however, is also met in *Sedum* sect. *Monanthoidea*. Although comprising only three species, their floral morphology varies from 6- to 10-merous, yellow, *Aichryson*-like flowers (*S. modestum, S. jaccardianum*) to *Monanthes*-like flowers with nectaries in *S. surculosum* (Maire 1976), which is one reason why Nyffeler (1995) placed this taxon in *Monanthes*. *Sedum modestum* is an annual plant while *S. jaccardianum* and *S. surculosum* are perennial herbs (Maire 1976). The differences in ecological space are somewhat difficult to evaluate. As described, habitats of MCS range on the Canary Islands from sea level to pine forest vegetation. *Monanthoidea*'s members also display a considerable ecological width by growing in rock vegetations in north-western Africa ranging from montane (limestone: *S. modestum, S. jaccardianum*) to high alpine altitudes (silicate: *S. surculosum*). We can summarise that there are differences in the morphological variation and ecological space between MCS and their continental sister, but there are clearly still many observations to be made.

Simply comparing the species numbers of MCS and their sister may not suffice to identify them as an example of an adaptively radiating group of species. To define the gradient of specification, we used a lineage through time plot (LTT) based on the phylogeny of MCS including all available nrITS sequences. Not surprisingly, the phylogenetic relationships are well in accordance with former investigations (Mes et al. 1996; Jorgensen and Frydenberg, 1999; Mort et al. 2002). However, some new aspects emerged. In addition to these studies, *Aeonium cuneatum* and *A. smithii* have been included. The latter is clearly part of "clade 2" in Mort et al. (2002); possibly at the base of this clade of mostly yellow-flowering taxa. In our analysis, the relationship of *Aeonium cuneatum* within the genus is not resolved, but based on morphological evidence (Liu 1989), it is likely part of Section Patinaria (Liu 1989) or "clade 1" (Mort et al. 2002) together with well-known species like *A. canariense* or *A. tabuliforme*. Our taxon selection represents a fairly complete species sampling (Fig. 2; 81% of the MCS group). Several excluded species like *Aeonium sedifolium* or *Monanthes* species or possible hybrids of *Aeonium* (cf. Bañares 1986, 1990) are unlikely to be of early origin and therefore

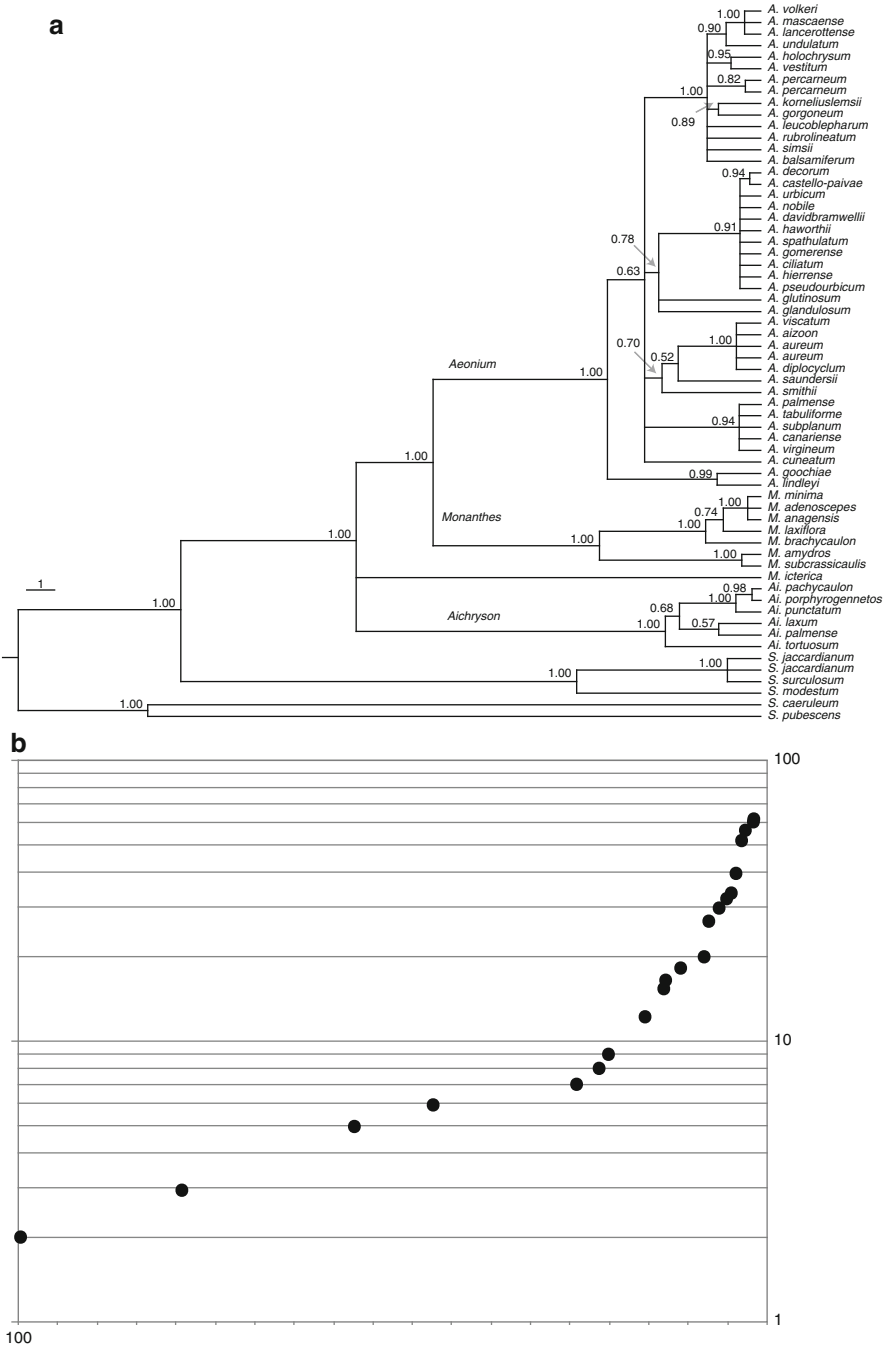


Fig. 2 (**a**) Ultrametric Bayesian consensus tree nrITS sequences of MCS produced using r8s. (**b**) Lineage/relative time plot based on these data. For dating estimates, see sect. 3.3

do not hamper our conclusions. In contrast, assuming a young age, the increase in speciation rate would be rather underestimated. By applying a maximum time frame of 20.7 mya, the age of the oldest Canarian island, Fuerteventura, MCS possibly evolved around 1.50 mya (SD = 1.76; confidence interval: 0.05–6.47). These estimates are younger than those by Kim et al. (2008) who suggested a Miocene origin of the group. This divergence could be due to the use of different methods, e.g. the use of a combined nrITS and cpDNA dataset and different calibration points by Kim et al. (2008). Irrespective of a precise age of MCS between the Lower Pleistocene and Miocene, it is striking, however, to notice a clear increase of diversification at the crown of *Aeonium* (Fig. 2), a node which is dated to 1.05 mya (SD = 1.26; confidence interval: 0.04–4.58) in our study. Of course, a higher extinction rate in the early phase of island colonisation seems possible. Still, one would expect some survivors at the base of MCS, perhaps *Monanthes icterica*, for example. Assuming equal extinction rates throughout time, this would indicate a constant speciation rate independent from the island colonisation event – according to our dating from 1.50 to 1.05 mya. Thus, the burst of speciation obviously happened at a later stage (ca. 0.5 my later) with the diversification of *Aeonium* (cf. Kim et al. 2008).

From these discussed points, we conclude that the MCS likely cannot be regarded as the outcome of an adaptive radiation in the strict sense of Schluter (1998), but basically form a younger radiation of *Aeonium* within the entire island lineage (cf. Jorgensen and Frydenberg 1999). This leaves the main question as to why such an increase in lineage diversification is found in *Aeonium*. Such a trigger of evolution may be caused by inner or outer factors, or a combination of both. In the following, we focus on the evolution of nuclear genes to contribute to solutions of this hypothesis.

3.2 Gene Evolution

3.2.1 Orthologous/Paralogous Copies

Orthologous gene copies are the result of a speciation event. In contrast, gene duplications within a species lead to paralogous copies. Gene duplications may be a primary source of raw material for the origin of evolutionary novelties like new gene function and expression patterns (Lynch and Conery 2000). Since duplicated genes often gain new, different functions, ideally these functions of genes are studied using an experimental approach which shows the impact of this gene on the phenotype (Theißen 2002).

In all three of our selected candidate genes, different gene copies were found. For the interpretation of gene copies, we used the Bayesian consensus trees and compared them with the nrITS trees as independently inferred phylogenies, and ran blast searches of the sequences (Genbank NCBI) for the possible functions of these copies. In total, we attempted to sequence at least five copies per accession (Mort

and Crawford 2004). We here, however, show the analyses of modified, reduced datasets with a single representative of each gene copy cluster of the studied species. These analyses may show slightly different topologies as those including all data (cf. Esfeld 2009). There is a relatively high chance that most of the copies of a species were targeted in this approach. Still, it seems possible that occasionally some small clades of paralogous copies of, e.g. *AP1*, may represent undersampled copies of a larger multigene family. For the time being, until other evidence is available, we interpret our gene clusters as belonging exclusively to certain taxa. The presented data include copies of *Monanthes* and *Aichryson*, which were only found in some clades.

PEPC: The tree reconstruction (Fig. 3) shows several clades of *PEPC* sequences indicating that there are several copies present in the Crassulaceae. At the base, a long branch group of *Sedum pubescens* (243, 1c43) and *S. surculosum* (34341) is found. Other copies of the same species (*S. pubescens* 343, *S. surculosum* 2c4391) connect to a core group of *Sedum Monanthoidea* and MCS. The pattern is relatively complex when several copies of *Aeonium* intermingle with sequences of *Monanthes* and *Aichryson*. We recognised at least three major copies within the island radiation as inferred from *A. nobile* indicated as A, B and C in Fig. 3, whereas the copies marked as A and B may possibly be interpreted each as a single orthologous cluster as indicated in an analysis which included all copies (Thiv et al., in preparation). Nonetheless, the higher level "phylogenetic" relationships inferred from *PEPC* contradict the nrITS patterns as our *PEPC* data suggest closest relationships between *Monanthes* and *Aichryson* in both clades A and C. This is another example where gene trees likely do not reflect phylogeny (Fortune et al. 2007). Within group A, *Aichryson pachycaulon* and *Monanthes anagensis* cluster together, and interestingly both grow in laurel forests also sharing an ecological character. We so far exclude a functional convergence because at least *A. pachycaulon* shares a copy of the C copies. The interspecific relationships of *Aeonium*, especially of clade C, as inferred from *PEPC*, do not coincide with the nrITS phylogeny. In contrast to the present knowledge, all yellow-flowered taxa (*A. saundersii*, *A. smithii*, *A. aureum*) can be found in a single group. Elsewhere in the tree, *Aeonium cuneatum* and *A. canariense* fall into the same clade, though with *Aeonium rubrolineatum* which has so far always been excluded from such grouping (Liu 1989; Mort et al. 2002).

The data indicate that the clusters are different gene copies rather than reflecting the strict phylogeny. Parts of the complex pattern found for *PEPC* can be explained by various functions of the different copies. According to BLAST search results, the basal clades of *Sedum surculosum* and *S. pubescens* most likely execute the function of PEPC isoform 4, as in *Lupinus*. *Sedum surculosum* 2c4391 and *S. caeruleum* were closest to *PEPC* isoform 2 of *Kalanchoe*. Otherwise, we likely sequenced *PEPC* isoform 1 for the large remainder of all MCS and *Sedum* Monanthoidea. Assuming that there are three major copies of *PEPC* isoform 1 within the MCS, we are left with the question of how they evolved. Because clades A and C include sequences of all three genera of MCS, they likely represent copies which evolved at an early stage in the MCS after the separation from *Sedum* sect. *Monanthoidea*. They could have been lost in *Monanthes* and *Aichryson* or

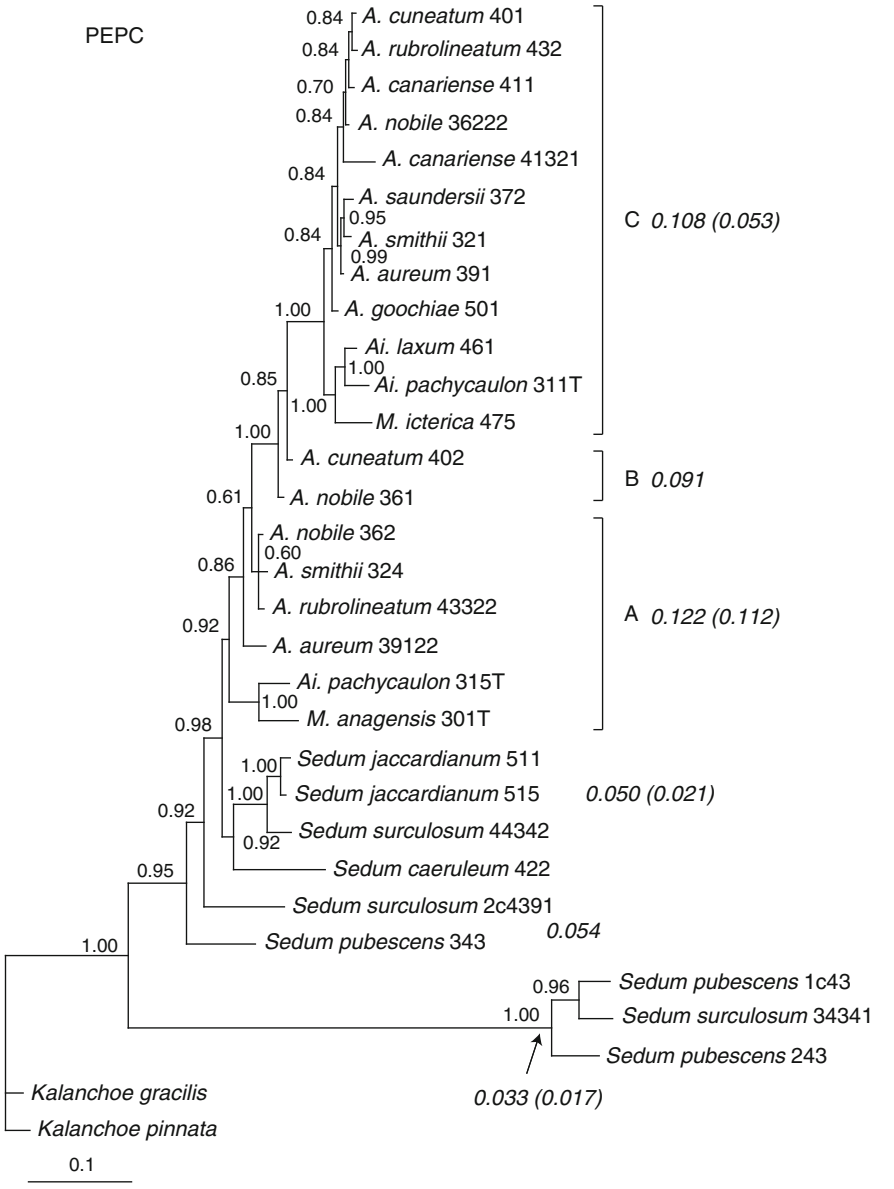


Fig. 3 Bayesian consensus tree of PEPC gene exon sequences of MCS. Posterior probabilities are given at the nodes. *Capitals* mark possible clusters of gene copies. Groups A and B were defined based on clusters of a larger analysis which yielded slightly different topologies (not shown). *Numbers in italics* indicate mean Ka/Ks values of the corresponding clades and the *numbers in parentheses* their standard deviation

exclusively gained in *Aeonium*. For the time being, we prefer the latter alternative since it seems more parsimonious that it was gained once, rather than lost twice.

AP3: In the Bayesian tree (Fig. 4), the mainland group, *Sedum* sect. *Monanthoidea*, is represented by an orthologous cluster with *S. jaccardianum* being nested in *S. surculosum*. For this lineage, BLAST searches indicated highest similarities to *AP3*-like genes of *Kalanchoe*.

The island group consists of two well-supported major clades (Fig. 4). A possible *DEFICIENS* (*DEF*)-like lineage (clade A in Fig. 4) belonging exclusively to *Aeonium* is sister clade to *AP3*-like sequences of *Aeonium*, *Monanthes* and *Aichryson* (clade B in Fig. 4). Whereas the relationships in clade A among *Aeonium* species appear difficult to interpret, the pattern of clade B is largely consistent with the nrITS phylogeny, where *Aichryson* is basal to *Monanthes* and *Aeonium*. In clade B, *Aichryson* and *Monanthes icterica* are found in a basal position, followed by *Monanthes anagensis* and *Aeonium*. As in the *PEPC* tree, *Aeonium saundersii*, *A. smithii*, and *A. aureum* group together. Close relationships between *Aeonium canariense* and *A. cuneatum* are also revealed, additionally in clade A, supporting the already mentioned morphological data. The particular position of *Monanthes icterica,* here sister to *Aichryson pachycaulon,* is also seen. In clade B, each species possesses an orthologous copy. As in *PEPC*, a *DEF*-like gene cluster exclusively attributed to *Aeonium* was found. Again, these genes could have evolved in *Aeonium* or were lost in *Monanthes* and *Aichryson*. Under the parsimony rule, it is conceivable that a gene duplication gave rise to *DEF*-like genes in *Aeonium*.

AP1: The most complex pattern was found for *AP1* (Fig. 5). When rooting the tree with *Sedum caeruleum*, a cluster of *Sedum Monanthoidea* and several clades of paralogous copies of MCS were detected. We define the first group A, consisting of *Aeonium goochiae* 23637 and *A. aureum* 1b3637, due to the fact that both cluster together as the most basal clade in a larger analysis (not shown). The same overall topology was found if *AP1* sequences of other rosids (e.g. Citrus) were added (not shown). The second clade B consists of only *A. smithii* 23637 and *A. saundersii* 33637 being close to *Sedum* sect. *Monanthoidea*. The third group, clade C, also comprises exclusively sequences of *Aeonium*. In clade D, sequences of *Monanthes* and *Aeonium* can be found. The patterns within these groups do not allow phylogenetic conclusions due to lack of resolution or it is also possible that there are few genes which are clustering together. Like that illustrated for *AP3* and *PEPC*, we are forced to interpret clades A-C as novelties for *Aeonium*.

General Pattern

In all candidate genes, increased numbers of copies were found exclusively for *Aeonium*. As described, it is more likely that single gains of these copies in *Aeonium* are present, rather than the exact same copies had independently disappeared in both

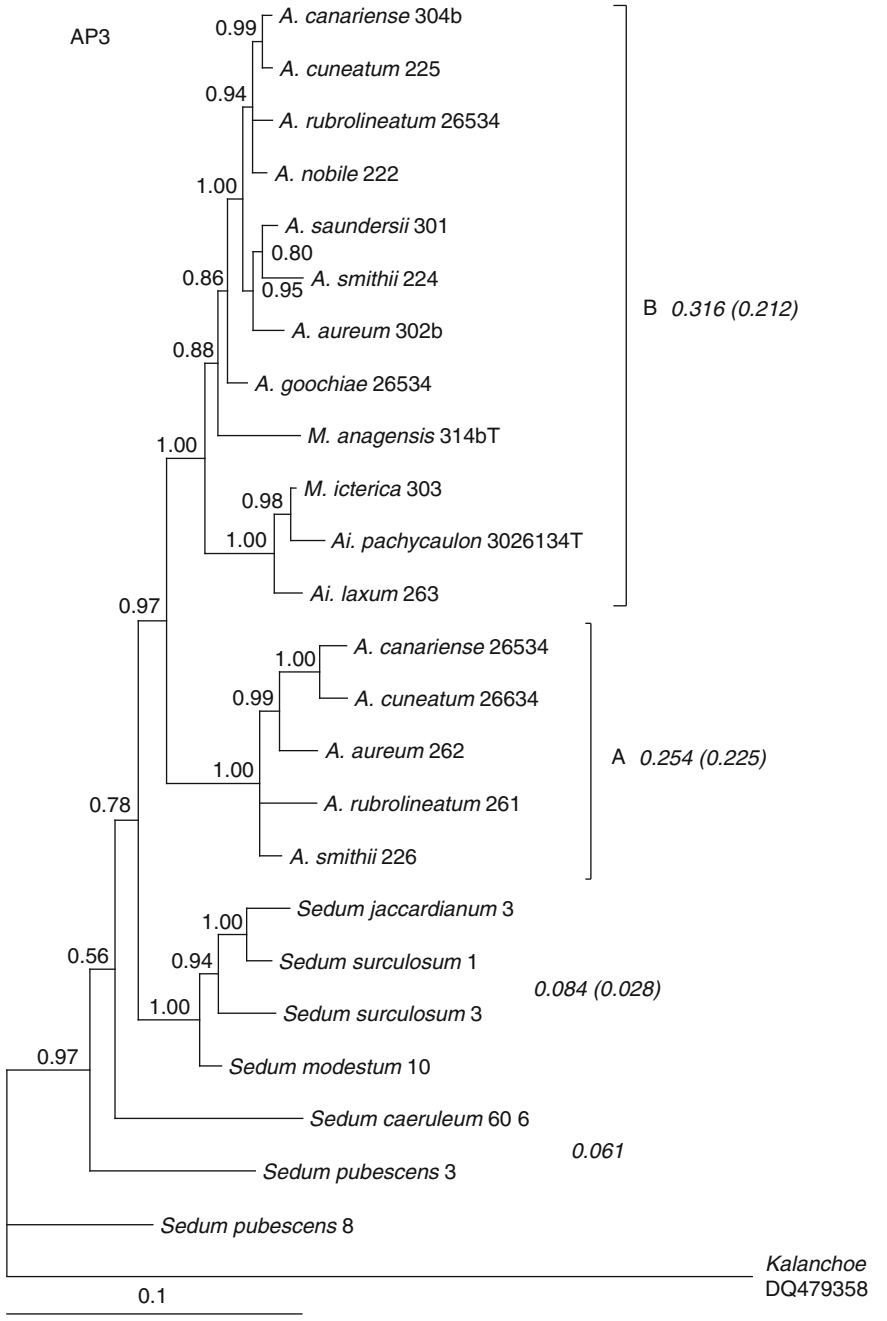


Fig. 4 Bayesian consensus tree of *AP3*-like exon sequences of MCS. Posterior probabilities are given at the nodes. *Capitals* mark possible clusters of gene copies. Number behind *Kalanchoe* is its EMBL no. *Numbers in italics* indicate mean Ka/Ks values of the corresponding clades and the *numbers in parentheses* their standard deviation

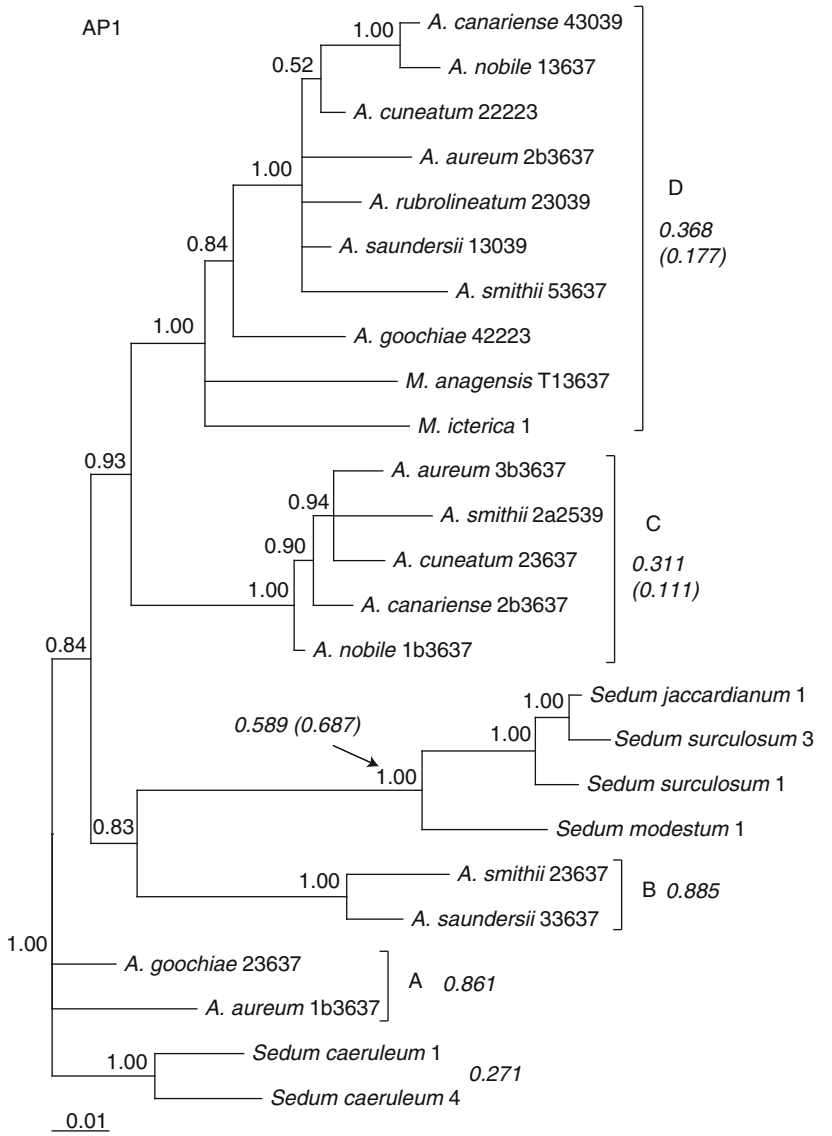


Fig. 5 Bayesian consensus tree of *AP1*-like exon sequences of MCS. Posterior probabilities are given at the nodes. *Capitals* mark possible clusters of gene copies. Group A was defined based on a larger analysis (not shown). *Numbers in italics* indicate mean Ka/Ks values of the corresponding clades and the *numbers in parentheses* their standard deviation

Aichryson and *Monanthes* at separate times, as indicated from the nrITS evidence. The general ability of creating several gene copies in our study group is already indicated by the fact that none of our candidate genes is a strict single copy gene. As

we accept that multiple gene duplications across three nuclear genes happened in *Aeonium*, which is, however, not mirrored in the exact phylogenetic order of our trees, it leaves us with the question on how this occurred. Gene duplications may be the result of anomalous homologous recombinations, retrotranspositions, or duplications of an entire chromosome or genome (Zhang 2003). Currently, there is no indication that PEPC gene, *AP3* and *AP1* are located on the same chromosome. In this case, gene duplications could have been caused by a single chromosome duplication event. Still, there are several arguments against such a hypothesis. Polyploidy is found across *Aichryson*, *Monanthes* and *Aeonium* (Uhl 1961; Liu 1989) and the latter two genera share the chromosome base number of $x = 18$. Cases of aneuploidy, possibly indicating single chromosome duplications, have only been reported for *Aichryson* (Uhl 1961). As we expect gene duplications at the begin of the evolution of *Aeonium*, the general chromosome numbers of $2n = 36$ and $2n = 72$ across this genus make a scenario of multiple single chromosome duplications unlikely. So far, there is no evidence for retrotransposons such as missing introns or poly A tracts in the MCS. Presently, unequal crossing over in *Aeonium* leading to gene tandems may be the best alternative explanation as to why there is an increased rate of gene duplications. Besides important chromosome counts by Uhl (1961) and Liu (1989), detailed karyological studies concerning chromosome pairing are still missing. This may be a promising task for the future.

Ohno (1970) and recently Zhang (2003) highlighted the importance of gene duplications as an evolutionary factor, a mechanism which is observed more frequently in plants rather than in animals (Li 1997; e.g. Aagaard et al. 2005). Our data support this view and show that gene duplications are likely linked to an increase of speciation in the MCS.

Although the increased number of copies in *Aeonium* is best interpreted as a result of gene duplications under the parsimony criterion, we consider an alternative explanation. They could also represent remaining copies of an initial polyploidy event in the MCS, while others underwent gene silencing. On the one hand, such scenario is supported by much higher chromosome base numbers in the island group compared with their mainland sisters, but on the other hand, it is less parsimonious. Until more data become available, none of these explanations is conclusive.

3.2.2 Phylogenetic Significance

In all candidate genes, several paralogous gene copies were detected. In many cases, there are conspicuous differences in their phylogenetic patterns compared to each other, but they often also differ from the nrITS tree. Considering the latter as relevant, independently inferred phylogenetic reconstruction, we assume that mutations in the coding regions of these candidate genes often overlay their phylogenetic signal. The discrepancies among the three genes may be partly caused by incomplete amplification of all copies across all taxa, e.g. missing *AP1* sequences for *Aichryson*. Still, there are congruencies among *PEPC* and *AP3* data yielding the same clades. Moreover, there is a phylogenetic signal in *AP3*-like sequences (clade B) reflecting the evolutionary history of MCS relatively well.

Accordingly, our data can contribute to a better systematic understanding of this group. The monophyly of *Sedum* sect. *Monanthoidea* is corroborated by candidate genes; *AP3* and *AP1* sequences of *S. jaccardianum* still appear nested inside those of *S. surculosum*. Such paraphyly seems also possible in the light of unresolved relationships among three accessions in the nrITS tree. The aberrant position of *Monanthes icterica* has been discussed intensively in the past (Nyffeler 1995; Mes et al. 1997; Mort et al. 2002) switching between *Monanthes* and *Aichryson*. Our results point towards close relationships to *Aichryson* rather than *Monanthes* represented by *M. anagensis* as also suggested by Mes et al. (1997) and Mort et al. (2002) based on morphology, RAPDs and cpDNA data. The use of candidate genes for phylogenetic reconstruction is supported by parts of our data. Future studies should extend the taxon selection, especially for *AP3*, to possibly solve these phylogenetic problems.

3.2.3 Evolution Rates of Candidate Genes

The ratio of nonsynonymous substitutions per nonsynonymous site (Ka) and synonymous substitutions per synonymous site (Ks) can deliver an estimation on the evolution rate of a gene by indicating the selection regime (Nei and Kumar 2000). A relative higher rate of nonsynonymous mutations, expressed in Ka/Ks, may lead to a change in the phenotype indicating positive selection (Nei and Kumar 2000).

The overall distribution pattern of evolutionary rates across the various lineages in MCS appears complex. When comparing DNA sequences of the island radiation relative to their continental counterparts, and much higher mean values of MCS can be observed for *AP3* and *PEPC*. Those differences are significant according to *t* tests ($p < 0.05$). For *PEPC*, no extreme differences among Ka/Ks values of clades A–C are found. For *AP3*, the *Aeonium* specific clade A shows lower values than clade B. This argues for an effect of both genes on the evolution of the entire island group which does not seem to be restricted to *Aeonium* alone. Without further experimental studies, it is, however, only possible to conjecture about sub- or neofunctionalisation of those copies (Zhang 2003). Quite surprising are patterns in *AP1*. Highest values are found for a few *Aeonium* sequences of groups A and B. The mainland cluster of *Sedum Monanthoidea* shows lower Ka/Ks values than these clades, but higher than those of clades C and D. There are examples where different copies execute varying functions. In orchids, multiple gene duplications led to copies which adopted different functions in the flower organisation (Mondragón-Palomino and Theißen 2008). In our case, it seems possible that the numerous copies of *Aeonium-AP1* can be divided into conservative ones (clades C and D), which are likely to have more or less kept their original functions, and clades A and B that took over other functions. At the same time, we keep in mind that nonfunctionalisation and the formation of pseudogenes of these sequences cannot be entirely excluded. Deriving from morphological and phenological variation in *Aeonium* and the effects of AP1 in *Arabidopsis* (Mandel and Yanofsky, 1995), those copies might possibly be involved in inflorescence branching and the flowering time. More studies are needed to resolve this.

Table 1 Results of *t* test indicating significant differences in Ka/Ks values among these copies (see Figs. 3–5). *Letters behind* the genes refer to the groups in Figs. 3–5

	PEPC-A	*PEPC*-C	*AP1*-C	*AP1*-D	*AP3*-A
PEPC-C	0.642				
AP1-C	0.001	0.000			
AP1-D	0.000	0.000	0.204		
AP3-A	0.113	0.072	0.482	0.157	
AP3-B	0.000	0.000	0.900	0.165	0.423

The complex patterns are mirrored when comparing Ka/Ks values using a *t* test between the gene copies of *PEPC*, *AP1* and *AP3* (Table 1). There is a significant difference in Ka/Ks values of *PEPC* compared to regulatory genes *AP1* and *AP3*. This is congruent with the wide observation that regulatory genes (*AP3*, *AP1*) evolve faster than structural genes (*PEPC*; White and Doebley 1998; Haag and True 2001; Barrier et al. 2001, 2003). Compared to the estimated mean Ka/Ks ratio among Brassicaceae genes of 0.14 (Tiffin and Hahn 2002), our values are raised. Our analysed regulatory genes contribute to tetrameric transcription factor complexes of MADS-box proteins which control other genes (Theißen and Saedler 2001). Therefore, an increase in Ka/Ks ratio of *AP1* and *AP3* is not surprising. In contrast, no significant differences could be found by *t* test analysis between the regulatory genes *AP1* and *AP3* and between the two copies A and C of *PEPC* (see Table 1 for details).

3.3 Evolution of MCS

Using our results and literature data, we attempt to reconstruct the evolutionary history of MCS. We focus on spatial and temporal aspects and on speciation modes.

Inferring from the close relationships to *Sedum Monanthoidea*, north-western Africa can likely be regarded as point of origin of the Macaronesian island radiation (Uhl 1961; Mes 1995; Mort et al. 2002). This is in accordance with assumed Mediterranean affinities of many other Canarian plants like, e.g., *Argyranthemum* (Asteraceae; Francisco-Ortega et al. 1997), *Sonchus* (Asteraceae; Kim et al. 1996), *Sideritis* (Lamiaceae; Barber et al. 2002) and *Ixanthus* (Gentianaceae; Thiv et al. 1999). Inferring from the basal positions of *Sedum modestum* within *Monanthoidea* and *Aichryson* within MCS, such an ancestor could have been an annual rock plant with a low number of yellow petals. On the Canary Islands, the MCS underwent the major radiation. In contrast to former views (Lems 1960; Bramwell 1976), occurrences outside this archipelago, e.g. Morocco, East Africa and Arabia) can clearly be interpreted as secondary (Mes et al. 1996; Mort et al. 2002).

In general, the speciation modes among MCS do not reflect uniform patterns and several speciation mechanisms seem to be active. Allopatric speciation can be easily inferred from phylogenetic patterns (Johannesson 2001). In the case that closely related species occur exclusively on single islands, gene flow is likely to be hampered or interrupted. Sometimes ecological vicariance where the taxa inhabit similar ecological spaces on various islands may be assumed, as in the case for Mort's et al. (2002) "clade 1" of *Aeonium*, largely corresponding to Sect. *Patinaria* (Liu 1989). Here, all species usually grow in slopes up to 1,000 m altitude (Liu 1989): *A. canariense* and *A. tabuliforme* on Tenerife, *A. virgineum* on Gran Canaria, *A. subplanum* on La Gomera, and *A. palmense* on La Palma and Hierro. In contrast to ecological vicariance, there may be only a few cases in which a radiation into different niches on a single island took place (Jorgensen and Frydenberg 1999). According to Mort et al. (2002), *Aeonium haworthii* (NW Tenerife, *Visnea-Dracaena* belt, laurel forests), *A. urbicum* (E Tenerife, partly laurel forests) and *A. pseudourbicum* (S Tenerife, succulent bush) form a moderately supported clade and all species grow on different parts of Tenerife, mostly in different habitats. If this pattern is not due to the geological history of Tenerife which formerly consisted of three segregated islands (Anchochea et al. 1990; Trusty et al. 2005), and given the correct phylogeny, this group may be an example of radiating into different habitats.

Although our molecular dating approach may suffer from imprecise calibration, the colonisation of the Canary Islands may have happened in the Upper Miocene-Pliocene-Pleistocene between 0.05 and 6.47 mya or perhaps even earlier (Kim et al. 2008, see Sect. 3.1). These periods are characterised by high volcanismic activities and uplifts on the Canary Islands (cf. Lösch 1990; Carracedo 1994; Trusty et al. 2005). This might have led to the formation of many new ecological niches which could be colonised by MCS. As Lösch (1990) and Mort et al. (2007) showed, there are specific physiological adaptations of MCS to their habitats. Carbon metabolism varies between strong CAM types to C_3 plants. Of our candidate genes, *PEPC* is strongest involved in such activities. As described earlier, the increased evolution rates of *PEPC*s in the island radiation may support the significant physiological adaptation in the MCS.

The highest evolution rate was found in *AP1*. As pointed out, this gene could have an effect on the flowering time. This outcome may explain our observation that the temporal floral activities of sympatric species of *Aeonium* often only overlap for a very short period (Esfeld et al. 2009). Moreover, different rewarding systems in *Aeonium*, offering pollen and/or nectar, likely have an impact on the pollinator behaviour and lead to much higher intra- than interspecific pollen transfer rates (Esfeld et al. 2009). Based on these results, this suggests that reproductive isolation may be a major trigger for the evolution of MCS. At the same time, the genetic separation is far from being complete. Therefore, this does not conflict with the observation of many natural (and artificial) hybrids based on morphological evidence (Praeger 1932; Liu 1989), but may even explain this phenomenon. These hybrids may become stable evolutionary lineages as has been suggested by Jorgensen and Frydenberg (1999), Jorgensen and Olesen (2001) and Mort et al.

(2002) who found inconsistencies between nuclear and plastid DNA data. We thus note that the MCS join several other island radiations where hybridisation is an important evolutionary driving factor (Seehausen 2004).

4 Summary

Conclusively, the evolution of the MCS was likely promoted by outer and inner factors. The availability of new niches formed by volcanismic activities likely met the opportunity of physiological adaptation. Our results indicate that nuclear gene duplications may be correlated with an increase in the speciation rate. The accelerated substitution rate of *AP1* may account for phenotypic changes which may cause reproductive isolation. Still, weak genetic barriers allow hybridisation to be a common phenomenon. Besides this, polyploidy is another evolutionary mechanism which also occurs in MCS, but is not as frequent as the other factors.

We could show that the analysis of candidate genes has contributed to better understanding of the evolution of diversity in the MCS. Experiments proving the effects in the phenotype of these genes in these taxa are still outstanding. In the future, other nuclear genes should be investigated. Of special interest would be genes involved in growth form plasticity. In the case that more gene duplications could be found for *Aeonium*, cytological studies could provide a deeper insight into these mechanisms. Another extremely promising future task would be the analysis of links and correlations between morphological, ecological and molecular data.

Acknowledgments We would cordially like to thank our collaborators; without their contributions this project could not have been implemented. For precious theoretical impact, we are grateful to Peter Linder, Philip Moline, Rolf Rutishauser (Zurich), Timo v.d. Niet (Pietermaritzburg, RSA), Joachim Thiede (Hamburg), Mark Mort (Kansas), Kerstin Neumann (Gatersleben) and Ulrich Kull (Stuttgart). Additionally, we thank Rainer Lösch (Düsseldorf), Urs Eggli (Zurich), Ray Stephenson (Northumberland), Stephen Jury (Reading) and Joël Lodé (Cuevas del Almanzora) for their access to plant material, Herbert Hohmann (Tenerife) for his help on Tenerife, Timo v.d. Niet, Aelys Humphrys, Valentin Grob, Philip Moline, Chloë Galley, Frédérique Pasquer (all Zurich), the laboratory of Martin Blum (Hohenheim) for precious laboratory help and Maia Gurushidze (Gatersleben) for help with r8s. Ingrid Jordon-Thaden helped with editorial input and many helpful suggestions to improve the manuscript. Particularly, we thank Frank Blattner (Gatersleben) and the ETX (Gatersleben) for their great support in many aspects, the Cabildo Insular (Tenerife) for permission to collect and conduct field work and the DFG for funding of this project to MT (Th 830/2-2).

References

Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408:796–815

Aagaard JE, Olmstead RE, Willis JH, Phillips PC (2005) Duplication of floral regulatory genes in the Lamiales. Am J Bot 92:1284–1293

Anchochea E, Fúster JM, Ibarrola E, Cendrero A, Coello J, Hernan F, Cantagrel JM, Jamond C (1990) Volcanic evolution of the island of Tenerife (Canary Islands) in the light of new K-Ar data. J Volcanol Geotherm Res 44:231–249

Baldwin BG (1997) Adaptive radiation of the Hawaiian silversword alliance: congruence and conflict of phylogenetic evidence from molecular and non-molecular investigations. In: Givnish TJ, Systsma KJ (eds) Molecular evolution and adaptive radiation. Cambridge University Press, Cambridge, UK, pp 104–128

Baldwin BG, Crawford D, Francisco-Ortega J, Kim S, Sang T, Stuessy T (1998) Molecular phylogenetic insights into the origin and evolution of island plants. In: Soltis DE, Soltis PS, Doyle JJ (eds) Molecular systematics of plants II. Kluwer, Boston, pp 410–441

Bañares A (1986) Híbridos interespecíficos del género *Aeonium* Webb&Berth. (Crassulaceae) en las Islas Canarias. Novedades y datos corológicos. Vieraea 16:57–71

Bañares A (1990) Híbridos de la familia Crassulaceae en las Islas Canarias. Novedades y datos corológicos. Vieraea 18:65–85

Barber JC, Francisco-Ortega J, Santos-Guerra A, Turner KG, Jansen RK (2002) Origin of Macaronesian *Sideritis* L. (Lamioideae: Lamiaceae) inferred from nuclear and chloroplast sequence datasets. Mol Phylogenet Evol 23:293–306

Barrier M, Bustamante CD, Yu J, Purugganan MD (2003) Selection on rapidly evolving proteins in the *Arabidopsis* genome. Genetics 163:723–733

Barrier M, Robichaux RH, Purugganan MD (2001) Accelerated regulatory gene evolution in an adaptive radiation. Proc Natl Acad Sci USA 98:10208–10213

Böhle U-R, Hilger HH, Martin WF (1996) Island colonization and evolution of the insular woody habit in *Echium* L. (Boraginaceae). Proc Natl Acad Sci USA 93:11740–11745

Bossuyt F, Milinkovitch MC (2001) Amphibians as indicators of early Tertiary "out-of-India" dispersal of Vertebrates. Science 292:93–95

Bramwell D (1968) Notes on the taxonomy and nomenclature of the genus *Aichryson*. Bol Inst Nac Invest Agron 28:203–213

Bramwell D (1976) The endemic flora of the Canary Islands. In: Kunkel G (ed) Biogeography and ecology in the Canary Islands. Junk, The Hague, pp 207–240

Carracedo JC (1994) The Canary Islands: an example of structural control on the growth of large oceanic island volcanoes. J Volcanol Geotherm Res 60:225–242

Chollet R, Vidal J, O'Leary MH (1996) Phosphoenolpyruvate carboxylase: a ubiquitous, highly regulated enzyme in plants. Annu Rev Plant Physiol Plant Mol Biol 47:273–298

Cooper A, Lalueza-Fox C, Anderson S, Rambaut A, Austin J, Ward J (2001) Complete mitochondrial genome sequences of two extinct moas clarify ratite evolution. Nature 409:704–707

Erbar C (2007) Current opinions in flower development and the evo-devo approach on plant phylogeny. Plant Syst Evol 269:107–132

Esfeld K (2009) The use of low-copy nuclear genes in the radiation of the Macaronesian Crassulaceae Sempervivoideae – phylogeny and evolutionary processes. Dissertation, University of Heidelberg

Esfeld K, Koch MA, van der Niet T, Seifan M, Thiv M (2009) Little interspecific pollen transfer despite overlap in pollinators between sympatric *Aeonium* (Crassulaceae) species pairs. Flora 204:709–717

Fairfield KN, Mort ME, Santos-Guerra A (2004) Phylogenetics and evolution of the Macaronesian members of the genus *Aichryson* (Crassulaceae) inferred from nuclear and chloroplast sequence data. Plant Syst Evol 248:71–83

Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol 17:368–376

Fortune PM, Schierenbeck KA, Ainouche AK, Jacquemin J, Wendel JF, Ainouche ML (2007) Evolutionary dynamics of *Waxy* and the origin of hexaploid *Spartina* species (Poaceae). Mol Phylogenet Evol 43:1040–1055

Francisco-Ortega J, Jansen RK, Santos-Guerra A (1996) Chloroplast DNA evidence of colonization, adaptive radiation, and hybridization in the evolution of the Macaronesian flora. Proc Natl Acad Sci USA 93:4085–4090

Francisco-Ortega J, Santos-Guerra A, Hines A, Jansen RK (1997) Molecular evidence for a Mediterranean origin of the Macaronesian endemic genus *Argyranthemum* (Asteraceae). Am J Bot 84:1595–1613

Futuyma DJ (2009) Evolution. Sinauer Associates, Sunderland, Massachusetts

Givnish TJ (1998) Adaptive radiation of plants on oceanic islands: classical patterns, molecular data, new insights. In: Grant P (ed) Evolution on islands. Oxford University Press, Oxford, pp 281–304

Gehrig H, Taybi T, Kluge M, Brulfert J (1995) Identification of multiple PEPC isogenes in leaves of the facultative crassulacean acid metabolism (CAM) plant *Kalanchoe blossfeldiana* Poelln. cv. Tom Thumb. FEBS Lett 377:399–402

Haag ES, True JR (2001) Perspective: from mutants to mechanisms? Assessing the candidate gene paradigm in evolutionary biology. Evolution 55:1077–1084

Heads M (2005) Dating nodes on molecular phylogenies: a critique of molecular biogeography. Cladistics 21:62–78

Hohenester A, Welss W (1993) Exkursionsflora für die Kanarischen Inseln. Ulmer, Stuttgart

Huelsenbeck JP, Ronquist F (2006) MrBayes: Bayesian inference of phylogeny v3.1.2. Distributed under the GNU general public license from the website: http://mrbayes.csit.fsu.edu

Hurst LD (2002) The Ka/Ks ratio: diagnosing the form of sequence evolution. Trends Genet 18:486–487

Johannesson K (2001) Parallel speciation: a key to sympatric divergence. Trends Ecol Evol 16:148–153

Jordan S, Simon C, Polhemus D (2003) Molecular systematics and adaptive radiation of Hawaii's endemic damselfly genus *Megalagrion* (Odonata: Coenagrionidae). Syst Biol 52:89–109

Jorgensen TH, Frydenberg J (1999) Diversification in insular plants: inferring the phylogenetic relationship in *Aeonium* (Crassulaceae) using ITS sequences nuclear ribosomal DNA. Nord J Bot 19:613–621

Jorgensen TH, Olesen JM (2000) Growth rules based on the modularity of the Canarian *Aeonium* (Crassulaceae) and their phylogenetic value. Bot J Linn Soc 132:223–240

Jorgensen TH, Olesen JM (2001) Adaptive radiation of island plants: evidence from *Aeonium* (Crassulaceae) of the Canary Islands. Perspect Plant Ecol Evol Syst 4:29–42

Juenger T, Purugganan MD, Mackay TFC (2000) Quantitative trait loci for floral morphology in *Arabidopsis thaliana*. Genetics 156:1379–1392

Kim S-C, Crawford DJ, Francisco-Ortega J, Santos-Guerra A (1996) A common origin for woody *Sonchus* and five related genera in the Macaronesian islands: molecular evidence for extensive radiation. Proc Natl Acad Sci USA 93:7743–7748

Kim S-C, McGowen MR, Lubinsky P, Barber JC, Mort ME, Santos-Guerra A (2008) Timing and tempo of early and successive adaptive radiations in Macaronesia. PLoS ONE 3(5): e2139

King MC, Wilson AC (1975) Evolution at two levels in humans and chimpanzees. Science 188:107–116

Kishino H, Thorne JL, Bruno WJ (2001) Performance of a divergence time estimation method under a probabilistic model of rate evolution. Mol Biol Evol 18:352–361

Kramer EM, Dorit RL, Irish VF (1998) Molecular evolution of genes controlling petal and stamen development: Duplication and divergence within the *APETALA3* and *PISTILLATA* MADS-box gene lineages. Genetics 149:765–783

Kull U (1982) Artbildung durch geographische Isolation bei Pflanzen – die Gattung *Aeonium* auf Teneriffa. Nat Mus 112:33–64

Lems K (1960) Botanical notes on the Canary Islands. II. The evolution of plant forms in the islands: *Aeonium*. Ecology 41:1–17

Li WH (1997) Molecular evolution. Sinauer, Sunderland, MA

Litt A, Irish VF (2003) Duplication and diversification in the *APETALA1/FRUITFULL* floral homeotic gene lineage: implications for the evolution of floral development. Genetics 165:821–833

Liu HY (1989) Systematics of *Aeonium* (Crassulaceae). National Museum of Natural Science, Taichung
Lösch R (1990) Funktionelle Voraussetzungen der adaptiven Nischenbesetzung in der Evolution der makaronesischen Semperviven. Diss Bot 146, Cramer, Berlin
Lösch R, Kappen L (1981) The cold resistance of Macaronesian Sempervivoideae. Oecologia 50:98–102
Lynch M, Conery JS (2000) The evolutionary fate and consequences of duplicate genes. Science 290:1151–1155
Mandel MA, Yanofsky MF (1995) A gene triggering flower formation in *Arabidopsis*. Nature 377:522–524
Maire R (1976) Flore de l'Afrique du Nord, vol 14. Lechevallier, Paris
Mes THM (1995) Phylogenetic and systematic implications of chloroplast and nuclear spacer sequence in the Macaronesian Sempervivoideae and related Sedoideae. In: 't Hart H, Eggli U (eds) Origin and evolution of the Macaronesian Sempervivoideae (Crassulaceae). Backhuys, Leiden, pp 31–44
Mes THM, 't Hart H (1996) The evolution of growth-forms in the Macaronesian genus *Aeonium* (Crassulaceae) inferred from chloroplast DNA RFLPs and morphology. Mol Ecol 5:351–363
Mes THM, van Brederode J, 't Hart H (1996) Origin of the woody Macaronesian Sempervivoideae and the phylogenetic position of the East African species of *Aeonium*. Bot Acta 109:477–491
Mes THM, Wijers G-J, 't Hart H (1997) Phylogenetic relationships in *Monanthes* (Crassulaceae) based on morphological, chloroplast and nuclear DNA variation. J Evol Biol 10:193–216
Mondragón-Palomino M, Theißen G (2008) MADS about the evolution of orchid flowers. Trends Plant Sci 13:51–59
Mort ME, Crawford DJ (2004) The continuing search: low-copy nuclear sequences for lower level plant molecular phylogenetic studies. Taxon 53:257–261
Mort ME, Soltis DE, Soltis PS, Francisco-Ortega J, Santos-Guerra A (2002) Phylogenetics and evolution of the Macaronesian clade of Crassulaceae inferred from nuclear and chloroplast sequence data. Syst Bot 27:271–288
Mort ME, Soltis DE, Soltis PS, Santos-Guerra A, Francisco-Ortega J (2007) Physiological evolution and association between physiology and growth form in *Aeonium* (Crassulaceae). Taxon 56:453–464
Nei M, Gojobori T (1986) Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol Biol Evol 3:418–426
Nei M, Kumar S (2000) Molecular evolution and phylogenetics. Oxford University Press, Oxford
Nyffeler R (1995) Die gattung monanthes haworth. Kakt and Sukk 46:157–165
Ohno S (1970) Evolution by gene duplication. Springer, Berlin
Pilon-Smits EAH, 't Hart H, Maas JW, Meesterburrie JAN, Kreuler R (1992) The evolution of the crassulacean acid metabolism in *Aeonium* inferred from carbon isotope composition and enzyme activities. Oecologia 91:548–553
Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. Bioinformatics 14:817–818
Purugganan MD (1998) The molecular evolution of development. Bioessays 20:700–711
Praeger RL (1932) An account of the *Sempervivum* group. Royal Horticultural Society, London
Reyes-Betancort JA, Santos-Guerra A, Guma IR, Humphries CJ, Carine MA (2008) Diversity, rarity and the evolution and conservation of the Canary Islands endemic flora. Anal Jard Bot Madrid 65:25–45
Richardson JE, Weitz MF, Fay MF, Cronk QCB, Linder HP, Reeves G, Chase MW (2001) Rapid and ancient origin of species richness in the Cape Flora of South Africa. Nature 412:181–183
Rutschmann F (2004) Bayesian molecular dating using PAML/multidivtime. A step-by-step manual. University of Zurich, Switzerland
Sanderson MJ (1998) Estimating rate and time in molecular phylogenies: beyond the molecular clock? In: Soltis DE, Soltis PS, Doyle JJ (eds) Molecular systematics of plants. Kluwer, Boston, pp 242–264
Sanderson S (2004) r8s, version 1.70. http://loco.biosci.arizona.edu/r8s/

Schluter D (1998) Ecological causes of speciation. In: Howard D, Berlocher S (eds) Endless forms: species and speciation. Oxford University Press, Oxford, pp 114–129
Schluter D (2000) The ecology of adaptive radiation. Oxford University Press, Oxford
Seehausen O (2004) Hybridization and adaptive radiation. Trends Ecol Evol 19:198–207
Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599
Theißen G (2001) Development of floral organ identity: stories from the MADS house. Curr Opin Plant Biol 4:75–85
Theißen G, Saedler H (2001) Floral quartets. Nature 409:469–471
Theißen G (2002) Orthology: secret life of genes. Nature 415:741
Thiv M, Struwe L, Kadereit JW (1999) The phylogenetic relationships and evolution of the Canarian laurel forest endemic *Ixanthus viscosus* (Ait.) Griseb. (Gentianaceae): evidence from matK and ITS sequence variation, and floral morphology and anatomy. Plant Syst Evol 218:299–317
Thiv M, Thulin M, Kilian N, Linder HP (2006) Eritreo-Arabian affinities of the Socotran Flora as revealed from the molecular phylogeny of *Aerva* (Amaranthaceae). Syst Bot 31:560–570
Thorne JL, Kishino H (2002) Divergence time and evolutionary rate estimation with multilocus data. Syst Biol 51:689–702
Thorne JL, Kishino H, Painter IS (1998) Estimating the rate of evolution of the rate of molecular evolution. Mol Biol Evol 15:1647–1657
Tiffin P, Hahn M (2002) Coding sequence divergence between two closely-related plant species–*Arabidopsis thaliana* and *Brassica rapa* ssp. *pekinensis*. J Mol Evol 54:746–753
Trusty JL, Olmstead RG, Santos-Guerra A, Sá-Fontinha S, Francisco-Ortega J (2005) Molecular phylogenetics of the Macaronesian-endemic genus *Bystopogon* (Lamiaceae). Paleo-islands, ecological shifts and interisland colonization. Mol Ecol 14:1177–1189
Uhl CH (1961) The chromosomes of the Sempervivoideae (Crassulaceae). Am J Bot 48:114–123
Voggenreiter V (1974) Geobotanische untersuchungen an der natürlichen vegetation der kanareninsel tenerife Diss Bot 26. Cramer, Lehre
Wagner WL, Funk VA (1995) Hawaiian biogeography: evolution on a hot spot archipelago. Smithsonian Institution, Washington
Weigel D (1998) From floral induction to floral shape. Curr Opin Plant Biol 1:55–59
White S, Doebley JF (1998) Of genes and genomes and the origin of maize. Trends Genet 14:327–332
Yang Z (1997) PAML: a program package for phylogenetic analysis by maximum likelihood. Comput Appl Biosci 13:555–556
Zhang J (2003) Evolution by gene duplication: an update. Trends Ecol Evol 18:292–298

Key Innovations Versus Key Opportunities: Identifying Causes of Rapid Radiations in Derived Ferns

Harald Schneider, Hans-Peter Kreier, Thomas Janssen, Elisabeth Otto, Haiko Muth, and Jochen Heinrichs

1 Introduction

Biological radiations, e.g., adaptive radiations and rapid radiations, are widely accepted as one of the major events contributing to the diversification of the tree of life, but many aspects of these events are poorly understood (Schluter 2000; Gavrilets and Losos 2009). The classical examples for biological radiations are adaptive radiations, in which a lineage occupies a range of niches by diversifying in a relative short time until all niches are filled. However, not all radiations necessarily fulfil this pattern and alternative scenarios such as rapid radiations are widely considered. A major setback is the lack of a generally accepted definition of biological radiations (Schluter 2000). Empirical studies on the frequency and contribution of radiation, however, need concise theoretical concepts and criteria such as temporal increase of diversification rate or the absolute number of species to define biological radiations (Schluter 2000; Gavrilets and Losos 2009). Thus, our knowledge is limited by both the disparity of applied concepts and the limited amount of studies scrutinizing evidence for as many lineages of organisms as possible. It is therefore impossible to estimate quantitatively the contribution of radiations to the diversity of life on earth today. A recent study on 101 phylogenies discovered evidence for a hypothesis that explains the constant accumulation of biodiversity through rare single speciation events instead of species radiations (Venditti et al. 2010).

H. Schneider (✉)
Department of Botany, Natural History Museum, London, UK
Albrecht-von-Haller Institut für Pflanzenwissenschaften, Georg-August Universität Goettingen, Göttingen, Germany
e-mail: h.schneider@nhm.ac.uk

H.-P. Kreier, T. Janssen, E. Otto, H. Muth, and J. Heinrichs
Albrecht-von-Haller Institut für Pflanzenwissenschaften, Georg-August Universität Goettingen, Göttingen, Germany

FU Berlin, Institut fuer Biologie, Angewandte Genetik 14195, Berlin, Germany

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_4,

Most studies on radiations focus on selected lineages, and the number of positive reports may overstate their contribution to the diversity of life (Schluter 2000; Gavrilets and Losos 2009) because many lineages were never studied for the existence of radiations especially those lineages lacking evidence for radiations. To overcome these limitations of our knowledge, we need studies screening various lineages of organisms for evidence on the occurrence of radiations. Here, we present a phylogenetic research approach to explore the frequency of radiations in a species-rich lineage of land plants. Such studies will likely discover many more examples for radiations than are currently recognized. Besides identifying putative radiations, we can then also address the question as to what is triggering radiations, either key innovations or key opportunities such as the colonization of oceanic islands, in a more comprehensive approach than by a few selected case studies (Hodges and Arnold 1995; Schluter 2000). However, this kind of study requires an operative definition of radiations. To achieve this, we use here a more restrictive definition that can be easily implemented in test procedures. The chosen approach simplifies the pattern of accumulation of diversity in a selected lineage as either created by a constant accumulation of diversity over time or as a punctuated pattern with a short period of increased diversification followed by a period of decreased diversification (Hodges and Arnold 1995; Schluter 2000; Ree 2005). This approach models more or less the situation of a rapid radiation or an adaptive radiation with an increasing amount of filled niches. This restrictive definition may not cover all evolutionary patterns currently considered to be a radiation. However, the advantages of the restrictive definition overcome this limitation. In the following, we use this definition to identify radiation events and to determine their trigger.

2 Identifying Evidence for Biological Radiations

Based on the applied definition, the accumulation of species diversity by radiations can be separated from accumulation of species diversity by randomly distributed speciation events in the phylogeny of a lineage. In the latter case, the diversification rate will be more or less constant, whereas rapid radiations are characterized by the sudden increase of the diversification rate followed by a decrease of this rate. The diversification rate is a composite of the speciation rate, the number of speciation events in a given time period, and the extinction rate, the number of extinction events in a given time period, as a result of either decreasing extinction rate or increased speciation rate. The change of the diversification rate can thus be the result of a change of the speciation rate or the extinction rate. The contribution of both has been discussed for radiation events. A radiation event may be caused by an increased speciation rate and a constant extinction rate but may also be the result of a decreased extinction rate and a constant speciation rate (Rabosky and Lovette 2008). The underlying statistic allows the test for significance of the pattern, and several methods have been developed to explore evidence for changes of diversification rates over time (Nee et al 1994; Pybus and Harvey 2000; Rabosky 2006).

Phylogenetic methods to detect variation of the diversification rates can be divided into procedures using temporal information such as divergence time estimates or whole-tree methods using imbalance of phylogenetic trees. The latter approach is implemented in the software SymmTREE (Chan and Moore 2002). The non-temporal approaches are particularly powerful to obtain a working hypothesis that can be subsequently explored using temporal frameworks. The most commonly used temporal approach is the generation of lineage through time plots (LTTP). The number of lineages is plotted against a time scale to visualize the accumulation of diversity through time. A major step forward was the introduction of gamma statistics (Pybus and Harvey 2000). These statistics are used to determine the significance of changes of rates. In recent years, advanced analytical processes have been established allowing comprehensive analyses of changes of diversification rates in the phylogenetic history of lineages (Rabosky 2006; Purvis et al. 2009; Ricklefs 2009). Here, we use LASER (likelihood analyses of speciation and extinction rates), developed by Rabosky (2006), in combination with APE (Paradis et al. 2004) and apTreeShape (Bortolussi et al. 2006). This maximum likelihood-based approach determines temporal shifts in the diversification rate based on a birth–death process (Nee et al. 1994). We use estimate speciation rates for clades using the calculations developed absolute diversification rates (Ricklefs 2009).

To be able to approach these analyses, robust phylogenetic hypotheses are required. They are obtained by generating sequence data of the plastid genome regions, such as the coding regions *rbcL* and *rps4*, the intergenic spacer region (IGS) *rps4-trnS* IGS, and the *trnL-trnF* region including the *trnL* intron and the *trnL-trnF* IGS for the studied lineages. It is important to consider a sampling that includes as many of the extant species as possible because incomplete sampling may create in misleading results. The sequence data are used to reconstruct the phylogeny using maximum likelihood (Schmidt and von Haeseler 2009), maximum parsimony (Swofford and Sullivan 2009), or Bayesian inference of phylogeny (Ronquist et al. 2009). These hypotheses provide information about the relationships of taxa and the phylogenetic distances among taxa. The latter are transformed into temporal distances by analyses using relaxed molecular clock models that allow lineages specific shifts in the rate of molecular evolution as implemented in r8s 1.71 (Sanderson 2006) and BEAST 1.5.3. (Drummond and Rambaut 2007). The BEAST program generates chronograms using a Bayesian inference of phylogeny under a model of molecular evolution and Yule-model of diversification. BEAST analyses take phylogenetic uncertainty by default into account, whereas in r8s, analysis of the uncertainty needs to be approached via bootstrap analyses. Estimates for the separation of the Davalliaceae and Polypodiacae (Schneider et al., 2004a, b; Schuettpelz and Pryer 2009) are used to calibrate the generated chronograms. These calibrations are used as fixed-point calibrations in analyses with r8s or as log-normal-distributed calibration intervals in BEAST analyses. The use of divergence time estimates was chosen to overcome the problem of the very limited fossil record of these ferns (van Uffelen 1991). However, the generated temporal hypotheses are consistent with the known fossil record.

3 Determining the Role of Key Innovations and/ or Key Opportunities

The first step is the identification of potential key innovations and/or key opportunities. This part of the study requires extensive knowledge on the morphology, distribution ranges and ecological preferences of the investigated lineage. The product of this initial investigation is a matrix in which the distribution of a character or geographic occurrences is scored for each taxon. The second step involves the reconstruction of the ancestral character state or ancestral distribution ranges. The employed applications differ between key innovations and key opportunities. Key innovations are usually scored as discrete character states and reconstructed using maximum parsimony, maximum likelihood, Bayesian character state reconstruction, and stochastic character state plotting as implemented in software such as Mesquite 2.72 (Maddison and Maddison 2009). Stochastic character state plotting is especially powerful, because lineages with key innovations are often isolated from their relatives for a long time and thus sitting at a long branch. This approach was carried out with Mesquite and with SIMMAP (Bollback 2006). Key opportunities require a different approach, because it involves the reconstruction of ancestral distribution ranges. Various methods have been proposed to pursue this kind of reconstruction (Clark et al. 2008). Some of these methods employ maximum parsimony reconstruction in which distribution ranges are managed the same way as for ancestral character state reconstructions. However, process-based models are expected to generate more accurate reconstructions by differentiating between the three main processes: local extinction, dispersal, and vicariance. DIVA (dispersal–vicariance analyses; Ronquist 1997) is one of the most powerful approaches despite this kind of analyses having problems with some of the assumptions. We also used DIVA (Ronquist, 1997) and Bayesian DIVA analyses (Nylander et al. 2008). This approach takes phylogenetic uncertainty into account by reconstructing the ancestral distribution for a group of phylogenetic hypotheses generated in a Bayesian inference, which samples trees of similar likelihoods. The third step includes the exploration for evidence concerning the coincidence of diversification rate shifts and the key innovation/key opportunity. This identification can be done visually by using the temporal distribution of the LTTP and the temporal occurrence of the key innovation/ key opportunity. The BiSSE model (Maddison et al. 2007) provides a more robust estimate to determine the correlation of character evolution and diversification rates of a lineage. The model and its application are implemented in Mesquite.

The outlined approach works well as long as the extant diversity has been sufficiently sampled, the phylogenetic tree is fully resolved, and the phylogenetic hypothesis is robust. A further issue is often our limited knowledge on some potentially important characters, e.g., physiological traits, because observations only exist for a small percentage of the taxa studied. Misleading results may be caused by frequent extinctions and diversity turnover in the lineage history. Improved models may be able to overcome this problem. A similar important limitation is the model of

character innovation. This is particularly the case when the lineage undergoing an increase of diversification is nested on a long branch. Thus, we cannot infer if the innovation of the character directly triggers the diversification rate shift or a time lapse is involved. A final issue to be raised is the problem of overlapping radiations. Two radiations in the same lineage may overlap and obscure the true pattern of diversification. In such a case, the employed tests may reject evidence for radiations because the generated pattern may not be distinguishable from a random distribution of speciation events in time.

4 Case Studies in the Epiphytic Polypodiaceae

All analyses were performed on a lineage of derived ferns. The Polypodiaceae (Schneider et al. 2004a, b). The lineage includes more than 1,200 species and only one other fern family may comprise more species (Smith et al. 2006b). The family shows a worldwide distribution but the majority of species are restricted to the tropical climatic regions. Epiphytic growth appears to be the ancestral life strategy in the family, but shifts in the ecological preferences, mainly to saxicolous and rheophytic habits, occur in several clades. In some species, a saxicolous habit replaced the epiphytic habit, but a few species have been adapted to rheophytic habitats. The Polypodiaceae lineage is among the youngest fern lineages with an age of about 20–50 million years to today for the most basal split within the family as indicated by divergence time estimates (Schneider et al. 2004a; Schuettpelz and Pryer 2009). The phylogeny of the lineage was studied extensively in the recent years, and sequence data of pastid genome DNA regions such as *rbcL* are now available for more than 600 out of approximately 1,200 species (e.g., Ranker et al. 2004; Schneider et al. 2004b, 2006a, b, 2008; Janssen and Schneider 2005; Kreier and Schneider 2006a, b; Smith et al., 2006b; Janssen et al. 2007; Kreier et al. 2007, 2008a, b; Salino et al. 2008; Otto et al. 2009; Wang et al. 2010a, b). The family is arguably the most extensively studied among the species-rich fern families, and DNA sequence data are available for more than 500 species. A global overview of the phylogeny of the lineage was obtained by a phylogenetic analyses of a *rbcL* dataset including the sister family Davalliaceae (48 species) and the family Oleandraceae (2 species), the latter as the outgroup, and more than 554 species of Polypodiaceae (Fig. 1). In general, the phylogeny is well resolved and the support values (high posterior and bootstrap values are indicated by ***), but the deeper nodes of the core Polypodiacae clades (numbers 1–3) lack any support and the relationships among these nodes are unclear. The branches connecting these nodes are extremely short, indicating an initial rapid radiation of the core Polypodiaceae. This hypothesis is also congruent with the chronogram shown in Fig. 2, which is based on a divergence time estimate of the lineage. According to our results the core Polypodiaceae (node 1) underwent a rapid diversification at around 25 mya.

The lineage established in this period includes more than 95% of the diversity of the Polypodiaceae. The resulting lineages are either mainly distributed in the

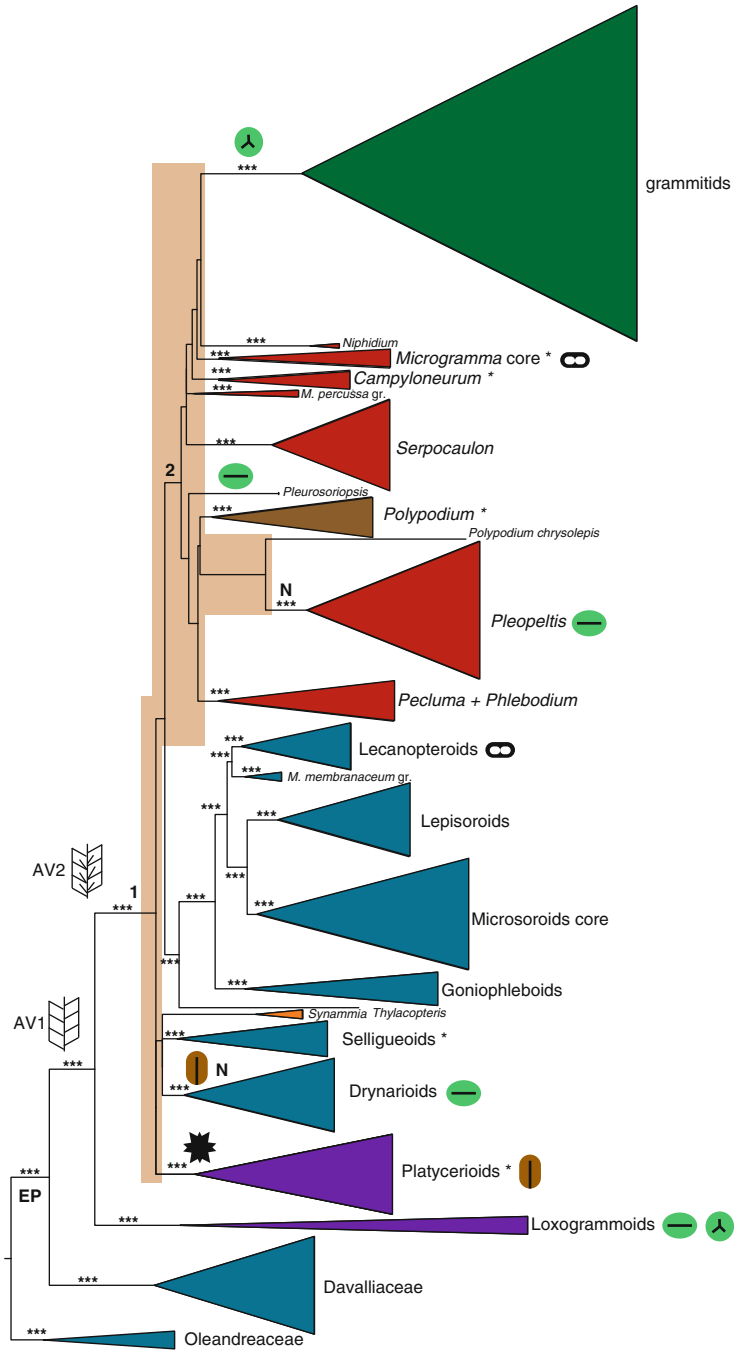


Fig. 1 Phylogeny of Polypodiaceae including the sister family Davalliaceae and as the out group the Oleandreaceae that are in turn sister to the Davalliaceae-Polypodiaceae clade. The phylogenetic hypothesis shown is based on the analyses of *rbcL* sequences of more than 600 species of

Neotropics or the Paleotropics. The grammitids are the only exception because this highly diverse group comprises similar numbers of Neotropical and Paleotropical species. However, the Paleotropical species are originated from Neotropical grammitids (Ranker et al 2004) and thus the nesting of the grammitids in an otherwise mainly Neotropical clade is consistent with the hypothesis of a segregation into mainly Neotropical and mainly Paleotropical lineages. The exploration for putative causes for this rapid diversification did not find any well-supported candidate for a key innovation. The only candidate is the apomorphy of leaf laminas with anastomosed venations with free veinlets in the areoles. However, this character is lost in the grammitids. The alternative is a correlation with a key opportunity. The Oligocene (23.5–33.7 mya) is characterized by cool global climates that contrast with the warm climates of the Eocene (38.7–53 mya). However, the transition from the Oligocene to the Miocene (5.30–23.5 mya) shows a global warming (Zachus et al. 2001; Mosburger et al. 2005) that may have offered tropical plants the opportunity to expand their ranges and perhaps also to rapid adaptive radiations. This hypothesis is currently under investigation.

A detailed analysis of all lineages of Polypodiaceae cannot be provided here because it will take up too much space. Instead, two examples of key innovations will be discussed as examples.

5 Key Innovations: Ants and Ferns

The first example concerns the ant fern mutualism as found in the Malesian genus *Lecanopteris* (Haufler et al. 2003) that is nested within the microsoroid ferns (Schneider et al. 2004b; Kreier et al. 2008b). The fern provides housing either

Fig. 1 (continued) *Polypodiaceae*. Maximum parsimony, maximum likelihood, and Bayesian inference of phylogeny recovered nearly identical topologies with the exception of some unsupported nodes. *Triangles* represent major lineages with the size of the *triangle* corresponding to the approximated number of species diversity of each lineage. The *color* of each *triangle* indicates its distribution range: *blue* paleotropical, *brown* northern temperate, *green* pantropical with an origin in the Neotropics, *orange* southern South America, *red* Neotropics with some species occurrences in Africa and southern India, *violet* paleotropical with some occurrences in the Neotropics. The *branch length* indicates the genetic distance among the clade. Nodes underlined with *brown squares* are poorly or not supported relationships. Nodes are indicated by numbers 1 = core Polypodiaceae, 2 = Neotropical Polypodiaceae plus grammitids. Other abbreviations and symbols above or below branches: *** strongly supported with bootstrap values of 100% and posterior value of $p \geq 0.95$, *AV1* innovation of regularly anatstomosed venation (drawing indicates pattern of veins), *AV2* innovation of regularly anastomosed venation with free-veinlets (drawing indicates pattern of veins), *EP* transition to epiphytic habit, *green ovals* occurrence of chlorophyllous (*green*) monolete spores, *green circles* occurrence of chlorophyllus (*green*) trilete spores, the *double star* occurrence of stelate hairs, *brown ovals* occurrence of litter collectors, *black ovals* with *white circles* occurrence of rhizome structures colonized by ants, *N* regular occurrence of nectaries on the mature leaf

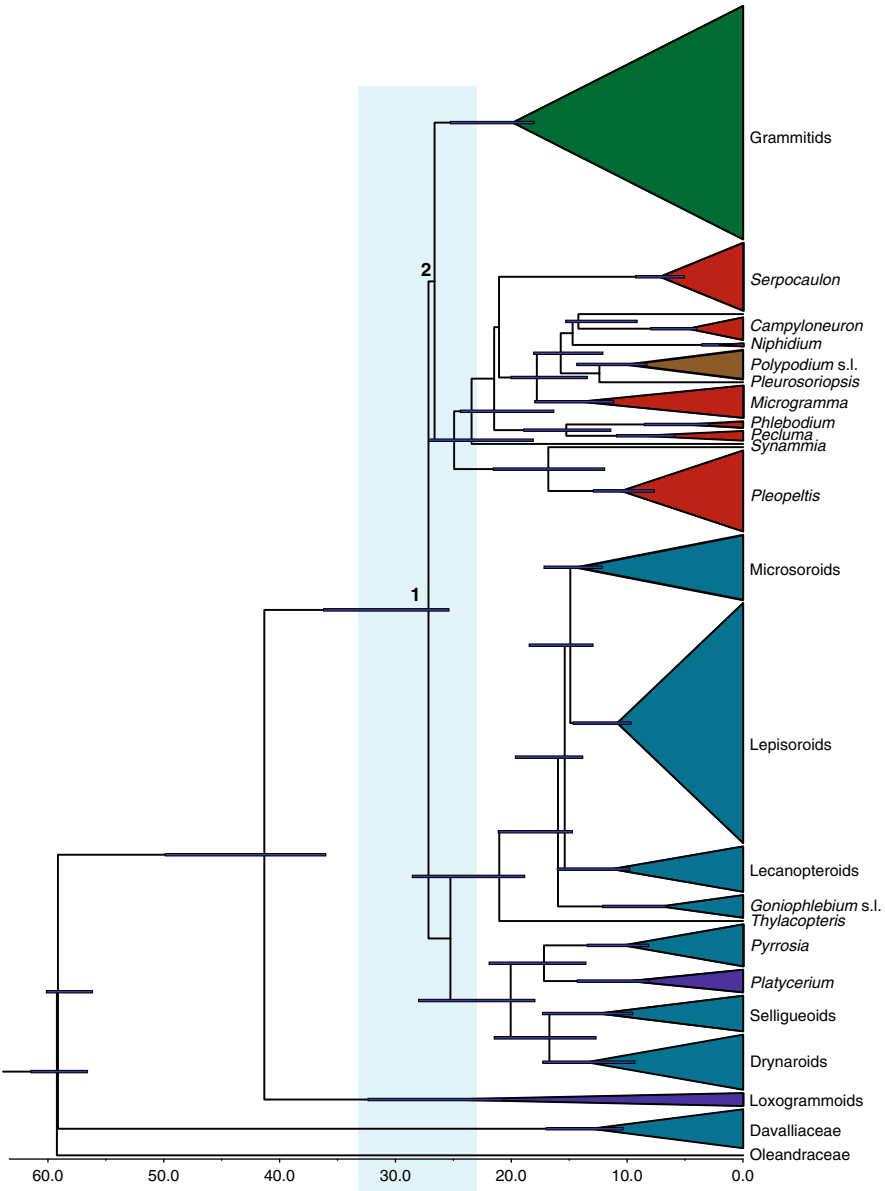


Fig. 2 Chronogram of the Polypodiaceae. The divergence estimates were performed using the same dataset as above with some changes in the sampling of lepisoroids. The analyses were performed using BEAST with the GTR + invgamma model for sequence evolution (parameters estimated during the analyses), lognormal relaxed molecular clock, and Yule process of diversification. *Blue bars* at nodes indicate the confidence interval of node ages. *Colors of triangles* are the same as in Fig. 1. The *blue square* indicates the Oligocene – a phase of relatively cool global climates in contrast to the generally warm global climates in the Eocene and Miocene

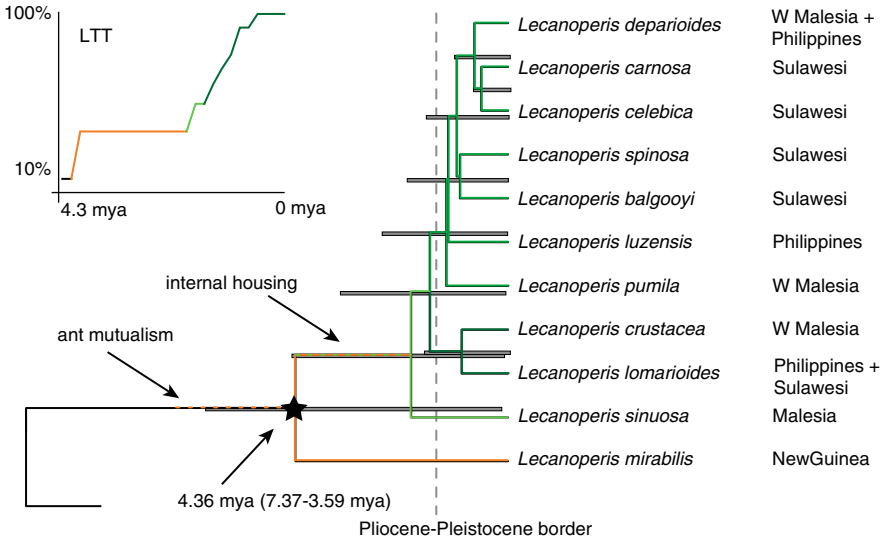


Fig. 3 Diversification of the ant-fern genus *Lecanopteris*. Chronogram estimates using BEAST and model parameters as in Fig. 2. *Gray bars* indicate the confidence intervals for each node. The *dashed line* indicates the border between the Pliocene and Pleistocene at 1.75 mya. The *color* of the *branches* shows the most parsimonious reconstruction of the evolution of ant dormancies in this fern genus. The common ancestor of *Lecanopteris* and its sister genus lacked the ant-fern mutualisms (*black lines*), *orange* indicates external housing, *green* internal housing. The *bright* to *dark shading* indicates the three different caveat structures in the rhizomes. *Dashed colored line* indicates uncertainty about the time of the innovation between the separation from the sister taxon without the character and the separation of the first clades with this character. The distribution of each species is given on the *right*. The *insert* on the *top left* shows the lineage through time plot (LTTP) plotting percentage of lineages versus time from 4.3 mya to today. The color of the graph corresponds to the character states of the most advanced clades present at the given time interval

inside the rhizomes in form of caves or below a flattened rhizome. In return, the epiphytic fern has access to a new source of nitrogen and phosphate (Gay 1993). Our divergence time estimates of the data set indicate a slow gradual evolution of the mutualism from about 4.36 mya onwards. The internal housing evolved in a period from ~4.36 mya to ~2.1 mya. Ancestral character reconstructing does not allow a more accurate estimate. However, the establishment of the internal housing model does not trigger the burst of speciation at about 1.75 mya (Fig. 3). Thus, we need to consider other explanations for the recent increase of the species richness in *Lecanopteris*. The BiSSE test did not find any significant evidence for a correlation of ant–fern mutualism or internal housing and changes in the diversification rates of *Lecanopteris*. Four out of 11 species are endemic to Sulawesi, and future studies need to infer the possibility of climatic fluctuations as the trigger of the speciation increase similar to the hypotheses concerning the explosive divergence of scaly tree ferns in Madagascar (Janssen et al. 2008).

6 Key Innovations and Key Opportunities

Litter collecting and the colonization of New Guinea by drynarioid ferns.

Drynarioid ferns are a small isolated lineage that is sister to the selligueid ferns (Figs. 1 and 2). Many species of drynarioid ferns show leaf structures adapted to collect litter. Access to nutrients is a major constraint to epiphytic plants, and litter collecting is one approach – see also ant mutualism – to obtain an advantage in the struggle for life (Janssen and Schneider 2005). Ancestral character reconstruction recovered the presence of structures for litter collecting as ancestral (Fig. 4a) but litter collectors are lost three times in the phylogeny of drynarioid ferns (*Christiopteris* and twice in *Agalomorpha*). Divergence time estimates recovered evidence for a initial rapid radiation of drynarioid which is likely linked with the innovation of leaf litter collectors. This hypothesis was supported by the BiSSE model analyses. However, we found evidence for a second radiation in the *Aglaomorpha* clade. This radiation is not correlated with changes of the leaf litter collectors (see Janssen and Schneider 2005). The derived *Aglaomorpha* clade (see Fig. 4b) shows a main distribution in Central and Eastern Malesia. Several species are endemic to the Philippines or New Guinea. The Malesian region is relatively young and formed by the collision of the Australian craton and south-east Asia around 10 mya (Hall 2002). The time estimates for the divergence of the *Agalomorpha* clade are within the predicted times of the formation of the New Guinea Alps and the mountains of the Philippines in the late Miocene and Pliocene (1.75–11 mya). A current study with an improved sampling supports the hypotheses of two radiations of drynarioid ferns, one coinciding with the innovation of litter collectors and the second one coinciding with the colonization of eastern Malesia.

7 Perspectives

The study found evidence for rapid radiations for the drynarioids and the core Polypodiaceae. No evidence for rapid radiations were found for several clades that were studied in detail, e.g., lepisoroids, loxogrammoidsn, microsoroids, and the *Platycerium*/*Pyrrosia* group (Kreier and Schneider 2006a, b; Kreier et al. 2008b; Wang et al. 2010a, b). Also, no evidence was found in the *Pleopeltis* clade (Otto et al. 2009). The grammitids, the most species-rich clade with more than 500 species, show an increased diversification rate and likely represent an adaptive radiation. However, the current sampling (Ranker et al. 2004; and this analysis) is insufficient to explore the hypothesis. In summary, adaptive radiations are likely the less common process of accumulation of biodiversity of these plants and the majority can be likely explained by the assembling through rare speciation events.

Only two examples for convincing key innovations were recovered. First, the innovation of litter collectors may have triggered the initial diversification of drynarioid ferns. However, a similar innovation of litter collectors resulted in a

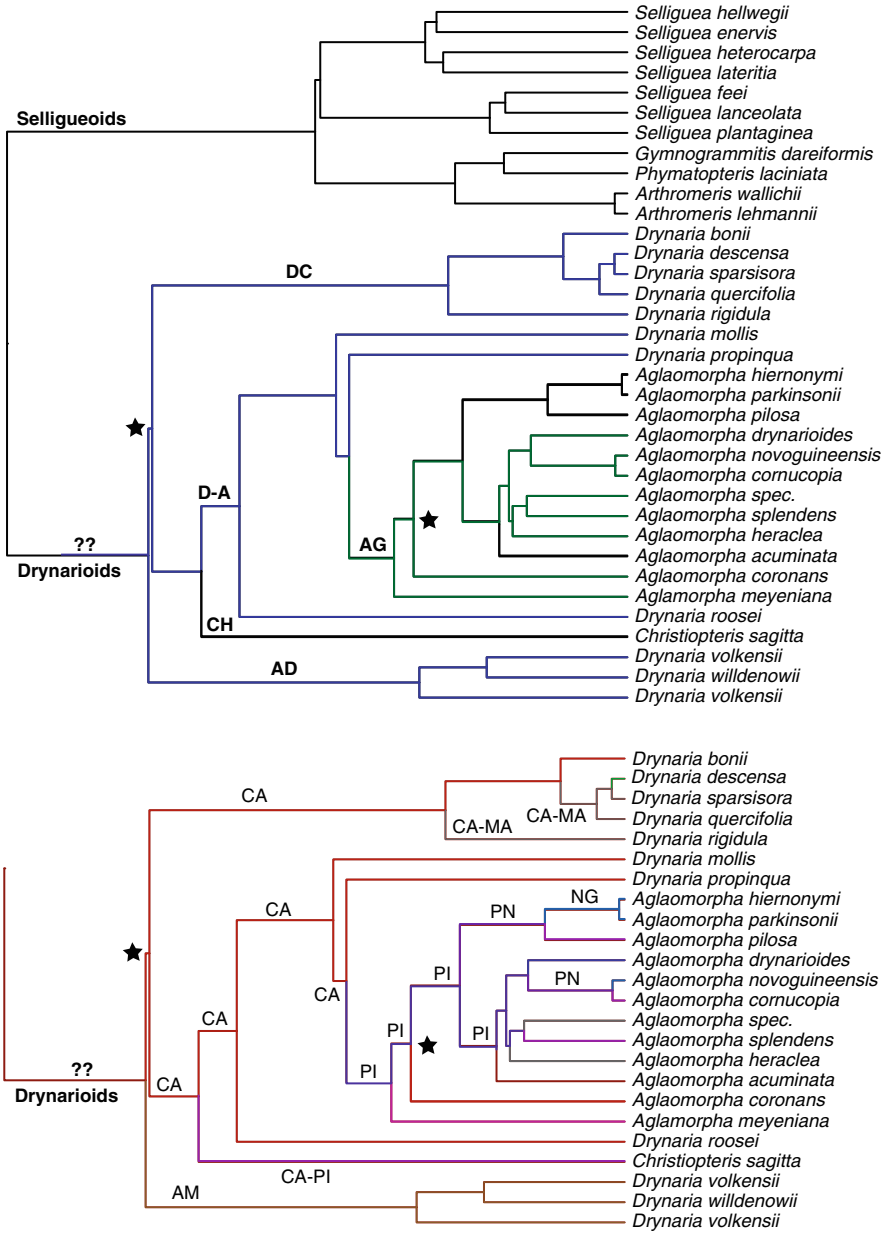


Fig. 4 Diversification of the drynarioid ferns. Chronogram estimates using BEAST and model parameters as in Fig. 2. The drynarioid radiation dates back to about 14 mya. (**a**) The character evolution of litter collectors. *Blue* leaf litter collected is a specialist humus collector leaf, *green* leaf litter collector is the basal part of a photosynthetic leaf, *black* leaf litter is absent. *AD* Afro-Drynaria, *AG* Aglaomorpha, *CH* Christiopteris, *DC* Drynaria core, *D-A* Drynaria-Aglaomorpha grade. *Stars* indicate radiations. (**b**) Ancestral distribution ranges as estimated using DIVA. *AM* Afromadagascar, *CA* continental Asia, *MA* Western Malesia, *NG* New Guinea, *PI* Philippines, *PN* Philippines/New Guinea. *Gray branches* indicate widespread taxa

gradual accumulation pattern in the genus *Platycerium*. The second candidate, the innovation of chlorophyllous spores may have triggered the grammitid radiation. Chlorophyllous (green) spores have differentiated chloroplasts in the dispersed spores whereas fern spores are usually dispersed with proplastids. Differentiated chloroplasts indicate the lack of a dormancy phase. It is not clear yet why this is an advantage for the grammitids, although we can speculate here about the adaptation to habitats without the potential to form spore banks. Barks of trees in wet tropical forests are a very unlikely substrate allowing the formation of spore banks. Instead, they are densely covered with liverworts and the fern gametophytes are likely growing in these liverwort mats. Grammitids often occur in these kinds of habitats but other ferns without chlorophyllous spores occur in the same habitat. In addition, chlorophyllous spores occur in a few other clades of Polypodiaceae without causing rapid/adaptive radiations.

We found some evidence for key opportunities triggering radiations. The radiation of the core Polypodiaceae has already been mentioned. The second example already mentioned is the genus *Aglaomorpha*. The third example is the Neotropical genus *Serpocaulon* (Smith et al. 2006b; Kreier et al. 2008a). The diversification of this genus is likely linked with the rise of the higher Andean mountain ranges from Bolivia to Colombia (Kreier et al. 2008a). A similar hypothesis was previously proposed for the Neotropical fern clade comprising the genera *Eriosorus* and *Jamesonia* (Sanchez-Baracaldo 2004).

In conclusion, our study found some evidence for rapid radiations in ferns, but the dominant process of species diversity accumulation is more likely similar to a model of rare speciation events that are not related in time (Venditti et al. 2010). Speciation bursts triggered by single innovations or opportunities occur but may contribute less than 20% to the overall species diversity of the Polypodiaceae. We also found some evidence for the impact of climatic fluctuations on the diversity of ferns but this needs further investigation.

Acknowledgements This project was financially supported by the Deutsche Forschungsgemeinschaft (SCHN 758/2-1, SCHN 758/2-2) under the Schwerpunk SPP 1127. We are in debt to all the colleagues who made material accessible to us, especially Alan Smith, Michael Kessler, Masahiro Kato, Chie Tsutsumi, Peter Hovenkamp. We also thank the curators and staff of several herbaria and botanical gardens for their support.

References

Bollback JP (2006) SIMMAP: stochastic character mapping of discrete traits on phylogenies. BMC Bioinformatics 7:88

Bortolussi N, Durand E, Blum MGB, Francois O (2006) APTreeshae: statistical analysis of phylogenetic tree shape. Bioinformatics 22:363–364

Chan KMA, Moore BR (2002) Whole-tree methods for detecting differential diversification rates. Syst Biol 51:855–865

Clark JR, Ree RH, Alfaro ME, King MG, Wagner WL, Roalson EH (2008) A comparative study in ancestral range reconstruction methods: retracing the uncertain histories of insulare lineages. Syst Biol 57:693–707

Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 7:214

Gavrilets S, Loso JB (2009) Adaptive radiation: contrasting theory with data. Science 323:732–737

Gay H (1993) Animal-fed plants: an investigation into the uptake of ant-derived nutrients by the far eastern epiphytic fern *Lecanopteris* (Polypodiaceae). Biol J Linn Soc 50:221–233

Hall R (2002) Cenozoic geological and plate tectonic evolution of SE Asia and the SW Pacific: computer-based reconstructions and animations. J Asian Earth Sci 20:353–434

Haufler CH, Grammer WA, Hennipman E, Ranker TA, Smith AR, Schneider H (2003) Systematics of the ant-fern genus *Lecanopteris* (Polypodiaceae): testing phylogenetic hypotheses with DNA sequences. Syst Bot 28:217–227

Hodges SA, Arnold ML (1995) Spurring plant diversification: are floral nectar spurs a key innovation? Proc R Soc Lond B 262:343–348

Janssen T, Schneider H (2005) Exploring the evolution of humus collecting leaves in drynarioid ferns (Polypodiaceae, Polypodiidae). Plant Syst Evol 252:175–197

Janssen T, Bystriakova N, Rakotondrainive F, Coomes D, Labat J-N, Schneider H (2008) Neoendemism in Madagascan scaly tree ferns results from recent, coincident diversification bursts. Evolution 62:1876–1889

Janssen T, Kreier H-P, Schneider H (2007) Origin and diversification of African ferns with special emphasis on Polypodiaceae. Brittonia 59:159–181

Kreier H-P, Schneider H (2006a) Phylogeny and biogeography of the staghorn fern genus *Platycerium* (Polypodiaceae, Polypododae). Am J Bot 93:217–225

Kreier H-P, Schneider H (2006b) Reinstatement of *Loxogramme dictyopteris*, based on phylogenetic evidence, for New Zealand endemic fern, *Anarthropteris lanceolata*. Aust Syst Bot 19:309–314

Kreier H-P, Rex M, Weising K, Kessler M, Smith AR, Schneider H (2008a) Inferring the diversification of the epiphytic fern genus *Serpocaulon* (Polypodiaceae) in South America using chloroplast sequences and AFLPs. Plant Syst Evol 274:1–16

Kreier H-P, Rojas-Alvarado SAR, Schneider H (2007) *Hyalotrichopteris* is indeed a *Campyloneurum* (Polypodiaceae). Am Fern J 97:127–135

Kreier H-P, Zhang X-C, Muth H, Schneider H (2008b) The microsoroid ferns: inferring the relationships of a highly diverse lineage of Paleotropical epiphytic ferns (Polypodiaceae, Polypodiidae). Mol Phylogenet Evol 48:1155–1167

Nee S, May RM, Harvey PH (1994) The reconstructed evolutionary process. Philos Trans R Soc Lond B 344:305–311

Maddison WP, Maddison DW (2009) Mesquite version 2.72. http://mesquiteproject.org

Maddison WP, Midford PE, Otto SP (2007) Estimating a binary character's effect on speciation and extinction. Syst Biol 56:701–710

Mosburger V, Utescher T, Dilcher DL (2005) Cenozoic contiental climatic evolution of Central Europe. Proc Natl Acad Sci USA 102:14964–14969

Nylander JAA, Olsson U, Alstrom P, Sanmartin I (2008) Accounting for phylogenetic uncertainty in biogeography: a Bayesian approach to dispersal-vicariance analysis of the trushes (Aves: Turdus). Syst Biol 57:257–268

Otto EM, Janssen T, Kreier H-P, Schneider H (2009) New insights into the phylogeny of *Pleopeltis* and related Neotropical genera (Polypodiacae, Polypodiopsida). Mol Phylogenet Evol 53:190–201

Paradis E, Claude J, Strimmer K (2004) APE: analyses of phylogenetics and evolution in R language. Bioinformatics 20:289–290

Purvis A, Orme CDL, Toomey NH, Peason PN (2009) Temporal patterns in diversification rates. In: Butlin Rm Bridle J, Schluter D (eds) Speciation and patterns of diversity. Cambridge University Press, Cambridge, pp 278–300

Pybus OG, Harvey PH (2000) Testing macro-evolutionary models using incomplete molecular phylogenies. Proc R Soc Lond B 267:2267–2272
Rabosky DL (2006) LASER: a maximum likelihood toolkit for detecting temporal shifts in diversification rates from molecular phylogenies. Evol Bioinform 2:247–250
Rabosky DL, Lovette IJ (2008) Explosive evolutionary radiations: decreasing speciation or increasing extinction through time? Evolution 62:1866–1875
Ranker TA, Smith AR, Parris BS, Geiger JMO, Haufler CH, Struab SCK, Schneider H (2004) Phylogeny and evolution of grammitid ferns (Grammitidaceae): a case of rampant morphological homoplasy. Taxon 53:415–428
Ree RR (2005) Detecting the historical signature of key innovations using stochastic modles of character evolution and cladogensis. Evolution 59:257–265
Ricklefs RE (2009) Speciation, extinction and diversity. In: Butlin Rm Bridle J, Schluter D (eds) Speciation and patterns of diversity. Cambridge University Press, Cambridge, pp 257–277
Ronquist F (1997) Dispersal-vicariance analysis: a new approach to the quantification of historical biogeography. Syst Biol 46:195–203
Ronquist F, van der Mark P, Huelsenbeck JP (2009) Bayesian phylogenetic analysis using MrBayes. In: Lemey P, Salemi M, Anne-Mieke V (eds) The phylogenetic handbook: a practical approach to phylogenetic analysis and hypothesis testing. Cambridge University Press, Cambridge, pp 219–236
Salino A, Almeide TE, Smith AR, Navarro-Gomez A, Kreier H-P, Schneider H (2008) A new *Micrograma* (Polypodiaceae) from Brazil and recircumscription of the genus based on phylogenetic evidence. Syst Bot 33:630–635
Sanchez-Baracaldo P (2004) Phylogenetics and biogeography of the neotropical fern genera *Jamesonia* and *Eriosorus* (Peridaceae). Am J Bot 91:274–284
Sanderson M (2006) r8s version 1.71. Analyses of rates ("r8s") of evolution. http://loco.biosci.arizona.edu/r8s/
Schluter D (2000) The ecology of adaptive radiation. Oxford University Press, Oxford
Schmidt HA, von Haeseler A (2009) Phylogenetic inference using maximum likelihood methods. In: Lemey P, Salemi M, Anne-Mieke V (eds) The phylogenetic handbook: a practical approach to phylogenetic analysis and hypothesis testing. Cambridge University Press, Cambridge, pp 181–198
Schneider H, Kreier H-P, Hovenkamp P, Janssen T (2008) Phylogenetic relationships of the fern genus *Christiopteris* shed new light onto the classification and biogeography of drynariod ferns. Bot J Linn Soc 157:645–656
Schneider H, Kreier H-P, Perrie L, Brownsey PJ (2006a) The relationships of *Microsorum* (Polypodiaceae) species occurring in New Zealand. N Z J Bot 4(4):121–127
Schneider H, Kreier H-P, Wilson R, Smith AR (2006b) The *Synammia* enigma: evidence for a temperate lineage of polygrammoid ferns (Polypodiacae, Polypodiidae) in southern South America. Syst Bot 31:30–40
Schneider H, Schuettpelz E, Pryer KM, Cranfill Rm Magallon S, Lupia R (2004a) Ferns diversified in the shadow of angiosperms. Nature 428:553–557
Schneider H, Smith AR, Cranfill R, Hildebrand TJ, Haufler CH, Ranker TA (2004b) Unraveling the phylogeny of polygrammoid ferns (Polypodiaceae and Grammitidaceae): exploring aspects of the diversification of epiphytic plants. Mol Phylogenet Evol 31:1041–1063
Schuettpelz E, Pryer KM (2009) Evidence for a Cenozoic radiation of ferns in an angiosperm-dominated canopy. Proc Natl Acad Sci USA 106:11200–11205
Smith AR, Kreier H-P, Haufler CH, Ranker TA, Schneider H (2006a) *Serpocaulon* (Polypodiaceae), a new genus segregated from *Polypodium*. Taxon 55:919–930
Smith AR, Pryer KM, Schuettpelz E, Korall P, Schneider H, Wolf PG (2006b) A classification of extant ferns. Taxon 55:705–731
Swofford DL, Sullivan J (2009) Phylogenetic inference based on parsimony and other methods using PAUP*. In: Lemey P, Salemi M, Vandamme A-M (eds) The phylogenetic handbook: a

practical approach to phylogenetic analysis and hypothesis testing. Cambridge University Press, Cambridge, pp 267–288

van Uffelen G (1991) Fossil Polypodiaceae and their spores. Blumea 36:253–272

Venditti C, Meade A, Pagel M (2010) Phylogeneis reveal new interpretation of speciation and the Red Queen. Nature 463:349–352

Wang L, Qi X-P, Xiang Q-P, Heinrichs J, Schneider H, Zhang X-C (2010a) Phylogeny of paleotropical fern genus *Lepisorus* (Polypodiaceae, Polypodiopsida) inferred from four chloroplast regions. Mol Phylogenet Evol 52:211–225

Wang L, Wu Z-Q, Xiang Q-P, Heirnichs J, Schneider H, Zhang X-C (2010b) A molecular phylogeny and a revised classification of tribe Lepisoreae (Polypodiaceae) based on an analysis of four plastid DNA regions. Bot J Linn Soc 162:28–38

Zachus J, Pagani M, Solan K, Thomas E, Billups K (2001) Trends, rhythms, and aberrations in global climate 65 MA to present. Science 292:686–693

Evolution of the Mating System in the Genus *Capsella* (Brassicaceae)

Melanie Paetsch, Sara Mayland-Quellhorst, Herbert Hurka, and Barbara Neuffer

Abstract Changes in the mating system are thought to be one of the main driving forces in the evolution of plants. Cross-fertilization provides genetic recombination; self-fertilization enables autonomous dispersal. The genus *Capsella* comprises one of the worlds most successfull colonizing weeds and is a promising new object in the study of the evolution of the self-incompatibility (SI) system in natural populations. The objective of the study was to get insight into the dynamics of the SI system in the genus *Capsella* in general by employing molecular evidence. We wanted to understand how the transition from self-incompatibility to self-compatibility, which neutralized the local restriction of the ancestral SI species *Capsella grandiflora*, was achieved. Here, we present implications for a remarkable degree of flexibility of mating systems inherited in *Capsella* that allow these plants to combine the advantages of outcrossing and selfing. We discuss the consequences of our findings for speciation process in this genus.

1 Introduction

The evolution of hermaphrodite flowers in the early angiosperms demanded the simultaneous development of mechanisms that avoid the negative effects of inbreeding. Angiosperms, therefore, are thought to have evolved early in their history a multitude of mechanisms to prevent fertilization of ovules with pollen from the same flower or plant (Weller et al. 1995). Morphological structures promoting outbreeding are based on temporal and/or spatial separation of the genders' maturity status. In nearly half the angiosperms, self-fertilization is prevented most effectively through the actions of the self-incompatibility (SI) system,

M. Paetsch (✉), S. Mayland-Quellhorst, H. Hurka, and B. Neuffer
Dept. of Botany, University of Osnabrueck, Barbarastraße 11, 49076 Osnabrueck, Germany
e-mail: Melanie.Paetsch@biologie.uni-osnabrueck.de

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_5,

which is described by Darwin (1862) as "one of the most surprising facts I have ever observed." Whitehouse (1950) stated that the evolution of SI mechanisms may have played a substantial role in diversification and success of the angiosperms. However, many genera include both, outcrossing and selfing species. Therefore, one has to assume that the transition from ancestral self-incompatibility (SI) to self-compatibility (SC) occured frequently and independently. It is estimated that approximately 30% of angiosperms are predominantly selfing (Müntzing 1956; Stebbins 1956) and so we must conclude that, in some cases, the advantages gained by self-fertilization outweigh the disadvantages, such as inbreeding depression and lack of gene recombination. Due to assurance of seed set, gene combinations which prove successful will ensure continuance and genes will become fixed. The fixing of variants may have a local adaptive advantage. Shimizu et al. (2005) assumed that "subsequent adaptations in floral morphology correlated with the evolution of self-pollination can quickly evolve after the loss of self-incompatibility." A classic example for the rapid diversification induced by selfing, are the "microspecies" of the *Erophila verna* complex (Brassicaceae; Winge 1940; Rich 1991). Thus, besides hybridization and polyploidization, changes in the mating system are thought to be one of the main driving forces in the evolution of the Brassicaceae (Hurka et al. 2005)

1.1 The Mating System in Brassicaceae Genera

The Brassicaceae family became a model for studies of the sporophytic SI system (Fig. 1) , which is currently best understood in *Brassica* (e.g., Takayama and Isogai 2005) and, more recently, in the *Arabidopsis* species (Charlesworth et al. 2000; Kusaba et al. 2001; Mable et al. 2005; Bechsgaard et al. 2006). The genes responsible for the recognition and rejection of self-pollen are located at the *S*-locus. Three

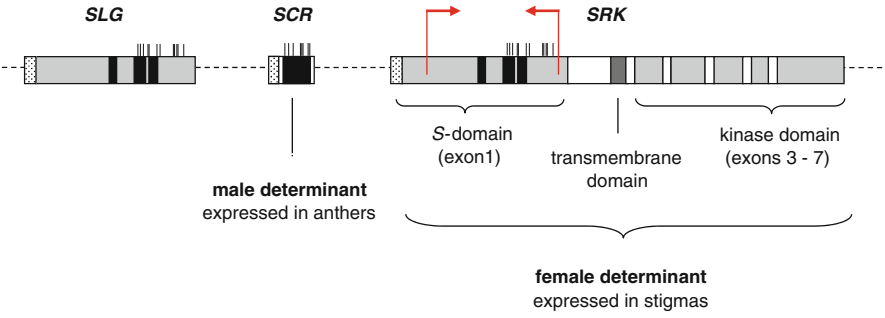


Fig. 1 Model of the *Brassica* S-locus. Designation, structure and arrangement of *S*-locus genes in *Brassica*. Hypervariable regions are shown by *black boxes*; *dotted boxes* mark the cleavable signal peptide, positions of the characteristic 12 conserved cystein residues are indicated by *black lines*. Approximate position of primers used in this study to amplify the *S*-domain in *Capsella* species partially is indicated by *red arrows*

highly polymorphic genes are involved in the SI response: *SLG* or *S*-locus glycoprotein (Nasrallah et al. 1985), *SRK* or *S*-locus receptor kinase (Stein et al. 1991), and *SCR*/SP11or *S*-locus cystein rich (Schopfer et al. 1999; Suzuki et al. 1999). The genes are tightly linked and behave as a single Mendelian locus, displaying multiple allelic versions, termed *S*-haplotypes (Nasrallah and Nasrallah 1993). The SI response is thought to occur when stigma and pollen share at least one allele. In the current model of the SI response, the pollen component *SCR* interacts with the extracellular domain of *SRK* protein in the stigma. The binding of the two proteins initiates a signaling cascade which leads to the inhibition of self-pollen by preventing hydration and further development of the pollen tube (e.g., Takayama and Isogai 2005). It has been demonstrated that *SLG* is not essential for the SI reaction, but it is thought to play a role in stabilizing the active *S*-receptor complex (Dixit et al. 2000) and may have evolved through partial duplication of *SRK* (Nasrallah 1994). *SRK* and *SLG* belong to the large family of *S*-genes, to the *S*-domain gene family or receptor-like kinase genes, respectively (Walker 1994). Members of this family share the structure shown in Fig 1. In *Brassica*, the majority of non-synonymous substitutions are clustered in three hypervariable regions which are believed to be involved in the determination of allele-specificity and thus might be an important factor in the generation of new *S*-haplotypes (Miege et al. 2001). *SRK* is known to be expressed in the papillar cells of the stigma 1–2 days before flower opening (Nasrallah et al. 1988).

The absence of an *SLG*-like gene in *Arabidopsis lyrata* suggests that the ancestral *S*-locus complex of crucifers consisted only of the two specificity determining genes *SRK* and *SCR* (Kusaba et al. 2001; Fobis-Loisy et al. 2004). In *Arabidopsis*, variable sites are not concentrated in three distinct hypervariable (HV) regions as in *Brassica* and the related genus *Raphanus* (Sakamoto et al. 1998; Lim et al. 2002), but are distributed more evenly over the complete *S*-domain sequence (Schierup et al. 2001). The *SRK*-alleles of *Brassica* and *Raphanus* are clearly subdivided into two classes, which represent dominant and recessive *S*-alleles (Chen and Nasrallah 1990; Kusaba et al 1997; Lim et al. 2002), whereas natural populations of *A. lyrata* have been shown to exhibit more complex dominance relationships (Mable et al. 2003; Prigoda et al. 2005). Furthermore, the chromosomal localization of the *S*-locus differs between Brassicaceae genera. In *Brassica*, the *S*-locus is located in a region that corresponds to a segment of *Arabidopsis thaliana* chromosome I (Conner et al. 1998), whereas in *A. lyrata* the position of the *S*-locus corresponds to *A. thaliana* chromosome IV (Kusaba et al. 2001). From this, it has been presumed that the *S*-locus was translocated in its entirety between two distant genomic locations (Kusaba et al. 2001).

1.2 The Genus Capsella *Is a Wild Relative of the Model Plant* Arabidopsis

The genus *Capsella* comprises three species, two diploids and one tetraploid. The diploid *C. grandiflora* (Fauché & Chamb.) Boiss. represents the ancestral obligate

outbreeding species from which *Capsella rubella* Reuter (2x) and *C. bursa-pastoris* (L.) Medik. (4x) originated (Hurka and Neuffer 1997). *C. grandiflora* is found only in western Greece and, rarely, in northern Italy. In *Capsella rubella*, the SI system broke down resulting in a predominantly selfing species with no change in the ploidy level. The breakdown of the SI system in *Capsella* coincides with colonizing ability and a wider distribution range (Fig. 2). *C. rubella* has colonized the Mediterranean climatic regions (Hurka and Neuffer 1997) and displays ecotypic differentiation with regard to such characters as the onset of flowering, prostrate versus erect types, and other features (Neuffer and Eschner 1995; Neuffer and Albers 1996). The most successful plant with the greatest colonizing ability in all man-made habitats, except for the humid tropics, is the tetraploid *C. bursa-pastoris*, which has adapted to a wide range of habitats and exhibits remarkable phenotypic plasticity (Hurka and Neuffer 1997; Hintz et al. 2006). The extensive genome conservation between the *Arabidopsis* species and *C. rubella* (Acarkan et al. 2000; Boivin et al. 2004; Koch and Kiefer 2005) allows the assumption that the *S*-locus in *Capsella* may also be comparable in both genera. As the SI research presented insufficient opportunity to compare findings in the *Arabidopsis* species to a closely related genus, the *Capsella* species received special attention. Thus, studies concerning the *Capsella* mating system have recently brought into focus (Paetsch et al. 2006; Nasrallah et al. 2007; Foxe et al. 2009). To date, at least 51 *S*-allele sequences have been identified in *C. grandiflora* (Bechsgaard and Schierup; Paetsch and Neuffer, unpublished data).

1.3 The Dynamic of the Mating System Provides Adaptive Potential

Mutations in the *SRK* gene as a source for the breakdown of the SI system were detected in both model genera *Brassica* (Goring et al. 1993) and *Arabidopsis* (Kusaba et al. 2001). Mutations affect the functionality of *S*-genes directly. A more subtle way of influencing the mating strategy is the ability to quantitatively vary the strength of the SI response. For example, in some self-incompatible species, older flowers tend to lose their ability to reject self-pollen. This observation has been termed "endseason-fertility" (Straub 1958) and is also known as a variant of pseudo-self-compatibility. Recently, Liu et al. (2007) succeeded in identifying a cryptic modifier which causes this phenomenon in *A. thaliana*. Several recent studies regarding variations in the strength of the SI response provide evidence, that the SI system has less rigid traits than predicted by the prevailing model (Mable et al. 2003, 2005; Mena-Ali and Stephenson 2007; Liu et al. 2007; Busch and Schoen 2008). This may be explained through the existence of a latent element of selfing in otherwise SI-species (Levin 1996; Brennan et al. 2005). On the other hand, in the predominantly selfing *C. bursa-pastoris*, outcrossing rates of up to 20% have been observed (Hurka and Neuffer 1997). Fobis-Loisy et al. (2004) termed

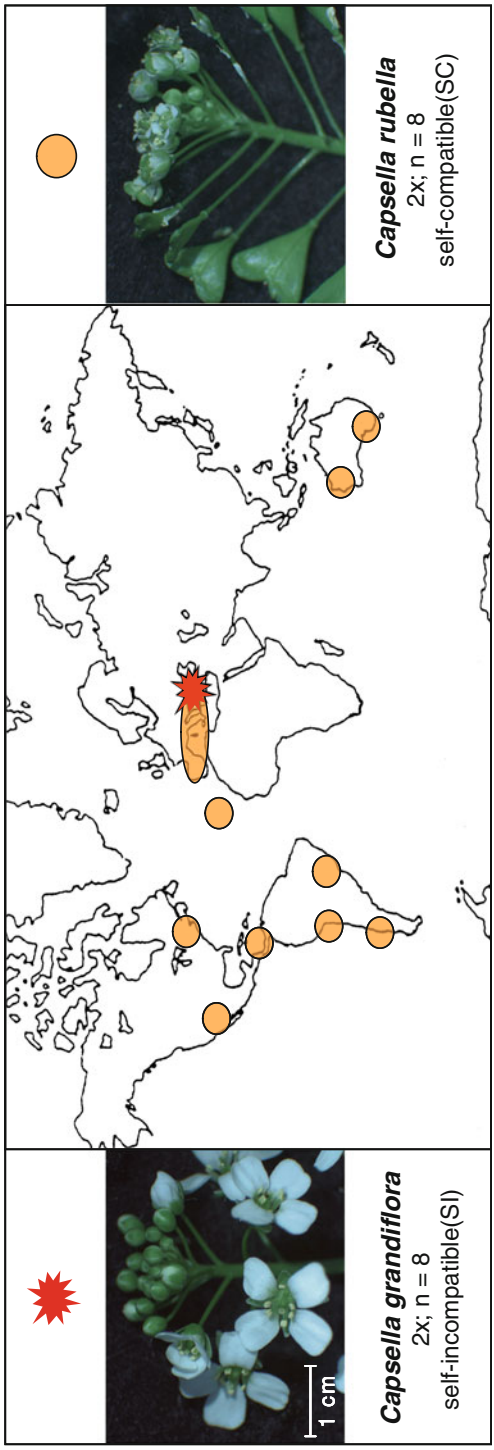


Fig. 2 Occurrence and flower morphology of diploid *Capsella*-species. Distribution of ancestral outcrossing species *Capsella grandiflora* is restricted to the western Balkans, the highly selfing species *Capsella rubella* has colonized worldwide in Mediterranean climates

this the "lable nature of the SI systems." Against this background, *Capsella* appears to be a promising new object and will contribute towards our understanding of the evolution of the SI system in natural populations, thereby provide insight into general speciation processes in the Brassicaceae.

2 Materials and Methods

2.1 Plant Material

The plant material used was collected individually from wild populations from different provenances throughout the distribution range of the *Capsella* species (*Capsella* Seed Collection, University of Osnabrueck; Table 1). Between 5 and 30 individuals of each population were raised in greenhouses.

2.2 Identification of S*-alleles in* C. grandiflora *and* C. rubella

2.2.1 DNA Extraction; PCR and Cloning

DNA was extracted from 0.5–1.0 g of leaf material using a CTAB protocol (Doyle and Doyle 1987, mod.) or the kit NucleoSpin Plant II (Macherey-Nagel). For PCR amplification, primers *SLG*f (5′-AGAACCTATGCATGGGTTGC-3′) and *SLG*r (5′-ATCTGACATAAAGATCTTGACC-3′) (Charlesworth et al. 2000) were used which were based on *Brassica S*-domain gene sequences and are known to amplify, among others, the *S*-domain of *SRK* in *A. lyrata*. After electrophoresis of PCR products in 1% agarose gel at 120 V, bands were stained with ethidiumbromid and

Table 1 Plant material

Population[a]	Provenance	Coordinates
Capsella grandiflora		
900	GR: Korfu: Acharavi	39°47′N, 19°48′E
923	GR: W-Peloponnes: Vrosina	39°39′N, 20°31′E
924	GR: W-Peloponnes: Botzaras	39°40′N, 20°35′E
926	GR: W-Peloponnes: Votonosi	39°46′N, 21°07′E
927	GR: W-Peloponnes: Metsovo	39°46′N, 21°10′E
934	GR: W-Peloponnes: Metsovo	39°46′N, 21°10′E
Capsella rubella		
774	I: Mt. Gargano	41°50′N, 16°00′E
1,241	ES: Sierra de Guadalupe	39°27′N, 5°19′W
1,249	P: Sintra near Lisboa	38°24′N, 7°23′W
1,504	ES: Canary Islands: La Palma	28°40′N, 17°52′W

[a]Population numbers according to *Capsella* Seed Bank (University of Osnabrueck)

examined under UV light. PCR fragments of the expected size (approximately 1 kb) were cloned in pCR 2.1 vector using the TA cloning kit (Invitrogen). Then, 10–50 separate clones were picked per individual, and plasmids were isolated using the NucleoSpin®Plasmid kit (Macherey-Nagel).

2.2.2 PCR-RFLP

PCR-fragments were cut from the vector through a restriction digest with EcoRI. Resulting bands were extracted from an agarose gel and re-amplified (see Sect. 2.2.1). Subsequently, the following restriction endonucleases were tested whether they had cut the isolated fragments in an *S*-allele-specific pattern: *Alu*I; *Apo*I; *Mbo*I; *Nde*I; *Pst*I; *Rsa*I; *Taq*$^{\alpha}$I; *Xba*I. Fragmentation was visualized by agarose gel electrophoresis. All restriction endonucleases were purchased from NEB (New England Biolabs) and treated as recommended.

2.2.3 Sequencing and Sequence Analyzes

Plasmids were sequenced in both directions using universal M13 primers. To avoid errors that may have occurred during PCR processing and sequencing, three independent clones obtained from the same individual that appeared to contain *SRK* candidate PCR fragments were sequenced. An ABI 377 automatic sequencer was used for DNA sequencing (Big Dye; Applied Biosystems). The resulting sequences were aligned to published *SRK*/*SLG* sequences of *Brassica* species, *Raphanus sativus*, *A. lyrata*, and *A. thaliana SRK* pseudogene as indicated by BLAST searches. A second member of the *S*-gene family, *ARK*3 of *Arabidopsis* species, which revealed similarities to *Capsella* sequences in BLAST searches was included in the alignment (Table 2). Sequences were aligned using BioEdit 5.0.9

Table 2 Accession numbers of Brassicaceae *S*-genes used in analyzes

Species	Genbank accession No.
Brassica	X79431, AB054815, AB326959 (*SLG*); X79432, AB054710, AB054718 (*SRK* classI) Y18259, Y18260, AB211197, AB211198 (*SRK* classII)
Raphanus	AB009677, AB009681, AB009684 (*SLG*); AB114846 AB114847, AB114849, AB114850, AB114852, AB114853 (*SRK*)
Arabidopsis lyrata	AB052755, AB052756, AF328990, AF328992, AF328994, AF328996-AF328998, AY186766- AY186768, AY186770, AY186777, DQ520281- DQ520285 (*SRK*)
Arabidopsis thaliana	NM118257 (ψ*SRK*); NM118258 (*ARK*3)
Capsella rubella	DQ534551 (*ARK*3)
Capsella grandiflora	DQ530637-DQ530642, EF530735, FJ613330-FJ3333 (*SRK*); DQ534558 (*ARK*3)

(Hall 1999), phylogenetic analyzes and distance analyzes were performed with the programs MEGA 2.1 (Kumar et al. 2001) and MrBayes 3.1.2 (Huelsenbeck and Ronquist 2003). Support for nodes in the tree was assessed by bootstrapping (1,000 replicates). Insight into the structure of sequences was gained through translation of DNA to amino acid sequences. The codon start of *Capsella* sequences is set in analogy to the known *SRK* sequences used in the alignment.

2.3 *Screening of BAC-Clones: Identification of* S-*locus in* C. rubella

A BAC library of *C. rubella* was kindly provided by J. Kroyman (MPI Jena). The library was screened by J. Kroymann with probes of the *SRK S*-domain of *C. grandiflora* and *ARK*3 *S*-domain of *C. rubella*, designed on the basis of our sequence information. Four BAC clones which have shown positive hybridization signals have been put at our disposal. The clones were digested with the restriction enzyme Hind III and fractionated through agarose gel electrophoresis. DNA fragments were transfered to a nylon membrane by Southern blotting. Digoxigenin-labeled probes (PCR DIG Labeling Mix; Roche Diagnostics) of the above-mentioned regions were used for the hybridization procedure. Hybridization signals were detected using anti-dig-antibodies conjugated with alkaline-phosphatase. Visualization was carried out colorimetrically using a BCIP/NBT-protocol. Positive fragments which hybridized with our probes were extracted from an agarose gel and subcloned in pGem3Z vector (Promega). After transformation (*E. coli* TOP10; Invitrogen), up to 50 clones per fragment were picked and digested with restriction enzymes to test for success of cloning and to reveal the inserts' size.

2.3.1 Analysis of the BAC Insert: Primer Walking

Hybridization signals marked a ~5-kb fragment of one BAC clone as a promising starting point for analysis of the BAC clone. Universal primers T7 and SP6 (Promega) were used for sequencing the fragment. Resulting sequences were aligned with corresponding genome regions of *A. thaliana* (BLAST). Based on this sequence information, a forward-primer was designed in order to sequence the 5 kb fragment step-by-step in its entirety. This primer has the sequence 5′-GAG GAA CAC AAG TCT TCG ATC-3′.

2.4 *Crossing Experiments*

2.4.1 Crossing Experiment I: Intraspecific Crossing

Thirty individuals of *C. grandiflora* population 934 (Table 1, Sect. 2.1) were raised in greenhouses. To avoid uncontrolled pollinations, plants were covered with permeable

plastic bags prior to flowering. The individuals were reciprocally cross-pollinated with each other as well as self-pollinated. Each cross was repeated three times. Success of crosses and the quality of seed set was assessed via measurement of fitness parameters such as fruit dimensions and seed counts. Measurement was performed using a stereomicroscope with the camera DFC 280 and the program IM50 (Leica, Germany). The crosses were accompanied by genotyping to identify the *S*-allelic composition of each individual. Genotyping was conducted as described in 2.2.

2.4.2 Crossing Experiment II: Interspecific Crossing

To test the hypothesis of Riley (1932, 1936) that in *Capsella* SC would be dominant over SI, we investigated the segregation of the F2 generation of an interspecific cross between *C. grandiflora* and *C. rubella*. Interspecific crossings between *C. grandiflora* (Pop. 926, Table 1, Sect. 2.1.1) and *C. rubella* (Pop. 1,504, Table 1, Sect. 2.1.1) had been performed previously (Hurka and Neuffer, unpublished). Six individuals of the F1 progeny were raised, which appeared to be almost fully self-compatible. The F2 seed material was sown and $\geq$16 F2 individuals were raised per F1 individual. To prevent uncontrolled pollinations, plants were covered with permeable plastic bags prior to flowering. Pollination with self-pollen was effected by carefully rubbing inflorescences within their plastic bags. Segregation of phenotypes in the F2 progenies was documented with regard to the compatibility status as well as size of flowers and fruits. The F2 progeny of one individual was subject to comparative studies of pollen tube growth. About 50 flowers per F2 individual were stained with decolorized aniline blue, following the method of Kho and Baër (1968). Slides were examined under the fluorescence microscope "Axioskop 20" (Zeiss, Germany) and development of pollen tubes was documented photographically with the camera RT Power Supply-SPOT and the program SPOT RT 3.0, SPOT Advanced (Zeiss, Germany).

3 Results

3.1 Identification of S-*Alleles*

Putative *SRK*-alleles in *C. grandiflora* were identified as previously described (Paetsch et al. 2006). Evidence for the identity of the sequences was gained through revelation of similarities in sequence and structure to publicized *S*-genes of other Brassicaceae taxa. The sequences show the 12 conserved cystein residues which are characteristic for *S*-domain genes. It became apparent that variable sites were not concentrated in three distinct HV regions, similar to the situation in *Arabidopsis* species (Schierup et al. 2001). Analyzes of sequence divergence revealed a high polymorphism between *Capsella S*-alleles, as expected for genes involved in

recognition processes. The mean pairwise distances of taxa groups (44 taxa; 769 sites) are 0.14–0.20 for *Raphanus*/*Brassica SRK*/*SLG*-alleles and 0.27–0.30 for *Arabidopsis*/*Capsella SRK*-alleles. The close relationship between *Capsella* and *Arabidopsis S*-alleles is reflected through the mixed clustering in the phylogenetic analysis (Fig. 3). To date, 18 *S*-alleles from *C. grandiflora* have been published

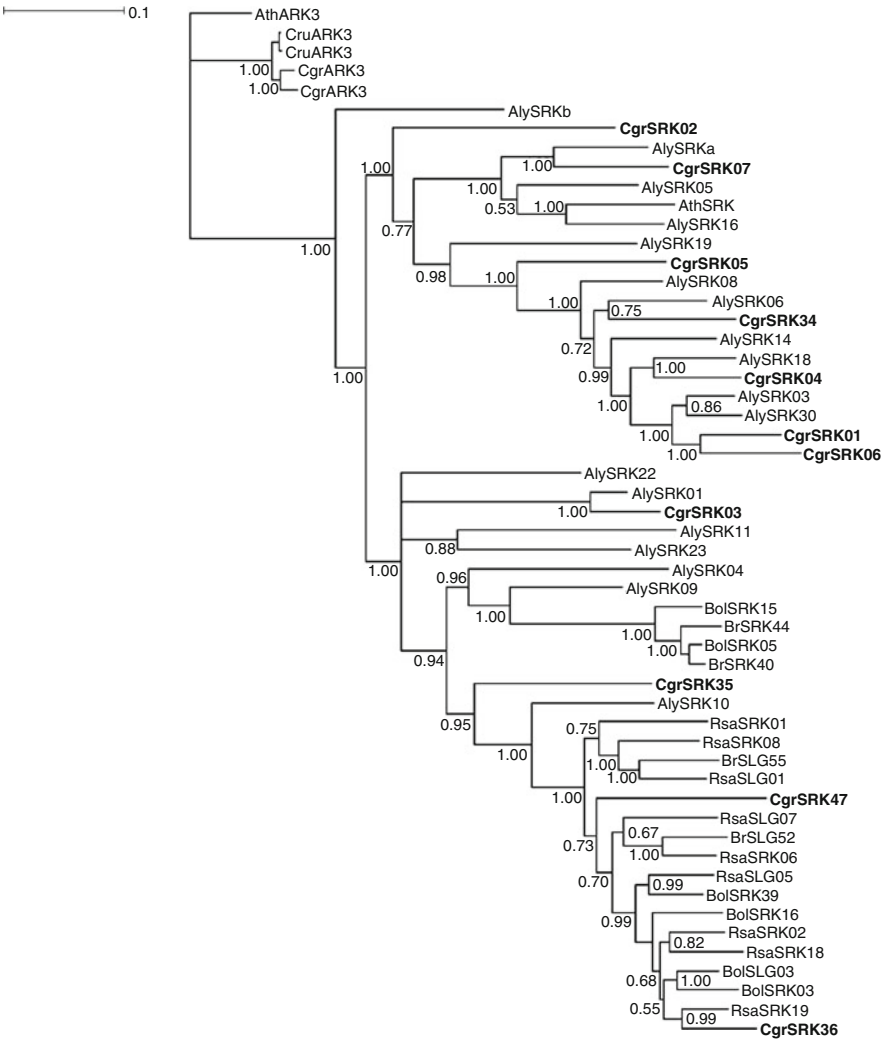


Fig. 3 Phylogram of Brassicaceae S-Genes. Phylogenetic relationships among *S*-genes of Brassicaceae taxa. The Bayesian 50% majority rule consensus tree is shown with mean posterior probability values (*clade credibilities*) on branches. The tree was rooted with *ARK*3 of *Arabidopsis* and *Capsella*. *S*-alleles of *C. grandiflora* (*bold letters*) cluster for the most part among those of *A. lyrata*, but a clear separation of the lineages *Brassica*/*Raphanus* and *Arabidopsis*/*Capsella* became blurred due to examples of trans-genus *S*-allele polymorphism (cf. Table 3)

(Paetsch et al. 2006; Nasrallah et al. 2007; Guo et al. 2009; this study). In *A. lyrata*, Prigoda et al. (2005) defined four dominance classes. Class A 1–3 *S*-alleles were shown to be dominant over Class B *S*-alleles, apart from a single exception, *AlySRK*01, which is recessive to all other *S*-alleles but is the most common *S*-allele in populations screened so far. Interestingly, the analysis of pairwise nucleotide distances between *S*-alleles of *Arabidopsis* and *Capsella* revealed that *AlySRK*01 and *CgrSRK*03 exhibit a very low sequence divergence of only 9% (Paetsch et al. 2006). Such a very low divergence is observed neither between *AlySRK*-alleles nor between *CgrSRK*-alleles. *C. grandiflora S*-alleles cluster in each of these dominance classes. And although analysis of the *Cgr S*-alleles dominance status is still outstanding, we conclude from these findings that both the *S*-allele polymorphism and the subdivision into dominance classes predated the separation of the genera *Arabidopsis* and *Capsella*. Most *SRK*-alleles of both genera are clearly separated from *Brassica* and *Raphanus S*-genes, whereas *CgrSRK*36 and *CgrSRK*47 are highly similar to *S*-genes of the *Brassica*/*Raphanus* lineage. Further cases of remarkably low *S*-allele sequence diversity among members of different genera have been reported recently (Nasrallah et al. 2007; Edh et al. 2009) and have been integrated into the NJ tree. Table 3 summarizes these examples of trans-genus *S*-allele polymorphism with regard to the sequence divergence (p-distances).

3.1.1 *S*-Allele Specific PCR-RFLP

PCR-RFLP has been shown to be a simple method for rapid differentiation between *S*-alleles and the identification of new *S*-alleles (Brace et al. 1994; Lim et al 2002). To establish the method in *Capsella*, several restriction enzymes were tested. Single digests with restriction enzymes *Apo*I and *Taq*$^{\alpha}$I were found to cut *S*-alleles in a characteristic pattern, which is exemplarily shown for *S*-alleles 1–5 in Fig. 4. These enzymes are also suitable for the differentiation between other *Capsella S*-alleles (data not shown).

3.2 *Screening of BAC-Clones: Identification of* S-*Locus Genes in* C. rubella

3.2.1 Identification of *C. rubella* 5-kb BAC fragment through comparison with *A. thaliana*

Restriction digests of *C. rubella* BAC clones and Southern blot analyzes lead to the identification of a ~5-kb restriction fragment that hybridized with DIG-labeled probes from the *SRK*/*ARK*3 *S*-domain of the *Capsella* species. The insert was partially sequenced using the primer walking technique. BLAST searches revealed high homologies to *Arabidopsis S*-genes. An alignment with *A. thaliana* genomic

Table 3 Trans-genus *S*-allele polymorphism

	***AlySRK*01**	*CgrSRK*03	***AlySRK*a**	*CgrSRK*07	***RsaSRK*19**	*CgrSRK*36	***RsaSRK*02**	*CgrSRK*47	***BolSRK*05**
***CgrSRK*03**	**0.08**	–	–	–	–	–	–	–	–
*AlySRK*a	0.29	0.31	–	–	–	–	–	–	–
***CgrSRK*07**	0.30	0.32	**0.15**	–	–	–	–	–	–
*RsaSRK*19	0.30	0.31	0.30	0.31	–	–	–	–	–
***CgrSRK*36**	0.32	0.33	0.32	0.31	**0.09**	–	–	–	–
*RsaSRK*02	0.29	0.31	0.31	0.30	0.09	0.10	–	–	–
***CgrSRK*47**	0.33	0.34	0.35	0.35	0.18	0.19	**0.17**	–	–
*BolSRK*05	0.31	0.30	0.33	0.33	0.28	0.29	0.28	0.30	–
***AlySRK*09**	0.26	0.27	0.30	0.30	0.26	0.26	0.25	0.28	**0.20**

[a]Examples for low sequence divergence between *S*-alleles of different Brassicaceae genera given in bold letters

[b]Pairwise distances; 801 bp

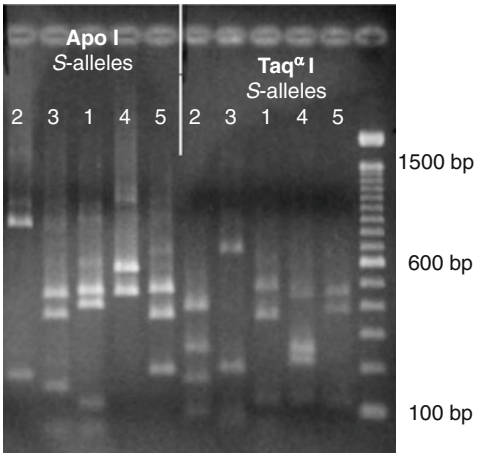


Fig. 4 S-allele-specific PCR-RFLP. Restriction enzymes *Apo*I and *Taq*$^{\alpha}$I cut *Capsella grandiflora S*-alleles *CgrSRK*01-05 in characteristic pattern

DNA showed that the ~5-kb insert of *C. rubella* corresponds to the *S*-locus containing region of *A. thaliana*, mainly to the gene At4g21380 (*ARK*3) which directly flanks the *SRK* pseudogene in *A. thaliana*. From the comparative mapping studies of Koch and Kiefer (2005), it is possible to deduce that the *S*-locus in the *Arabidopsis* species is located between the markers 60 and 61 (Fig. 5). These markers span the region between At4g27070 (marker 60: TSB2) and At4g12480 (marker 61:CH 42) (numbers of markers from Kuittinen et al. 2004). In *A. lyrata*, the region between these markers is located on linkage group 7, and in *C. rubella*, this region is found in its entirety on *C. rubella* linkage group G. We therefore conclude that the position of the *S*-locus in *Capsella* corresponds to that of *A. thaliana*, where it is located at chromosome IV.

3.3 Frequency and Dominance Relationships of S*-Allele*

3.3.1 Crossing Experiment I: Intraspecific Crossing

Extensive intraspecific crossings within one natural population of *C. grandiflora* were performed in order to search for further *S*-alleles, to determine *S*-allele frequencies and to reveal dominance relationships. Each combination of parents was pollinated reciprocally (repeated three times), including self-pollination of each individual. Phenotypic habit was documented, seed material of F1 was collected and analyzed for fertility parameters (fruit size, number of seeds per fruit; Fig. 6) to estimate the quality of compatibility. Parent plants were genotyped as described above (Sect. 2.1.2). The final analyzes comprise 18 individuals, in which we succeeded in identifying at least one *S*-allele. For correlation of crossings with genotypes, see Fig. 6. SI in this particular population appeared to function with a very low number of different *S*-alleles, even though we did not succeed in

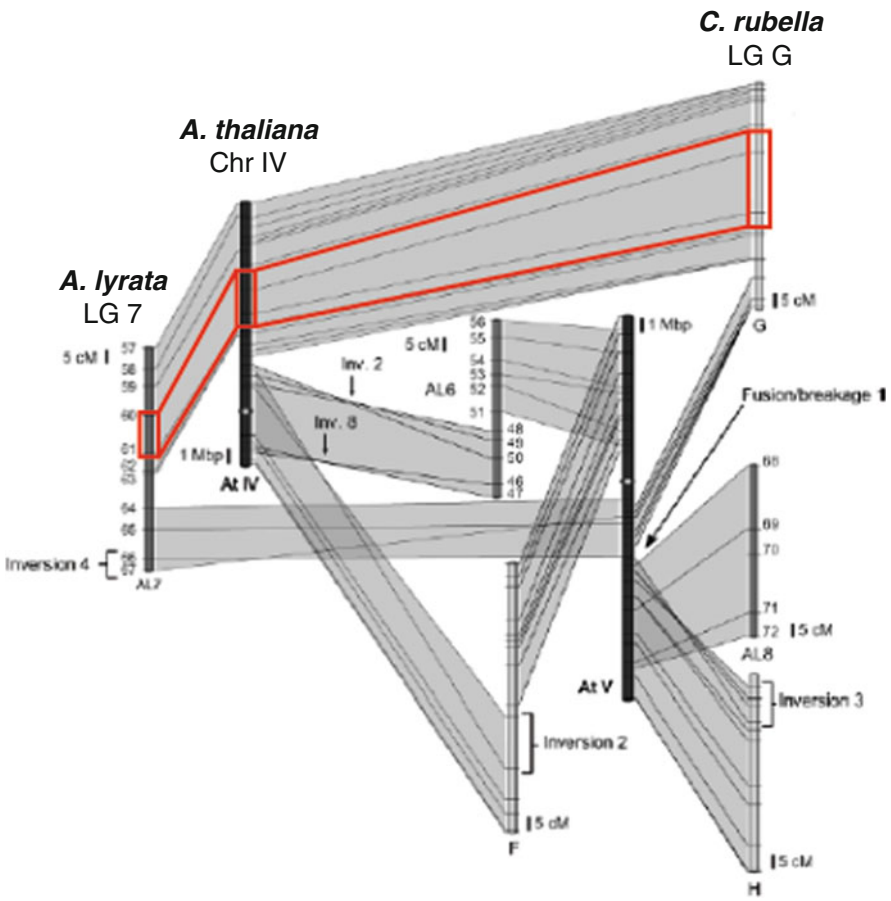


Fig. 5 Genome map of *Arabidopsis* species and *C. rubella* (Koch and Kiefer 2005, mod.). *Red frames* show the region between markers 60 and 61, which contains the *S*-locus in *Arabidopsis* species and which corresponds in its entirety to linkage group G of *C. rubella*

amplifying both *S*-alleles in all individuals. Five *S*-alleles have been identified so far: *SRK*01 (2x), *SRK*03 (4x), *SRK*05 (1x), *SRK*06 (9x), *SRK*34 (8x). A further interesting result of this study was that some individuals set seed when manually self-pollinated (pseudo-self-compatibility).

3.3.2 Crossing Experiment II: Interspecific Crossing

None of the six F2 progeny segregated clearly 3:1 for dominance of SC. All F2 progeny segregated significantly 1:2:1 (χ^2-tested) showing a high proportion of unusual intermediate phenotypes (Fig. 7). Thus, in natural populations of the *Capsella* species, Riley's hypothesis (1932; 1936; Sect. 2.4.2) could not be substantiated. Within the intermediates, two forms of appearance were observed: those

a

	maternal individual		2–1	2–3	2–4	3–1	3–2	3–3	9–1	9–3	12–3	13–1	14–3	15–3	19–1	25–4	26–1	28–1	32–1	32–2
	allele 1		SRK06	SRK01	SRK03	SRK06	SRK03	SRK06	SRK03	SRK34	SRK34	SRK34	SRK03	SRK06	SRK34	SRK06	SRK06	SRK34	SRK01	SRK06
paternal individual		allele 2	n.f	n.f	n.f	n.f	n.f	n.f	n.f	n.f	n.f	SRK06	n.f	SRK05	SRK06	SRK34	SRK34	n.f	n.f	SRK34
2–1	SRK06	n.f	−−−	−−−	+++	+++	+++	+++	+++	+++	#++	+#+	+++	−−−	−−+	+++	+++	+++	#−−	n.f
2–3	SRK01	n.f	−−−	−−−	n.f	−−0	−−	−−−	−−−	−−−	+++	−−	+	−~0	+++	~~~	+++	+++	−−0	+++
2–4	SRK03	n.f	+++	+++	−−−	+++	+++	+++	+++	+++	−−−	+++	+++	−−−	n.f	~##	+++	−0#	+++	+++
3–1	SRK06	n.f	++	−−−	+++	−−−	#+	n.f	+++	++	+++	#0#	+#+	−−−	+++	+++	−−−	#+#	#++	+++
3–2	SRK03	n.f	+++	−−−	0++	+++	0	+++	−−0	+++	+++	+++	−−+	−−−	#++	+++	+++	++	+##	+
3–3	SRK06	n.f	++	−−−	+++	+++	0+0	−−−	+++	+++	n.f	−−#	+++	0~~	+++	~~~	0++	−##	+++	0++
9–1	SRK03	n.f	+++	−+0	+++	++	−−−	+++	−−−	−−+	++	+++	−++	−−−	+++	+++	00−	+++	##+	+++
9–3	SRK34	n.f	+++	−0#	+++	+++	+++	−−−	+++	+++	+++	−−#	−−	−~~	+++	+++	+++	+++	+++	+++
12–3	SRK34	n.f	+++	−−	+++	+++	+++	+++	+++	+++	n.f	0+#	−−−	00~	+++	+++	−++	−00	+++	+++
13–1	SRK34	SRK06	n.f	−−−	−#+	#−−	++	+++	+#+	+++	+++	−−−	++	~~~	−++	++0	−−−	+++	#+	+++
14–3	SRK03	n.f	+++	+#+	++	+x	+++	+++	n.f	+++	−−−	+++	n.f	~~~	+++	#+#	−++	+++	+++	+++
15–3	SRK06	SRK05	n.f	−	−−−	−	−−0	−	−0	−−0	−−	n.f	n.f	−−−	−−	−−−	n.f	−−−	−##	n.f
19–1	SRK34	SRK06	+++	+++	+++	+++	+++	#−#	+++	+++	+++	##+	+++	~~−	n.f	+++	+++	−##	++#	+++
25–4	SRK06	SRK34	+++	−−−	+++	+++	+++	−−−	+++	#++	+++	+++	−++	−−−	++	−−−	+++	++	#+#	+++
26–1	SRK06	SRK34	#−−	+++	+++	−−0	+++	##	++	+++	+++	−−−	0++	−−	#++	###	−−0	+++	+++	+++
28–1	SRK34	n.f	+++	−	+++	0#0	+++	−−−	+++	+++	+++	++#	−++	−−−	+#−	+++	+++	n.f	##	+++
32–1	SRK01	n.f	−−−	−−−	#++	+++	n.f	+++	+++	+++	+++	+++	−−−	−~0	+++	+++	+++	+++	n.f	+−#
32–2	SRK06	SRK34	+++	+++	+++	+++	+++	++#	+++	+++	+++	+++	−−	−−0	+++	+++	−−0	+++	++	n.f

b

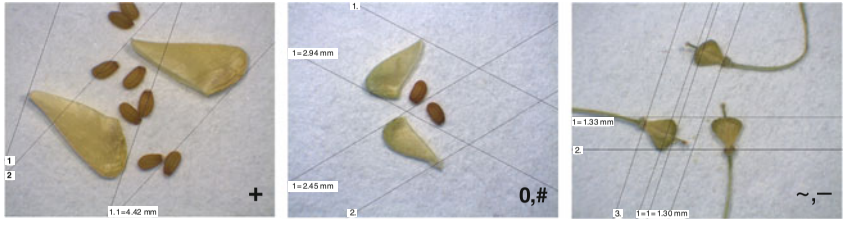


Fig. 6 Intraspecific crossing experiment within a natural *Capsella grandiflora* population. Eighteen individuals of *Capsella grandiflora* population no. 934 were crossed reciprocally with each other three times. (**a**) Individual *S*-allelic composition was determined as far as possible (*colored boxes*). (**b**) Success and quality of crosses between partners is assessed via parameters of seed set and fruit dimensions (+ compatible; o, #, x partially compatible; -, ∼ incompatible. Missing data are marked by "*n.f.*" Conspicuous is not only the unexpected high proportion of compatible crosses but also the low number of various *S*-alleles occurring at high frequencies

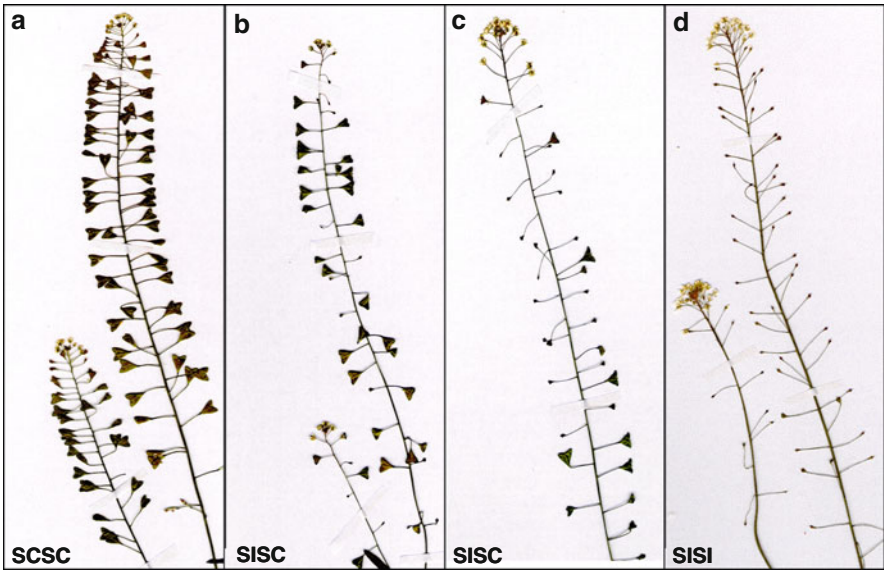


Fig. 7 Interspecific crossing experiment between *C. grandiflora* x *C. rubella***.** The F2 progeny segregated phenotypically 1:2:1 with (**a**) self-compatible, SCSC homozygous, (**b**) and (**c**) intermediate, SISC heterozygous, (**d**) self-incompatible, SISI homozygous phenotypes. Hypothetic *S*-genotypes of F2 hybrids were deduced from phenotypic habit and the ability to autonomous seed set

which had more sterile than fertile fruits, and those which had more fertile than sterile fruits. To analyze the basic process within the stigma and ovules, we performed histological examination of pollen tube growth within gynoeceum via fluorescence microscopy. In comparison to *C. rubella*, all F2 individuals show signs of interference in pollen tube development, namely in pollen tube guidance. Pollen tubes seemed to be coiled after leaving the stylus region and are wound around the stems of the ovules before they enter the micropyle (Fig. 8). This phenomenon accumulates in the intermediate phenotypes and might serve as an explanation for the irregular fertilization of ovules which causes the intermediate appearance. A further remarkable observation of this study was that at least one pollen tube developed in each F2 individual.

4 Discussion

The project described here was the first study which dealt with the SI system in the genus *Capsella* at the molecular level. Due to the close relationship between *Capsella* and the model plant *Arabidopsis*, our studies contributed fundamentally to the understanding of the evolution of the *S*-locus in the Brassicaceae. Initial evidence for the identification of putative *S*-alleles in *Capsella* has been gained

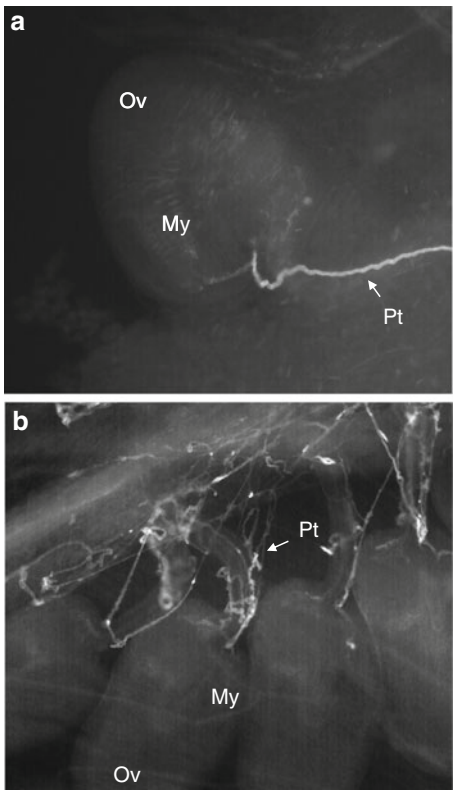


Fig. 8 Pollen tube growth after autonomous self-pollination in interspecific F2 hybrids. Normal pollen tube growth in (**a**) *Capsella rubella* and imperfect pollen tube guidance in (**b**) interspecific F2 hybrid between *C. grandiflora* x *C. rubella*. *Ov* ovule, *My* micropyle, *Pt* pollen tube

from similarities in sequence and structure of published Brassicaceae *S*-genes (Paetsch et al. 2006). The putative *Capsella S*-alleles exhibit the characteristic elements of *S*-locus receptor kinase proteins, and they cluster, for the most part, between those of *A. lyrata*. Brassicaceae SI systems were thought to evolve differently in at least two lineages: the Brassiceae lineage including species of the genera *Brassica* and *Raphanus*, and a lineage containing species of the genus *Arabidopsis*. Our results in *Capsella* SI research provide a decisive indication which confirms the existence of the two different lineages (Paetsch et al. 2006). It has been revealed that the chromosomal location of the *S*-locus, *S*-gene composition (presumably lacking the *SLG*), and *S*-allele polymorphism is highly similar in the genera *Capsella* and *Arabidopsis*. Furthermore, these genera appear to share ancient *S*-allels. On the basis of extensive sequence identity, it is possible that the *C. grandiflora SRK*03 and the *A. lyrata SRK*01 may have descended from one ancestral haplotype that existed before the divergence of *Capsella* and *Arabidopsis* (Paetsch et al. 2006). The studies of Nasrallah et al. (2007) regarding the low genetic diversity and the common genetic structure of *S*-genes in *C. grandiflora SRK*07 and *A. lyrata S*a haplotypes underlined this interpretation. Even more exciting is the finding that *C. grandiflora SRK*36 and *SRK*47 are highly similar to *SRK*-alleles of *R. sativus*. They may represent a common ancestor of the lineages of *Brassica*/

Raphanus and *Arabidopsis*/*Capsella*. Recently, Edh et al. (2009) concluded from the similarity between *AlySRK*09 and *Brassica* classII *S*-alleles that trans-generic polymorphisms at the *SRK* gene exist, which span the lineages of *Arabidopsis* and *Brassica*. All these examples of trans-genus *S*-allele polymorphism (Table 3) undermine the current clear separation of the lineages *Brassica*/*Raphanus* and *Arabidopsis*/*Capsella*, indicating that the polymorphism of *S*-haplotypes may even have predated the split of the lineages. This scenario is supported by the findings of Charlesworth et al. (2006) who found evidence for the maintenance of *S*-haplotypes for long evolutionary times as the putative result of extreme suppression of recombination in the S-locus containing region.

4.1 *The Chromosomal Location of the* Capsella S-*Locus is Similar to* Arabidopsis

We succeeded in identifying a restriction fragment of a *C. rubella* BAC clone that most likely contains parts of the *S*-locus, namely the region that corresponds to the *S*-locus genes At4g21390 to At4g21380 (=*ARK*3) on *A. thaliana* chromosome IV and *A. lyrata* LG 7. Considering the extensive genome conservation between *A. thaliana* chromosome IV and *C. rubella* linkage group G (Koch and Kiefer 2005) as well as the suppression of recombination at the *S*-locus (Kamau and Charlesworth 2005; Charlesworth et al. 2006), our findings allow a rough determination of the chromosomal position of *S*-locus genes in *C. rubella*. If the *Capsella S*-locus were more similar to that of the *Brassica*/*Raphanus* lineage, the sequences gained from the BAC fragment should correspond to *A. thaliana* chromosome I (Conner et al. 1998). As this is not the case, we conclude that the chromosomal location of the *S*-locus in *Capsella* is similar to that in *Arabidopsis*. This assumption was recently confirmed by studies of Nasrallah et al. (2007), who showed a similar genomic organization between two haplotypes of *A. lyrata* and *C. grandiflora*. They revealed similar positioning and spacing of *S*-genes in *C. grandiflora S*7 haplotype (i.e., *S*-genes *CgrSRK*07 and *CgrSCR*07) and the *A. lyrata S*a haplotype (Kusaba et al. 2001). Our findings could not conclusively unravel the question whether the translocation event occurred in the lineage of *Brassica* or that of *Arabidopsis*, but they show that the chromosomal position of the *S*-locus in *Arabidopsis* is not restricted to the genus *Arabidopsis* alone. This may support the prevailing opinion that the translocation event is more likely to have happened in the *Brassica* containing lineage, and that the *Arabidopsis S*-locus represents the ancestral condition (Kusaba et al. 2001; Edh et al. 2009).

4.2 *Natural Populations of* Capsella *Exhibit Flexible Mating Strategies*

We performed inter- and intraspecific crossings within and between *Capsella* species in order to determine number and frequency of *S*-alleles in a natural

population, in order to gain insight into dominance relationships of *S*-alleles, and to test the hypothesis whether SC is dominant over SI. The existence of an SC allele which is dominant over SI alleles was postulated by Riley (1932, 1936, see Sect. 2.4.2). To test this hypothesis, we performed interspecific crosses between individuals of *C. rubella* (SC) and *C. grandiflora* (SI) from natural populations. Six F1 hybrid plants were raised and all showed autonomous self-fertilization with nearly full seed set. The F1 seeds were used to raise F2 progeny individuals. F2 plants were expected to segregate 3:1 for self-compatibility if Riley's assumptions were correct. As this was not the case, we conclude that the hypothesis of SC being simply dominant over SI has not been corrobarated in our studies, which is in contrast to other reports (Nasrallah et al. 2007). The genetic disharmony between the genomes of the species, which became obvious in the interference of pollen tube guidance, prevent the segregation results from clear interpretation. However, one of the most interesting observations of this study is that at least one pollen tube developed successfully in each F2 individual. Even those individuals that were presumably homozygous for SI (Fig. 7d) could not prevent pollen grains from germinating in every case. We explained this phenomena by presuming "strong" and "weak" SI phenotypes, depending on the single *S*-allele carried by the F2 hybrids. These phenotypes are observed in general in natural *C. grandiflora* populations, where hybridization between the sympatrically occurring species happens in general (personal observation of the authors). The irregular fertilization of ovules in F2 hybrids shows that the SI response is occasionally passed by. The study of Foxe et al. (2009) implies that the split between these *Capsella* species had been a rather young event; their divergence estimate for the separation of the selfing *C. rubella* from the outcrossing *C. grandiflora* is 18,500 years. Here, the breakdown of the SI system appears to cause speciation directly. Thus, reproductive barriers between the species are likely to be established, but those did not lead to complete prevention of hybridization between diploid *Capsella* species.

In an intraspecific crossing experiment within a natural *C. grandiflora* population, genotyping provides evidence that SI in this particular population functions with a low number of various *S*-alleles (five *S*-alleles identified so far). Furthermore, we observed that more crosses resulted in seed set than should be expected when few *S*-alleles appear at high frequencies. Sharing of at least one allele between two crossing partners does not guarantee the triggering of the SI response, which is in contrast to the model genus *Brassica*. From this, we therefore assume complex dominance relationships with few *S*-alleles occuring at high frequencies, and a high proportion of more or less recessive *S*-alleles in *Capsella*. Our findings are in concordance with other studies in wild populations of genera with sporophytic SI (Mable et al. 2003, 2005; Brennan et al. 2003, 2005). Low numbers of *S*-alleles in natural populations have been shown to be sufficient to maintain populations of the self-incompatible species *Senecio squalidus* L. (Asteraceae) (Brennan et al. 2003). Rare *S*-alleles would have mating advantages, as self-incompatibility alleles underly negative frequency-dependent selection (Schierup and Vekemans 2008).

4.3 Evolutionary Consequences of Flexible Mating Strategies

The overall result of the crossing experiments is the finding, that the mating system in natural populations of *Capsella* reacts much more flexibly than predicted by the model system *Brassica*. This is to be assumed from the deduced high proportion of recessive *S*-alleles or "weak" *S*-alleles, whose SI-response is less strong. Accumulation of recessive *S*-alleles may lead to weakening of the SI-response up to and including for the occurance of selfing. From both the intra- and the interspecific crossing experiment, we conclude that the predominantly outcrossing *C. grandiflora* exhibits pseudo-self-compatibility, which is considered to be a mixed-mating strategy (e.g., Sherman-Broyles and Nasrallah 2008). The evolution of pseudo-self-compatibility or self-compatibility is a measure of ensuring reproductive success should a population dwindle away following a bottleneck (Levin 1996; Busch and Schoen 2008). Flexibility, being inherent in the *Capsella* mating system in general, may also be reflected in the finding that reproductive barriers in interspecific hybrids of *Capsella* are incomplete. Recently, Slotte et al. (2008) found evidence for hybridization and introgression after polyploidization in the predominantly selfing *Capsella bursa-pastoris*. They conclude that gene flow between *C. rubella* and *C. bursa-pastoris* might have contributed to the great genetic variability in *C. bursa-pastoris*. The maintainance of intraspecific outcrossing to a certain degree, and therewith the assurance for genetic recombination, would facilitate ecotypic differentiation, which is thought to have occurred recently with regard to flowering time in *Capsella* (Ceplitis et al. 2005). The potential to adapt the predominant mating strategy to the availability of mates or pollinators capacitates a flexible reaction to changes in the environment which would necessarily be an important trigger in subsequent speciation. Cheptou et al. (2002) reported on the partial breakdown of SI and subsequent full recovery of SI during the colonization history of *Crepis sancta* (Asteraceae). Summing up the findings of our inital SI studies we assume that in natural populations of the genus *Capsella* the transition from SI to SC does not inevitably lead to an "evolutionary dead end," and that flexibility of the mating system is likely to have played or is still playing an important role in the genus' speciation processes.

5 Summary

Our studies contributed to the understanding of the evolution of the *S*-locus in natural populations in general, as Brassicaceae SI research lacked a genus with which to compare the results gained from the model genus *Arabidopsis*. Molecular data from the *Capsella S*-locus filled this gap and corroborated the assumption that the SI system in the Brassicaceae evolves differently in at least two lineages, one containing *Brassica*/*Raphanus* and the other containing *Arabidopsis*/*Capsella*. This is to be concluded from the findings of close similarities to *Arabidopsis* species rather than to the model genus *Brassica* with regard to chromosomal location,

structure of the *S*-locus, *S*-allele polymorphism, and dominance interactions between *S*-alleles. Furthermore, the finding of trans-specific and trans-generic *S*-allele polymorphisms between both lineages underlines the antiquity and conservation of the *S*-locus containing region. The *Capsella* mating system appears to provide opportunities for mixed-mating, combining the advantages of both, and assurance of seed set through selfing, as well as genome recombination through maintainance of outcrossing and hybridization. This is especially true for the self-incompatible *C. grandiflora*, which exhibits pseudo-self-compatibility, and for the predominantly selfing polyploid *C. bursa-pastoris* showing striking phenotypic and genotypic variability.

Acknowledgments The authors thank Jürgen Kroymann for screening and providing *C. rubella* BAC clones, and Deborah Charlesworth, Marcus A. Koch, Walter Bleeker and Pieternella *C.* Luttikhuizen for helpful discussions. Thanks go to Ulrike Coja for technical assistance, Lucille Schmieding for language (english) editing and to the student assistants Sylvia Holdt and Sonja Bicker, who contributed valuable results, and to Andre Lipski, who made the use of the fluorescence microscope possible. Furthermore, we acknowledge the help from the team of the Botanical Garden Osnabrueck and we thank the DFG for financial support.

References

Acarkan A, Rossberg M, Koch M, Schmidt R (2000) Comparative genome analysis reveals extensive conservation of genome organisation for *Arabidopsis thaliana* and *Capsella rubella*. Plant J 23:55–62

Bechsgaard JS, Castric V, Charlesworth D, Vekemans X, Schierup MH (2006) The transition to self-compatibility in *Arabidopsis thaliana* and evolution within *S*-haplotypes over 10 million years. Mol Biol Evol 23:1741–1750

Boivin K, Acarkan A, Mbulu RS, Clarenz O, Schmidt R (2004) The *Arabidopsis* genome sequence as a tool for genome analysis in Brassicaceae. A comparison of the *Arabidopsis* and *Capsella rubella* genomes. Plant Physiol 135:735–744

Brace J, King GJ, Ockendon DJ (1994) A molecular approach to the identification of *S*-alleles in *Brassica oleracea*. Sex Plant Reprod 7:203–208

Brennan A, Harris S, Hiscock S (2003) The population genetics of sporophytic self-incompatibility in *Senecio squalidus* L. (Asteraceae): avoidance of mating constraints imposed by low S-allel number. Philos Trans R Soc Lond B 358:1047–1050

Brennan AC, Harris SA, Hiscock SJ (2005) Modes and rates of selfing and associated inbreeding depression in the self-incompatible plant *Senecio squalidus* (Asteraceae): a successful colonizing species in the British Isles. New Phytol 168:475–486

Busch JW, Schoen DJ (2008) The evolution of self-incompatibility when mates are limiting. Trends Plant Sci 13:128–136

Ceplitis A, Su Y, Lascoux M (2005) Bayesian inference of evolutionary history from chloroplast microsatellites in the cosmopolitan weed *Capsella bursa-pastoris* (Brassicaceae). Mol Ecol 14:4221–4233

Charlesworth D, Awadalla P, Mable BK, Schierup MH (2000) Population-level studies of multiallelic self-incompatibility loci, with particular reference to Brassicaceae. Ann Bot (Supp A) 85:227–239

Charlesworth D, Kamau E, Hagenblad J, Tang C (2006) Trans-specificity at loci near the self-incompatibility loci in *Arabidopsis*. Genetics 172:2699–2704

Chen CH, Nasrallah JB (1990) A new class of *S*-sequences defined by a pollen recessive self-incompatibility allele of *Brassica oleracea*. Mol Gen Genet 222:241–248

Cheptou PO, Lepart J, Escarre J (2002) Mating system variation along a successional gradient in the allogamous and colonizing plant *Crepis sancta* (Asteraceae). J Evol Biol 15:753–762

Conner JA, Conner P, Nasrallah ME, Nasrallah JB (1998) Comparative mapping of the *Brassica S* locus region and its homeolog in *Arabidopsis*. Implications for the evolution of mating systems in the Brassicaceae. Plant Cell 10:801–812

Darwin C (1862) On the two forms, or dimorphic condition, in the species of *Primula*, and on their remarkable sexual relations. J Proc Linn Soc Bot 6:77–96

Dixit R, Nasrallah ME, Nasrallah JB (2000) Post-transcriptional maturation of the *S* receptor kinase of *Brassica* correlates with co-expression of the *S*-locus glycoprotein in the stigmas of two *Brassica* strains an in transgenic tobacco plants. Plant Physiol 124:297–312

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull Bot Soc Am 19:11–15

Edh K, Widen B, Ceplitis A (2009) The evolution and diversification of *S*-locus haplotypes in the Brassicaceae family. Genetics 181:977–984

Fobis-Loisy I, Miege C, Gaude T (2004) Molecular evolution of the *S* locus controlling mating in the Brassicaceae. Plant Biol 6:109–118

Foxe JP, Slotte T, Stahl EA, Neuffer B, Hurka H, Wright SI (2009) Recent speciation associated with the evolution of selfing in *Capsella*. Proc Natl Acad Sci USA 106:5241–5245

Goring DR, Glavin TL, Schafer U, Rothstein S (1993) An *S* receptor kinase gene in self-compatible *Brassica napus* has a 1-bp deletion. Plant Cell 5:531–539

Guo YL, Bechsgaard JS, Slotte T, Neuffer B, Lascoux M, Weigel D, Schierup MH (2009) Recent speciation of *Capsella rubella* from *Capsella grandiflora*, associated with loss of self-incompatibility and an extreme bottleneck. Proc Nat Acad Sci USA 106:5246–5251

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41:95–98

Hintz M, Bartholmes C, Nutt P, Ziermann J, Hameister S, Neuffer B, Theissen G (2006) Catching a "hopeful monster": sheperd's purse (*Capsella bursa-pastoris*) as a model system to study the evolution of flower development. J Exp Bot 57:3531–3542

Huelsenbeck JP, Ronquist F (2003) MrBayes3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574

Hurka H, Neuffer B (1997) Evolutionary processes in the genus *Capsella* (Brassicaceae). Plant Syst Evol 206:295–316

Hurka H, Paetsch M, Bleeker W, Neuffer B (2005) Evolution within the Brassicaceae. In: Endress PK, Lüttge U, Parthier B (eds) From plant taxonomy to evolutionary biology. Nova acta Leopoldina NF, Bd. 92 Nr. 342:113–127

Kamau E, Charlesworth D (2005) Balancing selection and low recombination affect diversity near the self-incompatibility loci of the plant *Arabidopsis lyrata*. Curr Biol 15:1773–1778

Kho YO, Baër J (1968) Observing pollen tubes by means of fluorescence. Euphytica 17: 298–302

Koch MA, Kiefer M (2005) Genome evolution among cruciferous plants: a lecture from the comparison of the genetic maps of three diploid species: *Capsella rubella*, *Arabidopsis lyrata* subsp. *petraea*, and *Arabidopsis thaliana*. Am J Bot 92:761–767

Kuittinen H, de Haan AA, Vogl C, Oikarinen S, Leppälä J, Koch M, Mitchell-Olds T, Langley C, Savolainen O (2004) Comparing the linkage map of the close relatives *Arabidopsis lyrata* and *Arabidopsis thaliana*. Genetics 168:1575–1584

Kumar S, Tamura K, Jakobsen IB, Nei M (2001) MEGA2: Molecular evolutionary genetics analysis SOFTWARE, Version 2.1. Arizona State University, Tempe, Arizona, USA

Kusaba M, Nishio T, Satta Y, Hinata K, Ockendon DJ (1997) Striking sequence similarity in inter- and intraspecific comparisons of class I *SLG* alleles from *Brassica oleracea* and *Brassica* campestris: Implications for the evolution and recognition mechanism. Proc Natl Acad Sci USA 94:7673–7678

Kusaba M, Dwyer K, Hendershot J, Vrebalov J, Nasrallah JB, Nasrallah ME (2001) Self-incompatibility in the genus *Arabidopsis*: characterisation of the *S* locus in the outcrossing *A. lyrata* and its autogamous relative *A. thaliana*. Plant Cell 13:627–643

Levin DA (1996) The evolutionary significance of pseudo-self-fertility. Am Nat 148:321–332

Lim SH, Cho HJ, Cho YH, Kim BD (2002) Identification and classification of *S* haplotypes in *Raphanus sativus* by PCR-RFLP of the *S* locus glycoprotein (*SLG*) and the *S* locus receptor kinase (*SRK*) gene. Theor Appl Genet 104:1253–1262

Liu P, Sherman-Broyles S, Nasrallah ME, Nasrallah JB (2007) A cryptic modifier causing transient self-incompatibility in *Arabidopsis thaliana*. Curr Biol 17:734–740

Mable BK, Schierup MH, Charlesworth D (2003) Estimating the number, frequency, and dominance of *S*-alleles in a natural population of *Arabidopsis lyrata* (Brassicaceae) with sporophytic control of self-incompatibility. Heredity 90:422–431

Mable BK, Robertson AV, Dart S, Di Berardo C, Witham L (2005) Breakdown of self-incompatibility in the perennial *Arabidopsis lyrata* (Brassicaceae) and its genetic consequences. Evolution 59:1437–1448

Miege C, Ruffio-Chable V, Schierup MH, Cabrillac D, Dumas C, Gaude T, Cock JM (2001) Intrahaplotype polymorphism at the *Brassica S*-locus. Genetics 159:811–922

Mena-Ali JI, Stephenson AG (2007) Segregation analyses of partial self-incompatibility in self and cross progeny of *Solanum carolinense* reveal a leaky *S*-allele. Genetics 177:501–510

Müntzing A (1956) Chromosomes in relation to species differentiation and plant breeding. Conference of Chromosomes. Wageningen, The Netherlands, pp 1–37

Nasrallah JB, Kao T-H, Goldberg ML, Nasrallah ME (1985) A cDNA clone encoding an *S*-locus-specific glycoprotein from *Brassica oleracea*. Nature 318:263–267

Nasrallah JB, Yu S-M, Nasrallah ME (1988) Self-incompatibility genes of *Brassica oleracea*: expression, isolation, and structure. Proc Natl Acad Sci USA 85:5551–5555

Nasrallah JB, Nasrallah ME (1993) Pollen-stigma signaling in the sporophytic self-incompatibility response. Plant Cell 5:1325–1335

Nasrallah JB (1994) Evolution of the *Brassica* self-incompatibility locus: a look into *S*-locus gene polymorphism. Proc Natl Acad Sci USA 94:9516–9519

Nasrallah JB, Liu P, Sherman-Broyles S, Schmidt R, Nasrallah ME (2007) Epigenetic mechanisms for breakdown of self-incompatibility in interspecific hybrids. Genetics 175:1965–1973

Neuffer B, Eschner S (1995) Life-history traits and ploidy levels in the genus *Capsella* (Brassicaceae). Can J Bot 73:1354–1365

Neuffer B, Albers S (1996) Phenotypic and allozyme variability in *Capsella* populations with different ploidy levels from different continents. Bot Jahrb Syst 118:422–450

Paetsch M, Mayland-Quellhorst S, Neuffer B (2006) Evolution of the self-incompatibility system in the Brassicaceae: identification of *S*-locus receptor kinase (*SRK*) in self-incompatible *Capsella grandiflora*. Heredity 97:283–290

Prigoda NL, Nassuth A, Mable BK (2005) Phenotypic and genotypic expression of self-incompatibility haplotypes in *Arabidopsis lyrata* suggests unique origin of alleles in different dominance classes. Mol Biol Evol 22:1609–1620

Rich TCG (1991) Crucifers of Great Britain and Ireland, 6th edn, B.S.B.I. Handbook. Botanical Society of the British Isles, London

Riley HP (1932) Self-sterility in sheperd's purse. Genetics 17:231–295

Riley HP (1936) The genetics and physiology of self-sterility in the genus *Capsella*. Genetics 21:24–39

Sakamoto K, Kusaba M, Nishio T (1998) Polymorphism of the *S*-locus glycoproein gene (*SLG*) and the *S*-locus related gene (*SLR1*) in *Raphanus sativus* L. and self-incompatible ornamental plants in the Brassicaceae. Mol Gen Genet 258:397–403

Schierup MH, Mable BK, Awadalla P, Charlesworth D (2001) Identification and characterization of a polymorphic receptor kinase gene linked to the self-incompatibility locus of *Arabidopsis lyrata*. Genetics 158:387–399

Schierup MH, Vekemans X (2008) Genomic consequences of selection on self-incompatibility genes. Curr Opin Plant Biol 11:116–122

Schopfer CR, Nasrallah ME, Nasrallah JB (1999) The male determinant of self-incompatibility in *Brassica*. Science 286:1697–1700

Sherman-Broyles S, Nasrallah JB (2008) Self-incompatibility and evolution of mating systems in the Brassicaceae. In: Franklin-Tong VE (ed) Self-incompatibility in flowering plants: evolution, diversity, and mechanisms. Springer, Berlin Heidelberg, pp 123–142

Shimizu KK, Cork JM, Caicedo AL, Mays CA, Moore RC, Olsen KM, Ruzsa S, Coop G, Bustamante CD, Awadalla P, Puruggana MD (2005) Darwinian selection of a selfing locus. Science 306:2081–2083

Slotte T, Huang H, Lascoux M, Ceplitis A (2008) Polyploid speciation did not confer instant reproductive isolation in *Capsella* (Brassicaceae). Mol Biol Evol 25:1472–1481

Stein JC, Howlett BH, Boyes DC, Nasrallah ME, Nasrallah JB (1991) Molecular cloning of a putative receptor protein kinase gene encoded at the self-incompatibility locus of *Brassica oleracea*. Proc Natl Acad Sci USA 88:8816–8820

Straub J (1958) Das Überwinden der Selbststerilität. Zeitschrift für Botanik 46:98–11

Stebbins GL (1956) Artificial polyploidy as a tool in plant breeding. Brookhaven Symp Biol Genet Plant Breed 9:37–52

Suzuki G, Kai N, Hirose T, Fukui K, Nishio T, Takayama S, Isogai A, Watanabe M, Hinata K (1999) Genomic organization of the *S* locus: identification and characterization of genes in *SLG/SRK* region of an S9 haplotype of *Brassica campestris* (syn. rapa). Genetics 153:391–400

Takayama S, Isogai A (2005) Self-incompatibility in plants. Ann Rev Plant Biol 56:467–489

Walker JC (1994) Structure and function of the receptor-like protein-kinases of higher plants. Plant Mol Biol 26:1599–1609

Weller SG, Donoghue MJ, Charlesworth D (1995) The evolution of self-incompatibility in flowering plants: a phylogenetic approach. In: Hoch PC, Stephenson AG (eds) Experimental and molecular approaches to plant biosystematics. Missouri Botanical Garden, St. Louis, pp 355–382

Whitehouse HLK (1950) Multiple-allelomorph incompatibility of pollen and style in the evolution of angiosperms. Ann Bot 14:199–216

Winge Ö (1940) Taxonomic and evolutionary studies in *Erophila* based on cytogenetic investigations. Comp Rend Lab Carlsberg, ser physiol 23:41–47

Pollinator-Driven Speciation in Sexually Deceptive Orchids of the Genus *Ophrys*

Manfred Ayasse, Julia Gögler, and Johannes Stökl

Abstract *Ophrys* orchids mimic females of their pollinator species to attract male insects for pollination. Reproductive isolation is based on the specific attraction of males of usually a single pollinator species, mostly bees, by mimicking the female species-specific sex-pheromone. Sexually deceptive orchids are ideal candidates for studies of sympatric speciation, because key adaptive traits such as the pollinator-attracting scent are associated with their reproductive success and with premating isolation.

We have investigated processes of ecological speciation by using behavioural experiments and chemical, electrophysiological and population-genetic analyses. We show that minor changes in floral odour bouquets might be the driving force for pollinator shifts and speciation events. New pollinators act as an isolation barrier towards other sympatrically occurring species. Hybridisation occurs because of similar odour bouquets of species and the overlap of flowering periods. Hybrid speciation can also lead to the displacement of species by the hybrid population, if its reproductive success is higher than that in the parental species.

1 Introduction

Orchids include more than 20,000 species and comprise one of the largest families in the plant kingdom (Dressler 1993). Their remarkable variation of floral form and their diversity in pollination systems have always interested evolutionary biologists

M. Ayasse (✉) and J. Gögler
Institute for Experimental Ecology, University of Ulm, Albert-Einstein-Allee 11, 89069 Ulm, Germany
e-mail: manfred.ayasse@uni-ulm.de
J. Stökl
Institute for Experimental Ecology, University of Ulm, Albert-instein-llee 11, 89069 Ulm, Germany
Institut für Zoologie, AG Chemische Ökologie, Universität Regensburg, Universitätsstraße 31, 93053 Regensburg, Germany

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_6,

(Darwin 1885). Non-rewarding flowers are widespread among Orchidaceae, with one-third of the species employing deceit rather than food rewards to attract pollinators (Pijil and Dodson 1966; Dressler 1981; Ackerman 1986). The large number of deceptive species suggests an important role of floral deception and specialisation in species diversification (Cozzolino and Widmer 2005; but see Armbruster and Muchhala 2009; Schiestl and Schlüter 2009).

In this study, we have investigated, and discuss, the way that changes in floral odour bouquets that are responsible for pollinator attraction and species isolation might be the driving force for pollinator shifts and ecological speciation in *Ophrys* orchids. This review is far from complete as we have chosen to focus predominantly on sexually deceptive orchids of the genus *Ophrys*. The role of floral scent in the speciation of sexually deceptive orchids of Australia has been reviewed in a recent paper (Whitehead and Peakall 2009).

1.1 Sexual Deception

Pollination by sexual deception is thought to be the most remarkable mechanism of pollination exclusive to Orchidaceae and has independently evolved in Europe, Australia, South Africa and South America (Dafni 1984; Ackerman 1986; Nilsson 1992; Jersakova et al. 2006). Sexual deception is found in Europe in the genus *Ophrys* (Delforge 2006). *Ophrys* flowers imitate female insects in shape, colour and odour in order to attract males for pollination (Kullenberg 1961; Kullenberg and Bergström 1973; Fig. 1). Pollinators are mainly aculeate Hymenoptera, but also beetles and Diptera (Kullenberg 1961; Paulus 2006).

Male insects are lured to the orchid by volatile semiochemicals and visual cues. At close range, chemical signals from the flowers elicit sexual behaviour in males, which try to copulate with the flower labellum. In all species investigated so far, copulation attempts of the males can only be elicited by scent that is identical to the

Fig. 1 *Ophrys sphegodes* (head-pollinated) (*left*) and *O. lupercalis* (abdomen-pollinated) (*right*, Photo: H.F. Paulus) with pseudocopulating males of *Andrena nigroaenea*

female sex pheromone of the pollinating species (Schiestl et al. 1999, 2000; Ayasse et al. 2003; Ayasse 2006; Stökl et al. 2007, 2008), visual cues seeming to be less important. Chemical analyses have demonstrated that *Ophrys* flowers produce complex species-specific mixtures of more than 100 compounds (Borg-Karlson 1990; Ayasse et al. 2000, 2003; Stökl et al. 2005, 2007, 2008). Bioassays with synthetic compounds, however, have shown that not all the compounds have a function in pollinator attraction (reviewed in Ayasse 2006).

In *Andrena*-pollinated *Ophrys* species, a complex mixture of saturated and unsaturated hydrocarbons can define pollinator attraction (Schiestl et al. 1999; Stökl et al. 2005, 2007, 2008). Sympatrically occurring species are attracted by different mixtures of the same hydrocarbons (Schiestl and Ayasse 2002). Furthermore, both closely and distantly related *Ophrys* species pollinated by the same species of *Andrena* use the same compounds in a highly similar combination for pollinator attraction (Stökl et al. 2005). This indicates the convergent evolution of pollinator-attracting odours in *Ophrys* species.

1.2 Specialised Pollination and Floral Isolation

Sexual deception imposes strong specialisation in orchids as insect pheromones are generally highly species-specific (Ayasse et al. 2001). Most *Ophrys* species are visited and pollinated by males of usually only one pollinator species (Kullenberg 1961; Paulus and Gack 1990). Similar species-specificity is seen in the Australian orchid genera *Caladenia* (Stoutamire 1983) and *Chiloglottis* (Bower 1996) and their pollinators. The highly specific *Ophrys*–pollinator relationship represents the main mechanism of reproductive isolation between the often crossable *Ophrys* species (Ehrendorfer 1980; Paulus and Gack 1990; Schiestl and Ayasse 2002; Paulus 2006), and the species-specific scent is mainly responsible for premating isolation (Schiestl and Ayasse 2002; Schiestl 2005; Stökl et al. 2005; Ayasse 2006). In some cases, the different placements of the pollinia on the male provide a mechanical isolating mechanism between sympatric *Ophrys* species with the same pollinator (Fig. 1). Meanwhile, an example of postmating isolation has been reported (Scopece et al. 2007), which is thought to be acquired secondarily as a by-product of genetic divergence (Dobzhansky 1937; Mayr 1970).

1.3 Speciation

The genus *Ophrys* has extensively radiated throughout the Mediterranean region (Delforge 2006) and now encompasses more than 200 species. It has often been cited as a suitable model system for studying speciation events by pollinator shift. In *Ophrys*, flower visits are often rare and brief (Ayasse et al. 2000) and negative frequency-dependent selection in response to odour learning may favour variability

of the pollinator-attracting odour signals within orchid populations (Ayasse et al. 2000; Gigord et al. 2001). Odour changes as a result of genetic drift or selective pressure by pollinators, negative frequency-dependent selection or hybridisation may be the driving forces for speciation. Scent variation and the attraction of a novel pollinator by a group of *Ophrys* individuals in a population can, in principle, provide an immediate isolating factor, and speciation may occur rapidly in sympatry and as a progenitor-derivative speciation event in which the progenitor species remains entirely unaffected (Rieseberg and Brouillet 1994; Schlüter 2006; Schlüter and Harris 2006; Cozzolino and Scopece 2008). Because of selection by the new pollinator, such plants might adapt to a new ecological niche and a new species may evolve (Johnson 2007). In *Andrena*-pollinated *Ophrys* species that attract their pollinators with various mixtures of the same compounds, small changes in the odour bouquets of alkanes and alkenes result in the attraction of a different pollinator (Schiestl and Ayasse 2002; Ayasse 2006; Stökl et al. 2008, 2009).

Hybrid speciation is thought to be an important mechanism for speciation in many plants (Ehrendorfer 1980; Barton 2001; Seehausen 2004; Rieseberg and Willis 2007) and has also been suggested in *Ophrys* for the following reasons. First, the genus contains many closely related species that are sympatric to a great extent (Delforge 2006). Second, the chromosome number of all species is the same and hybrids are fertile (homodiploidy; Ehrendorfer 1980). Hybrid speciation is conceivable in two *Ophrys* taxa that occur in sympatry and attract the same pollinator (Ayasse 2006). The formation of hybrids in *Ophrys* may be stabilised, thus providing the potential for speciation, since the attraction of a new pollinator by the hybrids acts as a premating isolation barrier. Thus, the odour produced by hybrid plants is essential for the reproductive isolation of hybrids.

Despite the selective attraction of pollinators, false pollination does occur and hybrids can be occasionally found (Stebbins and Ferlan 1956; Danesch et al. 1975; Ehrendorfer 1980). For several species, such as *O. bertoloniformis*, *O. biancae*, *O. sitiaca* or *O. normanii*, a hybridogenic origin has been proposed based in the floral intermediacy between putative parental species (Stebbins and Ferlan 1956; Paulus and Gack 1995; Delforge 2006). Recent studies have suggested gene flow across species boundaries in sympatric species of the *O. sphegodes* group (Soliva and Widmer 2003; Mant et al. 2005) and the *O. fusca* group (Stökl et al. 2008, 2009).

The genetic control of morphological characters is unknown and, often, recognition of the different hybrids, such as F1 or later generation hybrids, backcrosses and introgressed individuals, becomes difficult (Rossi et al. 1992). Furthermore, distinguishing between morphological hybrids between two putative parents that already overlap in their morphology (Bateman et al. 1997) is often impossible. Therefore, a more powerful approach to the delimitation of species and intraspecific taxa is to combine molecular data and morphometric studies (reviewed in Bateman 2001).

Sexually deceptive orchids are ideal candidates for studies of radiation and sympatric speciation, because key adaptive traits such as the pollinator-attracting scent are associated with their reproductive success and also with premating isolation. The presence of many sympatric species with low levels of morphological

differences (Paulus 2001), differences in scent (Stökl et al. 2005) and molecular differences (Soliva et al. 2001; Soliva and Widmer 2003; Mant et al. 2005) between species suggest rapid adaptive radiation.

In the following, we present three case studies in which we have investigated the variation in floral traits and genotypic variation in hybrids and parental species of *Ophrys* on Sardinia and Majorca. Based on the available data concerning intra- and interspecific variation of the pollinator-attracting floral volatiles (Schiestl and Ayasse 2002; Stökl et al. 2005), we hypothesise the following evolutionary scenarios for speciation events in *Ophrys* (Fig. 2).

Two hypothetical *Ophrys* species occur in sympatry and attract their pollinators with different bouquets of the same chemical compounds (Fig. 2a). Odour changes as a result of genetic drift or negative frequency-dependent selection might result in an odour mutant of species 1 that produces a slightly different odour bouquet resembling more the odour bouquet of the second species (Fig. 2a) and that would thus attract both pollinators of the parental species.

Scenario 1: Resulting hybrids may produce intermediate odour bouquets of the same compounds. In this case, the ongoing attraction of both pollinators, formation of further hybrid generations and introgression can be expected (Fig. 2b, case study 1).

Scenario 2: The hybrid may replace one or both parental species (Stökl et al. 2008; case study 1) if its reproductive success is higher than in the parental species.

Scenario 3: The hybrid may attract a new pollinator species (Pollinator 3) if the scent, by chance, resembles the sex pheromone of a new pollinator species (Vereecken 2008). If the new pollinator is not attracted by the other available pollinators, speciation may occur (Fig. 2b).

Scenario 4: The mutant might be attractive for a new pollinator species (Pollinator 4; case study 2 and 3) and, again, if the new pollinator is not attracted by the other available pollinators, speciation may occur (Fig. 2b).

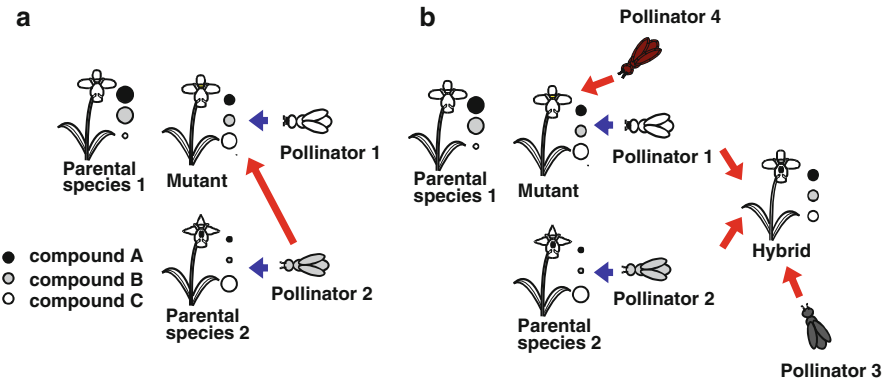


Fig. 2 Evolutionary scenario for speciation events in *Ophrys* orchids. Arrows indicate pollinator attraction, before hybridisation (*blue*) and after hybridisation (*red*)

Scenario 5: The hybrid might attract none of the available pollinators if the scent of the hybrids differs too greatly from its original pollinators and does not attract a new pollinator. In this case, the hybrids will disappear. This scenario probably often happened during the evolution of *Ophrys* orchids.

2 Case Study 1: Hybrid Speciation and Distinction of Species on Sardinia

Ophrys lupercalis Devillers-Terschuren & Devillers and *O. iricolor* Desfontaines are two species of the *O. fusca–O.lutea* group and are pollinated by *Andrena nigroaenea* Kirby 1802 and *A. morio* Brullé 1832, respectively. Paulus and Gack (1995) reported putative hybrids of *O. lupercalis* and *O. iricolor* on Sardinia; these were attractive for both pollinator species. Their flowers also showed an intermediate flower morphology. Both *O. lupercalis* and *O. iricolor* are widespread in the Mediterranean; however, hybrids have only been described in the western Mediterranean populations in Sardinia, Tunisia and Malta. Some authors consider the Sardinian form of *O. iricolor* as a distinct species (*O. eleonorae* Devillers-Terschuren & Devillers; Devillers and Devillers-Terschuren 1994), while others consider it as a geographical subspecies (*O. iricolor* subsp. *maxima* Terracciano; Paulus and Gack 1995). In this publication, we will use the name *O. iricolor*.

In behavioural field experiments in which we offered flowers of both species and hybrid plants to the males of *A. nigroaenea* and *A. morio*, we found that 36% of the intermediate flowers were attractive to each of the pollinators and 28% were attractive to both species (Stökl et al. 2008). Furthermore, flowers that showed the typical flower morphology of *O. lupercalis* or *O. iricolor* were attractive to the "false" pollinators. This raises doubts with regard to the hypothesis of the complete reproductive isolation of *Ophrys* species by selective attraction of a single pollinator species, as proposed by Paulus and Gack (1990).

Attraction of the non-legitimate pollinator should be more likely if the involved pollinator species are closely related and possess a similar female sex pheromone. In the next step, therefore, we collected solvent extracts of flowers and used them for electrophysiological (GC-EAD) and chemical (gas chromatography and mass spectroscopy) analyses. By coupling electroantennography with gas chromatography (GC-EAD), those compounds in an odour blend that can be perceived by the bee's antenna can be identified. In a previous study, this method has shown that *A. nigroaenea* males can perceive and are attracted by saturated and unsaturated hydrocarbons (Schiestl et al. 1999). The very same compounds also give reactions at the antenna of *A. morio* males (Stökl et al. 2007). Both orchid species therefore produce the same chemical compounds in their floral odour. Specific attraction is only achieved by different blends of the same compounds.

In such a case, a slight shift in the produced amount of same odour compounds can result in the attraction of a different pollinator species. To test this hypothesis,

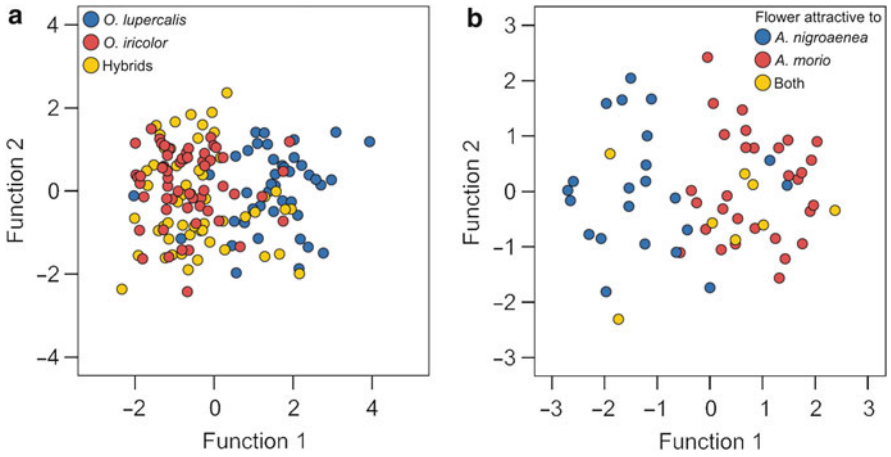


Fig. 3 Scatter plots of discriminant function analysis of GC-EAD active hydrocarbons in the floral odour of the *Ophrys* flowers (from Stökl et al. 2008). (**a**) Samples were grouped by *Ophrys* species (f1: $\chi^2 = 97.5$, $df = 8$, $P < 0.001$; f2: $\chi^2 = 3.1$, $df = 3$, $P = 0.379$). (**b**) Samples were grouped by their attractiveness for the pollinators (f1: $\chi^2 = 40.3$, $df = 8$, $P < 0.001$; f2: $\chi^2 = 1.8$, $df = 4$, $P = 0.618$)

we compared the pollinator-attracting odour bouquets of flowers from both species and also of flowers with intermediate morphology (hybrids). The analyses showed a limited separation of the odours of *O. lupercalis* and *O. iricolor* with a slight overlap between species (Stökl et al. 2008). The flowers with intermediate flower morphology did not form a separate group but were mostly placed with *O. iricolor* (Fig. 3a). This incomplete separation of the floral odours between species is in congruence with the observed attraction of the "false" pollinator species in behavioural experiments. To answer the question "When is a flower attractive for both pollinator species?", we compared the samples according to their attractiveness for pollinators, not according to orchid species (Fig. 3b). The odours of flowers attractive to either *A. nigroaenea* or *A. morio* were well separated. The flowers attractive to both species surprisingly were neither intermediate between the two other groups nor formed a third distinct group. Therefore, we could not explain in detail the attraction of the pollinators. Obviously, factors other than floral odour, such as age and the motivation of the males, might influence the males' behaviour.

An overlap in the two species, whereby hybrid individuals could not be separated from *O. iricolor*, was also obtained in an analysis of 17 morphological flower characters (Stökl et al. 2008). We therefore used genetic tools (AFLPs) to identify hybrid specimens and to assess the rate of hybridisation between the species. The results confirmed the breakdown of the reproductive isolation between species (Fig. 4) as expected from the observed cross-pollination events (Stökl et al. 2008). An almost complete transition occurs between the two species. This indicates not only hybridisation but also a high number of backcrosses of hybrids and species.

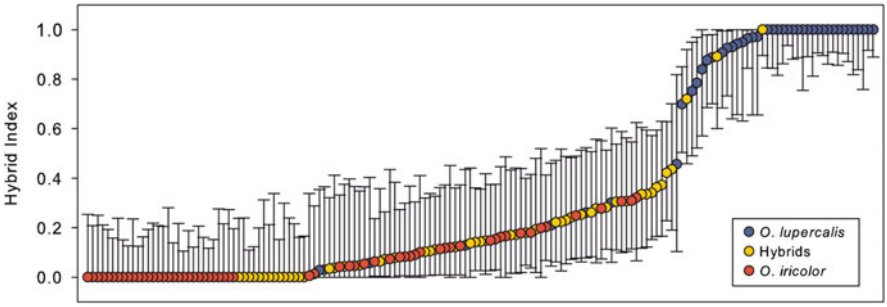


Fig. 4 Hybrid indices for *O. lupercalis*, *O. iricolor* and their hybrids (from Stökl et al. 2008). Indices vary from 0 = *O. iricolor* to 1 = *O. lupercalis*. Bars give 95% confidence interval

The AFLP data (Fig. 4) also show that most intermediate plants are more similar to *O. iricolor* than to *O. lupercalis* (Stökl et al. 2008). This suggests that *O. iricolor* has almost been replaced by an *O. iricolor x lupercalis* hybrid population on Sardinia. According to AFLP data, "pure" *O. lupercalis* can still be found. Competition for pollinators and the higher number of hybrid individuals make pollination events between pure individuals of *O. lupercalis* unlikely and rare. Eventually, *O. lupercalis* might also be completely displaced by the *O. iricolor* × *lupercalis* hybrid population on Sardinia.

3 Case Study 2: Pollinator-Driven Selection and Speciation in *Ophrys lupercalis*, *Ophrys bilunulata* and *Ophrys fabrella* on Majorca

Three species of *Ophrys* pollinated by three species of *Andrena* occur sympatrically on Majorca (Delforge 2006). *Ophrys lupercalis* Devillers-Terschuren & Devillers, which is widely distributed in the central and western Mediterranean, is pollinated by *A. nigroaenea* Kirby 1802. *Ophrys bilunulata* Risso is pollinated by *A. flavipes* Panzer 1799 and has a similar area of distribution as *O. lupercalis*. The third species, *O. fabrella* Paulus and Ayasse, which is pollinated by *A. fabrella* Perez 1903, is endemic to the Balearic Islands.

All three species bloom sympatrically and consecutively on Majorca. The flowering period of *O. bilunulata* overlaps with the flowering periods of both *O. lupercalis* and *O. fabrella*. Previous analyses have shown that *O. lupercalis* and *O. bilunulata* produce different bouquets of the same set of hydrocarbons to attract their pollinators, *A. nigroaenea* and *A. flavipes*, respectively (Schiestl and Ayasse 2002; Stökl et al. 2005). The overlapping flowering periods and a similarity in pollinator-attracting odours in at least two of the three species, viz. *Ophrys lupercalis* and *O. bilunulata* (Schiestl and Ayasse 2002), make this system an ideal candidate for studying hybridisation and speciation.

We therefore analysed the pollination system of the Majorcan species with the same methods that we had used in the study of the Sardinian species. First, we performed GC-EAD analyses with *O. fabrella* scent by using the antennae of *A. fabrella* males; such analyses had not previously been performed. The results showed that hydrocarbons and some non-hydrocarbons released EAD signals in the antennae of *A. fabrella* males. Most of those compounds are also EAD-active in males of *A. nigroaenea*, *A. flavipes* and *A. morio* (Schiestl et al. 1999; Schiestl and Ayasse 2002; Stökl et al. 2005, 2007).

A comparison of the GC-EAD active floral volatiles in a discriminant functional analysis showed a distinct odour bouquet for all three species, but with an overlap in the odour bouquets (Stökl et al. 2009; Fig. 5a). Unfortunately, because of bad weather conditions, we were unable to perform cross-attraction experiments with the Majorcan species as we had on Sardinia. We were only able to test flowers of *O. bilunulata* for their attractiveness to *A. flavipes*. Flowers from all location within the odour-space of *O. bilunulata* were attractive for males of *A. flavipes*. Even flowers with a scent from the overlapping areas with *O. lupercalis* and *O. fabrella* attracted males of *A. flavipes* (Fig. 5b). We therefore assume, although we have not directly observed, the cross-attraction of pollinators and, consequently, the hybridisation and introgression between species that we have found in genetic analyses.

Surprisingly, and contrary to the odour analysis, we could not find a distinct genotype of *O. bilunulata* in the genetic analysis by using AFLPs (Fig. 6). Most plants had either the genotype of *O. lupercalis* or the genotype of *O. fabrella*. This is in strong contrast to the distinct floral odour and flower morphology of *O. bilunulata* that we have found.

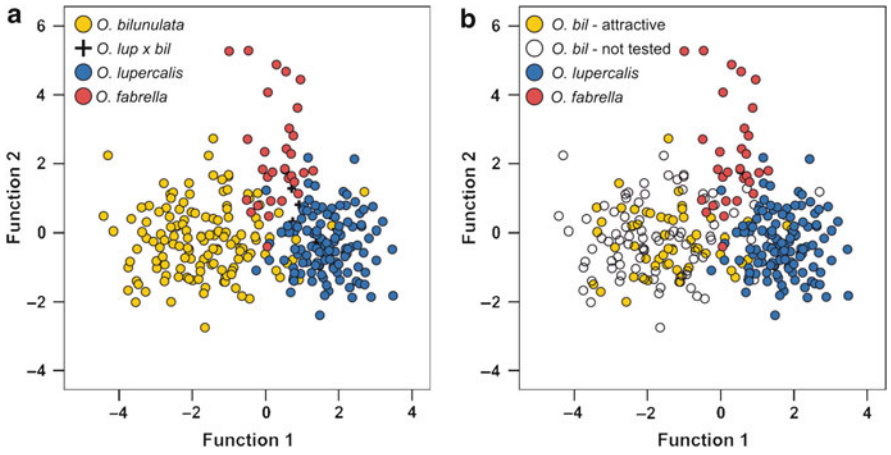


Fig. 5 Scatter plots of discriminant function analyses of GC-EAD active hydrocarbons in the floral odour of *Ophrys lupercalis*, *O. bilunulata* and *O. fabrella* (from Stökl et al. 2009) (f1: 77.6%, $\chi^2 = 535.9$, $df = 15$, $P < 0.001$; f2: 19.2%, $\chi^2 = 169.8$, $df = 8$, $P < 0.001$). (**a**) Samples grouped by species. (**b**) Flowers of *O. bilunulata* attractive to marked males of *A. flavipes*

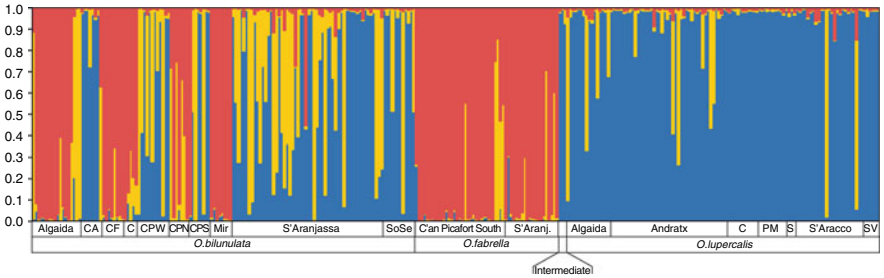


Fig. 6 Result of structure analysis (from Stökl et al. 2009). Stacked bars show the probability of each plant of *O. lupercalis*, *O. bilunulata* and *O. fabrella* originating from one of the three assumed species (*blue bars O. lupercalis*, *yellow bars O. bilunulata*, and *red bars O. fabrella*). Samples are sorted by species and population. *CA* Cala Agulla, *CF* Cala Figuera, *C* Capdella, *CPW* C'an Picafort West, *CPN* C'an Picafort North, *CPS* C'an Picafort South, *Mir* Mirador, *SoSe* Son Severa, *PM* Platjes de Mallorca, *S* Sa Grembla, *SV* Son Viguet. *Intermediate* flowers with a flower morphology intermediate between *O. bilunulata* and *O. lupercalis*

From the genetic data, we cannot tell whether the present situation results from the fusion or divergence of species. Indeed, the data can be interpreted in two different ways. Firstly, the hybridisation between the species is attributable to the similar odour bouquets of the species and an overlap of flowering periods. Distinct phenotypes of the species are maintained by selection by the pollinators. If the hybridisation is not completely compensated by the selective pressure of the pollinators, one species will finally displace the other. Secondly, the process of speciation is still underway, whereby one species is becoming separated from the other. Selection by the pollinators could create differences in odour bouquet that are not yet represented in the genetic structure.

In the case of *O. lupercalis* and *O. bilunulata*, a currently occurring speciation process can be excluded, because both species not only occur on Majorca but are also widespread throughout the Mediterranean (Delforge 2006). *O. lupercalis* is unlikely to have split from *O. bilunulata* on Majorca with this speciation process resulting in the same flower morphology and the same floral odour as in populations of *O. lupercalis* elsewhere.

In the case of *O. bilunulata* and *O. fabrella*, both explanations are plausible. *O. fabrella* is endemic to the Balearic Islands (Delforge 2006) and therefore most probably split from one of the other *Ophrys* species that co-occur on the Balearic Islands. Classic taxonomy based on floral characters place it close to *O. bilunulata* (Paulus and Ayasse, unpublished). Moreover, the result of our analysis of the non-hydrocarbons suggests a closer relationship to *O. bilunulata* than to *O. lupercalis* (Stökl et al. 2009). We thus consider that *O. fabrella* has evolved, possibly even sympatrically, from late-flowering *O. bilunulata* plants by recruiting a new pollinator, A. *fabrella*. The split into two species is almost complete in the odour phenotype but has not been completed at the genetic level, with backcrosses probably contributing to ongoing gene flow. However, we cannot exclude that *O. fabrella* has evolved differently and now hybridises with *O. bilunulata*.

4 Case Study 3: Speciation and Evolutionary Origin of Two Sympatrically Occurring Endemic Species, *O. chestermanii* and *O. normanii*, on Sardinia

O. normanii Wood and *O. chestermanii* Gölz & Reinhard occur in sympatry, are endemic on Sardinia (Baumann et al. 1995; Paulus and Gack 1995) and are pollinated by males of the social parasitic bumblebee species *Bombus vestalis* Geoffroy 1785, which removes the pollinia in both species with its head. This is in contrast to the situation in other sympatrically occurring species of *Ophrys* with the same pollinator where reproductive isolation is ensured by a mechanical barrier attributable to the different pollinia placement on the pollinator body (Agren et al. 1984; Paulus and Gack 1990).

The species origin of *O. normanii* and *O. chestermanii*, such as their taxonomical attribution, is controversial. Based on morphological similarities in flower traits, *O. chestermanii* has been attributed to the *O. bornmuelleri* group (Delforge 2006), which is also represented on Sardinia by another endemic species, *O. annae* Devillers-Terschuren & Devillers. In contrast, according to flower morphology, *O. tenthredinifera* Willdenow, which is widespread in the Mediterranean basin, is considered to be a closely related species of *O. normanii* (Paulus and Gack 1995). *O. tenthredinifera* is common on Sardinia where its local pollinator is *Eucera nigrilabris* Lepeletier 1841 as reported by Paulus and Gack (1995).

Based on morphological flower traits, *O. normanii* has been suggested to be an early generation hybrid between *O. chestermanii* and *O. tenthredinifera* (Wood 1983; Baumann et al. 1995). However, *O. normanii* populations are abundant in comparison with the parent species. Therefore, Paulus and Gack (1995) have proposed that *O. normanii* is either a hybridogenic species or is directly derived from *O. tenthredinifera*.

To investigate the evolutionary origin of *O. normanii* and *O. chestermanii*, we performed behavioural tests with odourless dummies of *B. vestalis* females that had been impregnated with labella extracts of the three *Ophrys* species. *O. chestermanii* and *O. normanii* extracts were both highly attractive for males of *B. vestalis*, whereas *O. tenthredinifera* samples rarely elicited copulations in the males (Gögler et al. 2009). Since pseudocopulatory events are necessary to remove pollinia and transfer them to another flower, and since a low rate of pollination is generally the rule in *Ophrys* (Ayasse et al. 2000), the likelihood of cross-pollination between *O. chestermanii* and *O. tenthredinifera* by males of *B. vestalis* is low and, consequently, so is the opportunity to hybridise. Furthermore, pollinator tests performed by Paulus and Gack (1995) have shown that *O. chestermanii* is not attractive to males of *Eucera nigrilabris*. These findings strongly reject the hypothesis that *O. normanii* is a present-day hybrid.

In another test series of behavioural experiments with polar and non-polar fractions of *O. normanii* and *O. chestermanii* labellum extracts, alcohols, esters and fatty acids, i.e. polar compounds, have turned out to play a major role in the attraction of *B. vestalis* males (Gögler et al. 2008). A cluster analysis performed

with GC-EAD active polar compounds has shown that both species attract male pollinators with the same odour bouquets (Gögler et al. 2009). *O. tenthredinifera* produces the same compounds but differs from these two species in odour compound composition (Fig. 7).The absence of attractiveness of *O. tenthredinifera* to *B. vestalis* males is based on the different relative amounts of the same compounds. To date, polar compounds have rarely been identified as playing a role in *Ophrys* pollinator attraction (Ayasse et al. 2003) and, in the most investigated species, hydrocarbons have been reported as stimulating male mating behaviour (Schiestl et al. 2000; Stökl et al. 2005, 2007; Vereecken et al. 2007).

A former study (Stökl et al. 2005) compared the floral compounds of several *Andrena* pollinated species of *Ophrys* and found that the similarity of odour bouquets of floral volatiles with no function in pollinator attraction reflects the phylogenetic relationship of species. Therefore, we performed a cluster analysis with floral compounds not involved in pollinator attraction. The results clearly showed a high similarity in the relative proportions of volatiles between *O. normanii* and *O. tenthredinifera*, whereas *O. chestermanii* and *O. normanii* strongly differed (Fig. 7). This result argues against the idea of a hybridogenic origin of *O. normanii* and correlates well with the results gathered in the population genetic analysis with AFLP markers. Since *O. chestermanii* was originally described as a subspecies of *O. holoserica* (Gölz and Reinhard 1990), we have included, in our genetic analyses, samples of *O. annae*, the segregate from Sardinia of the widespread *O. holoserica*. Genetic similarity in a cluster analysis based on a principal coordinate analysis

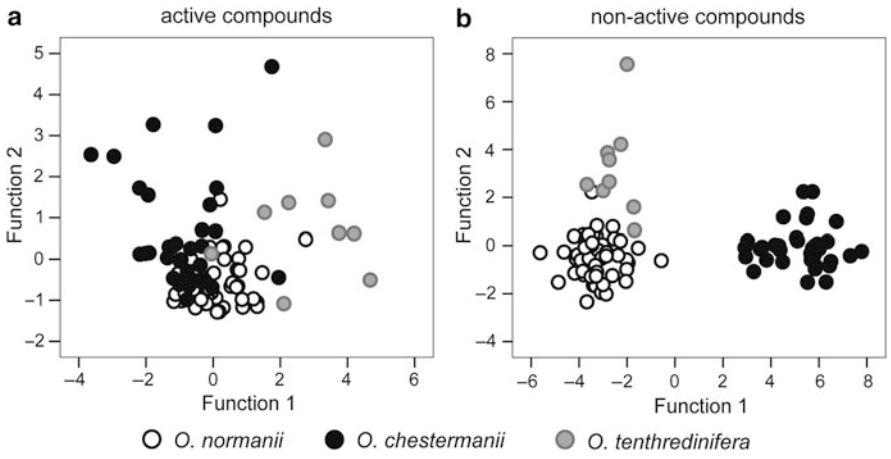


Fig. 7 Discriminant function analyses of (**a**) EAD-active polar compounds and (**b**) non-active compounds (from Gögler et al. 2009). The DFA performed with EAD-active polar compounds (**a**) shows an overlap between *O. chestermanii* and *O. normanii*, whereas *O. tenthredinifera* is separated from these two species. The first two functions accounted for 76.7% and 23.3% of the total variance (f1: $\chi^2 = 89.022$, $df = 8$, $P < 0.001$; f2: $\chi^2 = 24.784$, $df = 3$, $P < 0.001$). In a DFA with non-active compounds (**b**) the first function, explaining 93.7% of the variance, separates *O. chestermanii* from *O. normanii* and *O. tenthredinifera* (f1: $\chi^2 = 325.497$, $df = 18$, $P < 0.001$; f2: $\chi^2 = 68.056$, $df = 8$, $P < 0.001$)

performed with AFLP data (Fig. 8) indicates a close relationship between *O. normanii* and *O. tenthredinifera* and a clear separation from *O. chestermanii* and *O. annae*. A phylogenetic analysis performed with two different plastid regions has confirmed the patterns of genetic relatedness gathered with AFLP markers (Gögler et al. 2009).

With respect to the evolutionary origin of *O. normanii* and *O. chestermanii*, our results from behavioural experiments and from chemical and genetic analyses have shown that both species evolved independently from two different ancestors and converged to the same pollinator by producing the same odour bouquets of polar compounds. The genesis of *O.normanii* from the *O. tenthredinifera* clade, like that of *O. chestermanii* from the *O. annae-O. holoserica* clade, might have been the result of local changes in odour bouquets causing the attraction of a locally available, new pollinator such as *B. vestalis*. This pollinator shift acts as an isolation barrier towards related members of the same clade *(O. tenthredinifera* and *O. annae–O. holoserica,* respectively) and has occurred on Sardinia where *O. normanii* and *O. chestermanii* represent endemic species. Climate change might have caused shifted flowering periods and activity periods of the males of the new pollinator on Sardinia and may have been the reason for the attraction of a new pollinator.

Despite pollinator sharing, sympatrical occurrence and overlapping blooming periods, we have no indications for gene flow between these two species (Gögler

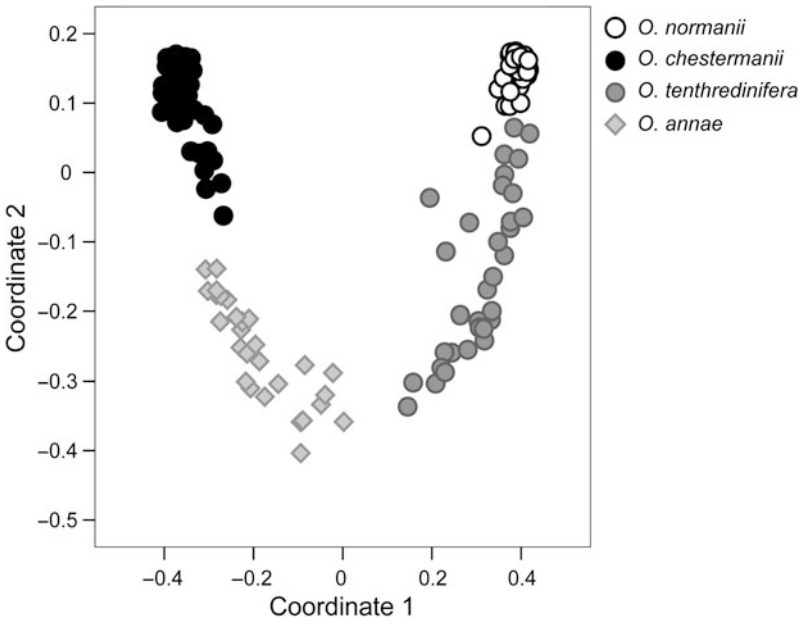


Fig. 8 Principle coordinate analysis performed with the first two coordinates based on AFLP data (from Gögler et al. 2009). Coordinate 1 separates *O. chestermanii* and *O. annae* from *O. normanii* and *O. tenthredinifera*. The first two coordinates explain 37.05% and 9.91% of the variance

et al. 2009). Further studies on pre- and postmating barriers might reveal the mechanisms of those barriers that completely prevent the development of hybrids between *O. chestermanii* and *O. normanii*.

5 Conclusions

In the sexually deceptive orchid genus *Ophrys*, floral scent is a key factor in the attraction of pollinators and, at the same time, represents a prezygotic isolation barrier between sympatrically occurring species (Kullenberg 1961; Paulus and Gack 1990; Scopece et al. 2007). The combination of behavioural experiments and chemical, electrophysiological and population genetic analyses that we have used has provided a suitable array of methods for describing the role of odour variation in species isolation, ecological speciation and even the distinction of species.

Our investigations have shown that minor changes in floral odour bouquets can be the driving force for pollinator shifts and speciation events. The comparative data from our studies in Sardinia and Majorca have shown that the males of all the species of *Andrena* that we have studied are attracted to *Ophrys* flowers by the same set of hydrocarbons (Schiestl et al. 1999; Schiestl and Ayasse 2002; Stökl et al. 2007, 2008, 2009) involving various alkenes that are thought to have a key function in pollinator attraction in *Andrena*-pollinated *Ophrys* species (Schiestl and Cozzolino 2008).This bouquet-based specificity of pollinator attraction was and is an important precondition for processes of speciation including hybrid speciation in the *Ophrys fusca-lutea* species group (Stökl et al. 2008, 2009). However, hybrid speciation can also lead to the displacement of species by the hybrid population (Stökl et al. 2008).

In *Ophrys* and other sexually deceptive orchids, adaptation to a new pollinator niche, mainly mediated by floral scent variation, can directly cause isolation by disruptive selection (Waser and Campbell 2004). In the highly specific pollination system, pollinators have been shown to learn and avoid flowers (Ayasse et al. 2000) or locations with flowers (Peakall 1990). Therefore, single plants may compete for pollinating males, with low frequency-dependent selection possibly circumventing this habituation and providing an advantage to rare odour phenotypes that, in combination with other ecological factors such as a shift in the activity period of potential pollinators, might recruit new pollinators.

If floral differences that affect pollinator attraction and cause premating isolation between species pairs are controlled by one or a few major quantitative trait loci, new species may evolve rapidly (Coyne and Orr 2004). In *Ophrys* species that attract their pollinators with a mixture of hydrocarbons, alkenes with certain double-bond positions are crucial in specific pollinator attraction and species isolation (Mant et al. 2005). Genes influencing the biosynthesis of a set of desaturases may be involved in regulating traits important for pollinator-mediated isolation (Schlüter and Schiestl 2008).

In future studies, the molecular bases for pollinator-attracting traits should be investigated and candidate genes underlying floral isolation should be identified. The investigated *Ophrys* species that use hydrocarbons for pollinator attraction have been well studied and are candidates for such kinds of analysis. However, we should not draw general conclusions based only on this group of *Ophrys* species and should also investigate other species such as *O. normanii* and *O. chestermanii*, in which chemicals other than hydrocarbons play a role in pollinator attraction and in which a different mechanism of scent variation and pollinator switch can therefore be expected (Gögler et al. 2009). For example *O. holoserica*, one of the rarely occurring species that attracts two different pollinators, the long-horned bee *Eucera longicornis* and the scarabid beetle *Phyllopherta horticola*, might be on its way to changing its pollinator. In this species, a pollinator switch might be brought about by changing the biosynthesis of qualitatively different compounds instead of switching the patterns of the same compounds. Most *Ophrys* species produce hundreds of chemical compounds (Borg-Karlson 1990; Erdmann 1996) and therefore have the evolutionary potential to switch pollinators, if necessary.

Acknowledgements We thank Pierluigi Cortis, Claudia Gack, Cesario Giotta, Manfred Kalteisen, Hannes Paulus, Marcello Piccitto and Antonio Scrugli for helping us to collect plant material. We are also grateful to Walther Vogel and Günter Assum, Institute of Human Genetics, University of Ulm, Philipp M. Schlüter and Tod F. Stuessy, Department of Systematic and Evolutionary Botany, University of Vienna and Salvatore Cozzolino, Department of Structural and Functional Biology, University of Naples for generous support and collaboration in the genetic analysis. Wittko Francke, Dirk Erdmann, Claudia Schulz and Robert Twele, Institute of Organic Chemistry, University of Hamburg are gratefully acknowledged for structure elucidation and synthesis of chemical compounds. Julia Gögler was supported by the FAZIT-Stiftung. Financial support was provided by DFG priority program SPP 1127(AY12/1-1, AY12/1-2).

References

Ackerman JD (1986) Mechanisms and evolution of food deceptive pollination systems in orchids. Lindleyana 1:108–113

Agren L, Kullenberg B, Sensenbaugh T (1984) Congruences in pilosity between 3 species of *Ophrys* Orchidaceae and their hymenopteran pollinators. Nova Acta Reg Soc Sci Ups Ser V:15–26

Armbruster WS, Muchhala N (2009) Associations between floral specialization and species diversity: cause, effect, or correlation? Evol Ecol 23:159–179

Ayasse M (2006) Floral scent and pollinator attraction in sexually deceptive orchids. In: Dudareva N, Pichersky E (eds) Biology of floral scent. CRC Press, Boca Raton, USA, pp 219–241

Ayasse M, Schiestl FP, Paulus HF, Löfstedt C, Hansson B, Ibarra F, Francke W (2000) Evolution of reproductive strategies in the sexually deceptive orchid *Ophrys sphegodes*: how does flower-specific variation of odor signals influence reproductive success? Evolution 54:1995–2006

Ayasse M, Paxton R, Tengö J (2001) Mating behavior and chemical communication in the hymenoptera. Annu Rev Entomol 46:31–78

Ayasse M, Schiestl FP, Paulus HF, Ibarra F, Francke W (2003) Pollinator attraction in a sexually deceptive orchid by means of unconventional chemicals. Proc R Soc Lond B 270:517–522

Barton NH (2001) The role of hybridization in evolution. Mol Ecol 10:551–568

Bateman RM (2001) Evolution and classification of European orchids: insights from molecular and morphological characters. J Eur Orch 33:33–119

Bateman RM, Pridgeon AM, Chase MW (1997) Phylogenetics of subtribe Orchidinae (Orchidoideae, Orchidaceae) based on nuclear ITS sequences. 2. Infrageneric relationships and taxonomic revision to achieve monophyly of *Orchis* sensu stricto. Lindleyana 12: 113–141

Baumann H, Giotta C, Künkele S, Lorenz R, Piccitto M (1995) *Ophrys holoserica* subsp. *chestermanii* J. J. Wood - eine gefährdete und endemische Orchidee von Sardinien. J Eur Orch 27:185–224

Borg-Karlson AK (1990) Chemical and ethological studies of pollination in the genus *Ophrys* (Orchidaceae). Phytochemistry 29:1359–1387

Bower CC (1996) Demonstration of pollinator-mediated reproductive isolation in sexually deceptive species of *Chiloglottis* (Orchidaceae:Caladeniinae). Aust J Bot 44:15–33

Coyne JA, Orr HA (2004) Speciation. Sinauer Associates, Sunderland, USA

Cozzolino S, Scopece G (2008) Specificity in pollination and consequences for postmating reproductive isolation in deceptive Mediterranean orchids. Philos Trans R Soc Lond B 363:3037–3046

Cozzolino S, Widmer A (2005) Orchid diversity: an evolutionary consequence of deception? Trends Ecol Evol 20:487–494

Dafni A (1984) Mimicry and deception in pollination. Annu Rev Ecol Syst 15:259–278

Danesch O, Danesch E, Ehrendorfer F, Ehrendorfer K (1975) Hybrids and hybrid taxa from *Ophrys bertolonii* and *Ophrys atrata* (Orchidaceae). Plant Syst Evol 124:79–123

Darwin C (1885) On the various contrivances by which Orchids are fertilized by insects. John Murray, London, UK

Delforge P (2006) Orchids of Europe, North Africa and the Middle East. A&C Black, London

Dobzhansky T (1937) Genetics and the origin of species. Columbia University Press, New York

Dressler RL (1981) The orchids: natural history and classification. Harvard University Press, Cambridge, MA

Dressler RL (1993) Phylogeny and classification of the orchid family. Dioscurides Press, Cambridge, Massachusetts

Ehrendorfer F (1980) Hybridisierung, Polyploidie und Evolution bei europäisch-mediterranen Orchideen. Die Orchidee Sonderheft:15–34

Erdmann D (1996) Identifizierung und Synthese flüchtiger Signalstoffe aus Insekten und ihren Wirtspflanzen. PhD thesis, University of Hamburg

Gigord LDB, Macnair MR, Smithson A (2001) Negative frequency-dependent selection maintains a dramatic flower color polymorphism in the rewardless orchid *Dactylorhiza sambucina* (L.) Soo. Proc Natl Acad Sci USA 98:6253–6255

Gögler J, Stökl J, Sramkova A, Twele R, Francke W, Cortis P, Scrugli A, Giotta C, Piccitto M, Ayasse M (2008) The role of pollinator attracting scent in the sexually deceptive orchids *Ophrys chestermanii, O. normanii,* and *O. tenthredinifera*. Mitt Dtsch Ges Allg Angew Entomol 16:175–178

Gögler J, Stökl J, Sramkova A, Twele R, Francke W, Cozzolino S, Cortis P, Scrugli A, Ayasse M (2009) Ménage à trois – two endemic species of deceptive orchids and one pollinator. Evolution 63(9):2222–2234

Gölz P, Reinhard HR (1990) Beitrag zur kenntnis der Orchideenflora sardiniens. Mitt Bl Arbeitskr Heim Orch Baden-Württ 22:405–510

Jersakova J, Johnson SD, Kindlmann P (2006) Mechanisms and evolution of deceptive pollination in orchids. Biol Rev 81:219–235

Johnson SD (2007) Pollinator-driven speciation in plants. In: Harder LD, Barrett SCH (eds) Ecology and evolution of flowers. Oxford University Press, Oxford, pp 295–310

Kullenberg B (1961) Studies in *Ophrys* pollination. Zool Bidr Upps 34:1–340

Kullenberg B, Bergström G (1973) The pollination of *Ophrys* orchids. Academic, Lidingo, Sweden

Mant J, Peakall R, Schiestl FP (2005) Does selection on floral odor promote differentiation among populations and species of the sexually deceptive orchid genus *Ophrys*? Evolution 59:1449–1463
Mayr E (1970) Populations, species and evolution an abridgment of animal species and evolution. Belknap, Cambridge, MA
Nilsson LA (1992) Orchid pollination biology. Trends Ecol Evol 7:255–259
Paulus HF (2001) Material zu einer Revision des *Ophrys fusca* s.str. Artenkreises I. – *Ophrys nigroaenea-fusca*, *O. colletes-fusca*, *O. flavipes-fusca*, *O. funera*, *O. forestieri* oder was ist die typische *Ophrys fusca* Link 1799 (Orchidaceae)? J Eur Orch 33:121–177
Paulus HF (2006) Deceived males – pollination biology of the Mediterranean orchid genus *Ophrys* (Orchidaceae). J Eur Orch 38:303–353
Paulus HF, Gack C (1990) Pollinators as prepollinating isolation factors evolution and speciation in *Ophrys* (Orchidaceae). Isr J Bot Basic Appl Plant Sci 39:43–80
Paulus HF, Gack C (1995) Zur Pseudokopulation und Bestäubung in der Gattung *Ophrys* (Orchidaceae) Sardiniens und Korsikas. Jber Naturwiss Ver Wuppertal 48:188–227
Peakall R (1990) Responses of male *Zaspilothynnus trilobatus* turner wasps to females and the sexually deceptive orchid it pollinates. Funct Ecol 4:159–168
Pijil LVD, Dodson CH (1966) Orchid flowers: their pollination and evolution. University of Miami Press, Coral Gables
Rieseberg LH, Brouillet L (1994) Are many plant species paraphyletic? Taxon 43:21–32
Rieseberg LH, Willis JH (2007) Plant speciation. Science 317:910–914
Rossi W, Corrias B, Arduino P, Cianchi R, Bullini L (1992) Gene variation and gene flow in *Orchis morio* (Orchidaceae) from Italy. Plant Syst Evol 179:43–58
Schiestl FP (2005) On the success of a swindle: pollination by deception in orchids. Naturwissenschaften 92:255–264
Schiestl FP, Ayasse M (2002) Do changes in floral odor cause speciation in sexually deceptive orchids? Plant Syst Evol 234:111–119
Schiestl FP, Cozzolino S (2008) Evolution of sexual mimicry in the orchid subtribe orchidinae: the role of preadaptations in the attraction of male bees as pollinators. BMC Evol Biol 8:27. doi:10.1186/1471-2148-8-27
Schiestl FP, Schlüter PM (2009) Floral isolation, specialized pollination, and pollinator behaviour in orchids. Annu Rev Entomol 54:425–446
Schiestl FP, Ayasse M, Paulus HF, Löfstedt C, Hansson BS, Ibarra F, Francke W (1999) Orchid pollination by sexual swindle. Nature 399:421–422
Schiestl FP, Ayasse M, Paulus HF, Löfstedt C, Hansson BS, Ibarra F, Francke W (2000) Sex pheromone mimicry in the early spider orchid (*Ophrys sphegodes*): patterns of hydrocarbons as the key mechanism for pollination by sexual deception. J Comp Physiol A 186:567–574
Schlüter PM (2006) Pollinator driven evolution in *Ophrys fusca* s.l. (Orchidaceae): insights from molecular studies with DNA fingerprint and sequence markers. Phd thesis, University of Vienna
Schlüter PM, Harris SA (2006) Analysis of multilocus fingerprinting data sets containing missing data. Mol Ecol Notes 6:569–572
Schlüter PM, Schiestl FP (2008) Molecular mechanisms of floral mimicry in orchids. Trends Plant Sci 13:228–235
Scopece G, Musacchio A, Widmer A, Cozzolino S (2007) Patterns of reproductive isolation in Mediterranean deceptive orchids. Evolution 61:2623–2642
Seehausen O (2004) Hybridization and adaptive radiation. Trends Ecol Evol 19:198–207
Soliva M, Kocyan A, Widmer A (2001) Molecular phylogenetics of the sexually deceptive orchid genus *Ophrys* (Orchidaceae) based on nuclear and chloroplast DNA sequences. Mol Phylogen Evol 20:78–88
Soliva M, Widmer A (2003) Gene flow across species boundaries in sympatric, sexually deceptive *Ophrys* (Orchidaceae) species. Evolution 57:2252–2261
Stebbins GL, Ferlan L (1956) Population variability, hybridization introgression in some species of *Ophrys*. Evolution (New York) 10:32–46

Stökl J, Paulus H, Dafni A, Schulz C, Francke W, Ayasse M (2005) Pollinator attracting odour signals in sexually deceptive orchids of the *Ophrys fusca* group. Plant Syst Evol 254:105–120

Stökl J, Twele R, Erdmann D, Francke W, Ayasse M (2007) Comparison of the flower scent of the sexually deceptive orchid *Ophrys iricolor* and the female sex pheromone of its pollinator *Andrena morio*. Chemoecology 17:231–233

Stökl J, Schlüter PM, Stuessy TF, Paulus HF, Assum G, Ayasse M (2008) Scent variation and hybridization cause the displacement of a sexually deceptive orchid species. Am J Bot 95:472–481

Stökl J, Schlüter PM, Stuessy TF, Paulus HF, Fraberger R, Erdmann D, Schulz C, Francke W, Assum G, Ayasse M (2009) Pollinator driven selection maintains discrete species in hybridizing sexually deceptive orchids of the genus *Ophrys*. Bot J Linn Soc 98:439–451

Stoutamire WP (1983) Wasp pollinated species of *Caladenia* Orchidaceae in Southwestern Australia. Aust J Bot 31:383–394

Vereecken NJ (2008) Pollinator-mediated selection, reproductive isolation and the evolution of floral traits in the genus *Ophrys* (Orchidaceae). PhD thesis, Free University of Brussels

Vereecken NJ, Mant J, Schiestl FP (2007) Population differentiation in female sex pheromone and male preferences in a solitary bee. Behav Ecol Sociobiol 61:811–821

Whitehead M, Peakall R (2009) Integrated floral scent, pollination ecology and population genetics. Funct Ecol 23(5):863–874

Waser NM, Campbell DR (2004) Ecological speciation in flowering plants. In: Dieckmann U (ed) Adaptive speciation. Cambridge University Press, Cambridge, pp 264–277

Wood JJ (1983) *Ophrys holoserica* (Burm. f.) Greuter subsp. *chestermanii* J. J. Wood and *O*. X *normanii* J. J. Wood. Orchid Rev 91:383–385

Population Genetics of Speciation and Demographic Inference Under Population Subdivision: Insights from Studies on Wild Tomatoes (*Solanum* sect. *Lycopersicon*)

Wolfgang Stephan and Thomas Städler

Abstract Multilocus sequencing studies assessing patterns of nucleotide polymorphism within and among closely related species provide valuable insights into the evolutionary processes involved in species divergence. We have employed the analytical framework of divergence population genetics in testing models of speciation in two species of wild tomatoes (clade *Lycopersicon*). However, all current implementations of divergence models assume panmixia within ancestral and extant species which introduces biases of potentially large magnitude, depending on the sampling scheme employed in empirical studies. Moreover, our coalescent simulations of samples from subdivided expanding populations confirm that, except at very high migration rates, sampling local populations is not equivalent to sampling from panmictic populations, with implications for studies spanning the range from *Drosophila* to humans. Within the constraints imposed by the complexities of the coalescent process in subdivided populations that are not accounted for in current divergence models, we found evidence for recent speciation ($\leq$0.55 million years) of the two wild tomato species, which based on patterns of linkage disequilibrium has occurred under residual gene flow.

W. Stephan (✉)
Department of Biology II, Section of Evolutionary Biology, University of Munich (LMU), Grosshaderner Strasse 2, 82152 Planegg-Martinsried, Germany
e-mail: stephan@bio.lmu.de

T. Städler
ETH Zurich, Institute of Integrative Biology, Plant Ecological Genetics, Universitätstrasse 16, 8092 Zurich, Switzerland
e-mail: thomas.staedler@env.ethz.ch

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_7,

1 Introduction

The mechanisms of species divergence have long been contentious. The importance of geographic isolation in facilitating evolutionary divergence as a consequence of mutation and genetic drift (or additionally, adaptive differentiation) was recognized early, and the process of allopatric speciation is uncontroversial on theoretical grounds (Coyne and Orr 2004, and references therein). If residual gene flow characterized the divergence of incipient species, however, modes *other* than strict allopatric speciation must be invoked, and these invariably require natural selection as one of the factors underlying species divergence. In principle, divergence under residual gene flow may proceed in sympatry or under parapatric conditions, i.e., geographically adjacent populations may be subject to directional selection incidentally conferring reproductive isolation (Endler 1977; Turelli et al. 2001). An initial period of divergence in allopatry may precede subsequent contact allowing gene flow and thus possibly direct selection for stronger interpopulation barriers ("reinforcement" of reproductive isolation; Rice and Hostert 1993; Coyne and Orr 2004). Some researchers posit that interspecific hybridization and postdivergence gene flow following secondary contact may promote novel advantageous gene combinations in populations of mixed ancestry, perhaps contributing to adaptive divergence and speciation (e.g., Seehausen 2004; Mallet 2005; Arnold 2006).

Multilocus DNA sequences collected within and among closely related species are useful to characterize the speciation process, as they contain a wealth of historical–demographic information and are particularly informative when considered in the framework of genealogical models. As an extension of population genetic procedures to the species level, the analytical framework of divergence population genetics (DPG) encompasses coalescent-based models to infer historical attributes of lineage divergence from a common ancestor (e.g., Wakeley and Hey 1997; Wang et al. 1997; Kliman et al. 2000). The DPG approach accommodates the stochastic nature of lineage sorting and thus the (gradually decreasing) segregation of shared ancestral polymorphism in the descendant species, as these become more differentiated through genetic drift and the accumulation of new mutations. One notable restriction of even the most mathematically sophisticated inference procedures implemented in current DPG programs (Nielsen and Wakeley 2001; Hey and Nielsen 2004, 2007) is the assumption of random mating within both ancestral and descendant (extant) species, i.e., the arguably important phenomenon of population subdivision is virtually ignored.

In our research program, we applied the DPG approach to wild tomatoes, a small monophyletic clade within the large genus *Solanum* (Solanaceae). Depending on taxonomic definitions, it consists of 9–13 species (Spooner et al. 2005; Peralta et al. 2008), of which the only cultivated species is *S. lycopersicum*. The geographic range of wild tomatoes extends from central Ecuador to northern Chile, mostly along the western slopes of the Andes and the coastal region of northwest South America (Moyle 2008). Although the tomato clade is relatively small, it spans a

wide range of climatic, biogeographical and environmental gradients, ranging from temperate deserts to wet tropical rainforests. Each species appears to display a characteristic geographical distribution pattern within this environmental variation (Rick 1979). Many traits that are putative adaptive responses to the environment have been identified, suggesting that abiotic ecological conditions play an important role in these species' evolution and speciation (Nakazato et al. 2008; Moyle 2008). As a consequence, wild tomato species exhibit great morphological, physiological, life history, and mating system variation.

Although the extant tomato species are phenotypically distinct, molecular evidence suggests that they are relatively young. Previous studies of wild tomatoes using a variety of molecular markers have generally found low levels of differentiation between species (e.g., Stephan and Langley 1998; Baudry et al. 2001), implying a fairly recent divergence of the tomato clade. In particular, our first comprehensive multilocus DNA study of three self-incompatible species demonstrated patterns of variation (including large numbers of shared polymorphisms in addition to fixed differences) that suggested the suitability of this clade as a plant speciation model under the DPG framework (Städler et al. 2005). However, this study was limited in that only single local population samples were used per species, thus ignoring possible complications due to population subdivision. Population substructure and its implications for historical and demographic inference became a major focus of our subsequent research on wild tomatoes, necessitating consideration of sampling schemes and their genealogical consequences. In addition to revealing more details of the speciation process, this line of work has yielded insights of general importance for many evolutionary and population genetic studies that have hitherto relied on the unrealistic assumption of random mating within species.

2 Materials and Methods

2.1 Population Sampling and Sequenced Loci

For the most detailed study of speciation in the tomato clade to date, we concentrated on two self-incompatible species that are very closely related: the widely distributed *Solanum peruvianum* and the southernmost tomato species, *S. chilense* (Fig. 1; Städler et al. 2005, 2008). The two morphologically differentiated species have partly overlapping ranges in the arid coastal regions of southern Peru and northern Chile, west of the continental divide (Rick 1979, 1986; Moyle 2008). It was recently proposed to recognize four species in what traditionally was regarded as the polymorphic *S. peruvianum* (Spooner et al. 2005; Peralta et al. 2008). According to this proposition, our sampling of three natural populations in central and southern Peru (see below) would encompass both the new entity *S. corneliomulleri* and *S. peruvianum* sensu stricto. However, there appear to be neither molecular data nor crossing results that would validate the proposed split of *S. corneliomulleri* from *S. peruvianum* s. str.; we thus treat all our new samples as *S. peruvianum*.

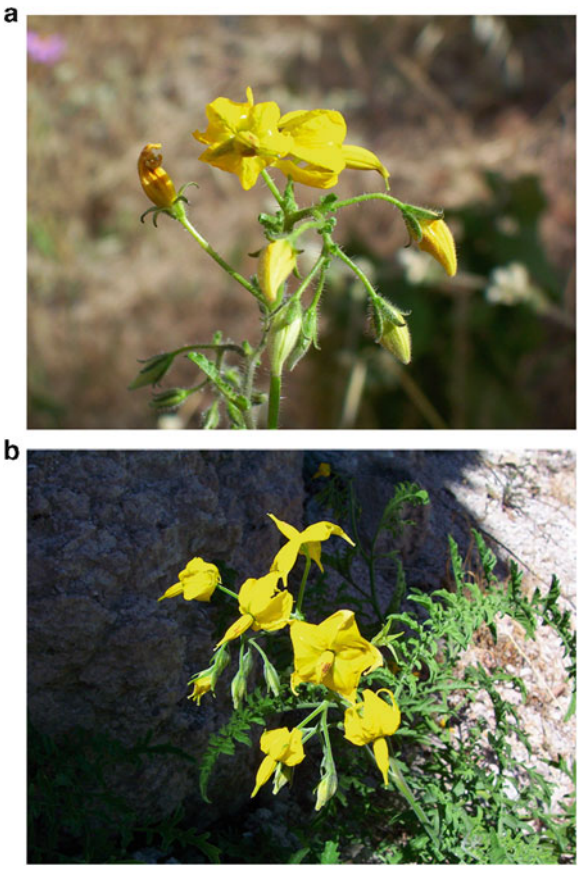


Fig. 1 Details of a *S. peruvianum* plant from the Canta population (**a**, *top*), contrasted with a *S. chilense* plant from the Moquegua population (**b**, *bottom*). Note the curved anther cone and more hairy stem in *S. peruvianum* versus the straight anther cone and erect growth form in *S. chilense*. The habitat of the Canta population is mesic whereas that of the Moquegua population is extremely arid; digital photographs by Gabriel Clostre

There is no published evidence for interspecific hybridization between *S. chilense* and *S. peruvianum* in their natural habitats, in concordance with strong postzygotic barriers uncovered in experimental crossing studies (Rick and Lamm 1955; Rick 1979). However, the limited amount of molecular data available for natural populations does not rule out undetected low or moderate levels of interspecific hybridization.

Six of the eight populations used in our study were sampled in southern and central Peru in May 2004 (Fig. 2): the *S. chilense* samples are from Quicacha, Moquegua, and Tacna (north to south), and those of *S. peruvianum* from Canta, Nazca, and Arequipa. For the other two populations, plant material was obtained from the Tomato Genetics Resource Center at the University of California Davis (*S. chilense* from Antofagasta, Chile, and *S. peruvianum* from Tarapaca, Chile). With the exception of the Canta population (*S. peruvianum*) and the population from Antofagasta

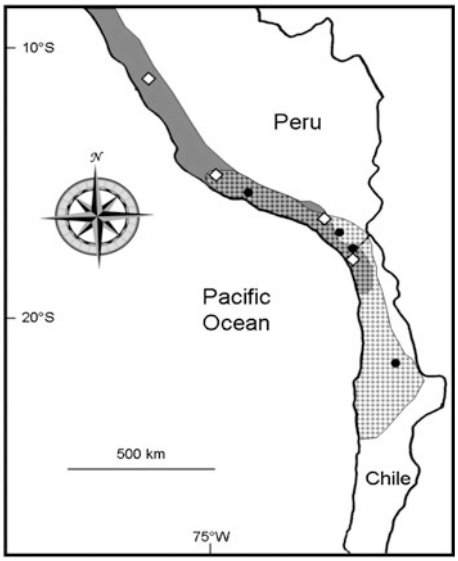


Fig. 2 Geographic ranges of *S. peruvianum* (*dark gray*) and *S. chilense* (*cross-hatched*) and approximate locations of the sampled populations. *S. peruvianum* populations from south to north (*white diamonds*): Tarapaca, Arequipa, Nazca, Canta; *S. chilense* populations from south to north (*black dots*): Antofagasta, Tacna, Moquegua, Quicacha. Note the region of sympatry in northernmost Chile and southern Peru. The former *S. peruvianum* sensu lato extends to northern Peru, but most populations north of ca. 9–10°S latitude are now regarded as the separate species *S. arcanum*. Reprinted, with permission, from Städler et al. (2008); copyright held by the Genetics Society of America

(*S. chilense*), all samples are from regions of sympatry with the other species, even though this may not be true at a local scale. The Canta population, however, is far north of the *S. chilense* species range, and the Antofagasta population is far south of the *S. peruvianum* distribution. Our sampling of several genuine populations represents one of two major sampling strategies that have been advocated for (mostly) different purposes, but we contend that the consequences and limitations of a given sampling scheme have not been sufficiently understood (see below).

We sequenced a subset of the loci used in our initial surveys (Städler et al. 2005; Roselius et al. 2005); CT066, CT093, CT166, CT179, CT198, CT208, CT251, and CT268 are eight anonymous, mapped single-copy cDNA markers. However, we found evidence of non-neutral evolution at locus CT208 where a clinal pattern of variation was detected in *S. chilense*, possibly reflecting an incomplete selective sweep (Arunyawat et al. 2007). Because the inclusion of loci under positive selection can mislead historical inference, our DPG analyses encompass only the seven remaining loci that show no obvious departures from neutral expectations. The nuclear sequence data were obtained from 5 to 7 plants per population (10–14 fully resolved alleles), and the sequence length concatenated over all eight loci is about 10.3 kb per "allele." All laboratory procedures and methods of population

genetic data analyses are provided in detail elsewhere (Arunyawat et al. 2007; Städler et al. 2008).

2.2 The Isolation Speciation Model

To analyze mainly demographic aspects of lineage divergence from a common ancestral population, we specifically used the isolation speciation model of Wakeley and Hey (1997), henceforth called the WH model (Fig. 3). This simple model of allopatric speciation assumes that an ancestral, panmictic species gave rise to two extant species at time τ in the past ($\tau = 2\,ut$, where t is the number of generations since speciation and u the mutation rate per generation per total genomic region sequenced). The ancestral species is characterized by the population mutation parameter θ_A and the two extant species by the parameters θ_1 and θ_2, respectively. The population mutation parameters are each given by $4N_e u$, where N_e denotes the effective population size. Hence, effective population size is assumed to be constant within species, but is allowed to change at the time of speciation. The model further assumes neutral evolution of molecular polymorphisms and no gene flow subsequent to the initial species divergence. As shown by Wakeley and Hey (1997), the expectations of the observable quantities S_{x1}, S_{x2}, S_s, and S_f (exclusive polymorphisms for species 1 and 2, shared polymorphisms and fixed differences, respectively) are functions of the four model parameters. Hence, by equating observations with expectations, their moment-based algorithm yields the parameter estimates from multilocus data, and goodness-of-fit with the model is assessed via coalescent simulations implementing the locus-specific recombination rates estimated from the empirical data (see Städler et al. 2008).

Most current applications of the DPG approach now use the more parameter-rich "isolation-with-migration" models (IM and IMa; Hey and Nielsen 2004, 2007) that incorporate bidirectional interspecific gene flow as parameters to be estimated

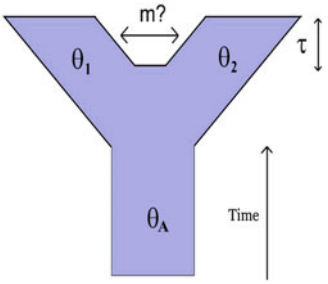


Fig. 3 Outline of the Wakeley-Hey isolation model of species divergence. Two descendant species diverge from a common ancestor at time τ in the past, with no interpopulation gene flow assumed since the onset of divergence. The population mutation parameters (θ_1, θ_2, θ_A, respectively) should primarily reflect differences in long-term effective population size between species, and they as well as τ are estimated from multilocus sequence data (see text). The possibility of introgression is indicated by "*m?*". Modified after Wakeley and Hey (1997)

from the multilocus sequence data, rather than assuming divergence in complete isolation. However, the IM/IMa algorithms share the undesirable feature of not being able to accommodate recombining data within loci; abundant evidence for high levels of recombination in the history of our wild tomato sequences (Arunyawat et al. 2007; Städler et al. 2008) has led us to "prefer" the WH model as our analytical framework, rather than to limit the analyses of sequence data to short stretches of apparently nonrecombining blocks.

2.3 *Sampling Schemes, Population Subdivision, and Range Expansions*

Motivated by our finding that the site frequency spectra (SFS) of segregating mutations are systematically biased toward low-frequency variants in "pooled" samples compared to the averages of local population samples (referred to as the "pooling effect"; Arunyawat et al. 2007), we initiated a series of coalescent simulations designed to evaluate the impact of different sampling schemes on the SFS under population subdivision and historical expansion of the total population. A pooled sample refers to all sequences obtained from the same species treated as a single entity, rather than as (in our case) four separate population samples that represent "local" samples. Consequently, sampling several populations per species allows one to contrast the average characteristics of local samples and those of the pooled sample. The third sampling scheme ("scattered") refers to single sequences obtained from each of many demes and is known to yield genealogies with similar properties as those from panmictic populations (Wakeley 1999, 2001; Wakeley and Aliacar 2001). The pooling effect appears to be widespread across diverse taxa, having been found in humans (Ptak and Przeworski 2002), *Drosophila* (Pool and Aquadro 2006), and several plant species (Ingvarsson 2005; Arunyawat et al. 2007; Moeller et al. 2007); however, a genuine understanding and consensus as to the underlying evolutionary processes has not been achieved.

Because we interpreted the common observation of lower/more negative Tajima's (1989) D values for pooled samples, compared to local samples, as genealogical signatures of population expansions (Arunyawat et al. 2007), our coalescent simulations focused on the establishment of population structure some time τ in the past (i.e., before time τ the population was panmictic) with concomitant increase in total population size. This scheme ought to be plausible under range expansions, as exemplified by migration out of Africa by both humans and *Drosophila melanogaster*, and subsequent colonization of expansive areas, or temperate-zone populations expanding from glacial refugia, or following a speciation event such as that inferred for the two wild tomato species, *S. peruvianum* and *S. chilense*. Specifically, we used a modified version of the coalescent simulation program ms (Hudson 2002) to simulate samples under both island-model and two-dimensional stepping-stone spatial structure, usually with 100 local subpopulations. These simulations encompassed a broad range of migration rates between

subpopulations, timing of the onset of population structure and simultaneous expansion, and magnitude of the overall expansion. Samples of size 20 were either distributed over 20 randomly chosen subpopulations ("scattered" sampling), four subpopulations ("pooled" sampling), or taken from a single subpopulation ("local" sampling). We computed average summary statistics based on the SFS (Tajima's *D* and Fu and Li's *D*) and evaluated the joint impact of the simulated sampling schemes and demographic history on these statistics. Further technical details of these simulations are described in Städler et al. (2009).

3 Results and Discussion

3.1 WH Model Parameter Estimation and the Impact of Population Subdivision

When all sequences obtained for *S. peruvianum* are compared with all *S. chilense* sequences (i.e., using the pooled data for each species), none of the loci exhibits any fixed interspecific differences, attesting to their very low level of molecular divergence. This, however, presents problems for the WH algorithm because both fixed differences and shared polymorphisms are required for robust parameter estimation and goodness-of-fit tests via coalescent simulations. To ameliorate this situation, we contrasted all nine combinations of interspecific pairs of high-diversity populations, as well as three non-arbitrary pooled samples (no more than two local samples per species) containing both high proportions (>85%) of the specieswide nucleotide diversity and a minimum of two fixed differences. Two features of the multilocus data characterize all such interspecific population comparisons: all loci exhibit multiple shared polymorphisms while there are only fixed interspecific differences at two of the loci.

Across all these interspecific comparisons (whether based on single populations or pooled samples), the estimates of ancestral population size (θ_A) are comparable to those for the *S. chilense* samples (θ_2), whereas the effective size of *S. peruvianum* (θ_1) is estimated to be about twice as large. As is customary for other such studies, the WH parameter estimates have broad confidence intervals. A notable result is that the divergence time estimates imply very recent divergence from a common ancestor, in the range of 0.22–0.40 units (= *S. peruvianum* N_e generations). Although the genealogical histories of our samples violate the model's assumption of within-species panmixia, coalescent simulations implementing the estimated recombination levels did not reject the simple WH isolation model. Summarizing these contrasts between observed data and expectations under the WH model, it is evident that our data fit the model surprisingly well, which is mainly due to the fairly even distribution of the polymorphic site classes across loci (Städler et al. 2008).

Because an alternative approach to estimate the size of the ancestral species and the scaled divergence time has been suggested for cases of very recent divergence

(i.e., lack of fixed interspecific differences; Wakeley and Hey 1997), we also calculated the minimum and average number of pairwise interspecific sequence differences, min (k_{ij}) and d_{12}, respectively. From the data summed over all loci, we obtained estimates of $\theta_A = 91.7$ and $\tau = 51$. This alternative estimate of θ_A is very close to the conventional WH estimates, whereas the alternative estimate of τ represents a roughly two-fold increase over most of the conventional WH estimates (Städler et al. 2008). Thus, using the estimate of the neutral mutation rate of 5.1×10^{-9} per site per year (Roselius et al. 2005), the divergence time of the two species is likely $\leq$0.55 million years.

An evaluation of multilocus studies using the WH approach suggests that only very recent or current introgression may allow formal rejection of the isolation model, making this a very conservative test. For example, several multilocus DPG studies were able to reject the isolation model due to large differences in patterns of shared polymorphisms and fixed differences among loci, a signature attributed to recent interspecific gene flow at some, but not all regions of the genome (e.g., Machado et al. 2002; Ramos-Onsins et al. 2004). However, unless multilocus studies happen to include both types of loci, the similar proportions of observed site categories across loci may indicate a good fit of the isolation model, even under postdivergence or recent interspecific gene flow at all loci, emphasizing the very conservative nature of the goodness-of-fit criteria. As noted above, the demographic estimates under the WH isolation model imply a modest population (or range) expansion for *S. peruvianum* and an effective size for *S. chilense* similar to that of the ancestral species. However, as we discuss in the following, these WH estimates of demographic history are likely to be biased, as other aspects of the multilocus data provide clear evidence for population expansions in both taxa. These biases may be jointly caused by particular properties of our dataset (lack of fixed differences) and the complexities of the coalescent process in subdivided populations that are not accounted for in current DPG implementations.

Wakeley (1999, 2000, 2001) introduced the distinction between the "scattering" and the "collecting" phase of the coalescent process in subdivided populations. The brief scattering phase traces the genealogy of a local sample until all remaining lineages (looking backwards in time) are located in different demes and is characterized by local coalescent events and migration events to different demes. The timescale of the ensuing, much longer, collecting phase depends on the rate of migration between demes and the number and size of demes in the total population, in that ancestral lineages can only coalesce when occupying the same deme. Because only scattered samples collected under equilibrium finite-island or two-dimensional stepping-stone spatial structure possess genealogical properties similar to those from panmictic populations (Wakeley 2000, 2001; Wakeley and Aliacar 2001; De and Durrett 2007), empirical studies employing other than truly scattered sampling have to consider effects of the scattering phase that would manifest themselves as deviations from panmixia.

Our high-diversity local samples contain significant proportions of the species-wide nucleotide diversity (three of four samples in both *S. peruvianum* and *S. chilense* with >80% of species-wide nucleotide diversity π; Arunyawat et al. 2007),

suggesting that local coalescent events during the scattering phase are fairly rare. However, local population samples contain substantial proportions of "private" low-frequency variants (mostly singletons) that hardly contribute to nucleotide diversity but fuel a large increase in the number of segregating sites in pooled samples. In other words, the θ estimator that is based on the number of segregating sites (Watterson 1975) increases markedly upon pooling whereas π only increases marginally. The features reflect the on average lower proportion of external branches in single-deme genealogies and explain why Tajima's *D* tends to be lower/more negative for pooled samples. There is thus evidence in our wild tomato data for the expected effects of (1) underestimating the within-species diversity using single-deme samples, as well as (2) underestimating divergence between species when considering scattered samples (Wakeley 2000); both are consequences of the distribution of diversity within and between local demes under population subdivision and the generally higher species-wide effective population size under low migration rates (Wakeley 2001; Pannell 2003).

We previously argued that our observations of significantly negative Tajima's *D* and/or Fu and Li's (1993) *D* values in pooled samples can only be explained by demographic/range expansion in both species (Arunyawat et al. 2007). When sampling has been local, signatures of a species-wide expansion are expected to be evident only under high rates of gene flow (Ray et al. 2003); using pooled population samples would enhance the detectability of such historical demographic changes roughly as a function of the number of demes over which the total sample is distributed (Städler et al. 2009). This follows from the genealogical principles discussed above, i.e., with decreasing levels of gene flow, local-sample genealogies are distorted due to scattering-phase coalescent events resulting in short external branches, whereas pooling has effects equivalent to adding migrants, i.e., unrelated individuals yielding sample genealogies with longer external branches (Ray et al. 2003; De and Durrett 2007; Arunyawat et al. 2007).

Clearly, the WH estimates for both extant species (θ_1 and θ_2) do not reflect the marked expansion inferred from other features of the data (see above). Assuming that the expansion time of both species mirrors their speciation time, part of the explanation could be that, given the low estimates of τ, there is not as much information about the descendant species in the data as about their common ancestor, unlike under much longer divergence times (Wakeley and Hey 1997). The discrepant demographic inferences can also partly be explained by our simulation findings, since the WH model treats any empirical sample as one from a panmictic population and hence picks up only attenuated signals of expansion in local or pooled samples, leading to smaller estimates of an expansion factor (see below; Städler et al. 2009). Another factor arguably contributing to underestimating θ_1 and θ_2 in our WH analyses is the lack of fixed nucleotide differences in many pooled sample comparisons; this lack of fixed differences also prevented systematic evaluation of randomly generated multideme samples that might otherwise have ameliorated some of the sampling-induced biases.

While this particular constraint would not be expected in comparisons of more diverged species, even scattered samples obtained from subdivided species do not

satisfy the critical assumption of panmixia that is underlying all current DPG algorithms, as divergence time τ is expected to be systematically underestimated. These caveats seem under-appreciated in empirical studies; recent plant studies using either the MIMAR (Becquet and Przeworski 2007), WH, or IM/IMa models of divergence typically pay little attention to the consequences of sampling design and tend to interpret the inferred parameter estimates at face value (e.g., Zhang and Ge 2007; Slotte et al. 2008; Strasburg and Rieseberg 2008; Foxe et al. 2009). Clearly, there is a need for more realistic models of speciation that explicitly take population subdivision into account in extracting signals of species' demographic history from sequence data.

3.2 Assessing Postdivergence Gene Flow by Linkage Disequilibrium (LD)

In addition to reflecting truly ancestral mutations as envisaged under the isolation model, shared polymorphisms between recently diverged taxa can arise through introgression subsequent to species divergence, a biologically plausible process under parapatric speciation or upon secondary contact after some divergence in allopatry. Machado et al. (2002) introduced a test of postdivergence gene flow based on patterns of LD among segregating sites. Under a scenario of gene flow, LD among pairs of shared polymorphisms in the recipient species should tend to be positive (i.e., preponderance of ancestral–ancestral and/or derived–derived SNP associations), and LD among pairs of sites where one member is a shared and the other an exclusive polymorphism should tend to be negative (i.e., preponderance of ancestral–derived SNP associations). Both expected effects can be seen as a consequence of insufficient time for recombination to erode LD (given introgression has occurred after initial species separation) compared to the situation where shared polymorphisms represent truly ancestral mutations, i.e., those preceding speciation.

Applying this test using coalescent simulations under the inferred WH demographic and divergence-time parameters, we found that locus CT066 exhibits significantly high LD values for all individual- and pooled-sample comparisons between both species, and locus CT166 shows significant or nearly significant LD values for several of the contrasts, reflecting stronger LD for a subset of intragenic LD than expected under isolation-model conditions (Städler et al. 2008). These results suggest bidirectional interspecific gene flow following initial species divergence, whereas there is no compelling evidence for such a scenario at the other loci. These coalescent simulations were run with what are likely underestimates of the true divergence time, making this test conservative, but the effects on LD of more drastic demographic expansions than those simulated are more difficult to predict. Moreover, our seemingly incongruent results (failure to reject speciation by isolation due to the good fit of the multilocus distribution of the four classes of segregating sites versus evidence for postdivergence gene flow based on patterns of intragenic

LD) are easily reconcilable, given the very conservative behavior of the goodness-of-fit tests and the different aspects of the data considered by each approach.

A second, more qualitative way to detect possible traces of past or present introgression looks for differences in the proportion of shared polymorphisms among all segregating sites between allopatric and sympatric interspecific sample comparisons. Our data indicate that shared polymorphisms generally are geographically widespread in both species (whereas many exclusive polymorphisms are geographically restricted and overall rare) and tend to occur in equivalent numbers in allopatric comparisons, inconsistent with expectations under very recent introgression. Moreover, the genealogical signals revealed by the LD-based test are not restricted to regions of current sympatry, as interspecific contrasts involving the allopatric Canta (*S. peruvianum*) and Antofagasta (*S. chilense*) populations also show evidence for postdivergence gene flow. These geographically dispersed signatures of postdivergence gene flow are consistent with introgression and subsequent spread through much of the species' ranges, either via post-speciation range expansions or intraspecific gene flow (Städler et al. 2008). Unlike other large-scale DPG studies that found sharing of entire haplotypes between species with partially overlapping ranges (e.g., Machado et al. 2002; Ramos-Onsins et al. 2004), our evidence for introgression is both more subtle and more difficult to uncover. The implications of having found evidence for a divergence-with-gene-flow model of speciation are, perhaps, also more interesting than for species that continue to exchange genes; for a discussion of geographical patterns of reproductive isolation between these species and their likely implications, see Städler et al. (2005, 2008).

3.3 Site Frequency Spectra in Samples from Nonequilibrium, Subdivided Populations

Using coalescent simulations, we evaluated three sampling schemes of sequences drawn from expanding subdivided populations under different levels of migration among local demes; all three sampling schemes have corresponding examples in empirical studies of DNA sequence diversity designed to infer aspects of demographic history and natural selection from summary statistics of the SFS and/or patterns of LD. Except for very low migration rates (<0.5–1 immigrants per generation), these simulations confirmed our earlier qualitative prediction that pooling several local samples is expected to increase the proportion of low-frequency polymorphisms compared to local samples, and that pooled samples are thus characterized by SFS that are intermediate (as quantified by statistics such as Tajima's D and Fu and Li's D) between those of local and scattered samples (Arunyawat et al. 2007; Städler et al. 2009). This is because as a function of decreasing levels of gene flow, local samples exhibit the distorting effects of scattering-phase coalescent events, i.e., their genealogies have lower external branch lengths; this effect impacts pooled samples to a lesser extent, depending on the exact composition of such samples.

We found effects of the sampling scheme on sample genealogies up to very high migration rates, especially under strong species-wide expansions. In other words, even under high migration rates (e.g., 25 immigrants per generation), local samples may not adequately reflect the species-wide demography. Our simulations predict a pooling effect over a wide range of migration rates, implying one way to test the null hypothesis of panmixia, i.e., that the genealogies of local samples are indistinguishable from those of scattered samples. Among the few published studies that have performed separate analyses of local population samples and the pooled sample consisting of several local samples, Pool and Aquadro (2006) demonstrated the pooling effect in sub-Saharan *Drosophila melanogaster*, as have several studies in plants (Ingvarsson 2005; Arunyawat et al. 2007; Moeller et al. 2007) and humans (e.g., Ptak and Przeworski 2002). In all these cases, the SFS of pooled samples were characterized by an excess of low-frequency variants, resulting in (more) negative Tajima's D values. So long as possible contributions of purifying selection to the SFS are accounted for, our simulation results support the interpretation that only global expansions and not the effects of population subdivision per se can generate substantially negative Tajima's D values in pooled samples (Städler et al. 2009).

It may seem surprising to observe the pooling effect even in highly mobile species such as *Drosophila melanogaster*, thus providing clear-cut evidence for deviations from panmixia at seemingly low levels of population differentiation, e.g., as quantified by F_{ST}. The impression of low levels of population substructure, however, largely stems from the classical interpretation of F_{ST} as a measure of differentiation. We have already explained above how the contrasting "dynamics" of segregating sites on the one hand and nucleotide diversity on the other hand in local versus pooled samples can explain the effect of pooling on the SFS under apparently low levels of population subdivision. Moreover, this also highlights the inherent dangers of equating low F_{ST} estimates with inconsequential levels of population subdivision.

In the framework of the infinite-alleles model, Jost (2008) recently argued that F_{ST} is not an appropriate measure of differentiation and pinpointed mathematical misconceptions underlying the standard approach. In particular, he identified the classical, additive partitioning of total heterozygosity into mean within-subpopulation heterozygosity and a between-subpopulation component as erroneous, because the latter two quantities are not truly independent but rather related through an incomplete partitioning (Jost 2008). Because classical results concerning the absolute number of migrants per generation required to prevent significant differentiation among local demes in the finite-island and stepping-stone models (e.g., $Nm > 1$) are based on the interpretation of F_{ST} as a measure of differentiation, Jost (2008) concluded that such "rules of thumb" must be considered invalid. Although he did not formally evaluate the infinite-sites model of sequence evolution, this conclusion goes hand in hand with our interpretations above. Even at moderate-to-high migration rates in our simulations, marked effects of the sampling scheme on the SFS were not restricted to expanding populations but were also found for subdivided populations at demographic equilibrium. Similar results were

previously obtained by De and Durrett (2007) for equilibrium populations and, implicitly, by Ray et al. (2003) for expanding populations.

Large-scale population genetic datasets are commonly evaluated in the framework of panmictic populations undergoing temporal changes in population size (for references, see Städler et al. 2009). Our results suggest that a more appropriate approach would acknowledge the subdivided nature of these species explicitly, which would require paying particular attention to sampling schemes and their genealogical consequences. Especially if sampling is from a local population, as has often been the case, it is inappropriate to compare patterns of diversity with expectations under the classical, neutral–equilibrium conditions embodied in commonly used tests of "neutrality." The traditional focus on population size changes through time to explain properties of local/regional samples should be questioned, especially in systems with moderate-to-high levels of gene flow where sample genealogies are not regionally monophyletic but rather embedded in species-wide genealogies. Our simulation scheme has been simplistic in assuming equal migration rates and deme sizes through time and across the entire metapopulation, but it is straightforward to explain empirical patterns, such as those found for various human population samples, in terms of differences in local population size and immigration rates during the recent past (Wakeley 2001; Wakeley and Aliacar 2001; Ray et al. 2003; Excoffier 2004).

Guided by notions of the special importance of population structure and the potential for local adaptation in plants, several recent studies have emphasized the need to include genuine local population samples, rather than relying exclusively on scattered samples (Wright and Gaut 2005; Moeller et al. 2007; Ross-Ibarra et al. 2008). While we agree that sampling locally has particular merit for studying signatures of local adaptation, our simulation results caution against analyzing such local samples naively, i.e., in the conventional framework of panmictic populations. In principle, sampling several local demes offers the opportunity to empirically contrast the properties of local samples with those of the pooled sample, analogous to two of our three simulation sampling schemes. At the very least, this exercise has the potential to infer general features of the species-wide demographic history despite the biases inherent in local samples. It would also partly mitigate against inferring spurious signatures of natural selection from levels and patterns of nucleotide polymorphism, or from the extent of haplotype structure (for examples, see De and Durrett 2007). However, only the genealogical structure of scattered samples is comparable to that of an elusive panmictic population with otherwise identical demographic history (e.g., species-wide expansion), whereas such samples preclude finding molecular evidence of local adaptation.

4 Summary

To obtain a more complete picture about species divergence in the tomato clade, which is considerably older than the estimated splitting time of *S. chilense* and *S. peruvianum*, we have sampled and analyzed two more self-incompatible species

(*S. arcanum* and *S. habrochaites*). Since these species may be more distantly related to *S. peruvianum* and *S. chilense*, this will allow us to study divergence patterns in the tomato clade in a rather comprehensive way. Of the self-incompatible species, only *S. pennellii* (closely related to *S. habrochaites*) and *S. huaylasense* (formerly part of *S. peruvianum*) are then not considered. Moreover, the sequence data that are currently available and those that will be available in the near future can only be comprehensively analyzed if new estimation procedures are developed that explicitly take population subdivision in both extant and ancestral species into account. To handle the complexity of the data, Approximate Bayesian methods appear to be most promising (for a recent example implementing panmixia assumptions, see Ross-Ibarra et al. 2009). Finally, a general conclusion of our analyses is that sampling schemes can influence genealogical signatures in sequence data even under high levels of migration. Given the panmixia assumption of current DPG algorithms, it follows that, to obtain more robust estimates of species' historical demography, one needs to collect data from geographically scattered samples. To this end, we are currently collecting sequence data from a large number of populations per species.

Acknowledgments We thank our past and present graduate students and technicians for their invaluable contributions to these studies: U. Arunyawat, G. Feldmaier-Fuchs, H. Lainer, T. Marczewski, C. Merino, and K. Roselius. The coalescent analyses benefitted from contributions by B. Haubold, S. Mousset, P. Pfaffelhuber, and A. Tellier. Our field work in Peru was greatly facilitated by administrative and logistical support from A. Cano and G. Clostre; permits to sample and export leaf material to Germany were granted by the Peruvian *Instituto Nacional de Recursos Naturales* (INRENA). Finally, we are grateful to the generous research funding from the *Deutsche Forschungsgemeinschaft* throughout the duration of the SPP-1127 "Radiations – Origins of Biological Diversity" (grants Ste 325/5-1 to 325-3).

References

Arnold ML (2006) Evolution through genetic exchange. Oxford University Press, New York

Arunyawat U, Stephan W, Städler T (2007) Using multilocus sequence data to assess population structure, natural selection and linkage disequilibrium in wild tomatoes. Mol Biol Evol 24:2310–2322

Baudry E, Kerdelhué C, Innan H, Stephan W (2001) Species and recombination effects on DNA variability in the tomato genus. Genetics 158:1725–1735

Becquet C, Przeworski M (2007) A new approach to estimate parameters of speciation models with application to apes. Genome Res 17:1505–1519

Coyne JA, Orr HA (2004) Speciation. Sinauer Associates, Sunderland, MA

De A, Durrett R (2007) Stepping-stone spatial structure causes slow decay of linkage disequilibrium and shifts the site frequency spectrum. Genetics 176:969–981

Endler JA (1977) Geographic variation, speciation, and clines. Princeton University Press, Princeton, NJ

Excoffier L (2004) Patterns of DNA sequence diversity and genetic structure after a range expansion: lessons from the infinite-island model. Mol Ecol 13:853–864

Foxe JP, Slotte T, Stahl EA, Neuffer B, Hurka H, Wright SI (2009) Recent speciation associated with the evolution of selfing in *Capsella*. Proc Natl Acad Sci USA 106:5241–5245

Fu Y-X, Li W-H (1993) Statistical tests of neutrality of mutations. Genetics 133:693–709
Hey J, Nielsen R (2004) Multilocus methods for estimating population sizes, migration rates and divergence time, with applications to the divergence of *Drosophila pseudoobscura* and *D. persimilis*. Genetics 167:747–760
Hey J, Nielsen R (2007) Integration within the Felsenstein equation for improved Markov chain Monte Carlo methods in population genetics. Proc Natl Acad Sci USA 104:2785–2790
Hudson RR (2002) Generating samples under a Wright-Fisher neutral model of genetic variation. Bioinformatics 18:337–338
Ingvarsson PK (2005) Nucleotide polymorphism and linkage disequilibrium within and among natural populations of European aspen (*Populus tremula* L., Salicaceae). Genetics 169:945–953
Jost L (2008) G_{ST} and its relatives do not measure differentiation. Mol Ecol 17:4015–4026
Kliman RM, Andolfatto P, Coyne JA, Depaulis F, Kreitman M et al (2000) The population genetics of the origin and divergence of the *Drosophila simulans* complex species. Genetics 156:1913–1931
Machado CA, Kliman RM, Markert JA, Hey J (2002) Inferring the history of speciation from multilocus DNA sequence data: the case of *Drosophila pseudoobscura* and close relatives. Mol Biol Evol 19:472–488
Mallet J (2005) Hybridization as an invasion of the genome. Trends Ecol Evol 20:229–237
Moeller DA, Tenaillon MI, Tiffin P (2007) Population structure and its effects on patterns of nucleotide polymorphism in teosinte (*Zea mays* ssp. *parviglumis*). Genetics 176:1799–1809
Moyle LC (2008) Ecological and evolutionary genomics in the wild tomatoes (*Solanum* sect. *Lycopersicon*). Evolution 62:2995–3013
Nakazato T, Bogonovich M, Moyle LC (2008) Environmental factors predict adaptive phenotypic differentiation within and between two wild Andean tomatoes. Evolution 62:774–792
Nielsen R, Wakeley J (2001) Distinguishing migration from isolation: a Markov chain Monte Carlo approach. Genetics 158:885–896
Pannell JR (2003) Coalescence in a metapopulation with recurrent local extinction and recolonization. Evolution 57:949–961
Peralta IE, Spooner DM, Knapp S (2008) Taxonomy of wild tomatoes and their relatives (*Solanum* sect. *Lycopersicoides*, sect. *Juglandifolia*, sect. *Lycopersicon*; Solanaceae). Syst Bot Monogr 84:1–186
Pool JE, Aquadro CF (2006) History and structure of sub-Saharan populations of *Drosophila melanogaster*. Genetics 174:915–929
Ptak SE, Przeworski M (2002) Evidence for population growth in humans is confounded by fine-scale population structure. Trends Genet 18:559–563
Ramos-Onsins SE, Stranger BE, Mitchell-Olds T, Aguadé M (2004) Multilocus analysis of variation and speciation in the closely related species *Arabidopsis halleri* and *A. lyrata*. Genetics 166:373–388
Ray N, Currat M, Excoffier L (2003) Intra-deme molecular diversity in spatially expanding populations. Mol Biol Evol 20:76–86
Rice WR, Hostert EE (1993) Laboratory experiments on speciation: what have we learned in forty years? Evolution 47:1637–1653
Rick CM (1979) Biosystematic studies in *Lycopersicon* and closely related species of *Solanum*. In: Hawkes JG, Lester RN, Skelding AD (eds) The biology and taxonomy of the Solanaceae. Academic, New York, pp 667–678
Rick CM (1986) Reproductive isolation in the *Lycopersicon peruvianum* complex. In: D'Arcy WG (ed) Solanaceae – biology and systematics. Columbia University Press, New York, pp 477–495
Rick CM, Lamm R (1955) Biosystematic studies on the status of *Lycopersicon chilense*. Am J Bot 42:663–675
Roselius K, Stephan W, Städler T (2005) The relationship of nucleotide polymorphism, recombination rate and selection in wild tomato species. Genetics 171:753–763

Ross-Ibarra J, Wright SI, Foxe JP, Kawabe A, DeRose-Wilson L, Gos G, Charlesworth D, Gaut BS (2008) Patterns of polymorphism and demographic history in natural populations of *Arabidopsis lyrata*. PLoS ONE 3:e2411

Ross-Ibarra J, Tenaillon M, Gaut BS (2009) Historical divergence and gene flow in the genus *Zea*. Genetics 181:1399–1413

Seehausen O (2004) Hybridization and adaptive radiation. Trends Ecol Evol 19:198–207

Slotte T, Huang H, Lascoux M, Ceplitis A (2008) Polyploid speciation did not confer instant reproductive isolation in *Capsella* (Brassicaceae). Mol Biol Evol 25:1472–1481

Spooner DM, Peralta IE, Knapp S (2005) Comparison of AFLPs with other markers for phylogenetic inference in wild tomatoes [*Solanum* L. section *Lycopersicon* (Mill.) Wettst.]. Taxon 54:43–61

Städler T, Roselius K, Stephan W (2005) Genealogical footprints of speciation processes in wild tomatoes: demography and evidence for historical gene flow. Evolution 59:1268–1279

Städler T, Arunyawat U, Stephan W (2008) Population genetics of speciation in two closely related wild tomatoes (*Solanum* section *Lycopersicon*). Genetics 178:339–350

Städler T, Haubold B, Merino C, Stephan W, Pfaffelhuber P (2009) The impact of sampling schemes on the site frequency spectrum in nonequilibrium subdivided populations. Genetics 182:205–216

Stephan W, Langley CH (1998) DNA polymorphism in *Lycopersicon* and crossing-over per physical length. Genetics 150:1585–1593

Strasburg JL, Rieseberg LH (2008) Molecular demographic history of the annual sunflowers *Helianthus annuus* and *H. petiolaris* – large effective population sizes and rates of long-term gene flow. Evolution 62:1936–1950

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595

Turelli M, Barton NH, Coyne JA (2001) Theory and speciation. Trends Ecol Evol 16:330–343

Wakeley J (1999) Nonequilibrium migration in human history. Genetics 153:1863–1871

Wakeley J (2000) The effects of subdivision on the genetic divergence of populations and species. Evolution 54:1092–1101

Wakeley J (2001) The coalescent in an island model of population subdivision with variation among demes. Theor Popul Biol 59:133–144

Wakeley J, Aliacar N (2001) Gene genealogies in a metapopulation. Genetics 159:893–905

Wakeley J, Hey J (1997) Estimating ancestral population parameters. Genetics 145:847–855

Wang RL, Wakeley J, Hey J (1997) Gene flow and natural selection in the origin of *Drosophila pseudoobscura* and close relatives. Genetics 147:1091–1106

Watterson GA (1975) On the number of segregating sites in genetical models without recombination. Theor Popul Biol 7:256–276

Wright SI, Gaut BS (2005) Molecular population genetics and the search for adaptive evolution in plants. Mol Biol Evol 22:506–519

Zhang L-B, Ge S (2007) Multilocus analysis of nucleotide variation and speciation in *Oryza officinalis* and its close relatives. Mol Biol Evol 24:769–783

Genetic Diversity, Evolution and Domestication of Wheat and Barley in the Fertile Crescent

Benjamin Kilian, William Martin, and Francesco Salamini

Abstract About 12,000 years ago, humans began the transition from hunter-gathering to a sedentary, agriculture-based society. From its origins in the Fertile Crescent, farming expanded throughout Europe, Asia and Africa, together with various domesticated plants and animals. Where, how and why agriculture originated is still debated. Progress has been made in understanding plant domestication in the last few years. The approach to understanding cereal domestication that we have taken in recent years has, in the main, involved the following five-pronged strategy: (1) the use of comprehensive germplasm collections covering the whole distribution area for each species and the collection of new germplasm for wild cereals from their primary habitats in nature; (2) the comparison of many wild and domesticated accessions for each species; (3) the identification of the wild progenitor in the wild gene pool and via comparison of genetic similarity across many loci with domesticate descendants; (4) the use of molecular fingerprinting techniques at

B. Kilian (✉)
Institute of Botany III, Heinrich-Heine-Universität Düsseldorf, Universitätsstrasse 1, 40225 Düsseldorf, Germany
Department of Plant Breeding and Genetics, Max Planck Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, 50829 Köln, Germany
Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Genebank/Genome Diversity, Corrensstrasse 3, 06466 Gatersleben, Germany
e-mail: kilian@ipk-gatersleben.de
W. Martin
Institute of Botany III, Heinrich-Heine-Universität Düsseldorf, Universitätsstrasse 1, 40225 Düsseldorf, Germany
F. Salamini
Fondazione Parco Tecnologico Padano, Via Einstein – Localita Cascina Codazza, 26900 Lodi, Italy
Department of Plant Breeding and Genetics, Max Planck Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, 50829 Köln, Germany

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_8,

many loci to compare wild and domesticate cereals; (5) the identification and cloning of genes involved in domestication. That work has provided some insights into the domestication process, insights that, placed in the archaeological context of human history in the Fertile Crescent, provide information about what humans were doing while domestication was taking place. This chapter reviews recent developments in our understanding of wheat and barley domestication history in the Fertile Crescent, events that forged the foundations of our present-day European culture.

During SPP 1127: Scope of this Study.

The main aim of the study during SPP1127 was to investigate and compare nucleotide diversity between wild and domesticated wheat and barley using large germplasm collections and different molecular markers to obtain new insights and to contribute to the ongoing discussion on the origin of agriculture and plant domestication in the Fertile Crescent.

Keywords Genetic diversity · Evolution · Domestication · Wheat · Barley · Fertile Crescent

1 Introduction

Cereals provide more than 50% of the worldwide crop production and are important renewable resources for food, feed, and industrial materials (http://faostat.fao.org). The Triticeae tribe within the Pooideae subfamily of the grass family Poaceae includes the crop genera *Triticum* (wheat), *Hordeum* (barley) and *Secale* (rye). Wheat is the primary cereal of temperate regions and the staple food for about 40% of the world's population. Globally, wheat is the second most widely grown crop, just recently superseded by maize, while barley ranks fourth after maize, wheat and rice (http://faostat.fao.org). Wheat and barley are the most important staple crops of Europe and of the western part of Asia. Wheat is mainly used for bread (*Triticum aestivum*, hexaploid) and pasta (*Triticum durum*, tetraploid), barley as fodder and for brewing beer, while rye is used for bread and fodder. Human history is closely interwoven with these three staple crops, because wheat and barley (and possibly also rye) belong to the Neolithic founder crops upon which western agriculture was built.

2 Origins of Cultivated Plants and Agriculture: A Brief Historical Overview

The origin of cultivated plants and their domestication have been of relevant interest beginning with the essays of Alexander von Humboldt (*Essai sur la géographie des plantes*, Humboldt von 1806; Fiedler and Leitner 2000), Charles

Darwin (*The origin of species*, Darwin 1859 and *The variation of animals and plants under domestication*, Darwin 1868), and Alphonse de Candolle (*Origine de plantes cultivées*, Candolle de 1883; Damania 1998).

In 1926, Nikolay Ivanovich Vavilov published his book *Centers of origin of cultivated plants* (Vavilov 1926). Vavilov noted that "the entire varietal and racial diversity of the field and vegetable crops is concentrated in mountainous districts". Vavilov summarized all his work on diversity in 1935 in *The phytogeographical basis for plant breeding* in which he describes eight centers, including a Mediterranean Center where wheats, barleys, vegetables and fruits originated (Vavilov 1992; Hawkes 1998). Two years later, the archaeologist and philosopher Vere Gordon Childe presented his "Oasis Theory" which proposed that agriculture began in the Near East when the climate changed at the end of the last glacial period, a process that he termed "Neolithic Revolution" (Childe 1928, 1936; Harris 1998). Subsequent work by Robert Braidwood who excavated Jarmo (Braidwood and Braidwood 1950) and Cayönü (Braidwood et al. 1969) led to the suggestion that agriculture began in the "hilly flanks of Breasted's 'Fertile Crescent'" (Braidwood and Braidwood 1950; Braidwood 1972; Braidwood, et al. 1983). The term "Fertile Crescent" (Fig. 1) stems in turn from James Henry Breasted (Breasted 1938; Braidwood 1972). Archaeological evidence, however, only tells part of the story about domestication, and contributions from (archaeo-) botany and genetics have significantly enriched our understanding of agriculture origins (Harlan and Zohary 1966; Harlan 1971, 1975, 1995; Hillman and Davies 1990; Nesbitt 1995, 2002; Nesbitt and Samuel 1996; Willcox 1996, 2005; Zohary 1999; Hillman 2000; Tanno and Willcox 2006).

For more than two decades, the use of molecular markers has been providing new information on genetic diversity of crop plants in relation to wild relatives, centers of domestication, time frame of the domestication process, and specific

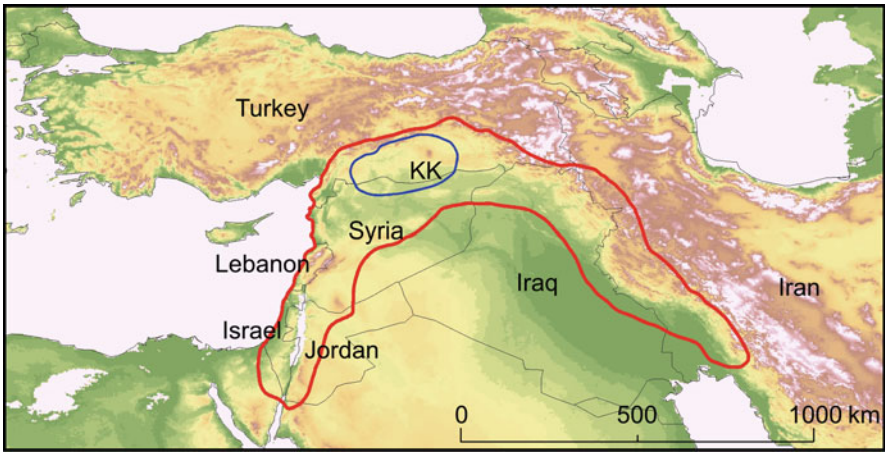


Fig. 1 Fertile Crescent and "core area" of plant domestication within the Fertile Crescent. The Fertile Crescent is indicated with a *red line* and the "core area" is shown with a *blue line*. *KK* Karacadag mountain range in south-eastern Turkey

alleles supporting domesticated traits. The connection between molecular markers and domestication geography took root in the paper by Heun et al. (1997), who found that, on the basis of AFLP (amplified fragment length polymorphism) markers, the closest wild relatives of domesticated einkorn (*Triticum monococcum*, diploid) occur in a very restricted area within the Karacadag mountain range in south-eastern Turkey (Fig. 1). From that, they concluded, not unreasonably, that this represents the site where humans first domesticated einkorn. Important contributions using different molecular markers for other species followed: barley (Badr, et al. 2000; Kilian et al. 2006; Morrell and Clegg 2007); einkorn (Kilian et al. 2007b); emmer (Ozkan et al. 2002, 2005; Mori et al. 2003; Luo et al. 2007); maize (Wright et al. 2005); rice (Londo et al. 2006), and sorghum (Hamblin et al. 2006).

3 Evolution and Domestication of *Triticeae*

Archaeological evidence documents the occurrence of plant remains at different excavation sites, in different stratigraphic layers that were analyzed and radiocarbon dated (Hillman 2000), from which a generally consistent picture emerges indicating that western agriculture originated in the Fertile Crescent after the last ice age, in aceramic Pre-Pottery Neolithic (PPN) from about 12,000 to 9,500 years ago (Zohary and Hopf 2000; Nesbitt 2002; Salamini et al. 2002). It is now widely held that Fertile Crescent agriculture originated in a "core area" in south-eastern Turkey to northern Syria (Fig. 1), where the distribution of wild forms (Fig. 2) are molecularly and cytologically closely related to the founder crops (Table 1) (Lev-Yadun et al. 2000; Abbo et al. 2006). From there, farming spread throughout

Fig. 2 Wild einkorn, wild emmer and *Aegilops* species in their natural habitat within the Karacadag mountain range. Picture taken by H. Özkan in early July 2004

Table 1 The founder crops of Neolithic agriculture and their wild progenitors

Name	Wild progenitor	Domesticated form
Einkorn wheat[a]	*Triticum boeoticum*	*T. monococcum*
Emmer wheat[b]	*Triticum dicoccoides*	*T. dicoccon*
Rye	*Secale vavilovii*	*S. cereale*
Barley	*Hordeum spontaneum*	*H. vulgare*
Lentil	*Lens orientalis*	*L. culinaris*
Pea	*Pisum humile*	*P. sativum*
Chickpea	*Cicer reticulatum*	*C. arietinum*
Bitter vetch	*Vicia ervilia*	*V. ervilia*
Flax	*Linum bienne*	*L. usitatissimum*

[a]It is still unclear if *Triticum urartu* was also collected, due to the fact that seeds of *T. urartu* and *T. boeoticum* cannot be distinguished

[b]Evidence supports that wild *Triticum araraticum* was the progenitor of *T. timopheevii,* but distinguishing plant remains of wild *T. dicoccoides* from those of wild *T. araraticum*, as well the two domesticates, is almost impossible

Europe, Asia and Africa (Ammerman and Cavalli-Sforza 1984; Nesbitt 2002). The domestication process lasted several centuries in the region (Tanno and Willcox 2006), always preceded by cultivation of wild populations before domestication (Weiss et al. 2006; Willcox et al. 2008).

Wild relatives differ from their crop descendants (Table 1) in several phenotypic characteristics (Fig. 3), collectively referred to as the "domestication syndrome" (Hammer 1984; Salamini et al. 2002; Kilian et al. 2009). The most important *Triticeae* traits modified during domestication were the free-threshing state and brittle rachis. Free threshing means that the seeds are released from the rachis at threshing, and brittle rachis means that the dried inflorescence (head) does not disarticulate at maturity (Fig. 3). Additional modifications taking place during domestication and subsequent breeding concerned seed size (larger seeds in domesticate forms), kernel row type (more rows in some domesticated species), plant height, grain hardness, tillering, seed dormancy, photoperiod, vernalization, and heading date. In addition, the spread of the domesticated cereals out of the Fertile Crescent required the adaptation to new environments supported by newly arisen favorable alleles at critical genetic loci (for more information, see Salamini et al. 2002; Kilian et al. 2009).

Wild and domesticated cereals are often designated as separate species, although this is as much for convenience sake, because their crossing progenies are usually fertile. However, it is common to refer to them, at least when describing archaeological and genetical events, as if they are different species, a formality which simplifies the discussion of domestication-related issues. Over the years, taxonomical classifications were developed by geneticists. For wheat, the latest comprehensive, systematic overview was completed in 1979 by Dorofeev and colleagues. In this chapter, the nomenclature and the genome formula given for *Triticum* by Dorofeev et al. (1979) and the *Aegilops* nomenclature based on van Slageren (1994) is mainly followed. In general, the Zohary and Hopf (2000) classification is accepted, with a few modifications where necessary (Table 2).

Fig. 3 Morphological differences between wild and domesticated einkorn wheat. Examples of dehiscent wild einkorn wheat ear (**a**), wild einkorn spikelet (**b**), detail of wild einkorn spikelet with smooth wild abscission scar (**c**) and wild einkorn seeds (**d**). Indehiscent domesticated ear (**e**), domesticated spikelet (**f**), detail of domesticated spikelet with jagged break (**g**) and domesticated seeds (**h**). This figure was kindly prepared by S. Kilian

Archaeological evidence indicates that plant remains of nine domesticated species very often appear together at common sites and times. It is therefore assumed that these species have been domesticated together as a "founder package" (Lev-Yadun et al. 2000). The wild and domesticated species of the Neolithic founder package are shown in Table 1.

Table 2 Comparative classification table for *Triticum* (modified from http://www.k-state.edu./wgrc). The traditional genome formulas are included

Genome	Dorofeev et al. (1979)	Mac Key (1988)	van Slageren (1994)	Kimber and Sears (1987)
A^u	*T. urartu*	*T. urartu*	*T. urartu*	*T. monococcum*
A^b	*T. boeoticum*	*T. monococcum* ssp. *boeoticum*	*T. monococcum* ssp. *aegilopoides*	*T. monococcum*
A^b	*T. monococcum*	*T. monococcum* ssp. *monococcum*	*T. monococcum* ssp. *monococcum*	*T. monococcum*
A^b	*T. sinskajae*			
AB	*T. aethiopicum*			
AB	*T. carthlicum*	*T. turgidum* ssp. *carthlicum*	*T. turgidum* ssp. *carthlicum*	*T. turgidum*
AB	*T. dicoccoides*	*T. turgidum* ssp. *dicoccoides*	*T. turgidum* ssp. *dicoccoides*	*T. turgidum*
AB	*T. dicoccon*	*T. turgidum* ssp. *dicoccum*	*T. turgidum* ssp. *dicoccum*	*T. turgidum*
AB	*T. durum*	*T. turgidum* ssp. *turgidum* conv. *durum*	*T. turgidum* ssp. *durum*	*T. turgidum*
AB	*T. ispahanicum*			
AB	*T. jakubzineri*			
AB	*T. karamyschevii*	*T. turgidum* ssp. *georgicum*	*T. turgidum* ssp. *paleocolchicum*	
AB	*T. polonicum*	*T. turgidum* ssp. *polonicum*	*T. turgidum* ssp. *polonicum*	*T. turgidum*
AB	*T. turanicum*	*T. turgidum* ssp. *turgidum* conv. *turanicum*	*T. turgidum* ssp. *turanicum*	
AB	*T. turgidum*	*T. turgidum* ssp. *turgidum* conv. *turgidum*	*T. turgidum* ssp. *turgidum*	*T. turgidum*
AG	*T. araraticum*	*T. timopheevii* ssp. *armeniacum*	*T. timopheevii* ssp. *armeniacum*	*T. timopheevii*
AG	*T. militinae*			
AG	*T. timopheevii*	*T. timopheevii* ssp. *timopheevii*	*T. timopheevii* ssp. *timopheevii*	*T. timopheevii*
ABD	*T. aestivum*	*T. aestivum* ssp. *aestivum*	*T. aestivum* ssp. *aestivum*	*T. aestivum*
ABD	*T. compactum*	*T. aestivum* ssp. *compactum*	*T. aestivum* ssp. *compactum*	*T. aestivum*
ABD	*T. macha*	*T. aestivum* ssp. *macha*	*T. aestivum* ssp. *macha*	*T. aestivum*
ABD	*T. petropavlovskyi*			
ABD	*T. spelta*	*T. aestivum* ssp. *spelta*	*T. aestivum* ssp. *spelta*	*T. aestivum*
ABD	*T. sphaerococcum*	*T. aestivum* ssp. *sphaerococcum*	*T. aestivum* ssp. *sphaerococcum*	*T. aestivum*
ABD	*T. vavilovii*	*T. aestivum*		
AAG	*T. zhukovskyi*	*T. zhukovskyi*	*T. zhukovskyi*	*T. zhukovskyi*

Molecular results, mainly concerning genome-wide measures of genetic similarity, have also traced the origins of domesticated cereals to wild populations of grasses that are still present in the Fertile Crescent (Heun et al. 1997; Ozkan et al. 2005; Luo et al. 2007; Kilian et al. 2007b), consistent with the independent archaeological evidence.

3.1 Wheat Evolution and Domestication

Studies of wheat evolution have attracted great attention over the past 100 years. Sakamura (1918), Sax and Sax (1924), and Kihara (1924) used cytogenetic methods and recognized that wheat species fall into three groups based upon their ploidy level: (1) diploid 2n = 14 = einkorn wheat; (2) tetraploid 4n = 28 = emmer wheats; (3) hexaploid 6n = 42 = bread wheats. Those cytogenetic studies led to the genome distinctions A, B, D, G, S, and so forth, that are still used today in wheat research. Importantly and uniquely among the cereals, hexaploid bread wheat has no direct hexaploid wild progenitor. It possesses three sets of homoeologous chromosomes, designated as BBA^uA^uDD, whose origins have differing degrees of certainty. The superscript "u" in the A^u genome designation indicates that the A genome is of the type found in *Triticum urartu*.

The D chromosomes stem from wild diploid *Aegilops tauschii* through alloploidization with the wild BBA^uA^u tetraploid *T. dicoccoides* (Kihara 1944). The A^u and B chromosomes derive from hybridization between the wild A^uA^u diploid *T. urartu* and a wild diploid B genome donor (Dvorak et al. 1993; Kilian et al. 2007a), frequently reported to belong to the *Sitopsis* section of *Aegilops*, which includes five species. Brandolini et al. (2006) quantified the genetic relationships among A genomes of wheats by AFLP fingerprinting. Seven AFLP primer combinations produced 239 genome A specific bands for analysis. The results indicate that: (1) the *T. urartu* genome is more closely related to the A genomes of polyploid wheats than to the genome of einkorn (*T. boeoticum*/*T. monococcum*); (2) *T. dicoccon* and *T. durum* cluster together supporting a common origin; (3) hexaploid hulled spelts cluster intermediate between tetraploid and hexaploid wheats; (4) GGA^uA^u wheats cluster distant from both diploid and other polyploid wheats; and (5) the *T. urartu* genome is about 20% more closely related to the A genomes of polyploidy wheats than the *T. boeoticum*/*T. monococcum* genome. *T. timopheevii* is equidistant from those of *T. urartu* and *T. monococcum*.

The tetraploid BBA^uA^u and GGA^uA^u wheats originated through independent allopolyploidization events between two wild diploid grasses. Strong evidence points to the wild outcrossing *Ae. speltoides* (SS) (or a genotype similar to it) as the female parent of tetraploid wheats and to wild *T. urartu* (A^uA^u) as the male parent (Dvorak and Zhang 1990; Huang et al. 2002; Zhang et al. 2002; Kilian et al. 2007a). Kilian et al. (2007a) used a 3-tiered approach. Using 70 amplified fragment length polymorphism (AFLP) loci, they sampled molecular diversity among 480 wheat lines from their natural habitats encompassing all S genome *Aegilops*, the putative progenitors of wheat B and G genomes. Fifty-nine *Aegilops*

representatives for S genome diversity were compared at 375 AFLP loci with diploid, tetraploid, and 11 nulli-tetrasomic *T. aestivum* Chinese Spring aneuploids lines: 6 nulliB–tetraD (N1BT1D, N2BT2D, N3BT3D, N4BT4D, N5BT5D, N6BT6D) and 5 nulliB–tetraA (N1BT1A, N2BT2A, N3BT3A, N5BT5A, N7BT7A) (Sears 1954). Nulli-tetrasomic lines are aneuploid genetic stocks that use the compensating ability of homoeologous chromosomes. For example, N1BT1D indicates nulli-tetrasomic lines missing a pair of chromosome 1B that is replaced by an extra pair of chromosome 1D. B genome-specific markers allowed pinning the origin of the B genome to S chromosomes of *Ae. speltoides*, while excluding other lineages.

The outbreeding nature of *Ae. speltoides* influences its molecular diversity and bears upon inferences of B and G genome origins. Haplotypes at nuclear and chloroplast loci *ACC1*, *G6PDH*, *GPT*, *PGK1*, *Q*, *VRN1*, and *ndhF* for ~70 *Aegilops* and *Triticum* lines (0.73 Mb sequenced) reveal both B and G genomes of polyploid wheats as unique samples of *Ae. speltoides* haplotype diversity. These have been sequestered by the BBA^uA^u *T. dicoccoides* and GGA^uA^u *T. araraticum* lineages during their independent origins. The hybridization which generated the BBA^uA^u wheats may have taken place between 0.25 and 1.3 Mya according to some estimates (Mori et al. 1995; Huang et al. 2002; Dvorak and Akhunov 2005), while the event that led to the GGA^uA^u wheats likely occurred later (Huang et al. 2002). The distinctly reticulate evolutionary relationships between wheats with different ploidy levels tracing to hybridization events are shown in Fig. 4. The corresponding inflorescence morphologies are shown in Fig. 5.

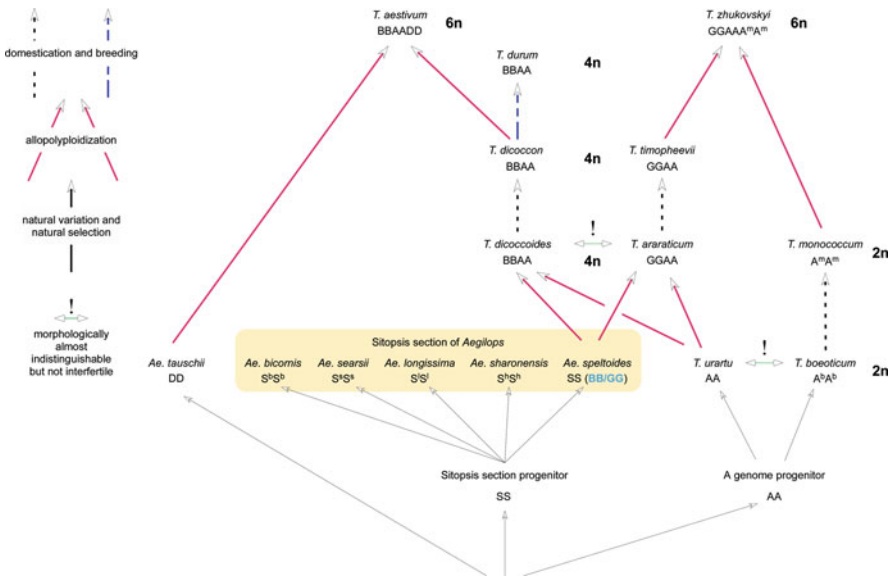


Fig. 4 Overview of wheat evolution and events. This figure has been originally published in Kilian et al. (2007a) (SI). Published with permission from Oxford University Press

Fig. 5 Ears of wheat species and *Aegilops* species involved in wheat evolution. Wheat: wild diploid (*T. urartu*; *T. boeoticum*), domesticated diploid (*T. monococcum*; *T. monococcum aegilopoides*, the feral form of *T. monococcum* is shown at the *right hand side* between *T. boeoticum* and *T. monococcum*), wild tetraploid (*T. dicoccoides*; *T. araraticum*), domesticated tetraploid (*T. dicoccon*; *T. durum*; *T. timopheevii*), hexaploid domesticated (*T. spelta*, *T. aestivum*) and *Aegilops* (*Ae. speltoides*; *Ae. tauschii*). See also Fig. 4

3.1.1 Diploid Wheats

Two wild diploid *Triticum* species are recognized: *T. boeoticum* (A^bA^b) and *T. urartu* (A^uA^u). They are separated by crossing barriers (Johnson and Dahliwal 1976), differ in plant morphology (Gandilian 1972; Dorofeev et al. 1979) and at biochemical and molecular marker loci (Johnson 1975; Dvorak et al. 1998a; Kilian et al. 2007b). The diploid einkorn wheat *T. monococcum* was among the first crops domesticated in the Fertile Crescent starting from the wild progenitor *T. boeoticum*. Domestication has been located to the geographic area of the volcanic Karacadag

Fig. 6 Massive stands of wild einkorn wheat (*T. boeoticum*) in the Karacadag mountain range. Picture taken by H. Özkan in early July 2004

mountain range in south-eastern Turkey (Heun et al. 1997; Fig. 6). The earliest archaeological records from domesticated einkorn are described from Abu Hureyra (Hillman et al. 1989), Cayönü (van Zeist and de Roller 1991–1992), and Nevali Cori (Pasternak 1998). Einkorn was the staple crop of the Sumer populations and has been found in the excavated layers of Troy (Nesbitt and Samuel 1996).

Today, einkorn is a relict crop with only marginal economic importance. During the last 5,000 years, einkorn was largely abandoned and replaced by tetraploid and hexaploid wheats, which deliver higher yields. This circumstance is of particular importance and makes einkorn an outstanding model system for investigating the history of crop domestication with genetic tools, in that domesticated einkorn germplasm is devoid of modern breeding bottlenecks. The term "breeding bottlenecks" refers to the circumstance that most modern cereal varieties are the product of intense breeding efforts particularly during the last 100 years. Because einkorn was never subjected to such breeding programs, Kilian et al. (2007b) reasoned that extensive sampling of genetic diversity among wild and domesticated accessions should discriminate between different hypotheses of cereal domestication, giving insights into the Neolithic domestication of this cereal that are not clouded by breeding bottlenecks that occurred in the last 100 years (the "green revolution").

In that study, Kilian et al. (2007b) investigated nucleotide variation at 18 loci from 92 domesticated einkorn lines compared to 321 lines from wild populations. Several insights into domestication history emerged from that study. One of the most important insights was that wild einkorn is not really a single homogeneous

Fig. 7 Ears of einkorn groups. Wild einkorn (*T. boeoticum*) groups are shown at the *bottom*: (race α, race β, race γ), domesticated einkorn (*T. monococcum*) and its feral form *T. monococcum aegilopoides* are shown at the *upper part*

population, rather wild einkorn underwent a natural process of genetic differentiation prior to domestication, resulting in three distinct *T. boeoticum* races (Fig. 7). These three races, which we designated α, β, and γ, are genetically distinct both at the level of their haplotypes across 18 loci studied and at the level of their AFLP fingerprints, but they are morphologically indistinguishable, or nearly so (Fig. 7). One of those races, wild race β, is genetically much more similar to domesticated einkorn, hence it is the race, or genotype, that was exploited by humans during domestication. Race β occurs only in the Karacadag and Kartal-Karadag Mountains. In this sense, the findings are consistent with those of Heun et al. (1997), but extend them significantly and open up further insights into the domestication process.

A second major surprise in the findings of Kilian et al. (2007b) was that nucleotide and haplotype diversity in domesticated einkorn was found to be higher

than in the β race. Nucleotide diversity, π, represents the average sequence divergence between all homologous sequences among all individuals in a given set for comparison. It is often used to infer the presence of past population bottlenecks in studies of domestication genetics, because when a population goes through a bottleneck, the allelic diversity in the population is diminished, and π is thus expected to be small. Several studies from the literature have reported evidence to suggest that nucleotide diversity is reduced in domesticated cereals, whence the concept domestication bottlenecks stems (Dubcovsky and Dvorak 2007). However, two points are critical here. First, most studies of cereal domestication entailed the analysis of highly inbred lines – how is one to infer the presence of an ancient domestication bottleneck if the real bottleneck occurred during breeding in the last 100 years? This issue looms over the domestication bottleneck concept in cereal domestication studies. Second, it was therefore all the more surprising that, when we looked for evidence of a breeding bottleneck in domesticate einkorn, a rare case of a cereal in which there have been no recent breeding bottlenecks, we found that there was no reduction of nucleotide diversity at all (Table 3). The absence of a domestication bottleneck is in contrast to the conclusions of studies of domestication in intensely bred crop species, where claims for domestication bottlenecks are commonplace. In nature, wild race β has been sampled only in the "core area" of agricultural development in south-eastern Turkey (Lev-Yadun et al. 2000; Bar-Yosef 2002; Lichter 2007), where the closest wild relatives of einkorn, emmer, barley, rye, chickpea, and lentil still grow (Ladizinsky 1985; Salamini et al. 2002; Ozkan et al. 2005; Abbo et al. 2006). Detailed archaeological reports by Hillman (2000), Willcox (2005), Weiss et al. (2006), and Willcox et al. (2008) describe how the pre-domestication cultivation of (wild) cereals lasted for centuries in the region, and how it was followed by gradual (Kislev 2002) and multiple (Gebel 2004) appearances of domesticated phenotypes. The genetic and cultural

Table 3 Haplotype and nucleotide diversity in wild and domesticate lines. See Kilian et al. (2007b) for further explanations

Species/ssp/race	n[a]	H[b]	Hd[c]	π_{tot}[d]	π_{sil}[d]	θ_{tot}[d]	θ_{sil}[d]
T. boeoticum (wild)	321	114	0.380	4.73	8.51	3.45	4.29
race α	230	77	0.242	3.48	6.37	2.72	3.23
race γ	49	75	0.362	4.59	7.81	4.20	5.27
race β	42	43	0.260	1.56	2.70	2.05	2.15
T. monococcum (domesticate)	84	61	0.278	2.97	5.44	2.45	2.89
T. m. aegilopoides (feral)	8	35	0.353	3.75	6.35	3.45	3.42
T. urartu (outgroup)	39	35	0.276	0.53	0.92	0.66	0.96

Hd Haplotype diversity for selfing species (Nei 1987); π_{tot} average number of nucleotide differences per site between two sequences calculated on the total number of polymorphic sites; π_{sil} average number of nucleotide differences per site between two sequences calculated on the silent sites (synonymous and noncoding positions); θ_{tot} Waterson's estimator per site calculated on the total number of polymorphic sites; θ_{sil} Waterson's estimator per site calculated at silent sites

[a]Number of lines

[b]Number of haplotypes found (gapped sites and the Lr10 locus excluded)

[c]Nei's unbiassed estimate of haplotype diversity for inbreeding species (Nei 1987)

[d]Values of nucleotide diversity Pi (π) and Waterson's estimator (θ) are given 10^3

mechanisms underlying the emergence of those phenotypes are remaining questions (Diamond and Belwood 2003).

If geographically distinct domestication events each entailed a random sampling from local genotypes, and if local populations can be identified based on molecular markers, domesticated lines should trace to different localities across the range of the wild progenitor (Jones 2004). This is not observed for einkorn: the wild race β described in Kilian et al. (2007b) appears to be the sister to domesticated einkorn in the absence of an evident reduction of genetic variation. This can be accommodated by a domestication model that we have called the "dispersed-specific" model (Fig. 8). In essence, in this new scenario, a sedentary society associated with the Mesolithic (12,500–9,500 BC) Natufian culture in the Levante (Bar-Yosef 2002) first harvested and then cultivated β race population(s) of wild einkorn, which was probably distributed to local human settlements in the "core area." Here, at these human settlements, favorable traits have been selected from the β race over the centuries and the β race became adapted to human needs. It is still unclear how much intermixture from other wild einkorn races occurred. In a later phase of agricultural expansion, the β race was transferred to other locations outside the

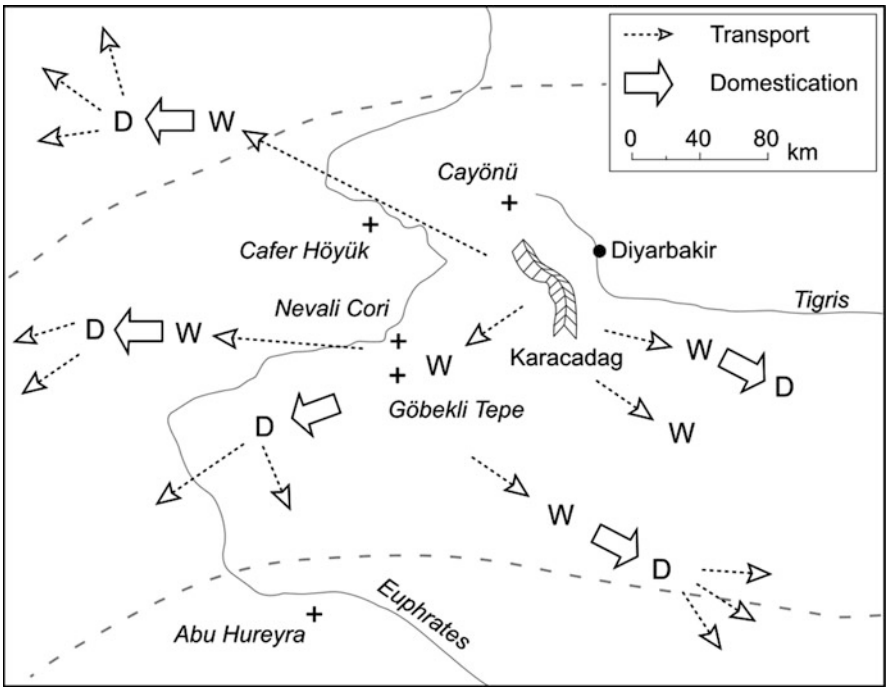


Fig. 8 The dispersed-specific model for einkorn domestication. Selected archaeological sites are indicated in *italics*. The Fertile Crescent is indicated with a *dotted line*. *W* Wild einkorn (β race); *D* domesticated einkorn. This figure has been originally published in Kilian et al. (2007b) (published with permission from Oxford University Press)

"core area," possibly already in a state of nascent domestication. Transport could have involved migrating farmers (Nadel 2002; Renfrew 2002) or exchange of seeds for other goods, as not all soils of the Fertile Crescent were adapted to cereal cultivation (Willcox 2005).

Concerning einkorn, in several areas, variants of the wild β race emerged having common domesticated traits. These domestication events occurred at several places within the "core area" These domestication events were connected with each other due to the same β race seed material used and due to the same human culture, and a genetic bottleneck would have occurred at each domesticating human settlement. However, domestication events at numerous villages would have allowed the newly domesticated lines to integrate a full arsenal of wild haplotypes; in essence, domestication bottlenecks at several human settlements starting from the same genetic pool of the wild β race would have resulted in no domestication bottleneck. In this scenario, the specificities still maintained at a molecular level of the wild β race make it possible to assign the adoption of the wild β race to the Karacadag core area, while the multiple extraction of domesticated lines from the β population has preserved their large genetic variation.

This hypothesis accounts for our molecular data and accommodates the results of archaeological excavations: tools for grinding seeds are present in the majority of Fertile Crescent sites well before the large seed remains of domesticated einkorn wheat (Bar-Yosef 2002), supporting the view that humans in the region were familiar with the harvest of wild seeds both in natural habitats and in cultivated fields (Weiss et al. 2006; Lichter 2007).

Further studies have been carried out on einkorn wheat during the SPP127 project.

Einkorn is cultivated today on a very small scale as feed for poultry and swine in some mountainous villages in Italy, Spain, and Turkey (Nesbitt and Samuel 1996; Perrino et al. 1996). This wheat has been re-discovered as a source of genetic variation for wheat breeding, and it is to some extent used by the food industry in Europe. One example is a recent study on natural variation and identification of micro-elements content in seeds of einkorn wheat (Ozkan et al. 2007). Micronutrient deficiencies in human beings are common problems, especially in developing countries. Among the micronutrient deficiencies, zinc (Zn) and iron (Fe) deficiencies are particularly important severely affecting the health of humans. A major reason for the widespread occurrence of micronutrient deficiencies in human beings is the high and monotonous consumption of cereal-based foods with very low content of micronutrients. An increase in concentration of Zn and Fe in grain is, therefore, a high-priority research area. Exploitation of large genetic variation for Zn and Fe existing in cereals germplasm is an important approach to minimize the extent of Zn and Fe deficiencies in developing countries. In the present study, the variation for seed content of micronutrients (Zn, Fe, Mn, and Cu) in 54 accessions of einkorn wheat (*T. monococcum*) was tested. Additionally, a mapping population comprising 168 recombinant inbred lines has also been tested for seed micronutrient variation and analyzed for QTL identification associated with micronutrient content. The results obtained showed large genotypic variation in micronutrient

contents. One major QTL, common to all four microelements and explaining from 10 to 30% of the variation, was observed on chromosome 5.

Wheat is the staple food for about 40% of the world's population. However, celiac disease (CD) is an inflammatory condition characterized by injury to the lining of the small intestine on exposure to the gluten of wheat, barley and rye. The prevalence of CD among Caucasians is in the range of 1:100–300 (Wieser and Koehler 2008). The involvement of gluten in the CD syndrome has been studied in detail in bread wheat, where a set of "toxic" and "immunogenic" peptides has been defined. For wheat diploid species, information on CD epitopes is poor. In a recent paper, Vaccino et al. (2009) have therefore adopted a genomic approach in order to understand the potential CD danger represented by storage proteins in diploid wheat, and they sequenced a sufficiently large number of cDNA clones related to storage protein genes of *T. monococcum*. Four bona fide toxic peptides and 13 immunogenic peptides were found. All the classes of storage proteins were shown to contain harmful sequences. The major conclusion is that einkorn has the full potential to induce the CD syndrome, as already evident for polyploid wheats. In addition, a complete overview of the storage protein gene arsenal in *T. monococcum* was presented, including a full-length HMW x-type sequence and a partial HMW y-type sequence. The information derived from that study strongly argues against the approach of breeding wheat species low in sequences noxious for CD patients by eliminating the immunogenic or toxic epitopes from their storage proteins arsenal; in fact, it seems hardly feasible to create new genotypes lacking all the 17 harmful peptides belonging to different loci. Accordingly, the silencing, via targeted mutagenesis of the genes giving rise to immunostimulatory sequences (Vader et al. 2003) also appears unrealistic.

The second wild diploid *Triticum* species, *T. urartu* (A^uA^u), occurs on basaltic rocks in some parts of the Fertile Crescent (Zohary and Hopf 2000). The species was never domesticated, but played a critical role in wheat evolution. *T. urartu* donated the A genome to all tetraploid and hexaploid wheats (Dvorak et al. 1993; Brandolini et al. 2006; Kilian et al. 2007a; Fig. 4).

3.1.2 Tetraploid Wheats

Two wild tetraploid wheat species are known, *T. dicoccoides* and *T. araraticum*. They are similar in morphology, but different in their genomic constitution: *T. dicoccoides*, has the genomic formula BBA^uA^u and *T. araraticum* GGA^uA^u (Zohary and Hopf 2000). *T. dicoccoides*, or wild emmer, belongs to the first cereals domesticated by humans in the Fertile Crescent and in its domesticated form is known as *T. dicoccon* (emmer, BBA^uA^u). This domestication step provided the key for subsequent bread wheat evolution (Fig. 4). Wild emmer was first discovered in nature in 1906 in eastern Galilee, Israel, by Aaron Aaronsohn (Aaronsohn and Schweinfurth 1906). His studies of geographic distribution and ecological requirements greatly contributed to our current understanding of emmer wheat distribution, diversity, and domestication. Wild emmer

nowadays has a more restricted distribution range than wild einkorn, and is recognized today in the western Fertile Crescent, the central part of south-eastern Turkey, and mountain areas in eastern Iraq and western Iran. Several issues concerning geography and domestication of wild emmer wheat were recently reviewed Özkan et al. (2010). The authors considered published molecular and archaeological data and re-analyzed the data of Ozkan et al. (2005). Wild emmer was probably domesticated in south-eastern Turkey (Ozkan et al. 2002, 2005, 2009; Mori et al. 2003; Luo et al. 2007). A reconsideration of the domestication geography of tetraploid wheats has been considered by Ozkan et al. (2005) and by Luo et al. (2007). Phylogenetic analysis indicate that two different races of *T. dicoccoides* exist, the western one, colonizing Israel, Syria, Lebanon, and Jordan, and the central-eastern one, which has been frequently sampled in Turkey and rarely in Iraq and Iran. It is the central-eastern race that has played the role of the progenitor of the domesticated germplasm. This is supported by the results from the collections of Ozkan et al. (2002), Mori et al. (2003), and Luo et al. (2007). A disagreement is nevertheless appearing at the local geographical scale: the chloroplast DNA data indicate the Kartal mountains at the western border of the "core area" (Abbo et al. 2006), while AFLP fingerprinting points to the Karacadag range as the putative site of tetraploid wheat domestication. From this area, emmer expanded across Asia, Europe, and Africa (Dubcovsky and Dvorak 2007). South-western expansion of domesticated emmer generated sympatry with the southern populations of *T. dicoccoides* and the rise of a secondary diversity center (Luo et al. 2007). This was followed by the subdivision of domesticated emmer into northern and southern subpopulations. North-east expansion allowed meeting the distribution of *Ae. tauschii* and, thus, the emergence of hexaploid *T. aestivum*. Genetic evidence suggests that the synthesis of hexaploid wheat took place within the corridor from Armenia to the south-western coast of the Caspian Sea (Dvorak et al. 1998b). Based on the D genome diversity, the synthesis of the hexaploid wheat has been estimated to have occurred at least twice (Dvorak et al. 1998b; Giles and Brown 2006).

Based on a direct estimation of mutation rate for microsatellite loci and re-sequenced candidate loci, Thuillet et al. (2002, 2005) and Haudry et al. (2007) have discussed the occurrence of bottlenecks during tetraploid wheat domestication and breeding. A continuous decrease of effective population sizes is reported, indicating the action of severe bottlenecks, associated in particular to breeding. However, Thuillet et al. (2005) reported that the bottleneck of domestication was relatively low, which in terms of Nei's heterozygosity correspond to the presence of 95% of the diversity in domesticated *T. dicoccon* compared to wild *T. dicoccoides* (but 63.2% of effective population size has been lost from wild to domesticated emmer wheat). This situation is remarkably similar to the one reported for einkorn by Kilian et al. (2007b). On the other hand, important losses of nucleotide diversity are reported at 21 loci from the comparisons of domesticated lines of *T. dicoccon* and *T. durum* with the wild *T. dicoccoides* (Haudry et al. 2007). However, in this experiment, it is difficult to separate recent bottlenecks from the loss of diversity due only to domestication.

Several cultivated tetraploid BBA^uA^u wheats were derived later from the domesticated emmer: *T. carthlicum* (Persian wheat), *T. polonicum* (Polish wheat), *T. ispahanicum, T. turanicum* (Khurasan wheat), and *T. turgidum* (English or pollard wheat). *Triticum dicoccon* was the favored crop for bread-making in ancient Egypt. Like einkorn, emmer wheat cultivation has declined today, and it can be found only in some traditional farming communities mainly in Russia and Ethiopia. Somewhat later, *T. durum* (macaroni or hard wheat) also originated from *T. dicoccon* (Damania 1998; Salamini et al. 2002; Ozkan et al. 2005, 2009), but different opinions exist on this point (Haudry et al. 2007). This naked wheat is widely cultivated today for pasta production.

In the eastern part of the Fertile Crescent, the wild tetraploid wheat *T. araraticum* (Araratian or Armenian wild emmer) substitutes *T. dicoccoides* (Johnson 1975; Zohary and Hopf 2000). While *T. dicoccoides* crosses easily with cultivated tetraploid wheats, *T. araraticum* does not, most probably due to relevant differences in the genome, like the existence of several translocations between B and G chromosomes (Feldman 1966). *Triticum araraticum* was also domesticated, but its cultivated form, *T. timopheevii* (GGA^uA^u; Timopheev's wheat), has been found only in west Georgia together with the hexaploid wheat *T. zhukovskyi* ($GGA^uA^uA^mA^m$; Zhukovskyi's wheat) (Dorofeev et al. 1979). It is speculated that, when emmer cultivation spread to Transcaucasia, local populations of *T. araraticum* were colonizing, as a weed, the fields of emmer crops and, by being incorporated into the agricultural cycle of harvest and sowing, became domesticated (Nesbitt and Samuel 1996).

3.1.3 Hexaploid Wheats–Bread Wheat

Economically, the most important wheat is *T. aestivum* or bread wheat (BBA^uA^uDD). Bread wheat is a temperate crop grown from 67°N in Norway, Finland, and Russia to 45°S in Argentina. *Triticum aestivum* comprises a number of free-threshing forms such as *T. compactum* (club wheat), *T. sphaerococcum* (Indian dwarf or shot wheat), *T. petropavlovskyi* (rice wheat), and *T. tibetanum* (Tibetan wheat). Other forms are hulled: *T. spelta* (Dinkel or large spelt), *T. macha*, *T. vavilovii*, and *T. yunnanense* (Dvorak et al. 1998a). No wild hexaploid wheat has ever been found, only a semi-wild weedy form of hulled and brittle hexaploid wheat, *T. tibetanum*, has been discovered in Tibet (Shao et al. 1983). It is accepted that *T. aestivum* originated from a cross between domesticated hulled tetraploid emmer *T. dicoccon* (or the free-threshing hard wheat *T. durum,* or the free-threshing *T. parvicoccum*) and the goat grass *Aegilops tauschii* (DD) (Kihara 1944; McFadden and Sears 1946; Kerber 1964; Kislev 1980; Dvorak et al. 1998a; Matsuoka and Nasuda 2004). This cross should have taken place after emmer or hard wheat cultivation spread east from the Fertile Crescent into the natural distribution area of *Ae. tauschii*. The cross occurred most probably south or west of the Caspian Sea about 8,000 years ago (Nesbitt and Samuel 1996; Salamini et al. 2002; Giles and Brown 2006). *Aegilops tauschii* encompasses several morphological varieties that

are roughly grouped into *Ae. tauschii* ssp. *tauschii* and *Ae. tauschii* ssp. *strangulata* (Kihara et al. 1965; Jaaska 1995; Dvorak et al. 1998a). Several studies show that *Ae. tauschii* ssp. *strangulata* provided the wheat D genome (at least twice), but contributions from both subspecies have also been discussed (Nishikawa et al. 1980; Jaaska 1981; Dvorak et al. 1998b; Talbert et al. 1998). If only a few *Ae. tauschii* genotypes participated in the origin of *T. aestivum*, this polyploidization should have been accompanied by a reduction of diversity (Haudry et al. 2007). However, high mutation rates, together with buffering effects caused by polyploidy, should enable hexaploid wheat to enhance diversity (Dubcovsky and Dvorak 2007).

One still unsolved important question is the origin of the hulled hexaploid wheat *T. spelta* or spelt (McFadden and Sears 1946; Kuckuck and Schiemann 1957; Kuckuck 1959; Nishikawa et al. 1980; Dvorak et al. 1998a; Salamini et al. 2002; Blatter et al. 2004). Two types of spelts are known: the Asian and European spelts. Whether *T. spelta* origin is monophyletic or polyphyletic is still open to debate (Nesbitt and Samuel 1996; Dvorak and Luo 2001; Blatter et al. 2004). Genetic data suggest that the hulled hexaploid wheats are more primitive than the free-threshing forms. This hypothesis, however, is not supported by archaeological findings because free-threshing forms occured earlier than the hulled ones. Hulled wheat appeared in central Europe in the Early Bronze Age (summarized in Nesbitt 2002) and 7,000-year-old remains are found in northern Iraq (Kislev 1984). Free-threshing wheat, in turn, has been found in Can Hassan III dating to 8,500 years ago (Hillman 1978) and at Cafer Höyük dating to 8,000–9,000 years ago (summarized in Salamini et al. 2002). The origin of hulled hexaploid wheat remains controversial.

3.2 Barley Evolution and Domestication

Hordeum vulgare (barley) was domesticated from its wild progenitor *H. spontaneum*. Like wheats, barley belongs to the oldest and most important crops of the Fertile Crescent (Takahashi 1955; Jaaska 1998; Zohary and Hopf 2000; Badr et al. 2000; Bothmer von et al. 2003; Pourkheirandish and Komatsuda 2007). Barley varieties are either two-rowed or six-rowed, based on type of ear. The species is more drought tolerant and much more salt tolerant than wheat. The crop was very important in some regions of the Fertile Crescent, the main crop in Mesopotamia, and a primary cereal in ancient Egypt (Harlan 1995).

Wild barley grains have been found in several pre-agricultural Pre-Pottery Neolithic sites. The earliest evidence is from Ohalo II, located at the shore of the Sea of Galilee, where 21,000-year-old wild remains were found in large amounts (Kislev et al. 1992). This supports the conclusion that wild barley has been collected from nature long before domestication. The earliest carbonized remains of domesticated barley are of the two-row type (van Zeist 1970; Hillman et al. 1989), but six-row types appear already at Ain Ghazal around 9,000–8,500 years ago (Rollefson et al. 1985; Willcox 1998). Domesticated barley later spread with

other crops through the Mediterranean to Europe and Africa, and eastwards through Iran and Afghanistan into India and China.

Wild barley *H. spontaneum* has a wider distribution than any wild wheat. It is present all over the Fertile Crescent because the species is a colonizer of disturbed agricultural habitats. The species occurs in the eastern Mediterranean and western Asia and reaches Turkmenia and Afghanistan in the east (Harlan and Zohary 1966). A few wild barley populations are also found in secondary habitats such as Morocco and Abyssinia.

Considerable studies have been invested in studying barley diversity and identifying the region of barley domestication. Badr et al. (2000) originally reported the monophyletic nature of barley domestication based on allelic frequencies at 400 AFLP polymorphic loci studied in 317 wild and 57 domesticated lines. The wild populations from Israel–Jordan were more similar than any others to the domesticated gene pool. The results supported the hypothesis that the Israel–Jordan area was the region in which barley was brought into culture. Moreover, the diagnostic allele I of the homeobox gene *BKn-3* [*Knotted-1*-like-homeobox (*Knox*) gene class], rarely but almost exclusively found in Israeli *H. spontaneum*, was pervasive in western landraces and modern cultivated varieties. In landraces from the Himalayas and India, the *BKn-3* allele IIIa prevails, indicating that an allelic substitution has taken place during the migration of barley from the Fertile Crescent to south Asia. Thus, the Himalayas can be considered a region of domesticated barley diversification. Other reports point to further domestication sites and to different origins (Schiemann 1939; Åberg 1940; Bekele 1983; Molina-Cano et al. 1987; Zohary and Hopf 2000; Molina-Cano et al. 2005; Morrell and Clegg 2007; Orabi et al. 2007; Azhaguvel and Komatsuda 2007; Saisho and Purugganan 2007). Allaby and Brown (2003, 2004) questioned the use of AFLP markers in phylogenetic studies addressing crop domestication. Subsequently, Salamini et al. (2004) cited several dozens of papers that correctly addressed domestication issues based on AFLP markers.

Studies based on molecular markers comparing wild to domesticated barley have shown that a large amount of nucleotide diversity has been lost in current domesticated varieties (Russell et al. 2004; Caldwell et al. 2006; Kilian et al. 2006; Morrell and Clegg 2007). Kilian et al. (2006) determined, for a representative sample of 20 domesticated barley (*H. vulgare*) lines and 25 wild *H. spontaneum* lines, the haplotypes at seven loci – *Adh2*, *Adh3*, *Amy1*, *Dhn9*, *GAPDH*, *PEPC*, and *WAXY*. The number of haplotypes, average nucleotide diversity, π, and Watterson's theta at silent sites was reduced in domesticated lines. Two loci, *Amy1* and *PEPC*, were monomorphic in domesticated lines, while *Amy1* and *GAPDH* produced significant values of Tajima's D when all domesticated and wild lines were considered. At *GAPDH*, π was slightly higher in domesticated than wild forms, due to divergent high-frequency haplotypes; for the remaining six loci, 87% of nucleotide diversity has been lost in the domesticated forms. Bottlenecks acting on neutrally evolving loci either during the domestication process or during subsequent breeding, or both, are sufficient to account for reduced diversity and the results of Tajima's test, without the need to evoke selection at these loci.

The domesticated varieties considered, although all sampled were among those currently cultivated in Turkey, were shown to share the same molecular and morphological variability as larger samples of barley genotypes, indicating that the conclusions reported are of general value for this crop.

Recent data have agreed with the conclusion that two-row and six-row genotypes may have different, independent origins (Zohary and Hopf 2000; Kilian et al. 2006; Komatsuda et al. 2007). These new findings are nevertheless in agreement with the previously inferred area of barley domestication in the Jordan valley (Badr et al. 2000). The new data open up the possibility that barley domestication might have occurred independently separate occasions. This conclusion would not be consistent with the conclusions of Badr et al. (2000), but is favored other authors, including Molina-Cano et al. (2005), Kolodinska Brantestam et al. (2004), Casas et al. (2005), Tanno and Takeda (2004); Komatsuda et al. (2004), Taketa et al. (2004), and Komatsuda et al. (2004). The particular matter concerning single versus multiple origins of barley is, however, complicated by the fact that: (1) multiple independent introgressions of genes from wild relatives to cultivated varieties can mimic multiple domestication events (Badr et al. 2000; Kanazin et al. 2002; Abdel-Ghani et al. 2004); and (2) splitting of domesticated genotypes in two alternative groups based on two- or six-rowed ears, hulled or naked caryopsis, western or eastern varieties, and brittleness of the rachis might have followed the domestication process, rather than being coeval with it.

4 Conclusions and Final Considerations

Archaeological and genetic evidence indicate that western agriculture began in the Fertile Crescent about 12,000 years ago (Zohary and Hopf 2000; Kilian et al. 2009). The present view is that the process of crop domestication was slow, spanned several centuries, and entailed repeated domestication events (Tanno and Willcox 2006). Local wild wheat populations were domesticated in a "core area" of the Fertile Crescent and were then gradually dispersed throughout the region (Abbo et al. 2006). Nearly all current domestication models predict a reduction in genetic diversity in domesticated forms compared to their wild progenitors (Doebley et al. 2006). However, recent work of Kilian et al. (2007b) with wild and domesticated einkorn wheat shows no reduction of diversity that could be attributable to the domestication process, raising the issue of whether previous inference of "domestication bottlenecks" in other cereals might in fact be instead breeding bottlenecks.

The keys to obtaining deeper insights to plant domestication are several-fold. First, comprehensive germplasm reference collections covering the whole distribution area for each species are needed, meaning that the natural habitats of the wild progenitors throughout the Fertile Crescent need to be more thoroughly sampled. Studies of domestication genetics need to encompass many wild and domesticated accessions for each species, otherwise sampling effects may severely bias the results. If the goal is to identify the closest relatives among wild progenitors, the

diversity and, in the case of einkorn, the previously unrecognized genetic structure of the wild gene pool needs to be accurately assessed. With the new generation of high throughput sequencing technologies in hand (Goldberg et al. 2006; Wicker et al. 2006), the pace of progress can be expected to accelerate. From the more practical perspective, new genomic resources for future plant breeding continually need to be developed, and agronomically important genes also need to be isolated from the wild genetic reserves, and it is clear that such activities will entail international consortia. Continued improvement of analytical methods addressing domestication issues based on mathematical and statistical models is needed (Pluzhnikov and Donnelly 1996; Thuillet et al. 2005; Haudry et al. 2007; Allaby et al. 2008), as are further excavation campaigns in different archeological sites in the Fertile Crescent, such as Göbekli Tepe or Jerf el Ahmar (Schmidt 2001; Neef 2003; Schmidt 2006; Willcox et al. 2008). In the not too distant future, we can expect new insights into the questions of how humans performed the most important breeding experiment in history 10 millennia before the discovery of genetics.

Acknowledgments We thank Sigi Effgen, Isabell Fuchs, Jutta Schütze, Charlotte Bulich, and Marianne Haberscheid for excellent technical assistance and Margit Pasemann, Birgit Thron, Marianne Limpert, Elke Bohlscheid, and Katiuscia Ceron for administrative support during the last years. We are grateful to the MPIZ sequence facilities (ADIS) headed by Bernd Weisshaar. We thank Maarten Koornneef, George Coupland, Moshe Feldman, Andrea Brandolini, Klaus Schmidt (DAI), and Andreas Graner for valuable suggestions. This research was supported by the Deutsche Forschungsgemeinschaft SPP 1127.

List of our publications resulting from the SPP 1127

Vaccino P, Becker H-A, Brandolini A, Salamini F, Kilian B (2009) A catalogue of *T. monococcum* genes encoding toxic and immunogenic peptides for celiac disease patients. Mol Genet Genom 281:289–300

Kilian B, Özkan H, Pozzi C, Salamini F (2009) Domestication of the Triticeae in the Fertile Crescent. In: Feuillet C, Muehlbauer G (eds) Genetics and genomics of the Triticeae. Plant genetics and genomics: crops and Models 7, Springer Science+Business Media, LLC, pp 81–119

Goncharov NP, Golovnina KA, Kilian B, Glushkov S, Blinov A, Shumny VK (2008) Evolutionary history of wheats - the main cereal of mankind. In: Dobretsov N, Kolchanov N, Rozanov A, Zavarzin G (eds) Biosphere origin and evolution. Springer, Berlin, pp 407–419

Altintas S, Toklu F, Kafkas S, Kilian B, Brandolini A, Özkan H (2008) Estimating genetic diversity in durum and bread wheat cultivars from Turkey using AFLP and SAMPL markers. Plant Breeding 127:9–14

Kilian B, Özkan H, Walther A, Kohl J, Dagan T, Salamini F, Martin W (2007) Molecular diversity at 18 loci in 321 wild and 92 domesticate lines reveal no reduction of nucleotide diversity during *Triticum monococcum* (einkorn) domestication: Implications for the origin of agriculture. Mol Biol Evol 24:2657–2668.

Ozkan H, Brandolini A, Torun A, Altintas S, Eker S, Kilian B, Braun H, Salamini F, Cakmak I (2007) Natural variation and identification of microelements content in seeds of einkorn wheat (*Triticum monococcum*). In: Buck HT, Nisi JE, Salomon N, (eds) Wheat production in stressed environments. Springer, Berlin, pp 455–462.

Kilian B, Özkan H, Deusch O, Effgen S, Brandolini A, Kohl J, Martin W, Salamini F (2007) Independent wheat B and G genome origins in outcrossing *Aegilops* progenitor haplotypes. Mol Biol Evol 24:217–227

Kilian B, Ozkan H, Kohl J, von Haeseler A, Barale F, Deusch O, Brandolini A, Yucel C, Martin W, Salamini F (2006) Haplotype structure at seven barley genes: relevance to gene pool bottlenecks, phylogeny of ear type and site of barley domestication. Mol Genet Genom 276:230–241

Brandolini A, Vaccino P, Boggini G, Ozkan H, Kilian B, Salamini F. 2006. Quantification of genetic relationships among A genomes of wheats. Genome 49:297–305

Invited Lectures and Data Presented from the SPP 1127

05/2003	Bad Honnef, DFG SPP1127 "Radiations: origins of biological diversity"
09/2003	Wernigerode, DFG SPP1127 "Radiations: origins of biological diversity"
05/2004	Bad Honnef, DFG SPP1127 "Radiations: origins of biological diversity"
07/2005	University of Cukurova, Adana, Turkey
08/2005	Udmurt State University, Izhevsk, Russia
09/2005	Bad Honnef, DFG SPP1127 "Radiations: origins of biological diversity"
09/2005	MPIZ Cologne
10/2006	5. Plant Genomics European Meetings, Venice, Italy
08/2007	MPIZ Cologne
11/2007	IPK Gatersleben
01/2008	University Kassel
04/2008	Systematics 2008, Göttingen, two talks
06/2008	Bad Honnef, DFG SPP1127 "Radiations: origins of biological diversity"
06/2008	EPSO Conference 2008, Toulon, France
09/2008	Harlan II (Biodiversity in Agriculture: Domestication, Evolution, & Sustainability) University of California, Davis, USA
10/2008	GPZ Göttingen (German Society of Plant Breeding), Kurt-von-Rümker Symposium
10/2008	University Halle, Department of Botany

Conferences Attended and Data Presented from the SPP 1127

10/2006	5. Plant GEMs Venice, Italy
04/2007	Aaronsohn-ITMI Conference, Tiberias, Israel
04/2008	Systematics 2008, Göttingen, Germany
06/2008	EPSO Conference 2008, Toulon, France
09/2008	Harlan II, US Davis, California, USA
10/2008	GPZ Symposium "Biodiversity in Plant Production" (German Society of Plant Breeding), Göttingen, Germany

Collaborations Resulted from the SPP 1127

Özkan H, Department of Field Crops, University of Cukurova, Adana, Turkey
Coupland G and von Korff M, Max Planck Institute for Plant Breeding Research, Köln, Germany
Pillen K, Department of Plant Breeding, Martin Luther University Halle-Wittenberg, Halle, Germany
Brandolini A and **Vaccino P**, CRA-Istituto Sperimentale per la Cerealicoltura, S. Angelo Lodigiano, Italy
David J, DIAPC Montpellier Supagro, France
Badaeva E and **Konovalov F**, N.I. Vavilov Institute of General Genetics, Moscow, Russia
Blattner F and **Jakob S**, IPK Gatersleben
Walther A, Regional Climate Group, Earth Sciences Centre, Göteborg University, Sweden

References

Aaronsohn A, Schweinfurth G (1906) Die Auffindung des wilden Emmers (*Triticum dicoccum*) in Nordpalästina. Altneuland Monatsschrift für die Wirtschaft. Erschliessung Palästinas 7–8:213–220

Abbo S, Gopher A, Peleg Z, Saranga Y, Fahima T, Salamini F, Lev-Yadun S (2006) The ripples of "the big (agricultural) bang": the spread of early wheat cultivation. Genome 49:861–863

Abdel-Ghani AH, Parzies HK, Omary A, Geiger HH (2004) Estimating the outcrossing rate of barley landraces and wild barley populations collected from ecologically different regions of Jordan. Theor Appl Genet 109:588–595

Åberg E (1940) The taxonomy and phylogeny of *Hordeum* L. sect. *Critesion* Ands. with special reference to Tibetian barleys. Symb Bot Upsaliensis 2:1–156

Allaby RG, Brown TA (2003) AFLP data and the origins of domesticated crops. Genome 46:448–453

Allaby RG, Brown TA (2004) Reply to the comment by Salamini et al. on "AFLP data and the origins of domesticated crops". Genome 47:621–622

Allaby RG, Fuller DQ, Brown TA (2008) The genetic expectations of a protracted model for the origins of domesticated crops. Proc Natl Acad Sci USA 105:13982–13986

Ammerman AJ, Cavalli-Sforza LL (1984) The neolithic transition and the genetics of populations in Europe. Princeton University Press, Princeton

Azhaguvel P, Komatsuda T (2007) A phylogenetic analysis based on nucleotide sequence of a marker linked to the brittle rachis locus indicates a diphyletic origin of barley. Ann Bot 100:1009–1015

Badr A, Müller K, Schäfer-Pregl R, El Rabey H, Effgen S, Ibrahim HH, Pozzi C, Rohde W, Salamini F (2000) On the origin and domestication history of barley (*Hordeum vulgare*). Mol Biol Evol 17:499–510

Bar-Yosef O (2002) The Natufian culture and the early Neolithic – Social and economic trends. In: Bellwood P, Renfrew C (eds) Examining the farming/language dispersal hyphothesis. McDonald Institute for Archaeological Research, Cambridge, pp 113–126

Bekele E (1983) A differential rate of regional distribution of barley flavonoid patterns in Ethiopia, and a view on the center of origin of barley. Hereditas 98:269–280

Blatter RHE, Jacomet S, Schlumbaum A (2004) About the origin of European spelt (*Triticum spelta* L.): allelic differentiation of the HMW Glutenin B1-1 and A1-2 subunit genes. Theor Appl Genet 108:360–367

Braidwood RJ, Braidwood L (1950) Jarmo: a village of early farmers in Iraq. Antiquity 24:189–195

Braidwood RJ, Cambel H, Watson PJ (1969) Prehistoric investigations in southwestern Turkey. Science 164:1275–1276

Braidwood RJ (1972) Prehistoric investigations in southwestern Asia. Proc Am Philos Soc 116:310–320

Braidwood LS, Braidwood RJ, Howe B, Reed CA, Watson PJ (1983) Prehistoric archeology along the Zagros flanks. Oriental Institute Publication 105, University of Chicago Press, Chicago

Brandolini A, Vaccino P, Boggini G, Ozkan H, Kilian B, Salamini F (2006) Quantification of genetic relationships among A genomes of wheats. Genome 49:297–305

Breasted JH (1938) The conquest of civilization. Literary guild of America, New York

Caldwell KS, Russell J, Langridge P, Powell W (2006) Extreme population-dependent linkage disequilibrium detected in an inbreeding plant species, *Hordeum vulgare*. Genetics 172:557–567

Candolle de, A (1883) (en fait, octobre 1882) Origine des plantes cultivées. Germer Baillière, Paris

Casas AM, Yahiaoui S, Ciudad F, Igartua E (2005) Distribution of MWG699 polymorphism in Spanish European barleys. Genome 48:41–45

Childe VG (1928) The most ancient east: the oriental prelude to European prehistory. Kegan Paul, London

Childe VG (1936) Man makes himself. Watts, London

Damania AB (1998) Diversity of major cultivated plants domesticated in the Near East. In: Damania AB, Valkoun J, Willcox G, Qualset CO (eds) The origins of agriculture and crop domestication. Proceedings of the Harlan Symposium. ICARDA, Aleppo, pp 51–64

Darwin C (1859) On the origin of species by means of natural selection, or the preservation of favored races in the struggle for life. John Murray, London

Darwin C (1868) The variation of animals and plants under domestication. John Murray, London

Diamond J, Belwood P (2003) Farmers and their languages: the first expansions. Science 300:597–603

Doebley JF, Gaut BS, Smith BD (2006) The molecular genetics of crop domestication. Cell 127:1309–1321

Dorofeev VF, Filatenko AA, Migushova EF, Udaczin RA, Jakubziner MM (1979) Wheat. In: Dorofeev VF, Korovina ON (eds) Flora of cultivated plants, vol 1. Leningrad, Russia

Dubcovsky J, Dvorak J (2007) Genome plasticity a key factor in the success of polyploid wheat under domestication. Science 316:1862–1866

Dvorak J, Zhang HB (1990) Variation in repeated nucleotide sequences sheds light on the phylogeny of the wheat B and G genomes. Proc Natl Acad Sci USA 87:9640–9644

Dvorak J, Diterlizzi P, Zhang H-B, Resta P (1993) The evolution of polyploid wheats: identification of the A genome donor species. Genome 36:21–31

Dvorak J, Luo MC (2001) Evolution of free-threshing and hulled forms of *Triticum aestivum*: old problems and new tools. In: Caligari PDS, Brandham PE (eds) The Linnean, Special issue No 3. Wheat taxonomy: the legacy of John Percival. Academic, London, pp 127–136

Dvorak J, Luo MC, Yang ZL (1998a) Genetic evidence on the origin of *Triticum aestivum* L. In: Damania AB, Valkoun J, Willcox G, Qualset CO (eds) The origins of agriculture and crop domestication. Proceedings of the Harlan symposium. ICARDA, Aleppo, pp 235–251

Dvorak J, Luo MC, Yang ZL, Zhang HB (1998b) The structure of the *Aegilops tauschii* genepool and the evolution of hexaploid wheat. Theor Appl Genet 67:657–670

Dvorak J, Akhunov E (2005) Tempos of gene locus delations and duplications and their relationship to recombination rate during diploid and polyploid evolution in the *Aegilops-Triticum* alliance. Genetics 17:323–332

Feldman M (1966) Identification of unpaired chromosomes in F1 hybrids involving *Triticum aestivum* and *T. timopheevii*. Can J Genet Cytol 8:144–151

Fiedler H, Leitner U (2000) Alexander von Humboldts Schriften. Bibliographie der selbständig erschienenen Werke. (= Beiträge zur Alexander-von-Humboldt-Forschung; 20). Berlin

Gandilian PA (1972) On wild growing *Triticum* species of Armenian SSR. Bot Zhur 57:173–181

Gebel HG (2004) There was no centre: the polycentric evolution of the near Eastern Neolithic. Neo-lithics 1/04:28–32

Giles RJ, Brown TA (2006) *GluDy* allele variations in *Aegilops tauschii* and *Triticum aestivum*: implications for the origins of hexaploid wheats. Theor Appl Genet 112:1563–1572

Goldberg SM, Johnson J, Busam D, Feldblyum T, Ferriera S, Friedman R, Halpern A, Khouri H, Kravitz SA, Lauro FM, Li K, Rogers YH, Strausberg R, Sutton G, Tallon L, Thomas T, Venter E, Frazier M, Venter JC (2006) A Sanger/pyrosequencing hybrid approach for the generation of high-quality draft assemblies of marine microbial genomes. Proc Natl Acad Sci USA 103:11240–11245

Hamblin MT, Casa AM, Sun H, Murray SC, Paterson AH, Aquadro CF, Kresovich S (2006) Challenges of detecting directional selection after a bottleneck: lessons from *Sorghum bicolor*. Genetics 173:953–964

Hammer K (1984) Das Domestikationssyndrom. Kulturpflanze 32:11–34

Harlan JR, Zohary D (1966) Distribution of wild wheats and barley. Science 153:1074–1080

Harlan JR (1971) Agricultural origins: centers and noncenters. Science 174:468–474

Harlan JR (1975) Our vanishing genetic resources. Science 188:618–621

Harlan JR (1995) The living fields: our agricultural heritage. Cambridge University Press, Cambridge

Harris DR (1998) The spread of neolithic agriculture from the Levant to western central Asia. In: Damania AB, Valkoun J, Willcox G, Qualset CO (eds) The origins of agriculture and crop domestication. Proceedings of the Harlan symposium. ICARDA, Aleppo, pp 65–82

Haudry A, Cenci A, Ravel C, Bataillon T, Brunel D, Poncet C, Hochu I, Poirier S, Santoni S, Glémin S, David J (2007) Grinding up wheat: a massive loss of nucleotide diversity since domestication. Mol Biol Evol 24:1506–1517

Hawkes JG (1998) Back to Vavilov: why were plants domesticated in some areas and not in others? In: Damania AB, Valkoun J, Willcox G, Qualset CO (eds) The origins of agriculture and crop domestication. Proceedings of the Harlan symposium. ICARDA, Aleppo, pp 5–8

Heun M, Schäfer-Pregl R, Klawan D, Castagna R, Accerbi M, Borghi B, Salamini F (1997) Site of einkorn wheat domestication identified by DNA fingerprinting. Science 278:1312–1314

Hillman GC (1978) On the origins of domestic rye – *Secale cereale*: the finds from Aceramic Can Hasan III in Turkey. Anatolian Stud 28:157–174

Hillman GC, Colledge SM, Harris DR (1989) Plant-food economy during the Epipalaeolithic period at Tell Abu Hureyra, Syria: dietary diversity, seasonality, and modes of exploitation. In: Harris DR, Hillman GC (eds) Foraging and farming: the evolution of plant exploitation. Unwin, London, pp 240–268

Hillman G, Davies S (1990) Measured domestication rates in wild wheats and barley under primitive cultivation, and their archaeological implications. J World Prehistory 4:157–222

Hillman G (2000) Plant food economy of Abu Hureyra. In: Moore A, Hillman G, Legge T (eds) Village on the Euphrates, from foraging to farming at Abu Hureyra. Oxford University Press, New York, pp 372–392

Huang S, Sirikhachornkit A, Su X, Faris J, Gill B, Haselkorn R, Gornicki P (2002) Genes encoding plastid acetyl-CoA carboxylase and 3-phosphoglycerate kinase of the *Triticum/Aegilops* complex and the evolutionary history of polyploidy wheat. Proc Natl Acad Sci USA 99:8133–8138

Jaaska V (1981) Aspartate aminotransferase and alcohol dehydrogenase isozymes: Intraspecific differentiation in *Aegilops tauschii* and the origin of the D genome polyploids in the wheat group. Plant Syst Evol 137:259–273

Jaaska V (1995) Isoenzymes in the evaluation of germplasm diversity in wild diploid relatives of cultivated wheat. In: Damania AB (ed) Biodiversity and wheat improvement. Wiley, Chichester, pp 247–257

Jaaska V (1998) On the origin and in statu nascendi domestication of rye and barley: A review. In: Damania AB, Valkoun J, Willcox G, Qualset CO (eds) The origins of agriculture and crop domestication. Proceedings of the Harlan Symposium. ICARDA, Aleppo, pp 210–217

Johnson BL (1975) Identification of the apparent B-genome donor of wheat. Can J Genet Cytol 17:21–39

Johnson BL, Dahliwal HS (1976) Reproductive isolation of *Triticum boeoticum* and *Triticum urartu* and the origin of the tetraploid wheats. Am J Bot 63:1088–1094

Jones MK (2004) Between fertile crescents: Minor grain crops and agricultural origins. In: Jones MK (ed) Traces of ancestry: studies in honour of Colin Renfrew. McDonald Institute for Archaeological Research, Cambridge, pp 127–135

Kanazin V, Talbert H, See D, DeCamp P, Nevo E, Blake T (2002) Discovery and assay of single-nucleotide polymorphisms in barley (*Hordeum vulgare*). Plant Mol Biol 48:529–537

Kerber ER (1964) Wheat: reconstitution of the tetraploid component (AABB) of hexaploids. Science 143:253–255

Kihara H (1924) Cytologische und genetische Studien bei wichtigen Getreidearten mit besonderer Rücksicht auf das Verhalten der Chromosomen und die Sterilität in den Bastarden. Mem Coll Sci Univ Kyoto Ser B 1:1–200

Kihara H (1944) Discovery of the DD-analyser, one of the ancestors of *Triticum vulgare*. Agric Hortic (Tokyo) 19:13–14

Kihara H, Yamashita H, Tanaka M (1965) Morphological, physiological, genetical, and cytological studies in *Aegilops* and *Triticum* collected in Pakistan, Afghanistan, Iran. Results of the Kyoto University scientific expedition to the Korakoram and Hindukush in 1955. In: Yamashita K (ed) Cultivated plants and their relatives. Kyoto. pp 4–41

Kilian B, Özkan H, Kohl J, von Haeseler A, Barale F, Deusch O, Brandolini A, Yucel C, Martin W, Salamini F (2006) Haplotype structure at seven barley genes: relevance to gene pool bottlenecks, phylogeny of ear type and site of barley domestication. Mol Gen Genom 276:230–241

Kilian B, Özkan H, Deusch O, Effgen S, Brandolini A, Kohl J, Martin W, Salamini F (2007a) Independent wheat B and G genome origins in outcrossing *Aegilops* progenitor haplotypes. Mol Biol Evol 24:217–227

Kilian B, Özkan H, Walther A, Kohl J, Dagan T, Salamini F, Martin W (2007b) Molecular diversity at 18 loci in 321 wild and 92 domesticate lines reveal no reduction of nucleotide diversity during *Triticum monococcum* (einkorn) domestication: Implications for the origin of agriculture. Mol Biol Evol 24:2657–2668

Kilian B, Özkan H, Pozzi C, Salamini F (2009) Domestication of the Triticeae in the fertile crescent. In: Feuillet C, Mühlbauer J (eds) Genetics and genomics of the Triticeae. Plant genetics and genomics: crops and models 7. Springer, New York, pp 81–119

Kimber G, Sears ER (1987) Evolution in the genus Triticum and the origin of cultivated wheat. In: Wheat and Wheat Improvement, 2nd Ed (Heyne EG, ed) Ameican Society of Agronomy, Madison, WI, pp 154–164

Kislev ME, Nadel D, Carmi I (1992) Epipalaeolithic (19, 000 BP) cereal and fruit diet at Ohalo II, Sea of Galilee. Isr Rev Palaeobot Palynol 73:161–166

Kislev ME (1980) *Triticum parvicoccum* sp. nov., the oldest naked wheat. Isr J Bot 28:95–107

Kislev ME (1984) Botanical evidence for ancient naked wheats in the Near East. In: von Zeist W, Casparie WA (eds) Plants and ancient man. Balkema, Rotterdam, pp 141–152

Kislev M (2002) Origin of annual crops by agro-evolution. Isr J Plant Sci 50:85–88

Kolodinska Brantestam A, von Bothmer R, Dayteg C, Rashal I, Tuvesson S, Weibull J (2004) Inter simple sequence repeat analysis of genetic diversity and relationships in cultivated barley of Nordic and Baltic origin. Hereditas 141:186–192

Komatsuda T, Maxim P, Senthil N, Mano Y (2004) High-density AFLP map of nonbrittle rachis 1 (*btr1*) and 2 (*btr2*) genes in barley (*Hordeum vulgare* L.). Theor Appl Genet 109:986–995

Komatsuda T, Pourkheirandish M, He C, Azhaguvel P, Kanamori H, Perovic D, Stein N, Graner A, Wicker T, Tagiri A, Lundqvist U, Fujimura T, Matsuoka M, Matsumoto T, Yano M (2007)

Six-rowed barley originated from a mutation in a homeodomain-leucine zipper I-class homeobox gene. Proc Natl Acad Sci USA 104:1424–1429
Kuckuck H, Schiemann E (1957) Über das Vorkommen von Speltz und Emmer (*Triticum spelta* L. und *T. dicoccum* Schubl.) im Iran. Z Pflanzenzüchtg 38:383–396
Kuckuck H (1959) Neuere Arbeiten zur Entstehung der hexaploiden Kulturweizen. Z Pflanzenzüchtg 41:205–226
Ladizinsky G (1985) Founder effect in crop-plant evolution. Econ Bot 39:191–199
Lev-Yadun S, Gopher A, Abbo S (2000) The cradle of agriculture. Science 288:1602–1603
Lichter C (ed) (2007) Die ältesten Monumente der Menschheit. Badisches Landesmuseum Karlsruhe. Theiss, Stuttgart
Londo JP, Chiang YC, Hung KH, Chiang TY, Schaal B (2006) Phylogeography of Asian wild rice, *Oryza rufipogon*, reveals multiple independent domestications of cultivated rice, *Oryza sativa*. Proc Natl Acad Sci USA 103:9578–9583
Luo MC, Yang ZL, You FM, Kawahara T, Waines JG, Dvorak J (2007) The structure of wild and domesticated emmer wheat populations, gene flow between them, and the site of emmer domestication. Theor Appl Genet 114:947–959
Matsuoka Y, Nasuda S (2004) Durum wheat as a candidate for the unknown female progenitor of bread wheat: an empirical study with a highly fertile F_1 hybrid with *Aegilops tauschii* Coss. Theor Appl Genet 109:1710–1717
McFadden ES, Sears ER (1946) The origin of *Triticum spelta* and its free-theshing hexaploid relatives. J Hered 37(81–89):107–116
Molina-Cano JL, Fra-Mon P, Salcedo G, Aragoncillo C, Roca de Togores F, Garcia-Olmedo F (1987) Morocco as a possible domestication center for barley: biochemical and agromorphological evidence. Theor Appl Genet 73:531–536
Molina-Cano JL, Russell JR, Moralejo MA, Escacena JL, Arias G, Powell W (2005) Chloroplast DNA microsatellite analysis supports a polyphyletic origin for barley. Theor Appl Genet 110:613–619
Mori N, Liu YG, Tsunewaki K (1995) Wheat phylogeny determined by RFLP analysis of nuclear DNA. 2. Wild tetraploid wheats. Theor Appl Genet 90:129–134
Mori N, Ishii T, Ishido T, Hirosawa S, Watatani H, Kawahara T, Nesbitt M, Belay G, Takumi S, Ogihara Y, Nakamura C (2003) Origin of domesticated emmer and common wheat inferred from chloroplast DNA fingerprinting. 10th international wheat genetics symposium. Paestum, pp 25–28
Morrell PL, Clegg MT (2007) Genetic evidence for a second domestication of barley (*Hordeum vulgare*) east of the Fertile Crescent. Proc Natl Acad Sci USA 104:3289–3294
Nadel D (2002) Ohalo II: a 23, 000-Year-old Fisher-Hunter-Gatherer's camp on the sea of galilee. University of Haifa, Haifa
Neef R (2003) Overlooking the steppe forest: preliminary report on the botanical remains from early Neolithic Göbekli Tepe (southern Turkey). Neolithics 2(03):13–15
Nei M (1987) Molecular evolutionary genetics. Columbia University Press, New York
Nesbitt M (1995) Plants and people in ancient Anatolia. Biblic Archaeol 58:68–81
Nesbitt M, Samuel D (1996) From stable crop to extinction? The archaeology and history of the hulled wheats. In: Padulosi S, Hammer K, Heller J (eds) Hulled wheats. International Plant Genetic Resources Institute, Rome, pp 41–100
Nesbitt M (2002) When and where did domesticated cereals first occur in southwest Asia? In: Cappers R, Bottema S (eds) The dawn of farming in the Near East. Ex Oriente, Berlin, pp 113–132
Nishikawa K, Furuta Y, Wada T (1980) Genetic studies on alpha-amylase isozymes in wheat. III. Intraspecific variation in Aegilops squarrosa and birthplace of hexaploid wheat. Jpn J Genet 55:325–336
Orabi J, Backes G, Wolday A, Yahyaoui A, Jahoor A (2007) The horn of Africa as a centre of barley diversification and a potential domestication site. Theor Appl Genet 114:1117–1127
Ozkan H, Brandolini A, Schaefer-Pregl R, Salamini F (2002) AFLP analysis of a collection of tetraploid wheats indicates the origin of emmer and hard wheat domestication in southeast Turkey. Mol Biol Evol 19:1797–1801

Ozkan H, Brandolini A, Pozzi C, Effgen S, Wunder J, Salamini F (2005) A reconsideration of the domestication geography of tetraploid wheats. Theor Appl Genet 110:1052–1060

Ozkan H, Brandolini A, Torun A, Altintas S, Eker S, Kilian B, Braun H, Salamini F, Cakmak I (2007) Natural variation and identification of microelements content in seeds of einkorn wheat (*Triticum monococcum*). In: Buck HT, Nisi JE, Salomon N (eds) Wheat production in stressed environments. Springer, Berlin, pp 455–462

Özkan H, Willcox G, Graner A, Salamini F, Kilian B (2010) Geographic distribution and domestication of wild Emmer wheat (*Triticum dicoccoides*)

Pasternak R (1998) Investigations of botanical remains from Nevali Cori PPNB, Turkey: a short interim report. In: Damania AB, Valkoun J, Willcox G, Qualset CO (eds) The origins of agriculture and crop domestication. Proceedings of the Harlan Symposium, pp 170–176

Perrino P, Laghetti G, D'Antuono LF, Al Ajlouni M, Kanbertay M, Szabo AT, Hammer K (1996) Ecogeographical distribution of hulled wheat species. In: Padulosi S, Hammer K, Heller J (eds) Hulled wheats. International Plant Genetic Resources Institute, Rome, pp 102–118

Pluzhnikov A, Donnelly P (1996) Optimal sequencing strategies for surveying molecular genetic diversity. Genetics 144:1247–1262

Pourkheirandish M, Komatsuda T (2007) The importance of barley genetics and domestication in a global perspective. Ann Bot 100:999–1008

Renfrew C (2002) The emerging synthesis': the archaeogenetics of farming/language dispersals and other spread zones. In: Bellwood P, Renfrew C (eds) Examining the farming language dispersal hypothesis. McDonald Institute for Archaeological Research, Cambridge, pp 3–16

Rollefson G, Simmons A, Donaldson M, Gillespie W, Kafafi Z, Kohler-Rollefson I, McAdam E, Ralston S, Tubb K (1985) Excavations at the pre-pottery Neolithic B village of 'Ain Ghazal (Jordan), 1983. Mitt Dtsch Orient-Ges Berlin 117:69–116

Russell J, Booth A, Fuller F, Harrower B, Hedley P, Machray G, Powell W (2004) A comparison of sequence-based polymorphism and haplotype content in transcribed and anonymous regions of the barley genome. Genome 47:389–398

Saisho D, Purugganan MD (2007) Molecular phylogeny of domesticated barley traces expansion of agriculture in the Old World. Genetics 177:1765–1776

Sakamura T (1918) Kurze Mitteilung über die Chromosomenzahlen und die Verwandtschafts-verhältnisse der *Triticum* Arten. Bot Mag Tokyo 32:151–154

Salamini F, Özkan H, Brandolini A, Schäfer-Pregl R, Martin W (2002) Genetics and geography of wild cereal domestication in the Near East. Nat Rev Genet 3:429–441

Salamini F, Heun M, Brandolini A, Ozkan H, Wunder J (2004) Comment on "AFLP data and the origins of domesticated crops". Genome 47:615–620

Sax K, Sax MJ (1924) Chromosome behaviour in a genus cross. Genetics 9:454–464

Sears ER (1954) The aneuploids of common wheat. Res Bull Missouri Agric Exp Stn 572:1–57

Schiemann E (1939) Gedanken zur Genzentrentheorie Vavilovs. Naturwissenschaften 27:377–401

Schmidt K (2001) Göbekli Tepe, southeastern Turkey. A preliminary report on the 1995–1999 excavations. Paleorient 26:45–54

Schmidt K (2006) Sie bauten die ersten Tempel. Beck, München

Shao Q, Li C, Basang C (1983) Semi-wild wheat from Xizang (Tibet). In: Sakamoto S (ed) Proceedings of the 6th international wheat genetics symposium, Kyoto, 1983. Plant Germ-Plasm Institute, Faculty of Agriculture, Kyoto University, Kyoto, Japan, pp 111–114

Slageren van MW (1994) Wild wheats: a monograph of *Aegilops* L. and *Amblyopyrum* (Jaub. and Spach) Eig (*Poaceae*). Agriculture University Papers, Wageningen

Takahashi R (1955) The origin of cultivated barley. In: Demerec M (ed) Advances in genetics. Academic, New York, pp 227–266

Taketa S, Kikuchi S, Awayama T, Yamamoto S, Ichii M, Kawasaki S (2004) Monophyletic origin of naked barley inferred from molecular analyses of a marker closely linked to the naked caryopsis gene (*nud*). Theor Appl Genet 108:1236–1242

Talbert LE, Smith LY, Blake NK (1998) More than one origin of hexaploid wheat is indicated by sequence comparison of low-copy DNA. Genome 41:402–407

Tanno K, Takeda K (2004) On the origin of six-rowed barley with brittle rachis, *agriocrithon* [*Hordeum vulgare* ssp. *vulgare* f. *agriocrithon* (Åberg) Bowd.], based on a DNA marker closely linked to the *vrs1* (six-row gene) locus. Theor Appl Genet 110:145–150

Tanno K, Willcox G (2006) How fast was wild wheat domesticated? Science 311:1886

Thuillet A-C, Bru D, David J, Roumet P, Santoni S, Sourdille P, Bataillon T (2002) Direct estimation of mutation rate for 10 microsatellite loci in durum wheat, *Triticum turgidum* (L.) Thell. Ssp durum desf. Mol Biol Evol 19:122–125

Thuillet A-C, Bataillon T, Poirier S, Santoni S, David JL (2005) Estimation of long-term effective population sizes through the history of durum wheat using microsatellite data. Genetics 169:1589–1599

Vaccino P, Becker H-A, Brandolini A, Salamini F, Kilian B (2009) A catalogue of *T. monococcum* genes encoding toxic and immunogenic peptides for celiac disease patients. Mol Genet Genom 281:289–300

Vader WL, Stepniak DT, Bunnik EM, Kooy YMC, De Haan W, Drijfhout JW, Van Veelen P, Koning F (2003) Characterization of cereal toxicity for celiac disease patients based on protein homology in grains. Gastroenterology 125:1105–1113

van Zeist W (1970) The oriental institute excavations at Mureybit, Syria: preliminary report on the 1965 campaign. Part III. Palaeobotany. J Near East Stud 29:167–176

van Zeist W, de Roller GJ (1991–1992) The plant husbandry of aceramic Cayönü, S.E. Turkey. Palaeohistorica 33/34:65–96

Vavilov NI (1926) Studies on the origin of cultivated plants. Institut Botanique Appliqué et d'Amelioration des Plantes, Leningrad

Vavilov NI (1992) Origin and geography of cultivated plants. (D. Love, transl.). Cambridge University Press, Cambridge, pp 316–366

von Bothmer R, van Hintum T, Knüpffer H, Sato K (eds) (2003) Diversity in barley (*Hordeum vulgare*). Elsevier, Amsterdam

von Humboldt A (1806) Ideen zu einer Geographie der Pflanzen nebst einem Naturgemälde der Tropenländer. Cotta'sche Buchhandlung, Tübingen

Weiss E, Kislev ME, Hartmann A (2006) Autonomous cultivation before domestication. Science 312:1608–1610

Wicker T, Schlagenhauf E, Graner A, Close TJ, Keller B, Stein N (2006) 454 sequencing put to the test using the complex genome of barley. BMC Genomics 7:275

Wieser H, Koehler P (2008) The biochemical basis of celiac disease. Cereal Chem 85:1–13

Willcox G (1996) Evidence for plant exploitation and vegetation history from three early Neolithic pre-pottery sites on the Euphrates (Syria). Veget Hist Archaeobot 5:143–152

Willcox G (1998) Archaeobotanical evidence for the beginnings of agriculture in southwest Asia. In: Damania AB, Valkoun J, Willcox G, Qualset CO (eds) The origins of agriculture and crop domestication. Proceedings of the Harlan Symposium. ICARDA, Aleppo, pp 25–38

Willcox G (2005) The distribution, natural habitats and availability of wild cereals in relation to their domestication in the Near East: multiple events, multiple centres. Veget Hist Archaeobot 14:534–541

Willcox G, Fornite S, Herveux L (2008) Early Holocene cultivation before domestication in northern Syria. Veget Hist Archaeobot 17:313–325

Wright SI, Vroh I, Schroeder SG, Yamasaki M, Doebley JF, McMullen MD, Gaut BS (2005) The effects of artificial selection on the maize genome. Science 308:1310–1314

Zhang W, Qu LJ, Gu H, Gao W, Liu M, Chen J, Chen Z (2002) Studies on the origin and evolution of tetraploid wheats based on the internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA. Theor Appl Genet 104:1099–1106

Zohary D (1999) Monophyletic vs. polyphyletic origin of the crops on which agriculture was founded in the Near East. Genet Res Crop Evol 46:133–142

Zohary D, Hopf M (2000) Domestication of plants in the old world. Oxford University Press, Oxford

Part II
Host-Plant Interaction

Mechanisms of Speciation in Southeast Asian Ant-Plants of the Genus *Macaranga* (Euphorbiaceae)

Kurt Weising, Daniela Guicking, Christina Fey-Wagner, Tim Kröger-Kilian, Tina Wöhrmann, Wiebke Dorstewitz, Gudrun Bänfer, Ute Moog, Miriam Vogel, Christina Baier, Frank R. Blattner, and Brigitte Fiala

Abstract The palaeotropic genus *Macaranga* (Euphorbiaceae) is an excellent model system to analyze co-evolutionary processes associated with myrmecophytism, a mutualistic interaction between plants and ants. Ant-plants like *Macaranga* provide nesting space and feed their partners, whereas the ants protect the plants from herbivores and competitors such as lianas. We used genome-based evidence to investigate speciation mechanisms in *Macaranga* ant-plants, and their co-evolution with ants from the genus *Crematogaster*. Our previous work had shown that myrmecophytic *Macaranga* species show little genetic differentiation, suggesting an adaptive radiation. We hypothesized that the obligatory symbiosis with ants may reduce gene flow among plant populations, eventually enhancing allopatric speciation. To test this hypothesis, we verified the monophyly of the investigated plant lineages by phylogenetic analyses, reconstructed parsimony networks based on chloroplast DNA (cpDNA) variation, and assessed population genetic parameters using nuclear microsatellites and cpDNA haplotypes. Our data provided evidence for vicariant events as well as for hybridization and cpDNA introgression among closely related *Macaranga* species. The extent of population differentiation within myrmecophytic versus non-myrmecophytic species proved to be in a similar range, indicating that our working hypothesis of enhanced allopatric speciation in myrmecophytes cannot be sustained by the present evidence. Nevertheless, the mutualistic interactions of *Macaranga* and associated ants may be a key innovation that opened an adaptive zone putatively exploited by the divergence of *Macaranga*.

K. Weising (✉), D. Guicking, C. Fey-Wagner, T. Kröger-Kilian, T. Wöhrmann, W. Dorstewitz, G. Bänfer, U. Moog, and M. Vogel
Plant Molecular Systematics, University of Kassel, 34109 Kassel, Germany
e-mail: weising@uni-kassel.de

C. Baier and F.R. Blattner
Leibniz Institute of Plant Genetics and Crop Science (IPK), 06466 Gatersleben, Germany

B. Fiala
Animal Ecology and Tropical Biology (Zoology III), Biocenter, University of Würzburg, 97074 Würzburg, Germany

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_9,

1 Introduction

1.1 Co-Evolution in Mutualistic Associations: A Challenge for Evolutionary Biology

One of the fundamental aims in evolutionary biology is a deeper understanding of the factors that determine the diversity of organisms. The ecological hypothesis of speciation is that reproductive isolation ultimately evolves as a consequence of divergent selection on traits between environments (Schluter 2000). Ecological interactions between species are particularly relevant in this respect (Rundle and Nosil 2005), and could well belong to the principal forces driving diversification and specialization between organisms. Mutualistic and symbiotic associations have been considered as important factors for creating biodiversity especially in the tropics (Schemske 2002). However, with a few recent exceptions (e.g., Elias et al. 2008), the co-evolutionary dynamics of mutualistic interactions have received comparatively little attention, and remain poorly explored (Herre et al. 1999; Heil and McKey 2003).

Systems in which species groups co-evolve are often complicated but have the inherent advantage that multiple, related speciation mechanisms can be tested simultaneously. One suitable system for such studies is formed by so-called myrmecophytes, i.e., plants that are permanently and regularly inhabited by ants (Davidson and McKey 1993; Jolivet 1996). Myrmecophytic ant–plant relationships are common constituents of tropical forest ecosystems, and they are usually mutualistic.

1.2 Radiation on Both Sides of a Myrmecophytic Interaction: The Macaranga–Crematogaster *System*

The most prominent ant–plant system in southeast Asia consists of the large genus *Macaranga* (Euphorbiaceae) and its manifold associations with ants from the genera *Crematogaster* and *Camponotus* (Fiala et al. 1989, 1999; Davies 2001). *Macaranga* comprises about 280 species with a range stretching from West Africa eastward to the southern Pacific islands (Whitmore 1975, 1982, 2008). *Macaranga* species are dioecious trees or shrubs with large leaves and often conspicuous stipules (Fig. 1a–d). Many of them are fast-growing pioneer trees. Inflorescences consist of numerous small and inconspicuous flowers that are commonly visited by thrips, which appear to be the main pollinators (Moog et al. 2002; Moog 2002; Fig. 1g). Fruits are capsules (Fig. 1e) that dehisce at maturity, exposing small and often colorful arillate seeds eaten by birds and small mammals that presumably act as primary dispersal agents.

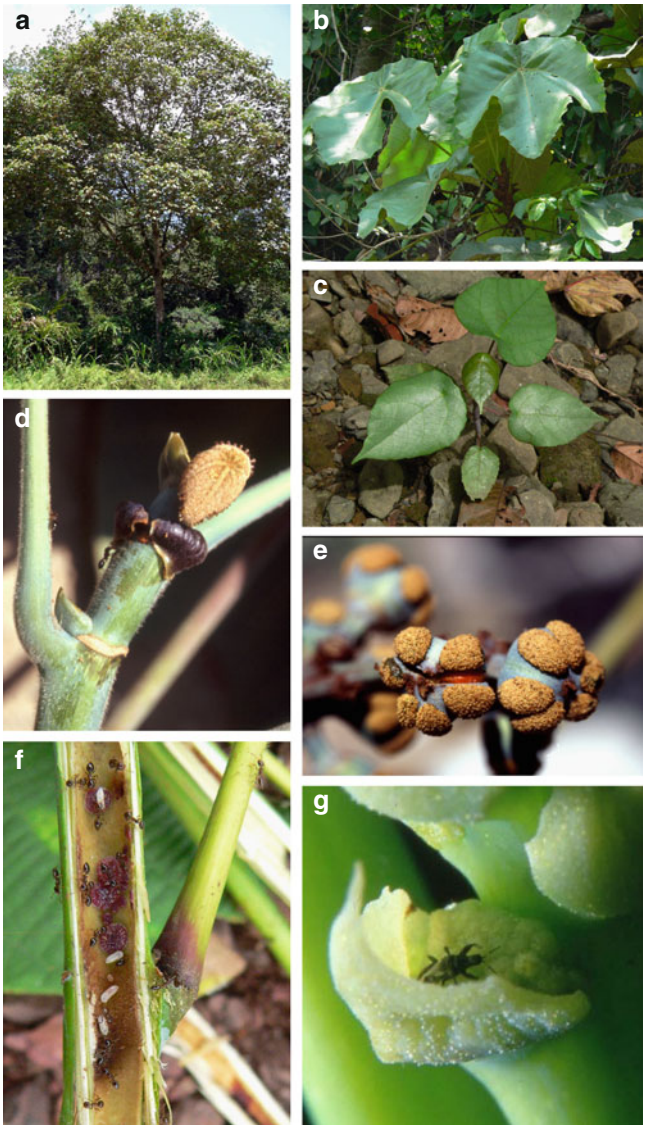


Fig. 1 (**a**) Habitus of *M. pearsonii*. (**b**) Peltate leaves of *M. gigantea*. (**c**) Seedling of *M. hypoleuca* in the stage of colonization. (**d**) Stipules of *M. indistincta*. (**e**) Dehiscing capsules of *M. hypoleuca* exposing arillate seeds. (**f**) Myrmecodomatium of *M. pearsonii* showing ant workers, larvae and symbiotic coccids. (**g**) Part of female inflorescence of *M. hullettii* with thrips

Within *Macaranga*, a wide spectrum of different ant–plant mutualisms exists. While extrafloral nectar and/or nutrient-rich food bodies produced by non-myrmecophytic species attract various opportunistic ants, obligate myrmecophytes also offer nesting space – so-called domatia – for specific ant partners that live exclusively on these plants

(Fiala and Maschwitz 1991, 1992a, b; Fig. 1f). In obligate myrmecophytes, the ants also harvest honeydew produced by scale insects maintained inside the internodes (Heckroth et al. 1998; Fig. 1f). In exchange for the provision of food and nesting sites, the ant partners effectively protect their host plants against herbivore damage, competition by climbers (Fiala et al. 1989), and apparently also against fungal infection (Heil et al. 1999, 2001a). Long-term experiments under field conditions have shown that obligate *Macaranga* myrmecophytes do not survive in the absence of their partner (Heil et al. 2001b). Also, specific partner ants have never been found nesting outside their plants, suggesting that both partners are highly dependent on each other.

With about 30 obligate myrmecophytes, *Macaranga* is the only genus in the palaeotropics that exhibits a substantial radiation of ant-plants (Whitmore 1975; Davies 2001). Phylogenetic trees based on sequence analyses of the nuclear ribosomal DNA internal transcribed spacer (ITS) showed that myrmecophytes are mainly confined to two clades, which correspond to sections *Pachystemon* and *Pruinosae*, respectively (Blattner et al. 2001; Davies et al. 2001). Only few members of these sections are not inhabited by ants. The ITS trees also showed good congruence with trees based on morphological data (Davies et al. 2001). One striking feature of the ITS dataset was the high sequence similarity among closely related *Macaranga* species, especially within section *Pachystemon*. This close genetic relationship stood in sharp contrast to the pronounced ecological and morphological differences among the members of these sections. We therefore hypothesized that the lack of genetic differentiation could be a consequence of a rapid and relatively recent radiation of *Macaranga* in pioneer habitats.

On a smaller scale, radiation has also occurred on the ant side, where at least eight distinct (morpho)species of *Crematogaster,* subgenus *Decacrema*, were found to colonize *Macaranga* myrmecophytes (Fiala et al. 1999; Feldhaar et al., this volume). In addition, one non-*Decacrema* ant and three species of *Camponotus* are involved (Maschwitz et al. 1996, 2004). Obligate *Macaranga* myrmecophytes and their partner ants are restricted to ever-wet, aseasonal rainforests of the Malay Peninsula, Sumatra, and Borneo (Fig. 2). Due to the ongoing decline of primary forests in these regions, myrmecophytic and non-myrmecophytic *Macaranga* pioneers now frequently dominate the landscape along roadsides, and *Macaranga* has become one of the most abundant tree genera in logged areas.

The various types of ant–plant interaction in the *Macaranga* complex and the radiation of both partners offer an exceptional model system for studying the evolution of myrmecophytism and speciation in mutualistic interactions. In our previous work dating back to the early 1990s, we collected detailed data on plant traits that are presumably adaptive in respect to the ant association. These include, for example, the relative numbers and chemical contents of food bodies (Heil et al. 1997, 1998; Fiala and Maschwitz 1992b), the characteristics of extrafloral nectaries and domatia (Fiala and Maschwitz 1991; 1992a), wax cover of the plants (Federle et al. 1997), and the occurrence of preformed thin zones of the stem (prostomata) that facilitate ant colony establishment (Federle et al. 2001). Many of these traits show interspecific differences which are coherent with their differential use by the ants and could therefore point to different selective pressures exerted by the ants

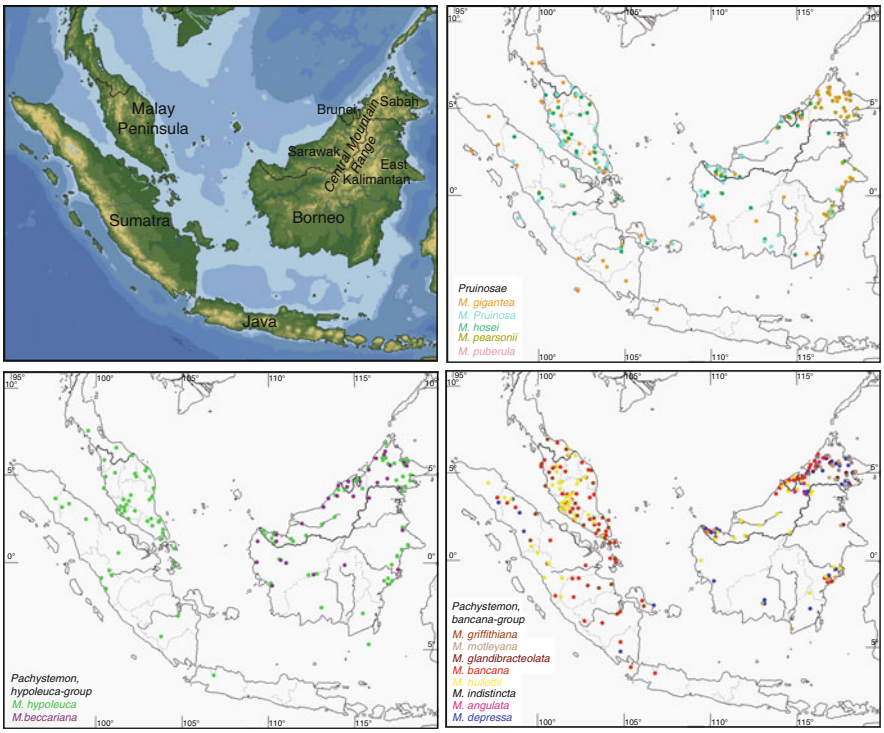


Fig. 2 Distribution patterns of *Macaranga* species of section *Pruinosae* and of the *bancana* and *hypoleuca* groups of section *Pachystemon*, analyzed in the present study. Localities are drawn according to information from Davies (2001), the National Herbarium of the Netherlands, Leiden, and own observations

(see Feldhaar et al., this volume). The degree of specificity of the colonization is not absolute and varies between species on both sides (Fiala et al. 1999). The question is whether these associations were reciprocally adaptive or just opened a new environment for both partners in which a radiation was possible.

1.3 Aims of Our Study

To shed light on the evolution of the *Macaranga–Crematogaster* system and to investigate whether and how the associated ants could have promoted speciation in their *Macaranga* host plants, we initiated a series of phylogenetic, phylogeographic, and population genetic studies on both sides of the interaction. We put our focus on three major topics:

1. To verify the monophyly of the radiating groups, our first aim was to reconstruct reliable molecular phylogenies for plants and ants. These are now available.

In Sect. 2, we describe how the molecular trees enabled us to define the relevant species groups for more detailed studies, and how the phylogenies allowed us to compare the patterns of evolutionary history of both partners. In particular, we aimed at solving the question whether the interaction between ants and plants can be better explained by strict co-speciation, or by co-adaptation and frequent host shifts.

2. Hybridization between closely related species is common in plants and had to be considered as a possible speciation mechanism. Evidence from our preliminary analyses suggested that closely related *Macaranga* species are connected by gene flow (Vogel et al. 2003). We therefore aimed to evaluate in more detail what roles hybridization and reticulate evolution could have played during the evolution of *Macaranga*. The results of these studies are summarized in Sect. 3.
3. One main question of our study asked for the reciprocal influences that ants and plants may exert upon the evolution of their respective partners. Could ants drive plant trait diversification by disruptive selection on adaptive traits? Or do ants exert a negative impact on the dispersal abilities of their host plants, due to the necessity of meeting the appropriate partners at a new colonization site? In Sect. 4, we summarize how these hypotheses were tested by means of comparative genetic analyses of myrmecophytic and non-myrmecophytic *Macaranga* species.

2 What Does Phylogeny Tell Us About the *Macaranga–Crematogaster* Co-Evolution?

2.1 Monophyly of Macaranga *Sections and Subsectional Groups with Myrmecophytes*

An important prerequisite for understanding the evolutionary history of any mutualism is the availability of robust phylogenies of the interacting organisms. As a first step to assess the co-evolution of ants and plants in the *Macaranga–Crematogaster* system, we aimed to set up a solid phylogeny of *Macaranga* myrmecophytes and their closest relatives. We therefore complemented our previous ITS trees (Blattner et al. 2001) with analyses of amplified fragment length polymorphisms (AFLPs; Bänfer et al. 2004) and networks of non-coding chloroplast DNA sequences (Vogel et al. 2003; Bänfer et al. 2006). Complementary phylogenetic work on the ant side has been done by our cooperation partners at the University of Würzburg (Feldhaar et al. 2003a; and see Feldhaar et al., this volume).

A similar overall pattern of relationships as in the ITS analysis, but much better resolution was achieved in an AFLP study (Bänfer et al. 2004; Fig. 3), which strongly supported the monophyly of each of the sections *Pachystemon* and *Pruinosae*. Moreover, four well-defined, clearly monophyletic groups were

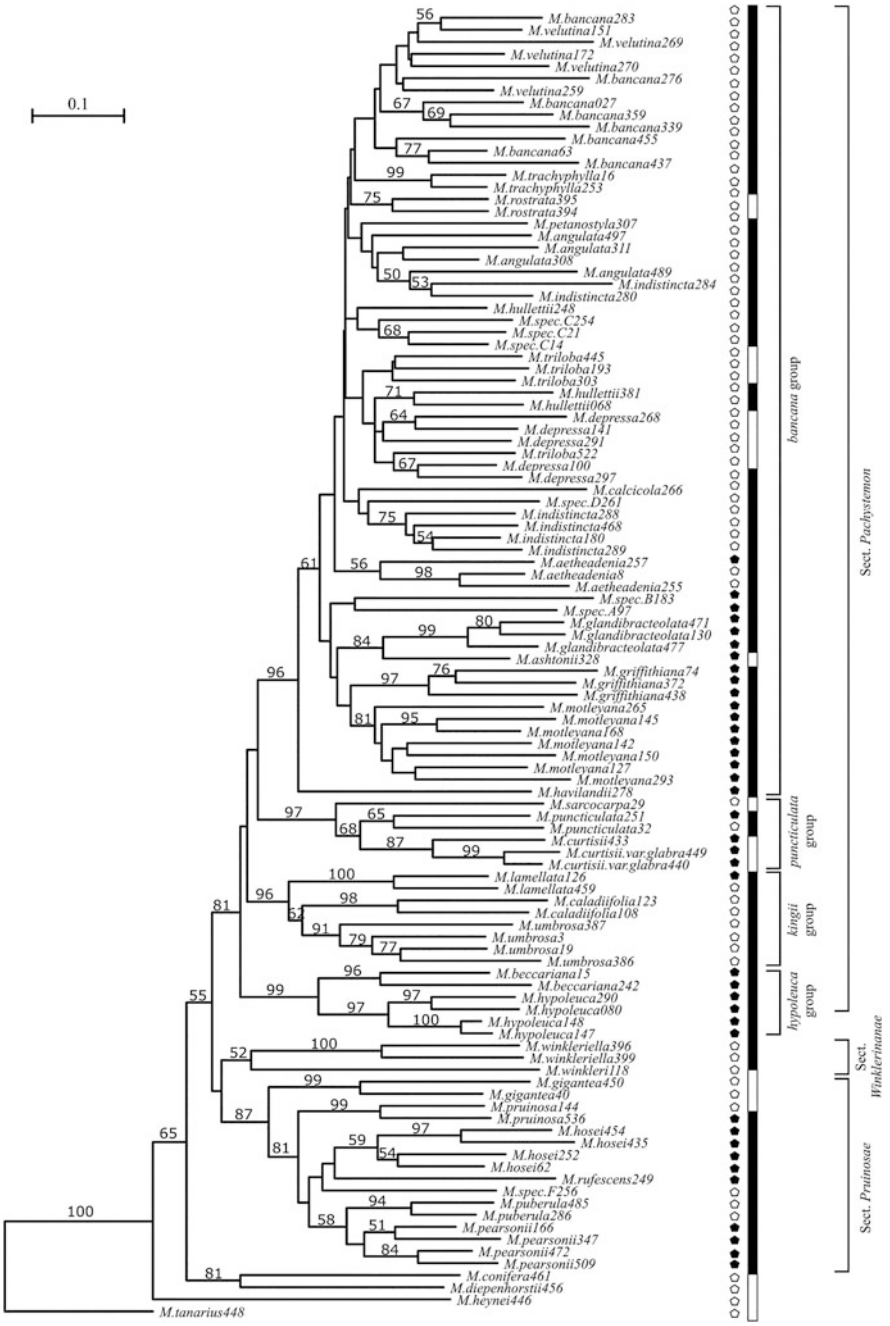


Fig. 3 Neighbor-joining tree of 108 *Macaranga* specimens based on 426 band positions obtained with eight AFLP primer pair combinations. The Nei and Li (1979) index of similarity was used to generate the underlying distance matrix. *Numbers* above the *branches* represent bootstrap values obtained from 1,000 replicates. Myrmecophytic specimens are indicated by *black shading* of the *vertical bar* to the *right* of the dendrogram. The presence versus absence of surface wax is indicated by *black* versus *white symbols*. Figure adopted from Bänfer et al. (2004), with permission

resolved within section *Pachystemon*, to which we refer as the *bancana*, *kingii*, *hypoleuca* and *puncticulata* groups, respectively (Bänfer et al. 2004; Fig. 3). Within section *Pruinosae*, the non-myrmecophytic *M. gigantea* was sister to a clade containing the myrmecophytes *M. pruinosa, M. hosei*, *M. rufescens, M. pearsonii*, and *M. puberula* (Fig. 3). The monophyly of section *Winklerianae*, including only two myrmecophytic species, *M. winkleri* and the rare and narrow endemic *M. winkleriella*, remained ambiguous in all studies (see also Kulju et al. 2007). Statistical parsimony networks of chloroplast haplotypes (Vogel et al. 2003; Bänfer et al. 2006), which are discussed in detail in Sect. 3, also supported sectional boundaries and sub-sectional groups within *Pachystemon*.

Our phylogenetic analyses provided good evidence that myrmecophytism evolved at least twice, and presumably even more often in *Macaranga* (see also Davies 2001). One independent origin is probably associated with section *Winklerianae*, a second one with section *Pruinosae*, a third with *M. puncticulata* of section *Pachystemon*, and at least one more origin within the remainder of the species-rich section *Pachystemon*, where myrmecophytism also appears to have been reversed one or several times. Each of these lineages exhibits a characteristic suite of morphological traits related to myrmecophytism (Fiala and Maschwitz 1992a, b; Federle et al. 1997, 2001; Heil et al. 1997, 1998; Feldhaar et al. 2003b), and each lineage is also characterized by a more or less specific set of ant partners (Fiala et al. 1999; Feldhaar et al. 2003b; see below). For example, in ant-inhabited species of section *Pachystemon*, the stems of seedlings swell at about 10 cm tall and the pith degrades automatically, whereas myrmecophytic species of section *Pruinosae* have solid stems that become hollow through excavation by the ants when about 75 cm tall.

2.2 *Co-Adaptation and Host Shift are Major Determinants of the* Macaranga–Crematogaster *Co-Evolution*

The general outcome of earlier studies on various ant–plant associations is that similar mutualisms often evolve independently, and that host switching within established mutualistic systems is more common than co-speciation (Davidson and McKey 1993; Ayala et al. 1996; Chenuil and McKey 1996; Brouat et al. 2001). The same situation apparently applies to the *Macaranga–Crematogaster* system as well. A direct comparison revealed that plant and ant phylogenies are not at all congruent with each other, strongly suggesting a scenario of host shift, i.e., repeated colonization of plants sharing similar ant-related characters by pre-adapted ants (Feldhaar et al. 2003a, b; see also Quek et al. 2004). This issue is treated in detail by Feldhaar et al. (this volume). Thus, while it is probably too early for a generalized concept, it appears that co-evolution in myrmecophytic systems predominantly occurred by facilitated host shift due to ecological fitting of pre-adapted partners, rather than by co-speciation and common descent.

3 Which Roles Did Hybridization and Reticulate Evolution Play During the Evolution of *Macaranga*?

3.1 *First Indications for Interspecific Gene Flow in* Macaranga

Whether interspecific hybridization is an important mechanism that generates biological diversity has long been a matter of controversy. Recently, Seehausen (2004) argued for a role of hybridization in adaptive radiations. The lack of genetic differentiation among closely related species within section *Pachystemon* and – to a smaller extent – also within section *Pruinosae* as revealed by our phylogenetic studies suggested that speciation processes in myrmecophytic *Macaranga* occurred only recently. Two questions were raised by this observation: (1) Are closely related *Macaranga* species still connected by gene flow? (2) Has reticulate evolution played a role in the evolution of *Macaranga*?

In preliminary experiments, we found that chloroplast haplotypes were sometimes shared between *Macaranga* species growing in the same geographical region (Vogel et al. 2003). This was mainly the case in the *bancana* group of section *Pachystemon*, where species also appeared to be genetically most closely related (Blattner et al. 2001). An association of chloroplast haplotypes with geographically circumscribed regions rather than with taxonomic boundaries has been detected in many other plant taxa (e.g., McKinnon et al. 2001), including tropical tree genera like *Lithocarpus* (Cannon and Manos 2003). Such patterns are generally explained by either incomplete lineage sorting, or more frequently, by "chloroplast capture" as a consequence of interspecific hybridization and successive backcrossing of a primary hybrid with the pollen parent. We concluded from our early experiments that the units of chloroplast gene flow may exceed species boundaries in *Macaranga*, and that a denser taxonomic sampling as well as more comprehensive sampling of populations would be necessary to uncover the phylogeographic structure of closely related species (see also Jakob and Blattner 2006).

3.2 *Development of Markers to Analyze Species Boundaries and Gene Flow among* Macaranga *Species*

To investigate the role that interspecific gene flow may have played in the evolution of myrmecophytic *Macaranga*, we initiated a comparative phylogeographic and population genetic analysis based on chloroplast and nuclear markers (see Schaal et al. 1998). For phylogeographic analysis, we successfully adopted the "consensus chloroplast microsatellite primer" (ccmp) approach of Weising and Gardner (1999), and also sequenced the *atp*B-*rbc*L spacer (Vogel et al. 2003; Bänfer et al. 2006). Variation at the target loci was identified either by sequencing or by single nucleotide sequencing (SNS) analysis, with details given in Guicking et al. (2008). Chloroplast haplotypes were defined from the combined information at several

loci, and used to construct statistical parsimony networks in a two-step procedure (for details see Bänfer et al. 2006). For population genetic analyses, 16 highly polymorphic nuclear microsatellite markers were developed from five *Macaranga* species (Guicking et al. 2006; Baier et al. 2009). The majority of these markers showed good cross-species transferability and proved to be useful tools for population genetic analyses in more than one *Macaranga* species.

3.3 *Evidence for Introgression and Incomplete Lineage Sorting Among* Macaranga *Ant-Plants*

To define the taxonomic entities among which introgression may occur, and to create a guideline for studying single species or closely related species groups, we first surveyed the haplotypic diversity across all three *Macaranga* sections harboring myrmecophytes (Bänfer et al. 2006). Included in the analysis were 143 individuals belonging to 41 species that covered all major evolutionary lineages within *Macaranga* sections *Pachystemon*, *Pruinosae*, and *Winklerianae*. A total of 88 chloroplast haplotypes were obtained and used to construct an extended chloroplast genealogy. In the network shown in Fig. 4, the three sections are well separated from each other as well as from the outgroups. Within section *Pachystemon*, two of the four species groups defined by our previous AFLP study (i.e., the *kingii* group and the *hypoleuca* group; Bänfer et al. 2004; see Fig. 3) also formed distinct branches in the cpDNA network, whereas the *puncticulata* group was nested within the *bancana* group.

The most striking result of this study was that, in the *bancana* group, chloroplast haplotypes were often shared among species, and that haplotype relationships often followed geographical rather than taxonomical patterns. Thus, chloroplast haplotypes in this group were split into three geographically defined branches, independent of species assignment and state of myrmecophytism. Group I comprised almost all specimens from Sabah, all samples of group II had been collected in Sarawak, Brunei, and East Kalimantan, and group III exclusively contained material from the Malay Peninsula (Fig. 4). Due to a limited number of samples, it was not possible to decide whether the same geographical patterning also occurred in the other taxonomic groups of *Macaranga*. We therefore decided to extend our sampling of section *Pruinosae* and the subsectional *hypoleuca* group of section *Pachystemon*.

In section *Pruinosae*, a total of 49 different chloroplast haplotypes were identified among 768 specimens from five species, including samples from northern and eastern Borneo (Sabah, Brunei, and East Kalimantan) as well as the Malay Peninsula (Guicking et al., submitted for publication). Only eight haplotypes were shared among two or more species and also took central positions in the network, suggesting their ancestral state. One particular haplotype was found in all but one species of the section, and probably represents the root of the *Pruinosae* clade. Contrary to the situation in the *bancana* group, none of the presumably derived

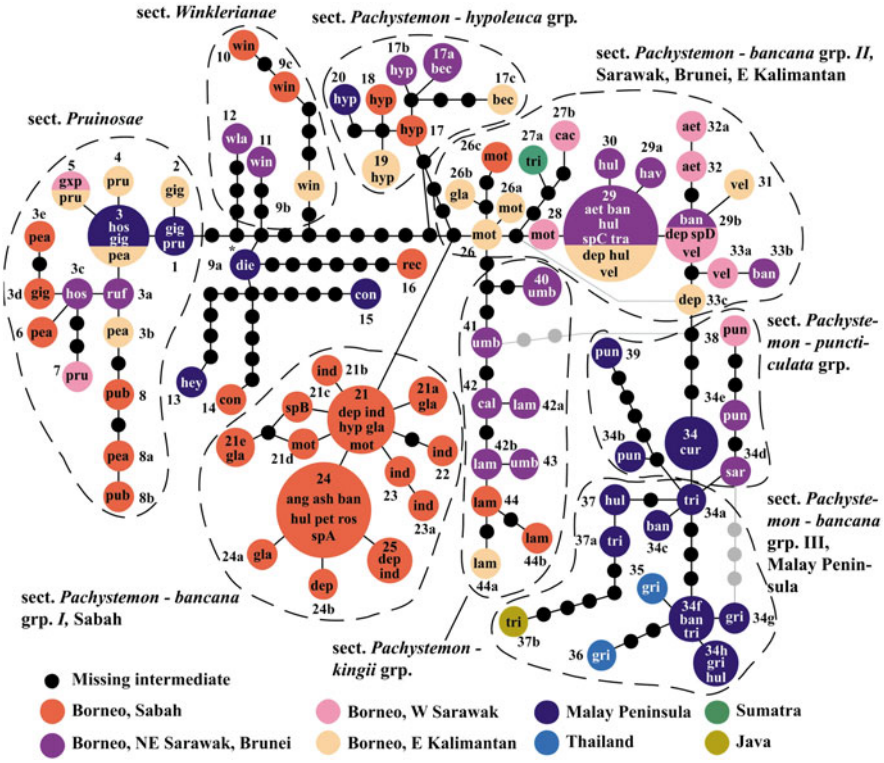


Fig. 4 Parsimony network of 88 chloroplast haplotypes of *Macaranga* species from the myrmecophytic sections or species groups, calculated from sequence variation of the *atp*B–*rbc*L intergenic spacer and the ccmp2 and ccmp6 loci. Except for *M. calcicola* (*cac*) and *M. winkleriella* (*wla*), species were abbreviated by the first three characters of their species epithets. *Circle* sizes represent the numbers of species sharing a specific chloroplast haplotype. *Numbers* depict arbitrary haplotype denominations of the backbone network (1–44) with character extensions (**a**–**f**) indicating haplotype subdivision due to length variation at six microsatellite loci and two indels. *Grey connections* between haplotypes are closed loops inferred by the TCS program (Clement et al. 2000), which represent less likely ways of chloroplast evolution. An *asterisk* indicates the probable position of the root of the network. Figure adopted from Bänfer et al. (2006), with permission

haplotypes located at tip positions of the network was shared among species. We also found no geographical patterning of haplotype distribution in this group. A similar picture arose for the two species of the *hypoleuca* group of section *Pachystemon*, where 18 chloroplast haplotypes were found among 449 samples originating from northern and eastern Borneo and from the Malay Peninsula (Kröger-Kilian 2006). Only three, presumably ancestral, haplotypes were shared between the two species.

Taken together, it appears that chloroplast haplotypes in section *Pruinosae* and in the *hypoleuca* group of section *Pachystemon* are much more species-specific

than those in the *bancana* group of section *Pachystemon*. While a pattern of geographical haplotype distribution as observed in the *bancana* group may be best explained by frequent hybridization and chloroplast capture, incomplete sorting of ancestral lineages most likely led to shared ancestral haplotypes in section *Pruinosae* and the *hypoleuca* group. This means that phylogeographic analyses of chloroplast haplotypes in individual species are possible and straightforward in the latter two groups. However, such an approach may produce misleading results in the *bancana* group of section *Pachystemon*, where extensive chloroplast haplotype sharing occurs.

3.4 *Reticulate Evolution in* Macaranga*: Frequent Hybridization and Introgression but Little Evidence for Stable Hybrids*

To evaluate the relative importance of introgression and incomplete lineage sorting and the role that reticulate evolution may play in the three study groups, we also performed population genetic studies based on nuclear microsatellite markers. Such studies allow the direct identification of recent hybrids by means of Bayesian inference (STRUCTURE; Pritchard et al. 2000). Of the *bancana* group of *Macaranga* section *Pachystemon*, we analyzed 761 specimens from nine species and two unspecified morphospecies (Dorstewitz 2008). For the population genetic analyses of section *Pruinosae* and the *hypoleuca* group, the same samples were included as in the phylogeographic studies. Allelic information from eight polymorphic microsatellite loci was considered in the analyses of the subsectional *bancana* and *hypoleuca* groups; seven loci (six in *M. pearsonii*) were included in the study of section *Pruinosae*.

Bayesian analyses of nuclear microsatellite data were performed using the program STRUCTURE, assuming an admixture model without prior information on species membership, and setting K (the number of groups) equal to the number of species considered. The results of these analyses showed that most of the investigated species were clearly distinct from each other (Fig. 5). However, STRUCTURE also pinpointed several recent hybrids in section *Pruinosae* and in the *bancana* group of section *Pachystemon*, whereas no evidence of recent hybridization was found within the *hypoleuca* group. In the *bancana* group, all specimens identified as hybrids by STRUCTURE also shared morphological characters from both putative parent species. Hybridization between the closely related myrmecophytic species *M. indistincta* and *M. angulata* was observed only once, whereas hybridization between *M. indistincta* and the non-myrmecophyte *M. depressa* seems to be a relatively frequent phenomenon. Putative hybrids of these two species were found at several locations throughout Sabah, some of them in fruit. Germination of the seeds in the greenhouse was successful, and the F1 generation showed the same intermediate morphology as the parent trees.

Despite the much lower number of chloroplast haplotypes shared among species in section *Pruinosae* as compared to the *bancana* group (see Sect. 3.3), analyses of

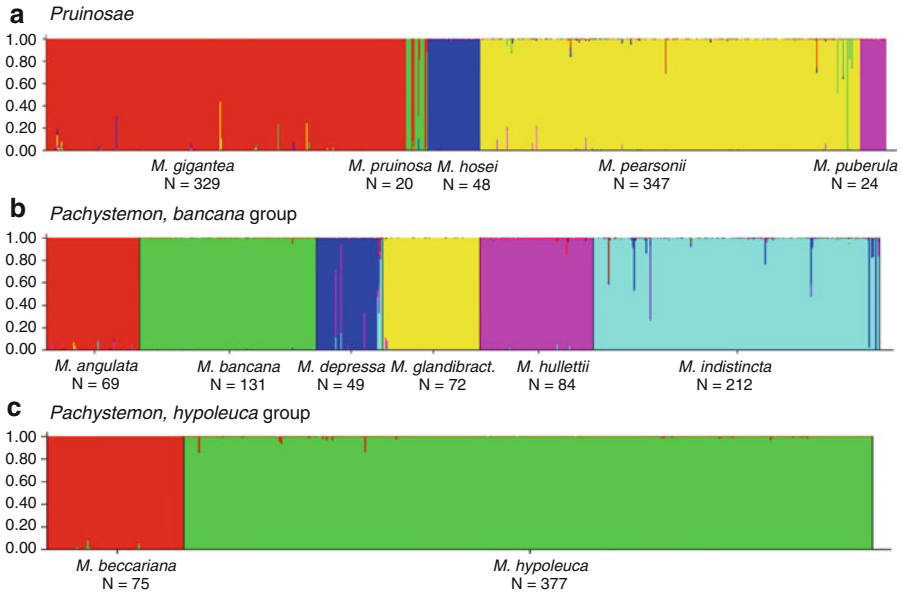


Fig. 5 Identification of putative hybrids among species in section *Pruinosae*, and the *bancana* and *hypoleuca* group of section *Pachystemon* using Bayesian analysis as implemented in the program STRUCTURE 2.2 (Pritchard et al. 2000). Each individual is represented as a *thin vertical line*, which is partitioned into colored segments that indicate the individual's membership fractions in the assumed species

nuclear microsatellites yielded a similar proportion of putative hybrids (Fig. 5). Hybridization in section *Pruinosae* was particularly frequent between *M. gigantea* and *M. pruinosa*, as was also suggested by Davies (2001) based on morphological observations. Both species are non-myrmecophytes in Borneo, whereas *M. pruinosa* is often inhabited by ants outside Borneo. Putative hybrids were also detected between *M. pearsonii* and *M. pruinosa* in East Kalimantan, where both species occur in sympatry. One individual that was clearly *M. gigantea* by morphological criteria could be identified as a hybrid between *M. gigantea* and *M. pearsonii* by genetical means.

Taken together, hybridization between closely related species appears to be quite frequent in the *bancana* group of section *Pachystemon* and in section *Pruinosae*, but little evidence was found for the formation of stable hybrid zones. With the exception of the presumable hybrids between *M. indistincta* and *M. depressa*, which were found at several locations and produced fertile seeds, no further observations exist that would support the idea of an important role of reticulate evolution in *Macaranga*. Apparently, mechanisms that keep related sympatric species apart nowadays are quite efficient in *Macaranga*, despite the presumably low genetic differentiation between the species. As possible candidates for such mechanisms, different pollinators and asynchronous flowering times have been suggested (Moog 2002; Moog et al. 2002).

In myrmecophytic systems, the outcome of hybridization may also be influenced by the ant partners. In a recent study of the African myrmecophyte genus *Leonardoxa,* Léotard et al. (2008) found phenotypically intermediate F1 hybrids that had only poorly developed domatia and were not colonized by the ant partners. Apparently, these hybrids presented ecological maladaptations to mutualistic ants, eventually resulting in hybrid inviability. *Macaranga indistincta* and *M. angulata* have similar ant-related traits and are inhabited by the same *Crematogaster* species, so hybrids between these two species should not pose a problem to the ants. Interestingly, in the same location (Poring Hot Springs, Sabah), a bimodal hybrid zone between the two inhabiting ant species was found, both of which also colonized another *Macaranga* species from section *Pachystemon* (*M. glandibracteolata*). This may point to a rather flexible host plant resource use of these ant species (see Feldhaar et al., this volume).

The hybrids found between non-myrmecophytic *M. depressa* and myrmecophytes either developed the domatia of their parent species – and were also colonized – or they appeared morphologically as *M. depressa* with solid stems, and were then not ant-inhabited. Thus, fitness consequences for *Macaranga* hybrid plants or hybrid-inhabiting ants cannot be ruled out, but long-term studies in the field which would be necessary to achieve conclusive results are difficult to conduct.

4 How Could the Ants Have Influenced Speciation Processes in Their Plant Partners?

4.1 The "Allopatric Speciation Hypothesis": Limited Effective Seed Dispersal Could Enhance Genetic Differentiation in Myrmecophytes

When thinking about possible mechanisms that could have triggered radiation of myrmecophytic *Macaranga*, we recalled our earlier observation that both partners, ants and plants, do not mature without their specific symbionts (Heil et al. 2001b). It may therefore be expected that *Macaranga* seeds germinating far away from the next source of their specific ant partner will have a problem in establishing a new population. This problem will be further enhanced by the dioecious breeding system of the plants. The obligate requirement of a partner organism may therefore reduce the ability for successful colonization of new areas, a feature which has been shown to be important in other mutualists (Pierce 1987). As a consequence of limited effective dispersal ability, populations which accidentally became dissected due to geographical or climatic changes might readily become genetically isolated from each other. Climatic changes have in fact occurred in the Quaternary, and have caused major habitat changes in southeast Asia (Whitmore 1987). Differentiation is supposed to occur after genetic isolation due to habitat differences that result in different selectional forces on the fragmented populations. Thus, the obligate

association with ants could eventually have facilitated allopatric speciation in their partner plants. In a second hypothesis, the colonization by ants is considered as a key innovation that opened a new adaptive zone. Both partners can then be viewed as biotic selective forces acting upon each other. We would then expect that speciation may also have happened sympatrically through disruptive selection due to ecological factors. In some cases, abiotic factors such as adaptation to edaphic conditions or higher altitudes might also have led to reproductive isolation.

In our experiments, we have so far focused on the allopatric scenario. Given that our "allopatric speciation hypothesis" was correct, then gene flow among populations of myrmecophytic species should generally be reduced as compared to related, non-myrmecophytic species, which do not depend on the presence of ants when establishing a new stand. Consequently, we would expect that myrmecophytic species also show a more pronounced population substructuring as compared to non-myrmecophytes. This structuring should mainly be measurable on a large geographical scale (especially when populations are separated by mountain ranges), whereas little differentiation is expected where no geographic barriers exist. Moreover, differences between myrmecophytes and non-myrmecophytes should be much more pronounced at the chloroplast DNA level, because only seed-mediated gene flow is expected to be influenced by the partner ant. In principle, this prediction can be tested by a comparative population genetic analysis in myrmecophytic versus non-myrmecophytic *Macaranga* species. As results obtained by analyzing just one species of each type cannot easily be generalized, several pairs of species need to be studied, where each pair should belong to the same phylogenetic clade. Optimally, the species that will be compared should also show similar ecological preferences, distribution, and life history patterns. These requirements are, however, difficult to meet.

4.2 Population Structure of Myrmecophytic Versus Non-Myrmecophytic Macaranga *Species: No Support for the "Allopatric Speciation Hypothesis"*

To test our hypothesis, we analyzed the intraspecific genetic structure of several species from section *Pruinosae*, and of both the *bancana* and *hypoleuca* group of section *Pachystemon*, based on frequency distributions of nuclear microsatellite alleles and chloroplast haplotypes (for details, see the legend of Table 1). To further characterize intraspecific differentiation, nuclear microsatellite data were also analyzed by a Bayesian assignment approach as implemented in the program STRUCTURE 2.2 (Pritchard et al. 2000), assuming an admixture model.

Section *Pruinosae* appears to be the best-suited model system for a comparative analysis of myrmecophytes and non-myrmecophytes. In this section, the abundant and widely distributed non-myrmecophyte *M. gigantea* is sister to a set of several myrmecophytes (see Bänfer et al. 2004; Fig. 3). The latter show more restricted ranges, which are often parapatric (Fig. 2). Such a situation would be perfectly explained by

Table 1 Compilation of Phi_{ST} values calculated from an analysis of molecular variance (AMOVA) of nuclear microsatellite and chloroplast haplotype data, respectively, indicating population structure in myrmecophytic versus non-myrmecophytic *Macaranga* species of sections *Pachystemon* and *Pruinosae*. Calculations were performed with the program ARLEQUIN 3.11 (Excoffier et al. 2005). *C*. Crematogaster *PM* Peninsula Malaysia, *S* Sumatra, *SB* Southern Borneo, *EK* East Kalimantan, *Sw* Sarawak, *Br* Brunei, *Sb* Sabah (east of Crocker Range), *CR* Crocker Range. Origins of sampled populations are shown in *bold*. *N Ind.*: total number of individuals analysed, *N Pop.*: number of populations considered for AMOVA; *regional scale*: including *all* populations (with $n > 5$) sampled from the entire Malesian region with; *local scale*: including only populations (with $n > 5$) from eastern Sabah; *** $p < 0.001$

	Myrmecophyte	Ant partners	Distribution	Habitat	N Ind.	N Pop.	Phi_{ST} (nc)	Phi_{ST} (cp)	N Pop.	Phi_{ST} (nc)	Phi_{ST} (cp)
						Regional scale			Local scale		
Section *Pruinosae*											
M. gigantea	No	Non-myrmecophyte	**PM,** S,SB,**EK**, **Sw**,**Br**,**Sb**	Lowland	329	20	0.160***	0.704***	6	0.063***	0.462***
M. pruinosa	No/Yes	msp. 2, msp.1	PM,S,SB,**EK**,**Sw**	Lowland	20	2	0.203***	0.674***			
M. hosei	Yes	msp. 2, msp.1	**PM**,S,SB,**EK**, Sw,**Br**	Lowland	48	5	0.158***	0.391***			
M. pearsonii	Yes	msp. 2, msp. 1	**EK**,**Sb**	Lowland	347	15	0.082***	0.561***	11	0.037***	0.506***
M. puberula	Yes	msp. 2, msp. 1	**CR**	Altitudinal	24	2	0.090***	0.724***			
Section *Pachystemon, hypoleuca*-group											
M. hypoleuca	Yes	*C. decamera, msp. 1,7,9*	**PM**,S,SB,**EK**, Sw,**Br**,**Sb**	Lowland	377	19	0.109***	0.563***	8	0.031***	0.521***
M. beccariana	Yes	*C. decamera*	SB,**EK**,Sw, **Br**,**Sb**	Lowland	72	6	0.145***	0.519***			
Section *Pachystemon, bancana*-group											
M. griffithiana	Yes	msp., 1, msp. 5	**PM**,S	Lowland	31	3	0.091***	n.a.			
M. motleyana	Yes	msp. 1,7	SB,**EK**,Sw,**Sb**	Lowland	96	10	0.173***	n.a.	6	0.173***	
M. glandibracteolata	Yes	*C. captiosa,* msp. 10, 1, 7	EK,**Sb**	Lowland	72	5	0.075***	n.a.	5	0.075***	
M. bancana	Yes	*C. captiosa,* msp. 3	**PM**,S,**EK**,**Sw**,Br	Lowland	131	10	0.079***	n.a.			
M. hullettii	Yes	msp. 3, *captiosa*	**PM**,S,SB,**EK**, Sw,**Br**	Lowland	84	3	0.145***	n.a.			
M. indistincta	Yes	*X. captiosa*, msp. 10, 1	**Sb**	Lowland	171	10	0.058***	n.a.	10	0.058***	
M. angulata	Yes	msp. 10, *C. captiosa, 7*	**CR**	Altitudinal	69	5	0.134***	n.a.			
M. depressa	No	Non-myrmecophyte	S,SB,EK,Sw,**Sb**	Lowland	49	4	0.125***	n.a.	4	0.125***	

the above allopatric speciation hypothesis. However, our analyses revealed a similar number of chloroplast haplotypes and comparative levels of genetic diversity in *M. gigantea* and *M. pearsonii* (and three other less comprehensively sampled myrmecophytes; Guicking et al., submitted for publication). Likewise, nuclear microsatellite-based Phi_{ST} values among populations were in the same order of magnitude in all species of section *Pruinosae* or were even lower in myrmecophytes (Table 1). Thus, contrary to our expectations, myrmecophytic species did not show a stronger population structure than non-myrmecophytic species, neither on a regional nor on a local scale (considering only populations from eastern Sabah, a region that is not dissected by conspicuous geographic barriers). In fact, the only example in which the myrmecophytic *M. pearsonii* showed slightly higher levels of among-population variation than the non-myrmecophytic *M. gigantea* was in the case of chloroplast haplotypes on a local scale (Table 1).

The situation is more complicated in the *bancana* group of section *Pachystemon*. First, chloroplast data of individual species were not applicable because of the geographical instead of taxonomical distribution of chloroplast haplotypes in this group (see above and Fig. 4). Second, there are only few non-myrmecophytes in the *bancana* group, and phylogenetic trees suggest that these are in a derived rather than basal position (Bänfer et al. 2004; Fig. 3), pointing to a secondary loss of myrmecophytism. It was thus difficult to find suitable species pairs for comparison, and *M. depressa* appears to be the only non-myrmecophyte of the group that is sufficiently abundant for this kind of investigation. Again, none of our population genetic analyses based on nuclear microsatellite data showed any significant difference between *M. depressa* on the one hand and seven different myrmecophytic species on the other that could be related to the association with symbiotic ants (Dorstewitz 2008; Table 1). *Macaranga depressa* showed an intermediate level of among population variation, both on the regional and local scale. One also has to keep in mind that we do not know when myrmecophytism was lost in this species, or how fast changes in genetic population structure have been manifested after the loss. The level of among-population variation in the two myrmecophytic species of the *hypoleuca* group was in the same order of magnitude as in the other taxonomical groups (Kröger-Kilian 2006; Table 1).

Apparently, the plants successfully colonize any locality where a specific *Crematogaster* (*Decacrema*) ant species is available that is able to colonize them. In other words, if they "cannot find the ants they like, they have learnt to like the ants they find." Consequently, the ants are specific to host plant clades rather than to individual host species, and there are ant species with a rather broad host plant spectrum. We therefore assume that the majority of *Crematogaster (Decacrema)* ants commonly found in *Macaranga* may enable a plant to successfully colonize a new habitat, even if the interaction is not optimal. Even if one particular ant species would offer better protection and be more "cost-effective" than others (see Feldhaar et al., this volume), plants would suffer fitness losses if they are too selective, thus favoring a generalization of the association. Dispersal into completely ant-free habitats was probably a rather rare phenomenon or perhaps just delayed the colonization process, but did not lead to the isolation of populations. We may

thus conclude that our working hypothesis of allopatric speciation does not apply in the form described above.

The question also remains as to how long it takes before reduced gene flow is mirrored in the genetic population parameters analyzed here. We have to consider that continuous rainforest covered the whole of Borneo over a long time, and some rain forest refugia certainly remained even in periods of climatic fluctuations. As a pioneer plant, *Macaranga* may have been influenced by shrinking/expansion of rain forests during Pleistocene glaciations and by the severe anthropogenic forest destruction and fragmentation during the last 50 years. Until recently, *Macaranga* plants have probably been widespread and abundant in gaps and along rivers, which are their original habitats in undisturbed forests. As long as populations have been in contact throughout continuous forest, gene flow has probably been as efficient in myrmecophytes as in non-myrmecophytes. Even after barriers have formed, it may take some time before the interruption of gene flow results in a measurable population differentiation. In *M. griffithiana*, for instance, populations from the Malaysian Peninsula and a disjunct area in continental eastern Thailand were only weakly differentiated from each other, despite being separated by thousands of kilometres with no forest cover left (Maschwitz et al. 2004).

4.3 Putative Role of Vicariance

As outlined in the previous section, our data did not reveal any obvious association between the population structure of myrmecophytic *Macaranga* species and their association with ants. The level of population structure was also not predictable from taxonomic affiliation, altitude, distribution range, or the associated ant partners. We conclude that the extent of population structure in individual *Macaranga* species most likely depends on a number of interacting factors that are difficult to disentangle.

The only factors that clearly play a general role for population differentiation (and speciation) in *Macaranga* are geographic barriers. In all species investigated by Bayesian inference with STRUCTURE 2.2, we found a strong differentiation between populations from (1) the Malay Peninsula and Borneo, separated by the South China Sea, and (2) between Sabah, Brunei/Sarawak, and East Kalimantan on Borneo, separated by the central mountain range and mountains in southern Sabah (data not presented). The same geographic barriers separate a number of allopatric or parapatric *Macaranga* species pairs (Fig. 2). Thus, there is little doubt that vicariance played a major role during species differentiation in *Macaranga*. Within each of these regions, population differentiation was mostly negligible. There is also quite a good congruence in the split of the major groups of ants with the main lineages in *Macaranga* (see Feldhaar et al., this volume). These biogeographic parallels are an indication that contemporary distributions of plants and ants are largely products of the same historical events.

4.4 The Relative Role of Seed and Pollen Dispersal

Another general result from our study was that, for all *Macaranga* species, a much stronger genetic structure was revealed by chloroplast haplotypes as compared to nuclear markers. At least in part, this is certainly a consequence of the smaller effective population size of the chloroplast genome (Ennos et al. 1999). However, this observation may also indicate more efficient pollen dispersal in *Macaranga* than previously anticipated. Initially, we assumed that the restricted flying ability of the main pollinators (thrips; Moog et al. 2002; Moog 2002) should enhance population isolation, because pollen-mediated gene flow between remote populations was supposed to be low. Taking into account the much weaker population structure revealed by nuclear versus chloroplast data, we revised this assumption. One possible scenario is that thrips are passively dispersed by the wind, and in this way should be able to travel long distances (Lewis 1973; Mound and Marullo 1996). If this holds true, thrips actually represent highly efficient pollinators by combining passive long-distance dispersal with active search for adequate target plants at a local scale. Unexpectedly high pollination distances of 5–16 km were also demonstrated for tiny fig wasps (Nason et al. 1998). Extensive outcrossing, as in dioecious plants, can easily homogenize population substructuring. Thus, if rates of pollen flow as monitored by nuclear markers are high enough, they may obscure the effects of other restricting factors (such as limited seed dispersal) on the genetic differentiation of populations.

5 Conclusions

Probably, two main phases have occurred in the evolution of myrmecophytic *Macaranga*: a first phase involved a split into several main ancestral lineages due to shifts in life history and ecological specialization as a consequence of the association with ants. Later, disjunction events have triggered allopatric (and partly) vicariant speciation within each of these lineages. Basically, the colonization of *Macaranga* plants by ants can be viewed as a key innovation, which opened a new adaptive zone for both partners because it made new resources and habitats available. Ants entering such an association circumvent competition with sympatric arboreal ants for foraging grounds and nesting space. On the other hand, plants that recruited ants will grow more successfully in clearings and other disturbed forest areas because their partners protect them from herbivores and from the numerous vines that act as competitors in open habitats. Myrmecophytes therefore have an immediate advantage in pioneer habitats over plants that are not ant-inhabited.

Adaptive radiation in both systems could therefore have followed a different scenario. *Macaranga* plants could have diversified in the particular ecological context of the selective pressures in their pioneer habitats – as a self-reinforcing system of adaptive traits, evolving by reciprocal feedback among the partners. Ants could have driven plant differentiation by changes in their behaviour, morphology,

and physiology. In turn, changes in those plant traits that are relevant for the mutualism, e.g., nest site and food resources as well as epicuticular waxes, could have driven differentiation of the ant partners. However, as both partners were restricted by their host's distributions as they were not able to live independently, availability of partners might have been more relevant than perfect adaptation.

Acknowledgments We acknowledge financial support by Deutsche Forschungsgemeinschaft (grants We 1830/4, Fi 606/5 and Bl 462/2) within priority program SPP1127. Permission to conduct research in Malaysia was kindly granted by the Economic Planning Unit (EPU) of the Prime Minister´s Office, Kuala Lumpur and EPU in Kota Kinabalu, Sabah, as well as the Danum Valley Management Committee and the Forest Department, Sarawak. The permit to work in Brunei was obtained by Universiti Brunei Darussalam and Brunei Museum. We thank our counterparts and colleagues in Malaysia and Brunei for their cooperation and support, especially Prof. Datin Dr. Maryati Mohamed, Dr. Rosli bin Hashim, Dr. Kamariah Abu Salim, and members of Sabah Parks, namely Dr. Jamili Nais and Dr. Maklarin bin Lakim. Logistic support and help in many other ways by Prof. Dr. K. Eduard Linsenmair is gratefully acknowledged. We thank Dr. Ferry Slik and Prof. Dr. Ulrich Maschwitz for the kind supply of plant material, and Dr. Heike Feldhaar for fruitful discussions on ant–plant interactions. Two anonymous reviewers provided valuable suggestions.

References

Ayala FJ, Wetterer JK, Longino JT, Hartl DL (1996) Molecular phylogeny of *Azteca* ants (Hymenoptera: Formicidae) and the colonization of *Cecropia* trees. Mol Phylogenet Evol 5:423–428

Baier C, Guicking D, Prinz K, Fey-Wagner C, Wöhrmann T, Weising K, Debener T, Schie S, Blattner FR (2009) Isolation and characterization of eleven new microsatellite markers for *Macaranga* (Euphorbiaceae). Mol Ecol Resour 9:1049–1052

Bänfer G, Fiala B, Weising K (2004) AFLP analysis of phylogenetic relationships among myrmecophytic species of *Macaranga* (Euphorbiaceae) and their allies. Plant Syst Evol 248:213–231

Bänfer G, Moog U, Fiala B, Mohamed M, Weising K, Blattner FR (2006) A chloroplast genealogy of myrmecophytic *Macaranga* species (Euphorbiaceae) in southeast Asia reveals hybridization, vicariance and long distance dispersals. Mol Ecol 15:4409–4424

Blattner FR, Weising K, Bänfer G, Maschwitz U, Fiala B (2001) Molecular analysis of phylogenetic relationships among myrmecophytic *Macaranga* species (Euphorbiaceae). Mol Phylogenet Evol 19:331–344

Brouat C, Gielly L, McKey D (2001) Phylogenetic relationships in the genus *Leonardoxa* (Leguminosae: Caesalpinioideae) inferred from chloroplast *trn*L intron and *trn*L-*trn*F intergenic spacer sequences. Am J Bot 88:143–149

Cannon CH, Manos PS (2003) Phylogeography of the Southeast Asian stone oaks (*Lithocarpus*). J Biogeogr 30:211–226

Chenuil A, McKey DB (1996) Molecular phylogenetic study of a myrmecophyte symbiosis: did *Leonardoxa*/ant associations diversify via cospeciation? Mol Phylogenet Evol 6:270–286

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. Mol Ecol 9:1657–1660

Davidson DW, McKey D (1993) The evolutionary ecology of symbiotic ant-plant relationships. J Hym Res 2:13–83

Davies SJ (2001) Systematics of *Macaranga* sects *Pachystemon* and *Pruinosae* (Euphorbiaceae). Harv Paper Bot 6:371–448

Davies SJ, Lum SKY, Chan RKG, Wang LK (2001) Evolution of myrmecophytism in western Malesian *Macaranga* (Euphorbiaceae). Evolution 55:1542–1559

Dorstewitz W (2008) Populationsgenetik von Arten der Gattung *Macaranga*, Sektion *Pachystemon* in Südostasien. Diploma Thesis, University of Kassel, Germany

Elias M, Gompert Z, Jiggins C, Willmott K (2008) Mutualistic interactions drive ecological niche convergence in a diverse butterfly community. PLoS Biol 6(12):e300

Ennos RA, Sinclair WT, Hu XS, Langdon A (1999) Using organelle markers to elucidate the history, ecology and evolution of plant populations. In: Hollingsworth PM, Bateman RM, Gornall RJ (eds) Molecular systematics and plant evolution. Taylor & Francis, London, pp 1–19

Excoffier L, Laval G, Schneider S (2005) ARLEQUIN ver. 3.0: An integrated software package for population genetics data analysis. Evol Bioinformatics Online 1:47–50

Federle W, Maschwitz U, Fiala B, Riederer M, Hölldobler B (1997) Slippery ant-plants and skillful climbers: Selection and protection of the specific partner ants by epicuticular wax blooms in *Macaranga* (Euphorbiaceae). Oecologia 112:217–224

Federle W, Fiala B, Zizka G, Maschwitz U (2001) Incident daylight as orientation cure for hole-boring ants: prostomata in *Macaranga* ant-plants. Insect Soc 48:165–177

Feldhaar H, Fiala B, Gadau J, Mohamed M, Maschwitz U (2003a) Molecular phylogeny of *Crematogaster* subgenus *Decacrema* ants (Hymenoptera: Formicidae) and the colonization of *Macaranga* (Euphorbiaceae) trees. Mol Phylogenet Evol 27:441–452

Feldhaar H, Fiala B, Rosli BH, Maschwitz U (2003b) Patterns of the *Crematogaster-Macaranga* association: the ant partner makes the difference. Insect Soc 50:9–19

Fiala B, Maschwitz U (1991) Extrafloral nectaries in the genus *Macaranga* (Euphorbiaceae) in Malaysia: comparative studies of their possible significance as predispositions for myrmecophytism. Biol J Linn Soc 44:287–305

Fiala B, Maschwitz U (1992a) Domatia as most important preadaptations in the evolution of myrmecophytes in a paleotropical tree genus. Plant Syst Evol 180:53–64

Fiala B, Maschwitz U (1992b) Food bodies and their significance for obligate ant-association in the tree genus *Macaranga* (Euphorbiaceae). Bot J Linn Soc 110:61–75

Fiala B, Maschwitz U, Tho YP, Helbig AJ (1989) Studies of a South East Asian ant-plant association: protection of *Macaranga* trees by *Crematogaster borneensis*. Oecologia 79:463–470

Fiala B, Jakob A, Maschwitz U, Linsenmair KE (1999) Diversity, evolutionary specialization and geographic distribution of a mutualistic ant–plant complex: *Macaranga* and *Crematogaster* in South East Asia. Biol J Linn Soc 66:305–331

Guicking D, Tikam SR, Blattner FR, Weising K (2006) Microsatellite markers for the palaeotropic pioneer tree genus *Macaranga* (Euphorbiaceae) and their cross-species transferability. Mol Ecol Notes 6:245–248

Guicking D, Kröger-Kilian T, Weising K, Blattner FR (2008) Single nucleotide sequence analysis: a cost- and time-effective protocol for the analysis of microsatellite- and indel-rich chloroplast DNA regions. Mol Ecol Resour 8:62–65

Heckroth HP, Fiala B, Gullan PJ, Idris AHJ, Maschwitz U (1998) The soft scale (Coccidae) associates of Malaysian ant-plants. J Trop Ecol 14:427–443

Heil M, McKey D (2003) Protective ant-plant interactions as model systems in ecological and evolutionary research. Annu Rev Ecol Evol Syst 34:425–453

Heil M, Fiala B, Linsenmair KE, Zotz G, Menke P, Maschwitz U (1997) Food body production in *Macaranga triloba* (Euphorbiaceae): a plant investment in anti-herbivore defence via symbiotic ant partners. J Ecol 85:847–861

Heil M, Fiala B, Kaiser W, Linsenmair KE (1998) Chemical contents of *Macaranga* food bodies: physiological adaptations to their role in ant attraction and nutrition. Funct Ecol 12:117–122

Heil M, Fiala B, Boller T, Linsenmair KE (1999) Reduced chitinase activities in ant plants of the genus *Macaranga*. Naturwissenschaften 86:146–149

Heil M, Koch T, Hilpert A, Fiala B, Boland W, Linsenmair KE (2001a) Extrafloral nectar production of the ant-associated plant, *Macaranga tanarius*, is an induced, indirect, defense response elicited by jasmonic acid. Proc Natl Acad Sci USA 98:1083–1088

Heil M, Fiala B, Maschwitz U, Linsenmair KE (2001b) On the benefits of indirect defence: short- and long-term studies in antiherbivore protection via mutualistic ants. Oecologia 126: 394–403

Herre EA, Knowlton N, Mueller UG, Rehner SA (1999) The evolution of mutualisms: exploring the paths between conflict and cooperation. Trends Ecol Evol 14:49–53

Jakob SS, Blattner FR (2006) A chloroplast genealogy of *Hordeum* (Poaceae): long-term persisting haplotypes, incomplete lineage sorting, regional extinction, and the consequences for phylogenetic inference. Mol Biol Evol 23:1602–1612

Jolivet P (1996) Ants and plants: an example of coevolution (enlarged edition). Backhuys, Leiden

Kröger-Kilian T (2006) Vergleichende Untersuchungen zur genetischen Diversität und Populationsstruktur der Ameisenpflanzenarten *Macaranga hypoleuca* und *Macaranga beccariana* (Euphorbiaceae) in Südostasien. Diploma Thesis, University of Kassel, Germany

Kulju KKM, Sierra SEC, Draisma SGA, Samuel R, van Welzen PC (2007) Molecular phylogeny of *Macaranga*, *Mallotus*, and related genera. Am J Bot 94:1726–1743

Lewis T (1973) Thrips: their biology, ecology and economic importance. Academic, New York

Léotard G, Saltmarsh A, Kjellberg F, McKey D (2008) Mutualism, hybrid inviability and speciation in a tropical ant-plant. J Evol Biol 21:1133–1143

Maschwitz U, Fiala B, Davies SJ, Linsenmair KE (1996) A south-east Asian myrmecophyte with two alternative inhabitants: *Camponotus* or *Crematogaster* as partners of *Macaranga lamellata*. Ecotropica 2:29–40

Maschwitz U, Fiala B, Dumpert K (2004) An unusual myrmecophytic *Macaranga* association, occurring in a disjunct area in the monsoon zone of Southeast Asia: phenology and description of the ant species. Ecotropica 10:33–49

McKinnon GE, Vaillancourt RE, Jackson HD, Potts BM (2001) Chloroplast sharing in the Tasmanian eucalypts. Evolution 55:703–711

Moog U (2002) Die Reproduktion von *Macaranga* (Euphorbiaceae) in Südostasien: Bestäubung durch Thripse und Kastration durch Pflanzenameisen. PhD thesis, University of Frankfurt

Moog U, Fiala B, Federle W, Maschwitz U (2002) Thrips pollination of the dioecious ant plant *Macaranga hullettii* (Euphorbiaceae) in Southeast Asia. Am J Bot 89:50–59

Mound LA, Marullo R (1996) The thrips of Central and South America: an introduction. Mem Entomol Int 6:1–488

Nason JD, Herre EA, Hamrick JL (1998) The breeding structure of a tropical keystone plant resource. Nature 391:685–687

Nei M, Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76:5269–5273

Pierce NE (1987) The evolution and biogeography of associations between lycaenid butterflies and ants. Oxford Surv Evol Biol 4:89–116

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

Quek SP, Davies SJ, Itino T, Pierce NE (2004) Codiversification in an ant-plant mutualism: stem texture and the evolution of host use in *Crematogaster* (Formicidae: Myrmicinae) inhabitants of *Macaranga* (Euphorbiaceae). Evolution 58:554–570

Rundle HD, Nosil P (2005) Ecological speciation. Ecol Lett 8:336–352

Schaal BA, Hayworth DA, Olsen KM, Rauscher JT, Smith WA (1998) Phylogeographic studies in plants: problems and prospects. Mol Ecol 7:465–474

Schemske DW (2002) Ecological and evolutionary perspectives on the origins of tropical diversity. In: Chazdon RL, Whitmore TC (eds) Foundations of tropical forest biology. University of Chicago Press, Chicago, pp 163–173

Schluter D (2000) The ecology of adaptive radiation. Oxford University Press, Oxford

Seehausen O (2004) Hybridization and adaptive radiation. Trends Ecol Evol 19:198–207

Vogel M, Bänfer G, Moog U, Weising K (2003) Development and characterization of chloroplast microsatellite markers in *Macaranga* (Euphorbiaceae). Genome 46:845–857

Weising K, Gardner RG (1999) A set of conserved PCR primers for the analysis of simple sequence repeat polymorphisms in chloroplast genomes of dicotyledonous angiosperms. Genome 42:9–19
Whitmore TC (1975) *Macaranga*. In: Airy Shaw HK (ed) The Euphorbiaceae of Borneo. Kew Bull Add Ser 4:140–159
Whitmore TC (1982) *Macaranga* Thou. Kew Bull 36:312–323
Whitmore TC (1987) Biogeographical evolution of the Malay Archipelago. Clarendon, Oxford
Whitmore TC (2008) The genus *Macaranga* - a prodromus. Kew Publishing, Kew

Speciation in Obligately Plant-Associated *Crematogaster* Ants: Host Distribution Rather than Adaption Towards Specific Hosts Drives the Process

Heike Feldhaar, Jürgen Gadau, and Brigitte Fiala

Abstract Ecological interactions among organisms may be an essential factor facilitating speciation processes. In one of the most species-rich ant–plant symbiotic systems worldwide pioneer trees of the euphorb genus *Macaranga* are inhabited by specific partner ants, mostly of the genus *Crematogaster* subgenus *Decacrema*. Both groups underwent radiation, with 30 species of *Macaranga* being colonized by eight species of *Crematogaster*. In this obligate association, the ants rely solely on their host for nutrition and nesting space. Hosts are distributed patchily in disturbed sites or gaps in primary forest. Association patterns are non-random in spite of the often sympatric occurrence of several host-plant species. Generally, each ant species colonizes two to seven different host species over its whole distributional range. Speciation processes in the ants may thus be driven either by adaptation towards alternative host species or by spatial patterns of host distribution, or by both factors. Limited dispersal of queens and nest site limitation due to the obligate association with a host were found to lead to significant isolation by distance on a small spatial scale in primary forest. Extremely high intraspecific genetic variation of mitochondrial markers was in contrast to the low genetic variability of nuclear markers, also pointing towards small population sizes of the

H. Feldhaar (✉)
Behavioural Physiology and Sociobiology (Zoology II), Biocenter, Am Hubland, University of Würzburg, 97074 Würzburg, Germany
Behavioural Biology, University of Osnabrück, Barbarastr. 11, 49076 Osnabrück, Germany
e-mail: feldhaar@biologie.uni-osnabrueck.de

J. Gadau
Behavioural Physiology and Sociobiology (Zoology II), Biocenter, Am Hubland, University of Würzburg, 97074 Würzburg, Germany
School of Life Sciences, Arizona State University, PO Box 874501, Tempe, AZ 85287 - 4501, USA

B. Fiala
Animal Ecology and Tropical Biology (Zoology III), Biocenter, Am Hubland, University of Würzburg, Germany

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_10,

ants and the importance of genetic drift in the diversification processes. Adaptation towards alternative hosts may occur as a by-product when different *Macaranga* hosts are colonized in different regions.

1 Introduction

> If we are to gain insights into the nature of selective forces, it must come, I think, from a study of ecology. In particular, it must come from a study of the co-evolution of interacting species, because the main selective forces acting on a species are likely to come from changes in its competitors, its predators, and its parasites (Maynard Smith 1998) – and we may add "symbionts".

Mutually beneficial interactions between different organisms are widespread in nature, in spite of the presumed instability of such interactions. In theory, mutualisms should be unstable due to the possibility that cheaters may evolve that exploit the mutualism without providing a beneficial commodity to the partner organism. Over evolutionary time scales, conflict in mutualistic associations may be reduced by coupling the reproduction of the respective organisms, which is most effectively done by vertical transmission of a symbiont (Bronstein et al. 2006; Douglas 2008).

In ant–plant associations, the transmission of the partners is horizontal. Ant queens and plants reproduce and disperse independently and need to come together again in each generation. To prevent cheaters from exploiting such obligate ant–plant associations, it is assumed that the host plants have evolved characters that act as selective filters allowing only those ant species to access the plant that enhance the host's fitness (Bronstein et al. 2006; Davidson and McKey 1993). Thus, a co-evolutionary process may be triggered where mutualists will adapt to hosts, resulting in a high degree of phenotype matching between the ant and the plant host (Brouat et al. 2001; Davidson and McKey 1993; Davidson et al. 1989; Federle et al. 2001; Federle et al. 1997).

In one of the most species-rich ant–plant symbiotic associations worldwide, ca. 30 species of pioneer trees of the genus *Macaranga* (Euphorbiaceae) are inhabited by specific partner ants, mostly by eight species of ants of the genus *Crematogaster* (subgenus *Decacrema*) (Bänfer et al. 2004; Blattner et al. 2001; Davies et al. 2001; Feldhaar et al. 2003a; Fiala et al. 1999). In these mutualistic associations, the ants defend the trees against herbivores and vines (Federle et al. 2002; Fiala et al. 1994) in return for nesting space in hollow stems as well as food in the form of food-bodies (Fig. 1) (Fiala et al. 1989). All hosts associated with *Decacrema* species belong to the sections *Pachystemon* and *Pruinosa* within the genus *Macaranga* (Blattner et al. 2001; Davies et al. 2001). The associations of ants and plants are not strictly species-specific, however, as each of the eight species of *Crematogaster* (*Decacrema*) inhabits between two to seven different *Macaranga* hosts and – from the plants' point of view – each species of *Macaranga* obligately associated with *Crematogaster* (*Decacrema*) ants may be colonized by up to three

Fig. 1 *Crematogaster* (*Decacrema*) ants on *Macaranga*. (**a**) Workers of *C. captiosa* walking on self-made trails on the thinly wax-covered surface of *M. glandibracteolata*. (**b**) Queen of *C. decamera* gnawing an entrance hole into an internode of *M. hypoleuca*. (**c**) Workers of

different ant species (Feldhaar et al. 2003a; Fiala et al. 1999). Nonetheless, recurring association patterns between the two groups that are stable over a wide geographic range can be observed (Feldhaar et al. 2003b; Fiala et al. 1999). *Macaranga* hosts generally occur over a smaller geographic range in comparison to the ants, i.e., more endemic species are found, whereas four out of the nine species of *Crematogaster* (*Decacrema*) occur over the whole distributional range of this ant–plant association (Fiala et al. 1999). These widespread species of *Crematogaster* (*Decacrema*) often inhabit different species of *Macaranga* in different regions and – like the endemic ant species – may also colonize more than one host plant species in sympatry (Feldhaar et al. 2003a, b; Fiala et al. 1999). Myrmecophytism, the obligate association of host plants with permanent ant inhabitants has evolved independently not only in the sections *Pachystemon* and *Pruinosae* but in another group of *Macaranga*, the section *Winklerianae* (Blattner et al. 2001; Davies et al. 2001; Weising et al., this volume). The two myrmecophytic species within this group are both endemic to Borneo (*M. winkleri* and the rare *M. winkleriella*) and are also inhabited by a species of *Crematogaster* (*Crematogaster* species 8), that does not, however, belong to the subgenus *Decacrema* (Fiala et al. 1999).

The association is obligate for ants and plants alike. However, the ant partner relies more heavily on the plant partner, as the ants depend exclusively on the food provided directly by the plant (Fiala and Maschwitz 1992; Heil et al. 1997) or indirectly via scale insects within the domatia (Heckroth et al. 1998) and have to date never been found nesting or foraging away from their hosts. In contrast, saplings of host plants have to survive without the protection of their partner ants until they have reached a size where they grow internodes large enough for an ant queen to enter. Large *Macaranga* trees that have lost their partner ant due to the comparatively shorter life-span of the ant colony, will often also survive the lag in time until they are recolonized (Feldhaar et al. 2003b).

Speciation processes in the obligate *Crematogaster* (*Decacrema*) plant–ants may thus be driven by several different forces: On the one hand, the obligate association with their hosts may influence diversification processes in the ant partner as the plant–ants are constrained in their distribution by their hosts and cannot exist without them. Thus, the number of saplings of hosts available in a population imposes an upper limit to the population size of the ant species in the respective habitat, with each host being inhabited by a single colony (Feldhaar et al. 2003b; Fiala et al. 1999). On the other hand, the association with several different *Macaranga* hosts may drive diversification processes by specialization and adaptation towards alternative host species. Local adaptation may occur, especially when

Fig. 1 (continued) *C. captiosa* on *M. indistincta*. (**d**) Workers of *C. decamera* collecting food-bodies from the underside of a leaf of *M. hypuleuca*. (**e**) Workers of *C. captiosa* on *M. indistinca* (picture courtesy of S. Frohschammer). (**f**) Queen of *C. captiosa* with brood and workers in the stem of *M. glandibracteolata* (picture courtesy of K.E. Linsenmair). (**g**) Leaves of *M. indistinca* from an uncolonized plant (*a*) and a plant protected by ants (*b*) (picture courtesy of S. Frohschammer)

different host species are colonized in different regions. In addition, the ant–plant association is restricted to constantly wet tropical rainforest and has undergone repeated range expansions and reductions due to climatic changes during the Pleistocene (Quek et al. 2007).

2 Species Delimitation of *Crematogaster* (*Decacrema*) Ants Associated with *Macaranga*

A prerequisite to inferring speciation processes is to delimit species (Sites and Marshall 2003). To date, most of the eight *Macaranga*-associated *Crematogaster* (*Decacrema*) species have not been formally described, but have been assigned to morphospecies (msp.) (Fiala et al. 1999). The species can be distinguished based on queen morphology and life history characters such as the onset of reproduction or constancy of host-choice (Feldhaar et al. 2003b; Fiala et al. 1999). Two species, *Crematogaster* msp. 4 and *C*. msp. 6, can be synonymised with *C*. *captiosa* and *C*. *decamera* described by Forel (1910, 1911), respectively. Phylogenetic analysis of the *Decacrema*-species associated with *Macaranga* based on mitochondrial DNA (mtDNA) has mostly confirmed the morphospecies, but several mismatches between morphology and mtDNA haplotypes imply polyphyly of some morphospecies (Feldhaar et al. 2003a). In addition, genetic distances within morphospecies were much higher than expected, with pairwise distances exceeding 5% within *C*. *captiosa* or 7% within *C*. *decamera* (Feldhaar et al. 2003a). Such high within-species distances are unusual for most insect groups (but see Magnacca and Danforth 2007), but have been found for ants before, even when excluding the possibility of cryptic species or hybridization (Brandt et al. 2007). In order to distinguish between different processes leading to the observed pattern, such as introgression due to hybridization, presence of cryptic species, or elevated substitution rates in mitochondrial DNA, more taxa were added to our mtDNA phylogeny (86 taxa; Fig. 2), and a phylogenetic analysis of a 579-base-pair fragment of the nuclear gene elongation factor 1-α (EF1-α) was also conducted (parsimony network; Fig. 3). EF1-α is frequently used for genus level phylogenetic analysis of hymenoptera (Danforth et al. 1999; Degnan et al. 2004).

The enlarged phylogeny of *Crematogaster* (*Decacrema*) did not result in better resolution of the designated morphospecies (Fig. 2). Again, *C*. msp. 3 and *C*. msp. 7 were monophyletic, albeit with huge within-species variation (Table 1). *C*. *decamera* is paraphyletic with respect to *C*. msp. 9, which is a very rare and endemic species found only in Peninsular Malaysia (Feldhaar et al. 2003a; Fiala et al. 1999). All species belonging to the derived *captiosa*-group are polyphyletic.

In contrast to the mtDNA, tree topology of EF1-α was poorly resolved due to very low variation in this nuclear gene among *Crematogaster* (*Decacrema*) that inhabit *Macaranga* (Table 1). We therefore constructed a parsimony network (Fig. 3). *C*. *decamera* and *C*. msp. 9 form a distinct cluster that is separated from all other species by six steps. In contrast, *C*. msp. 3 that clusters with *C*. *decamera*

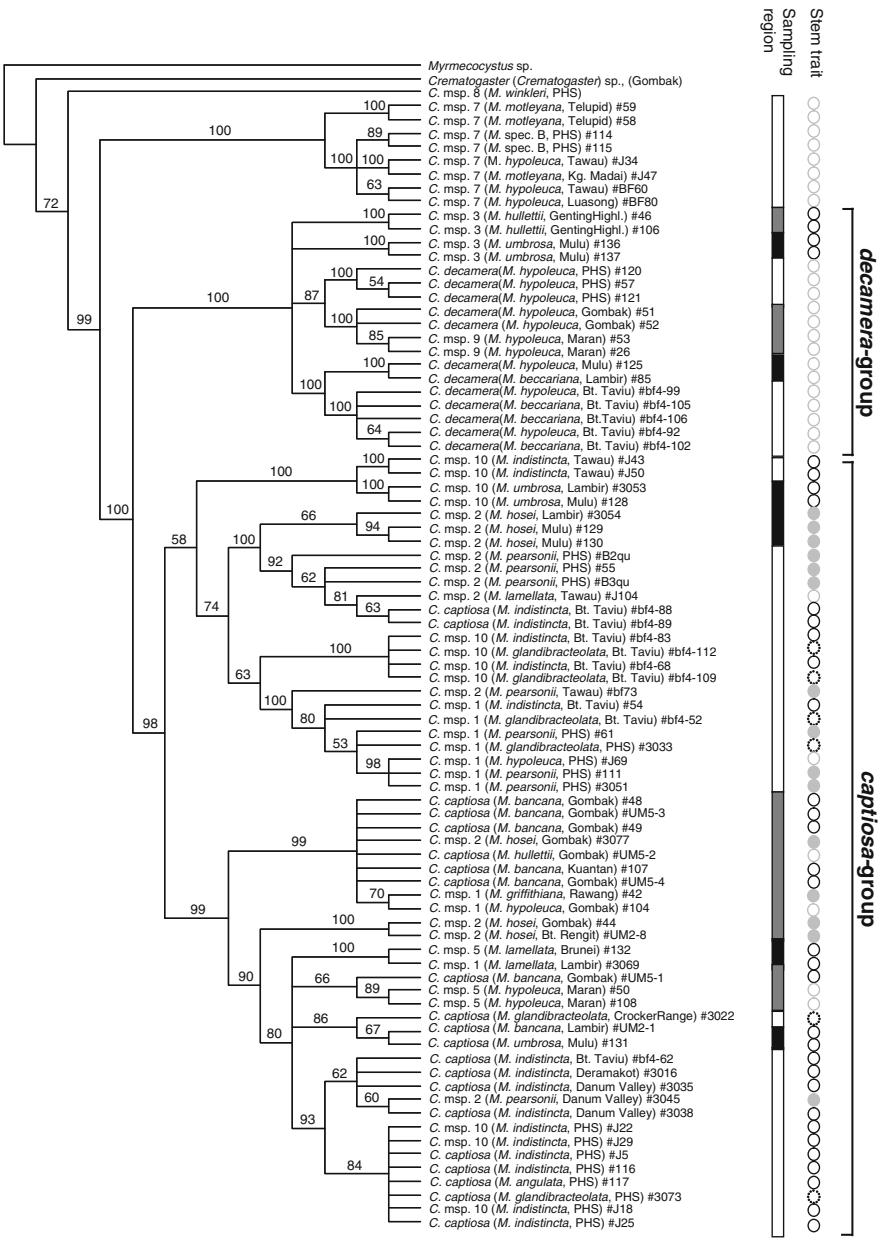


Fig. 2 Majority rule consensus tree (of 216 most parsimonious trees) obtained by maximum parsimony analysis of *Macaranga*-associated *Crematogaster* (*Decacrema*) ants based on 924 bp of mitochondrial DNA (484 bp of COI/ 440 bp of COII). Maximum likelihood analysis (PHyML version 2.4.4; Guindon and Gascuel, 2003) yielded similar tree-topologies. Bootstrap values $\geq$50 are given above nodes (maximum parsimony analysis, 1,000 pseudoreplications of heuristic search, with ten random-sequence addition replicates per pseudoreplicate and TBR as branch

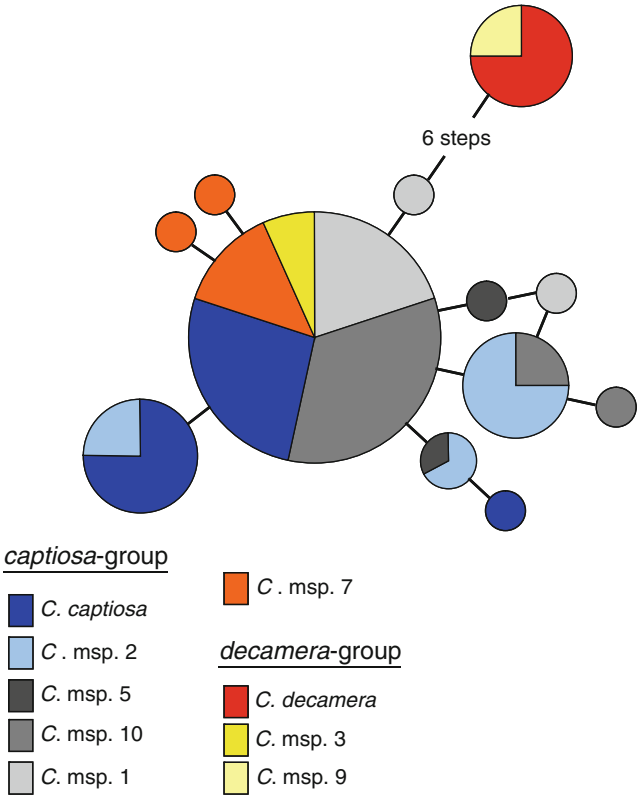


Fig. 3 Parsimony network of *Macaranga*-associated *Crematogaster* (*Decacrema*) ants based on 579 base pairs of the nuclear gene elongation factor-1α. Species are color-coded. Size of *circles* are proportional to number of sequences contained in this haplotype

and and *C*. msp. 7 that forms a clade well separated from all other *Decacrema* species in the mtDNA phylogeny are both included in the most common haplotype of the *captiosa*-group (Fig. 3). All haplotypes of the *captiosa*-group are separated from the most common haplotype by two steps at most and often comprise samples of two morphospecies. Interestingly, shared haplotypes of the *captiosa*-group comprise samples from both major clades of the group in the mtDNA phylogeny, e.g., the haplotype on the lower left of the haplotype network (Fig. 3) contains the

Fig. 2 (continued) swapping mode). Geographic regions that samples were collected in are represented by *bars* on the right of the taxa: *Gray bars* Peninsula Malaysia, *white bars* Sabah, *black bars* Sarawak and Brunei. Stem traits of the respective hosts the ants were collected from are represented by *circles*: *black circles* non–waxy surface, *gray circles* wax–covered, *dotted circle* only slight wax-cover; *open circles* pith degrades by itself, *filled circles* pith needs to be actively excavated by the ant partner

Table 1 Comparison of within-species corrected pairwise-distances (Kimura-2 parameter) of a 924-base-pair fragment of Cytochromeoxidase 1 (COI: 484 bp)/Cytochromeoxidase 2 (COII: 440 bp) and a 576-base-pair fragment of the nuclear gene elongation-factor 1α. Among the *Crematogaster* species (subgenus *Decacrema*) queens of the *decamera*-group are smaller than queens of the *captiosa*-group. *Crematogaster* msp. 8 that does not belong to this subgenus has larger queens

Species	Max. pairwise distances (%)		Region	Status of species	High pairwise distance (mtDNA) due to
	COI/COII	EF-1α			
C. msp. 7	14 (10)	0.51 (6)	Sabah	Monophyletic	Accelerated substitution rates
decamera-group					
C. decamera	8.5 (16)	0.0 (5)	PM, Sabah	Paraphyletic	Accelerated substitution rates
C. msp. 3	5.4 (4)	–	PM, Sarawak	Monophyletic	Accelerated substitution rates
captiosa-group					
C. msp. 1	10 (10)	0.17 (6)	PM, Sabah	Polyphyletic	Hybridization/ancient haplotypes/accelerated substitution rates
C. msp. 2	9 (10)	0.3 (9)	PM, Sabah, Sarawak	Polyphyletic	Hybridization/ancient haplotypes/accelerated substitution rates
C. captiosa	8 (25)	0.5 (11)	PM, Sabah	Polyphyletic	Hybridization/ancient haplotypes/accelerated substitution rates
C. msp. 10	8 (8)	0.0 (8)	Sabah	Polyphyletic	Hybridization/ancient haplotypes/accelerated substitution rates
non-*Decacrema*					
C. msp. 8	2 (136)	0.0 (2)	Sabah, Brunei, Sarawak	Monophyletic	

samples #J5 and #bf4-62 of *C. captiosa* (lower clade of *captiosa*-group in Fig. 2) as well as #3054 of *C. msp. 2* (upper clade in Fig. 2, respectively).

As a third method aside from mitochondrial and nuclear sequence analysis, a Bayesian clustering method as implemented in STRUCTURE 2.2 (Pritchard et al. 2000) was used to test whether assignment of individuals to species within the *captiosa*-group based on morphology was congruent with the assignment based on multilocus microsatellite genotypes. Queens from small colonies of all species belonging to the *captiosa*-group (*C*. msp. 1, *C*. msp. 2, *C. captiosa*, and *C*. msp. 10, that is endemic to southeast Borneo) were analyzed using five highly polymorphic microsatellite loci that were developed for *C*. msp. 2 (Feldhaar et al. 2004). At least four loci amplified in each species and none was fixed for a single allele in any species. The assignment test confirmed our morphospecies concept unambiguously, as most specimens were assigned to the "right" species with high posterior probability (>95%) when using species affiliation based on queen morphology as prior

population information (Fig. 4). However, about 2% of the individuals of *C. captiosa* and *C.* msp. 10 studied show mismatches between phenotype and multilocus-genotype. Individuals from these two species also share mtDNA haplotypes at one sampling area (Poring Hot Spring, Sabah), strongly suggesting ongoing hybridization (Fig. 4, individuals marked with an arrow) (Feldhaar et al. 2008). These hybrids may still be fertile, as the genotyped individuals were queens that had already produced a number of workers. Interestingly, evidence for hybridization can be found mostly in ants that colonize the same *Macaranga*-species in a given habitat (e.g., *C. captiosa* and *C.* msp. 10 colonize *M. indistincta* and *M. glandibracteolata* in Poring Hot Spring).

In conclusion, the obligately *Macaranga*-associated species of the *Crematogaster* (*Decacrema*) complex can be delimited only when using a combination of microsatellite markers and morphology. Polyphyly of species within the *captiosa*-group at the mtDNA-level and low resolution of the nuclear gene EF1-α significantly hampers the use of such data for this purpose. The problem may be circumvented by only using phylotypes that are not linked to a species concept (Quek et al. 2004, 2007), which, though, will not uncover processes such as hybridization in these ants. High within-species genetic variation and polyphyly of species in the mtDNA dataset can be explained, at least in part, by ongoing hybridization processes. Other mismatches of phenotype and mtDNA haplotype may be due to past hybridization events or shared ancestral haplotypes. Another reason for the high sequence divergence found within species that form a monophyletic cluster may be accelerated substitution rates in mtDNA in contrast to the nuclear gene (Magnacca and Danforth 2007). For DNA-based approaches of species identification or delimitation, such as DNA barcoding or DNA taxonomy, that rely solely on mtDNA the *Crematogaster* (*Decacrema*) ants

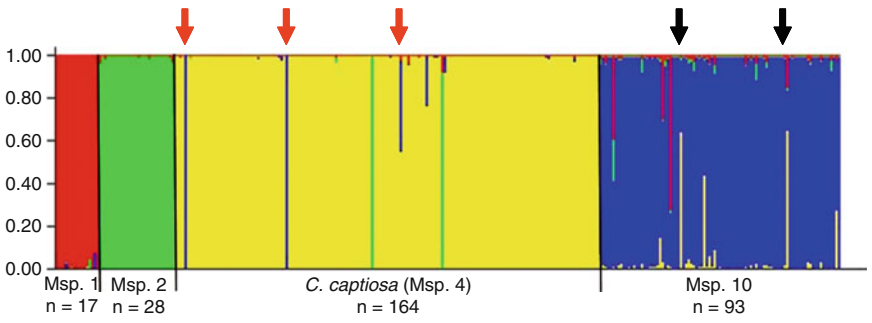


Fig. 4 Species delimitation and identification of hybrids (marked with *red* and *black arrows*) within the *captiosa*-group using the assignment program Structure 2.2 (Pritchard et al. 2000) based on five microsatellite loci. Samples were all collected in Sabah (northeast Borneo). Morphological data of the queens was used as a prior for the assignment of species. To allow for hybridization among species, the admixture model was used. Hybrids were identified by mismatches in morphology and genotype. The *arrows* mark individuals from *C.* msp. 10 and *C. captiosa* that share the same mitochondrial DNA haplotype. Individuals are represented as *vertical lines* with the coloration of the line indicating assignment probability towards the cluster with the respective color

would be very challenging. Like other groups with high intraspecific genetic variability on the mtDNA-level and in part very low interspecific variability due to introgression, species assignment will often fail (e.g., Meier et al. 2006). The number of species within the genetically highly diverse *decamera*-group and *C*. msp. 7 would be overestimated, whereas the number of lineages found in the *captiosa*-group may not be altered dramatically. In the latter, however, species may often be assigned wrongly due to shared haplotypes among species.

3 Co-evolution Between *Crematogaster* (*Decacrema*) and *Macaranga* Hosts: Adaptations of the Ants Towards Their Hosts

The obligate and exclusive association of *Crematogaster* (*Decacrema*) ants with their *Macaranga* hosts suggests that diversification of the ants may have been driven by a co-evolutionary process. A comparison of the phylogenies of the *Crematogaster* ants with their plant host showed that phylogenies are not congruent, pointing towards frequent host switches of the ants (Feldhaar et al. 2003a). Strict co-evolution between pairs of ants and plants is anyway unlikely, since all *Decacrema* species colonize more than one host, and even host species from different clades of *Macaranga* where myrmecophytism, the obligate association with ants, has evolved independently (Blattner et al. 2001; Davies et al. 2001; Feldhaar et al. 2003a).

Nonetheless, when ant species colonize more than one host species, a non-random subset of *Macaranga* species is chosen that matches morphological characters of the ants and life-history traits, suggesting adaptation of the ants towards their hosts (Fig. 2; Feldhaar et al. 2003b; Quek et al. 2004). We hypothesized that the acquisition of ant symbionts enabled the *Macaranga* trees to spread into new habitats and subsequently to diversify due to this ant-mediated habitat extension. The ant symbionts could then have radiated on the background of this increasing species diversity of their plant partners. Host recognition is a prerequisite to specialization and adaptation towards suitable hosts and enabling ants to return to the same species or suit of species from generation to generation. The most important characters of the hosts exerting selection pressures on the ants are expected to be the quantity and quality of food and nesting space provided by the plant, since the obligate plant-ants are entirely dependent on their host.

3.1 Choosing and Finding a Host

Choice experiments showed that foundresses are able to distinguish between different *Macaranga* species based on chemical (Inui et al. 2001; Jürgens et al. 2006), and possibly also tactile (Jürgens et al. 2006), cues, and that they prefer their

natural host plants. We hypothesized that imprinting on the natal host may be a possible mechanism to enhance host recognition of queens (Davis and Stamps 2004). However, queens of *C. captiosa* and *C.* msp. 10 raised on *M. indistincta* showed no preference for this host when given the choice between this species and their second potential host *M. glandibracteolata* in Sabah (northeast Borneo) (Feldhaar and Fiala, unpublished results).

Chemical cues of the hosts may also play a role for mating of these specialized plant-ants. Unlike other ants, mating flights in these *Crematogaster* (*Decacrema*) species are not synchronized and foundresses can be found all year round (Feldhaar et al. 2005). Thus, attraction towards mating partners may be enhanced by the hosts if mating takes place in vicinity of the hosts, which is not yet known. If this was the case, it may also explain hybridization between species that colonize the same *Macaranga* species in a given habitat (see above).

3.2 Wax Running

Macaranga hosts can be subdivided into plants whose stems are covered with a wax-bloom and non-waxy hosts, with the exception of a few species that have only a slight wax-cover (*M. glandibracteolata,* Fig. 1a) or produce wax-blooms only as large trees (*M. indistincta*). Only "wax runners" (*C.* msp. 1, *C.* msp. 2, *C. decamera*, *C.* msp. 7 and *C.* msp. 9) are able to move on such waxy plant surfaces, whereas the other species will drop off the plant or can only walk very slowly on such host stems (Federle et al. 1997). The wax running capacity of the ants is based on a combination of morphological (longer legs), locomotory and behavioral adaptations (Bruening and Federle 2005).

3.3 Entering the Host: Queen Size Matters

Size of the queen seems to matter with respect to strength of mandibles and the ability to gain entrance to the hosts. Both small queens of the *decamera*-group and large queens of the *captiosa*-group can easily enter saplings of their respective host species. However, only the larger queens of the *captiosa*-group were found to enter branches of abandoned trees, whose tissue is much thicker and harder in comparison to saplings. We found a positive correlation between hardness of tissue and time needed to gnaw entrance holes into the stem. Queens of the *decamera* group did not succeed in producing a hole at all (Feldhaar and Fiala, unpublished results). Size of the queen and strength of the mandibles will similarly matter when the domatia of the host plants have to be excavated by the ants, as is the case in several host species of the section *Pruinosae* such as *M. hosei* and *M. pearsonii* (see Weising et al., this volume; Quek et al. 2004). Thus, *C.* msp. 2, the largest species among the *Decacrema* on *Macaranga*, is highly specialized on this group of hosts (Fig. 2).

3.4 Available Food and Nesting Space

Characteristics of *Macaranga* hosts related to the amount and quality of food as well as nesting space should be most important for the ants and may thus exert strong selection pressures. For *M. bancana*, an estimated 5% of the biomass produced by the plant is invested into the production of food-bodies (Heil et al. 1997). Worker density of ant colonies of *C. captiosa* on *M. bancana* correlated positively with the amount of food offered by the host plant (Heil et al. 2001). Production of food-bodies was in turn dependent upon nutrient availability to the plant. Ants of the *decamera*-group and *C.* msp. 7 have smaller queens and workers than ants of the *captiosa*-group, and colonies of the former group start reproduction at a much smaller colony size, when colonies comprise only approximately 500 workers. In comparison, ants of the *captiosa*-group produce reproductive offspring when colony size exceeds 3,000 workers (Feldhaar et al. 2003b). Food limitation may thus play a stronger role in ants belonging to the *captiosa*-group than the *decamera*-group. Species with early onset of reproduction would potentially be able to reach the reproductive stage in all hosts species. Species of the *captiosa*-group may have a competitive disadvantage compared to smaller species in slow growing and less productive plants in primary forest and are sometime replaced by them (Feldhaar and Fiala, personal observations).

Differences in availability of food and nesting space may be only minor among the suite of *Macaranga* hosts colonized by a particular ant species. The amount and quality of food produced by e.g., *M. indistincta* and *M. glandibracteolata* did not differ significantly, measured as the number of food-bodies produced by the plant and their amino acid content. We found no significant differences in density of workers or mortality of queens in colonies of either *C. captiosa* or *C.* msp. 10 on these hosts (Feldhaar and Fiala, unpublished results). However, mismatches in food availability of plants and needs of the ant colony do sometimes occur, and may lead to conflicts in the associations. *C. captiosa* was observed to sometimes castrate flowers of *M. hullettii* but not of *M. bancana* within the same area (Moog 2002). When *Macaranga* hosts flower, the stipules producing the food-bodies are abscised by the plant and the ants will face a period of low food availability until the stipules re-grow after the flowering period. Thus, flowers may be destroyed to ensure constant availability of food. Interestingly, the ants did only show castration behavior on relatively small plants and when they had not yet reached the reproductive stage (Moog 2002).

3.5 Are All Ant Partners Equal? – The Plant Perspective

Apart from sometimes being castrated by the specific partner ants, hosts benefit from the *Crematogaster* (*Decacrema*) colonies. However, from the host's point of view, those ants should be favored that maximize the cost-benefit ratio, e.g., when

smaller bodied ants with lower density of workers provide equal services to larger ants. Strength of pruning behavior – the clipping of overgrowing vegetation by workers – differs between the *Decacrema* species. This behavior on the one hand keeps competing ants off the plant and on the other hand is important for the plant in the competition for light. Generally "wax runners" prune less in comparison to non-wax runners, presumably due to the fact that the former face fewer ant competitors on their hosts since only a few species are able to walk on such waxy surfaces (Federle et al. 2002). Colonies of *C. captiosa* and *C.* msp. 10 were found to prune vines less efficiently on the thinly wax-covered *M. glandibracteolata* than on *M. indistincta*, in spite of colonies having the same density of workers. However, *C.* msp. 10 was still significantly more active on the surface of the first host species than *C. captiosa* and is therefore the more desirable partner ant. However, to date, no real "cheater" is known for the *Crematogaster–Macaranga* association that reaps all the benefits from its host without paying the costs. Due to the monophyly of the *Crematogaster* (*Decacrema*), differences between ant partners may be less pronounced in comparison to the more diverse ant partners inhabiting other myrmecophytes that often comprise ants from different genera (Davidson and McKey 1993; Dejean et al. 2004; Frederickson 2005; Heil and McKey 2003; Yu and Davidson 1997).

4 Radiation in *Crematogaster* (*Decacrema*) Ants: Is It Driven By Adaptation Towards Different Host Species?

4.1 Population Genetic Studies on A Local Scale

The obligate association of the *Crematogaster* (*Decacrema*) ants with their *Macaranga* hosts may be a double-edged sword. On the one hand, the specialized ants benefit from a relatively competition-free space since non-specialized ants are not able to utilize the nesting space and food offered (Federle and Rheindt 2005) unless the host has been abandoned by a colony due to death of the queen (Feldhaar et al. 2003b). On the other hand, *Crematogaster* (*Decacrema*) species are now restricted by their hosts in distribution and abundance since they can no longer nest independently away from their hosts. The number of suitable hosts present in a given area therefore sets the upper limit of the population size of the ants. In gaps of primary forest, one may find only a handful of saplings to several dozen at a time (Feldhaar, personal observation), whereas the number of hosts may be much larger in cleared areas. Speciation processes in the *Crematogaster* (*Decacrema*) ants may thus be driven either by adaptation towards different host species, as all *Decacrema* species colonize more than one host species, or by the spatial distribution of their hosts.

If host plants exert strong selection pressures on their partner ants, forcing them to specialize on a particular host, then this should be reflected in their genetic population structure, i.e., ants may form host races. This hypothesis was tested in

two populations (Poring Hot Spring and Kampung Monggis) in secondary forest habitat in Sabah, northeast Borneo (approximately 35 km linear distance), where *C. captiosa* colonizes both *M. indistincta* (population 1: $n=51$ founding queens; population 2: $n=9$, respectively) and *M. glandibracteolata* (population 1: $n=37$ founding queens; population 2: $n=13$) in sympatry. We expected that if *C. captiosa* forms host races, then genetic variation found within each habitat among queens collected from the different hosts should exceed variation between habitats on the same host. Genetic differentiation between populations was studied by two three-level hierarchical analyses of molecular variance (AMOVA) based on five microsatellite loci using the program ARLEQUIN 3.11 (Excoffier et al. 2005). Two nesting orders were used: individuals in hosts in sites and individuals in sites in hosts. Genetic differentiation was significant within populations (among individuals) in both hierarchical orders (97.1 and 95.8% of respective genetic variance). Variation between sites was also significant in both hierarchical orders (3.4 and 2%), whereas differentiation among hosts could not be detected (−0.5 and 2.01%). Pairwise comparisons of F_{ST} of the four populations (*C. captiosa* separated by site and host within sites) showed that differentiation between *C. captiosa* collected from *M. glandibracteolata* in Kampung Monggis towards all other populations was mostly responsible for the genetic differentiation detected (range of F_{ST} from 0.044 to 0.079 in contrast to all other comparisons which ranged from 0.012 to 0.017).

It was striking, however, that when the population of *C. captiosa* from secondary forest in Poring Hot Spring from both hosts was compared to a small population within primary forest at higher elevation, which inhabits a third host, *M. angulata*, genetic differentiation was significantly higher (pairwise F_{ST} of 0.12 to *C. captiosa* from *M. indistincta* and 0.1 from *M. glandibracteolata*). This third population comprises only few *M. angulata* hosts along the edge of a stream. Of the estimated 50 hosts present, only a minority was colonized by *C. captiosa* whereas most were colonized by *C.* msp. 7. The strong differentiation may not be the result of adaptation towards the host, however, but rather due to genetic drift in an isolated small population. However, populations of hosts restricted to a gap or a river edge such as that of *M. angulata* may represent the natural population structure of most *Macaranga* species (Whitmore 1969). Due solely to recent anthropogenic destruction of the forest, several species of *Macaranga* hosts have become quite common trees in cleared areas, with *M. pearsonii, M. hypoleuca*, *M. indistincta,* and *M.bancana* sometimes dominating stands in disturbed habitats (Fiala and Feldhaar, personal observation). Thus, nowadays, the population structure of the obligate plant ants may be strongly influenced by such anthropogenic disturbances and may not reflect natural conditions. We therefore conducted a second population genetic analysis in a primary forest habitat (Danum Valley) in Sabah, northeast Borneo.

To exclude potential influence on population structure due to colonization of and potentially adaptation towards different hosts, we chose *C. decamera*, which only inhabits *M. hypoleuca* in the respective area. In total, 190 founding queens or queens of incipient colonies were collected from their host *M. hypoleuca* from an area of ca. $1{,}500 \times 2{,}000$ m that is situated on the edge of the primary forest and is

partitioned by a few trails. Foundress queens were collected from 28 naturally occurring gaps due to treefalls, with each gap containing between 2 and 46 saplings (mean ± standard deviation 4.5 ± 8.9). In addition, 24 queens were collected in adjoining secondary forest less than 1 km distant from the main study site. Over all samples from gaps containing more than five individual queens (maximum distance approximately 3.5 km), we found a slight but significant isolation by distance (Fig. 5) (following Rousset 2000), i.e., an increase in genetic distance (based on five microsatellite loci) was correlated with increasing geographic distance. We estimated dispersal distance of foundresses by assigning them to their natal colony based on their multilocus genotypes. The 22 foundresses (out of 190) that could be unambiguously assigned had a mean dispersal distance of 475 m from their natal colony (range, 1–900 m). Among foundresses, full-sisters were more likely to occur at shorter distances (up to 400 m distance between them) than in saplings that were further apart. In addition, colonization rate of uninhabited saplings in gaps was negatively correlated with the distance to the next potential mature colony releasing sexuals. All colonization events observed ($n = 39$) were on saplings that were less than 1 km from all potential colonies with reproductives (Feldhaar, Türke and Fiala, unpublished results).

These results suggest that queens only rarely disperse over more than a few kilometres, although longer distance dispersal occurs as queens are able to find and

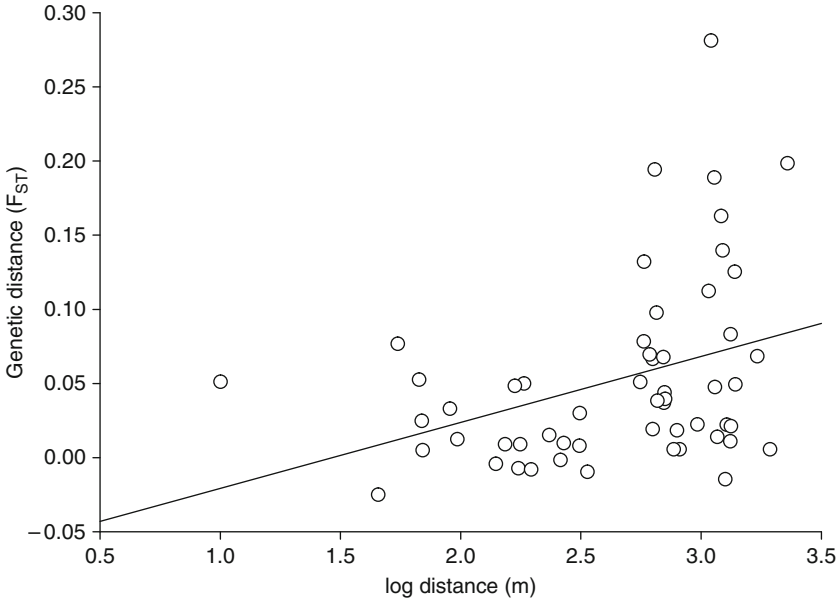


Fig. 5 Small–scale genetic structure of *Crematogaster decamera* based on five microsatellite loci. Gaps containing five or more foundress queens were included in the analysis. Foundresses were collected in gaps in a primary forest habitat and an adjoining patch of secondary forest

colonize isolated plants within primary forest. These findings may be explained either by limited dispersal abilities of the queens and high mortality with increasing dispersal distance or by philopatry. Queens may benefit from not dispersing too far from their natal nest, as the survival of their particular host plant up to the reproductive stage of the inhabiting ants should be a good indicator that the local habitat is suitable for their host plants. Therefore, foundress queens may first search for a suitable sapling in the vicinity of their natal nest and only in the case that they do not find any are forced to fly further. That sapling availability is very limited is obvious from the fact that most saplings are colonized by several queens before one colony finally occupies the plant alone (Fiala and Feldhaar, personal observations). However, since inbreeding in ants with complementary sex determination may incur considerable costs due to the production of diploid males (Ratnieks 1990), males may be forced to disperse further away from their natal nest, which could be achieved by active flying or possibly wind drift. This problem may be enhanced in small populations. For the males, mortality risk during dispersal may be even higher due to their smaller body size, but for the same reason they are less costly for the colony to produce.

Limited dispersal or philopatry by queens in contrast to higher levels of gene-flow among populations due to male dispersal would also explain the observed pattern of strongly diverged mtDNA in comparison to limited differentiation in nuclear markers (microsatellites and EF-1α). If both sexes stay close to their natal nest for mating and colony founding, our expectations would be that local population substructure should translate into a larger number of (cryptic) ant species in comparison to their hosts – and not eight species of *Crematogaster* (*Decacrema*) colonizing approximately 30 *Macaranga* host species.

4.2 Vicariant Evolution – The Broader Geographic Scale

The deepest genetic splits within ant species are most often not correlated with changes in host association, but with geographic barriers similar to those of their *Macaranga* hosts (Weising et al., this volume; Bänfer et al. 2006). We found strong differentiation between populations separated by the South China Sea (Peninsula Malaysia vs Borneo) and, within Borneo, the Crocker Range separated the north-eastern populations (Sabah) from the western populations (Brunei and Sarawak) – a biogeographic pattern that is consistent with the pattern found by Quek and co-workers (Quek et al. 2007). Individuals from the larger geographic regions rarely clustered together (Fig. 2). Divergence of populations on a large geographic scale is also supported by a higher genetic divergence at microsatellite loci (results not shown). Diversity of mtDNA lineages was found to be highest in *Crematogaster* (*Decacrema*) ants in mountain ranges by Quek et al. (2007), especially the Crocker Range. Mountain ranges have been rainforest refugia during the drier parts of the Pleistocene and thus harbor a large number of endemic plants, including

myrmecophytic *Macaranga* species (Ashton 2003; Bänfer et al. 2006). In addition to a larger number of potential hosts, spatial heterogeneity should be higher in primary forest from lowland to higher altitudes, and may facilitate the coexistence of more lineages (Yu et al. 2001).

5 Conclusion and Outlook

Speciation processes in the *Crematogaster* (*Decacrema*) ants can only be inferred when utilizing several different genetic markers in conjunction with morphological data as well as field experiments and observations. In contrast to our expectations when we set out on this project, speciation in the *Crematogaster* (*Decacrema*) plant-ants does not seem to be driven by adaptation towards specific hosts. When *Macaranga* hosts diversified initially, selection pressure exerted by certain host-types on morphological and life-history characters may have been stronger for the *Decacrema* ants, e.g., for smaller and larger queens as well as ants that are able to utilize hosts with a waxy stem surface. Once these "types" of ant-partners had evolved, adaptation towards the often rather similar hosts of the same taxonomic section (or clade) may have played a minor role and limited dispersal of the ant partner may have become more important in driving diversification processes. The *Crematogaster* (*Decacrema*) ants were found to be genetically structured on small spatial scales, as reflected by the studies on population structure in primary forest. Subpopulations in primary forest gaps have very small population sizes (often below 50 individuals), and hosts are often widely scattered. The strong genetic differentiation at the mtDNA-level supports the notion that population sizes may be small, as the likelihood of DNA substitutions becoming fixed due to genetic drift is much greater than in larger populations. In addition, hybridization among ant species has been shown to often occur when homospecific mating partners are scarce for queens and population sizes are small, such as in social parasites (Feldhaar et al. 2008). On a regional scale, however, this substructure did not translate into strong differentiation in nuclear markers.

In previous studies (Fiala et al. 1999) and again here, we found that all *Crematogaster* (*Decacrema*) species colonize several different host species, albeit with similar ant-related traits (such as nesting space and food production or wax-cover of stems; see also Quek et al. 2004, 2007). Such a suite of hosts is unlikely to exert selection on their ant partners strong enough to specialize further, since fitness-relevant characters such as food and nesting space provided do not differ strongly among those hosts. The ability to recognize and colonize more than one host may be beneficial to the ants even when hosts do differ to a certain extent. Fitness lost by switching to a suboptimal host (e.g., one providing less food) that may be colonized when the "right" host is not available incurs only relatively little cost in comparison to the total loss of fitness when queens are not able to found a colony at all. From the plant's point of view, being too choosy does

not pay for the same reason: if partner ants do not differ too much in the cost-benefit ratio for their host, fitness gains would be small in comparison to the risk of not being colonized when trying to exclude too many *Decacrema* ant species. The spatial heterogeneity of distributions of hosts and ants alike may therefore limit further specialization on the ant and the plant side, but may also facilitate species coexistence in this ant–plant association (Yu et al. 2001).

Whereas *Macaranga* hosts can be dispersed over medium to long distances by birds or small mammals (Weising et al., this volume; Moog et al. 2002) and may thus reach remote habitats, the *Crematogaster* queens will not usually be able to "follow" the plant from the same source over longer distances. The hosts may be colonized by ant queens of the respective species from source populations close to the new habitat of the plant, or ants colonizing different hosts may switch to the new host. This may happen primarily in populations where ants face high intra-specific competition though, since queens otherwise show high levels of host constancy.

The host populations and subsequently the populations of the ant partners may have been altered strongly in the past two decades due to human disturbance of forests. We cannot exclude that this disturbance has already had an impact on the association patterns and local genetic diversity found in this study and others on this system (Feldhaar et al. 2003a; Quek et al. 2004, 2007). When fragmentation began, cleared patches may have first facilitated a rise in abundance of the pioneer *Macaranga* plants, enhancing gene flow among ant populations. With progressive conversion of primary or secondary forest into large areas of agricultural land or oil palm plantation, stands of *Macaranga* are now often separated by many kilometres and may thus strongly restrict geneflow among ant populations or result in the loss of source populations of foundresses. A comparative study in the future may therefore yield interesting results on the dynamics of this ant–plant association, e.g., we would expect host shifts to occur more often and to find a more pronounced genetic substructure of the *Crematogaster* (*Decacrema*) ants on regional scale if ant populations become more isolated.

Acknowledgments We are very grateful to David Nash and two anonymous reviewers for comments on an earlier version of this manuscript. This work was supported by Deutsche Forschungsgemeinschaft (grants Ga661/2-1, Fe631/1-1, Fe631/1-2 and Fi606/5) within priority program SPP 1127 "Adaptive Radiations". Permission to conduct research in Malaysia was kindly granted by the Economic Planning Unit (EPU) of the Prime Minister´s Office, Kuala Lumpur, and EPU in Kota Kinabalu, Sabah, as well as the Danum Valley Management Committee. Our permit to work in Brunei was obtained by Universiti Brunei Darussalam and Brunei Museum. We thank our counterparts and colleagues in Malaysia and Brunei for their cooperation and support, especially Prof. Datin Dr. Maryati Mohamed, Dr. Rosli bin Hashim, Dr. Kamariah Abu Salim, and members of Sabah Parks, namely Dr. Jamili Nais and Dr. Maklarin bin Lakim. Logistic support and help in many other ways by Prof. Dr. K. Eduard Linsenmair is gratefully acknowledged. We thank Prof. Dr. Ulrich Maschwitz for the kind supply of ant material. Barbara Feldmeyer, Sabine Frohschammer and Manfred Türke contributed greatly to field work. We are grateful for support in sampling and especially discussing speciation processes in this ant–plant association to Daniela Guicking, Frank Blattner, Kurt Weising, Ute Moog and Christina Baier from the accompanying *Macaranga* project.

References

Ashton PS (2003) Floristic zonation of tree communities on wet tropical mountains revisited. Perspect Plant Ecol Evol Syst 6(1):87–104

Bänfer G, Fiala B, Weising K (2004) AFLP analysis of phylogenetic relationships among myrmecophytic species of *Macaranga* (Euphorbiaceae) and their allies. Plant Syst Evol 249:213–231

Bänfer G, Moog U, Fiala B, Mohamed M, Weising K, Blattner FR (2006) A chloroplast genealogy of myrmecophytic *Macaranga* species (Euphorbiaceae) in Southeast Asia reveals hybridization, vicariance and long-distance dispersals. Mol Ecol 15:4409–4424

Blattner FR, Weising K, Banfer G, Maschwitz U, Fiala B (2001) Molecular analysis of phylogenetic relationships among myrmecophytic *Macaranga* species (Euphorbiaceae). Mol Phylogenet Evol 19:331–344

Brandt M, Fischer-Blass B, Heinze J, Foitzik S (2007) Population structure and the co-evolution between social parasites and their hosts. Mol Ecol 16:2063–2078

Bronstein JL, Alarcon R, Geber M (2006) The evolution of plant–insect mutualisms. New Phytol 172:412–428

Brouat C, Garcia N, Andary C, McKey D (2001) Plant lock and ant key: pairwise coevolution of an exclusion filter in an ant–plant mutualism. Proc R Soc Lond B 268:2131–2141

Bruening T, Federle W (2005) Biomechanics of 'waxrunning' in *Crematogaster* ant–partners of *Macaranga* trees. Comp Biochem Physiol A Comp Physiol 141:S153

Danforth BN, Sauquet H, Packer L (1999) Phylogeny of the bee genus *Halictus* (Hymenoptera: Halictidae) based on parsimony and likelihood analyses of nuclear EF-1 alpha sequence data. Mol Phylogenet Evol 13:605–618

Davidson DW, McKey D (1993) The evolutionary ecology of symbiotic ant–plant relationships. J Hym Res 2:13–83

Davidson DW, Snelling RR, Longino JT (1989) Competition among ants for myrmecophytes and the significance of plant trichomes. Biotropica 21(1):64–73

Davies SJ, Lum SKY, Chan R, Wang LK (2001) Evolution of myrmecophytism in western Malesian *Macaranga* (Euphorbiaceae). Evolution 55:1542–1559

Davis JM, Stamps JA (2004) The effect of natal experience on habitat preferences. Trends Ecol Evol 19:411–416

Degnan PH, Lazarus AB, Brock CD, Wernegreen JJ (2004) Host-symbiont stability and fast evolutionary rates in an ant-bacterium association: cospeciation of *Camponotus* species and their endosymbionts, *Candidatus* Blochmannia. Syst Biol 53:95–110

Dejean A, Quilichini A, Delabie JHC, Orivel J, Corbara B, Gibernau M (2004) Influence of its associated ant species on the life history of the myrmecophyte *Cordia nodosa* in French Guiana. J Trop Ecol 20:701–704

Douglas AE (2008) Conflict, cheats and the persistence of symbioses. New Phytol 177:849–858

Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evol Bioinform Online 1:47–50

Federle W, Fiala B, Zizka G, Maschwitz U (2001) Incident daylight as orientation cue for hole-boring ants: prostomata in *Macaranga* ant-plants. Insectes Soc 48:165–177

Federle W, Maschwitz U, Fiala B, Riederer M, Hölldobler B (1997) Slippery ant-plants and skilful climbers: selection and protection of specific ant partners by epicuticular wax blooms in *Macaranga* (Euphorbiaceae). Oecologia 112:217–224

Federle W, Maschwitz U, Hölldobler B (2002) Pruning of host plant neighbours as defence against enemy ant invasions: *Crematogaster* ant partners of *Macaranga* protected by "wax barriers" prune less than their congeners. Oecologia 132:264–270

Federle W, Rheindt FE (2005) *Macaranga* ant-plants hide food from intruders: correlation of food presentation and presence of wax barriers analysed using phylogenetically independent contrasts. Biol J Linn Soc Lond 84:177–193

Feldhaar H, Fiala B, Gadau J (2004) Characterization of microsatellite markers for plant–ants of the genus *Crematogaster* subgenus *Decacrema*. Mol Ecol Notes 4:409–411

Feldhaar H, Fiala B, Gadau J (2005) A shift in colony founding behaviour in the obligate plant-ant *Crematogaster* (*Decacrema*) morphospecies 2. Insect Soc 52:222–230

Feldhaar H, Fiala B, Gadau J, Mohamed M, Maschwitz U (2003a) Molecular phylogeny of *Crematogaster* subgenus *Decacrema* ants (Hymenoptera: Formicidae) and the colonization of *Macaranga* (Euphorbiaceae) trees. Mol Phylogenet Evol 27:441–452

Feldhaar H, Fiala B, Hashim RB, Maschwitz U (2003b) Patterns of the *Crematogaster–Macaranga* association: The ant partner makes the difference. Insect Soc 50:9–19

Feldhaar H, Foitzik S, Heinze J (2008) Lifelong commitment to the wrong partner: hybridization in ants. Philos Trans R Soc Lond B 363:2891–2899

Fiala B, Grunsky H, Maschwitz U, Linsenmair KE (1994) Diversity of ant-plant interactions: protective efficacy in *Macaranga* species with different degrees of ant association. Oecologia 97:186–192

Fiala B, Jakob A, Maschwitz U (1999) Diversity, evolutionary specialization and geographic distribution of a mutualistic ant–plant complex: *Macaranga* and *Crematogaster* in South East Asia. Biol J Linn Soc Lond 66:305–331

Fiala B, Maschwitz U (1992) Food bodies and their significance for obligate ant-association in the tree genus *Macaranga* (Euphorbiaceae). Bot J Linn Soc 10:61–75

Fiala B, Maschwitz U, Pong TY, Helbig AJ (1989) Studies of a South East Asian ant–plant association: protection of *Macaranga* trees by *Crematogaster borneensis*. Oecologia 79:463–470

Forel A (1910) Note sur quelques fourmis d'Afrique. Ann Soc Entomol Belg 54:421–458

Forel A (1911) Fourmis de Bornéo, Singapore, Ceylan, etc. récoltées par MM. Haviland, Green, Winkler, Will, Hose, Roepke et Waldo. Rev Suisse Zool 19:23–62

Frederickson ME (2005) Ant species confer different partner benefits on two neotropical myrmecophytes. Oecologia 143:387–395

Guindon S, Gascuel O (2003) PhyML - A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52:696–704

Heckroth HP, Fiala B, Gullan PJ, Idris AH, Maschwitz U (1998) The soft scale (Coccidae) associates of Malaysian ant-plants. J Trop Ecol 14:427–443

Heil M, Fiala B, Linsenmair KE, Zotz G, Menke P, Maschwitz U (1997) Food body production in *Macaranga triloba* (Euphorbiaceae): a plant investment in anti-herbivore defence via symbiotic ant partners. J Ecol 85:847–861

Heil M, Hilpert A, Fiala B, Linsenmair KE (2001) Nutrient availability and indirect (biotic) defence in a Malaysian ant-plant. Oecologia 126:404–408

Heil M, McKey D (2003) Protective ant–plant interactions as model systems in ecological and evolutionary research. Annu Rev Ecol Evol Syst 34:425–553

Inui Y, Itioka T, Murase K, Yamaoka R, Itino T (2001) Chemical recognition of partner plant species by foundress ant queens in *Macaranga–Crematogaster* myrmecophytism. J Chem Ecol 27:2029–2040

Jürgens A, Feldhaar H, Feldmeyer B, Fiala B (2006) Chemical composition of leaf volatiles in *Macaranga* species (Euphorbiaceae) and their potential role as olfactory cues in host-localization of foundress queens of specific ant partners. Biochem Syst Ecol 34:97–113

Magnacca KN, Danforth BN (2007) Low nuclear variation supports a recent origin of Hawaiian *Hylaeus* bees (Hymenoptera: Colletidae). Mol Phylogenet Evol 43:908–915

Maynard Smith J (1998) Evolutionary genetics. Oxford University Press, Oxford

Meier R, Shiyang K, Vaidya G, Ng P (2006) DNA barcoding and taxonomy in diptera: a tale of high intraspecific variability and low identification success. Syst Biol 55:715–728

Moog U (2002) Die Reproduktion von *Macaranga* (Euphorbiaceae) in Südostasien: Bestäubung durch Thripse und Kastration durch Pflanzenameisen. PhD thesis, University of Frankfurt

Moog U, Fiala B, Federle W, Maschwitz U (2002) Thrips pollination of the dioecious ant plant *Macaranga hullettii* (Euphorbiaceae) in Southeast Asia. Am J Bot 89:50–59

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

Quek SP, Davies SJ, Ashton PS, Itino T, Pierce NE (2007) The geography of diversification in mutualistic ants: a gene's-eye view into the Neogene history of Sundaland rain forests. Mol Ecol 16:2045–2062

Quek SP, Davies SJ, Itino T, Pierce NE (2004) Codiversification in an ant-plant mutualism: stem texture and the evolution of host use in *Crematogaster* (Formicidae: Myrmicinae) inhabitants of *Macaranga* (Euphorbiaceae). Evolution 58:554–570

Ratnieks FLW (1990) The evolution of polyandry by queens in social hymenoptera - the significance of the timing of removal of diploid males. Behav Ecol Sociobiol 26:343–348

Rousset F (2000) Genetic differentiation between individuals. J Evol Biol 13:58–62

Sites JW, Marshall JC (2003) Delimiting species: a renaissance issue in systematic biology. Trends Ecol Evol 18:462–470

Swofford DL (2002) PAUP*: Phylogenetic analysis using parsimony. Ver. 4.0b10. Sinauer Associates, Sunderland, MA

Weising K et al. (2010) Mechanisms of speciation in Southeast Asian ant-plants of the genus *Macaranga* (Euphorbicaceae) associated with *Crematogaster* ants. In: Glaubrecht M (ed) Evolution in Action. Springer, Berlin

Whitmore TC (1969) First thoughts on species evolution in Malayan *Macaranga*. Biol J Linn Soc Lond 1:223–231

Yu DW, Davidson DW (1997) Experimental studies of species-specificity in *Cecropia*-ant relationships. Ecol Monogr 67:273–294

Yu DW, Pierce NE (1998) A castration parasite of an ant–plant mutualism. Proc R Soc Lond B 265:375–382

Yu DW, Wilson HB, Pierce NE (2001) An empirical model of species coexistence in a spatially structured environment. Ecology 82:1761–1771

Radiation, Biological Diversity and Host–Parasite Interactions in Wild Roses, Rust Fungi and Insects

Annette Kohnen, Roland Brandl, Roman Fricke, Friederike Gallenmüller, Katrin Klinge, Ines Köhnen, Wolfgang Maier, Franz Oberwinkler, Christiane Ritz, Thomas Speck, Günter Theissen, Teja Tscharntke, Andrea Vaupel, and Volker Wissemann

Abstract One of the major tasks in evolutionary ecology is to explain how interspecific interactions influence the dynamics of evolutionary processes and enable radiation and genesis of biological diversity. The bewildering diversity of dog roses is generated by a heterogamous reproductive system. Genetic distance between rose taxa was analysed as base line for the explanation of subsequent radiation of the two host-dependent parasite groups, rust fungi and insects. We investigated the interaction between each host–parasite system and between the parasite groups. We learned that the functional diploidy at the meiotic level is not reflected at the phenotypic level in dog roses. The phytophagous insect community shows only minor differences in composition on different rose species. These invertebrates seem not to be negatively affected by glandular trichomes, but for the rust fungi, *Phragmidium* glandular trichomes matter, because they are negatively correlated with the infection. The abundance of two rose specialists, the

V. Wissemann (✉)
Institut für Allgemeine Botanik & Pflanzenphysiologie, AG Spezielle Botanik, Justus-Liebig-Universität Giessen, Heinrich-Buff-Ring 38, 35390 Gießen, Germany
e-mail: volker.wissemann@bot1.bio.uni-giessen.de

A. Kohnen, R. Brandl, R. Fricke, and A. Vaupel
Philipps-Universität Marburg, Marburg, Germany

F. Gallenmüller and T. Speck
Albert-Ludwigs-Universität-Freiburg, Freiburg im Breisgau, Germany

K. Klinge and T. Tscharntke
Georg-August Universität Göttingen, Göttingen, Germany

W. Maier
Ruhr-Universität Bochum, Bochum, Germany

F. Oberwinkler
Eberhard Karls Universität Tübingen, Tübingen, Germany

I. Köhnen and G. Theissen
Friedrich Schiller Universität Jena, Jena, Germany

C. Ritz
Justus-Liebig-Universität Giessen, Giessen, Germany

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_11,

rose hip fly *Rhagoletis alternata* Fall. and the rose gall wasp *Diplolepis rosae* L., differed on rose species, but *Rh. alternata* showed neither any genetic differentiation on host species nor geographical differentiation. As a basic result, we detected that genetic diversity of dog roses is not translated into a host-specific radiation of the parasites. We assume that intensive reticulate evolution of dog roses prevents co-speciation.

1 Introduction: Radiation, Biodiversity and Host–Parasite Interaction in the *Rosa*-System

A key topic in evolutionary ecology is to explain how interspecific interactions influence the dynamics of evolutionary processes and enable radiation and unfolding of biological diversity. In host–parasite interacting systems, the most important questions are: how does the radiation and diversity of the hosts translate into the radiation and diversity of the parasites, and what is the role of parasite interactions?

Such analyses are extremely rare due to the complexity of these systems (see, e.g., Clay 1989; Pirozynski and Hawksworth 1989). In this study, we investigate the host–parasite net of dog roses (Rosaceae, Rosoideae, *Rosa* L., sect. *Caninae* (DC.) Ser.) rust fungi (*Phragmidium*) and insects. By analysing the genetic distance, variability and phylogenetic relationships between rose taxa, we determine a base line for the explanation of the subsequent radiation processes of two host-dependent parasite groups, rust fungi and insects (Fig. 1). Understanding the dog roses' radiation process enables us to unravel the levels of interaction (co-evolution, co-speciation, individualistic interaction) on which rust fungi and insects act. Dog roses are thought to have evolved during the Pliocene (5.3–1.8 mya) as a result of a single event into which the peculiar mode of *Canina*-meiosis developed and then colonised Central Europe by a very fast and explosive radiation during the Pleistocene and Holocene (Zielinski 1986). Wissemann (2000b) and the study by Ritz et al. (2005a) showed that dog roses are permanent allopolyploids that have arisen by multiple hybridisation events. The high genetic variability due to allopolyploidy and great homology between the different chromosome sets enabling interfertility between any dog rose species are the reasons for the morphological variation and the existence of numerous local forms.

Subsequent to the *Rosa* radiation, numerous pathogens interacted with their hosts. At present both the analysed species of rose rusts and insects are found on any of the investigated dog rose species. Nevertheless, little is known about the genetic diversity of the two main (and commercially important) rust fungi on roses, *Phragmidium mucronatum* (Pers.) Schltdl. and *P. tuberculatum* J. Müller. We do not know anything about the radiation process of subspecific taxa (races, strains) and we do not know the level at which the fungi became host specific. The same the situation occurs in the numerous rose-specific insect species. One group is the gall-forming insects, which are highly susceptible to plant resistance and are adapted to specific resources in most cases.

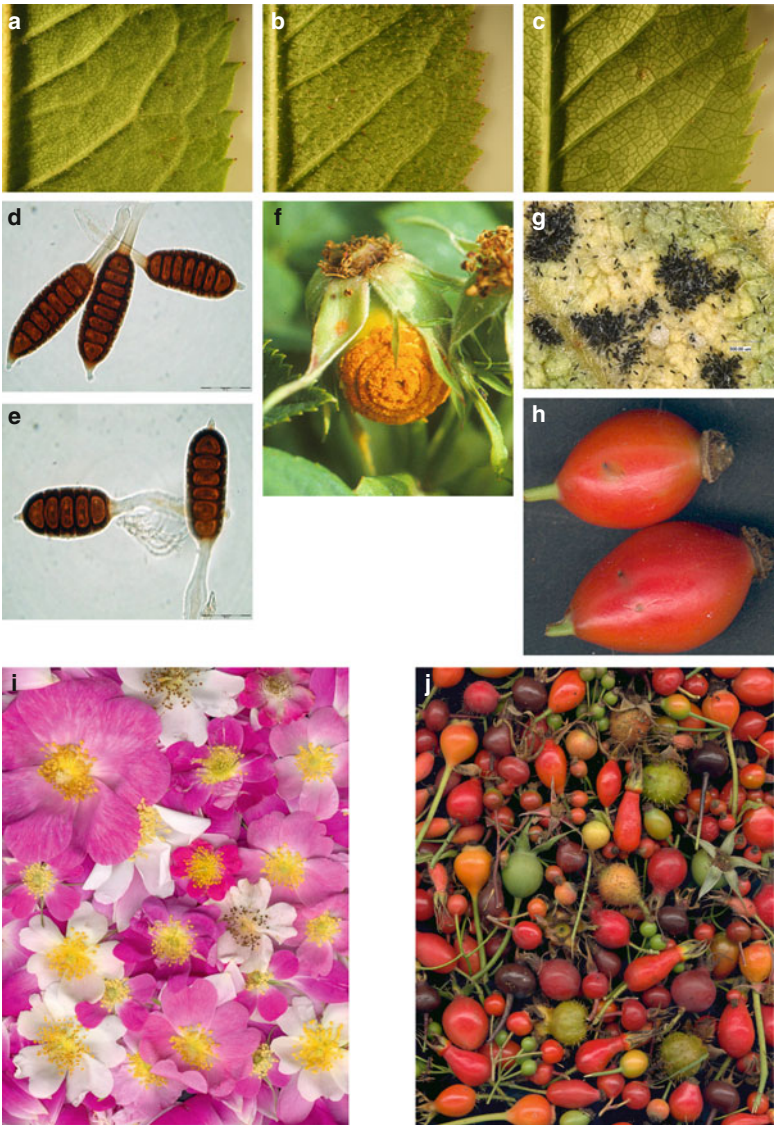


Fig. 1 (**a**) Lower surface of *R. corymbifera* (hairy), (**b**) *R. rubiginosa* (glandular), (**c**) *R. canina* (glabrous, eglandular). (**d**) Spores of the rose rust fungus *Phragmidium mucronatum*. (**e**) Spores of the rose rust fungus *Phragmidium tuberculatum*. (**f**) Rust infection on a hip of *R. canina* (**g**) Teliospores (**h**) *Rhagoletis alternata* infected hip of *R. canina*. (**i**) Flower diversity in *Rosa*. (**j**) Hip diversity

2 Dog Roses are Allopolylpoids: Genetic Constitution of Section *Caninae*

The genus *Rosa* consists of about 200 species following the classification by Wissemann (2003) and Wissemann and Ritz (2005). Based on morphological characters, four subgenera are recognised: *Hulthemia* (Dumort.) Focke, *Platyrhodon* (Hurst) Rehder, *Hesperhodos* Cockerell and *Rosa*. The subgenus *Rosa* comprises ten sections with more than 150 species of various ploidy levels, distributed mainly in the temperate regions of the northern hemisphere. Studies involving excessive cloning of nuclear genes (ribosomal spacers and low copy genes) revealed that hybridisation played an important role in the evolution of polyploid rose taxa (Wissemann 2000b; Ritz et al. 2005a; Joly and Bruenau 2006; Joly et al. 2006). Within subgenus *Rosa*, members of the polyploid section *Caninae* are of particular interest, not only because of their unique meiotic behaviour but also for the readiness with which members of the section can hybridise (Feuerhahn and Spethmann 1995; Wissemann and Hellwig 1997; Reichert 1998; Nybom et al. 2004, 2006; Werlemark and Nybom 2001; Werlemark et al. 1999). Early studies analysing nrITS-1 sequences revealed the existence of non-concerted evolution of the nrITS-region and thus confirmed the allopolyploid constitution of dog roses (Eigner and Wissemann 1999; Wissemann 1999, 2000b, 2002). Based on these studies, we showed that the tetra-, penta- or hexaploid dog roses arose by multiple hybridisations across the genus *Rosa* (Fig. 2; Ritz et al. 2005a).

Dog roses are cytologically characterised by a specific meiosis (Täckholm 1920, 1922; Klášterská 1969, 1971; Klášterská and Natarajan 1974; Roberts 1975). This meiosis leads to heterogamous reproduction with (in the case of pentaploid roses, $2n = 5x = 35$) haploid pollen grains ($n = 1x = 7$) and tetraploid egg cells ($n = 4x = 28$; reviewed in Wissemann and Ritz 2007). By microsatellite analysis (Ritz and Wissemann, submitted) we added further support to the first results of Nybom et al. (2004, 2006) that always the same chromosome sets pair during meiosis (bivalents) and the same three sets are unpaired (univalents). These findings support the hypothesis that dog roses are functional diploids: The bivalents are meiotically recombined and transmitted through the eggcell and the pollengrain,

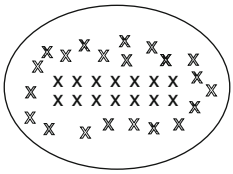


Fig. 2 Hypothetic genetic constitution of an allopolyploid pentaploid dog rose ($2n = 5x = 35$, $x = 7$) with parental genomes from multiple hybridisation after Ritz et al. (2005a) and Kovarik et al. (2008). *Black* chromosomes: diploid bivalent forming Protocaninae genome; *grey white* and *checked* chromosomes: *woodsii*-, *rugosa*- and *gallica*-univalent-forming genomes of other sections of the genus *Rosa*

but the univalents are inherited apomictically by the eggcell only (Fagerlind 1945; Zielinski 1986; Nybom et al. 2004, 2006; Lim et al. 2005). Thus, the canina meiosis combines two modes of reproduction: The interacting bivalents generate variability via sexual recombination and the apomictic univalents conserve information which allow for the tremendous variability and radiation possibilities. This unique meiosis of dog roses is presumably connected to a particular chromosome set characterised by the *canina*-nrITS type, which is not known to exist anymore in a diploid species (Ritz et al. 2005a). Present results show that the *canina*-nrITS type has at least two copies in each dog rose and is involved in bivalent formation (Kovarik et al. 2008; Ritz et al., unpublished). We assume this *canina*-nrITS type to be a trace for the existence of the hypothetic Protocaninae genome. The diploid Protocaninae are probably extinct but rescued as the bivalent-forming set during meiosis (Fig. 2). On the other hand, the canina genome could also have evolved by mutation as it was shown in the evolution from teosinte to mayze in which the existence of a "Proto-mayze" has also been proposed (Doebley 2004). A polyphyletic origin of dog roses seems improbable keeping the uniqueness and complexity of canina meiosis in mind. However, phylogenetic trees based on chloroplast DNA sequences are polyphyletic with respect to section *Caninae* because members of subsect. *Caninae* are not sister to the glandular species of subsect. *Rubigineae* H. Christ and *Vestitae* H. Christ but to non-dog roses of sections *Indicae* Thory, *Rosa* and *Synstylae* DC. (Wissemann and Ritz 2005; Bruneau et al. 2007).

3 Character Inheritance in the Heterogamous System of Dog Roses

As a consequence of the canina-meiosis the proportion of genetic information contributed by the maternal parent to the offspring is four times larger than that of the pollen parent. Thus, offspring are largely matroclinal with respect to most morphological characters of leaves, flowers and hips (reviewed in Wissemann and Ritz 2007). This pattern of character inheritance is also expressed at the anatomical, biochemical and genetic level, since studies on epicuticular waxes and flower volatiles (Wissemann 2000a, b, 2007; Wissemann and Degenhardt, unpublished) and on microsatellites and random amplified polymorphic DNA (RAPD) bands (Werlemark et al. 1999, Werlemark and Nybom 2001; Nybom et al. 2004, 2006; Ritz and Wissemann, submitted) demonstrated a strong matrocliny of interspecific hybrids.

However, some morphological traits do not match these observations, because Wissemann et al. (2006) showed that the growth habit of dog roses is dominantly inherited. Interspecific reciprocal hybrids between *R. canina* L. and *R. rubiginosa* L. were characterised by the lax and arching branches in contrast to dense erect branches of *R. rubiginosa*. This pattern could be the result of (1) a heterosis effect, (2) the inheritance of dominant allele encoding growth form, or (3), which is favoured by the authors, multiple factors of which some are not inevitably subjected to inheritance but are responsible for the syndrome growth form.

Moreover, the taxonomic important characters, sepal persistence and the diameter of the orifice, are paternally expressed and possibly controlled by genomic imprinting (Gustafsson 1944; Ritz and Wissemann 2003). The paternal inheritance of these characters results in the morphological identity between interspecific hybrids and described dog rose species (Fig. 3). Ritz and Wissemann (submitted) demonstrated that *R. micrantha* Borrer ex Sm. and the corresponding interspecific hybrid *R. rubiginosa* × *R. canina* are genetically not completely identical. Microsatellite alleles of *R. micrantha* corresponded to those of the potential parents; however, all investigated samples of *R. micrantha* were in contrast to the pentaploid hybrids hexaploid. The authors assumed that the initial interspecific hybrid giving rise to *R. micrantha* was established by an increase of ploidy level to maintain two highly homologous chromosome sets for correct bivalent formation during canina meiosis.

The matrocliny of the majority of characters points despite the functional diploidy of the meiosis system to the functionality of genetic information stored on univalent genomes. In contrast, Lim et al. (2005) assumed that the lacking recombination between the univalents leads to gene degradation due to a relaxed selection pressure. However, a study on gene expression of single copy genes did not point to degradation or silencing of alleles on univalents (Ritz et al. unpublished).

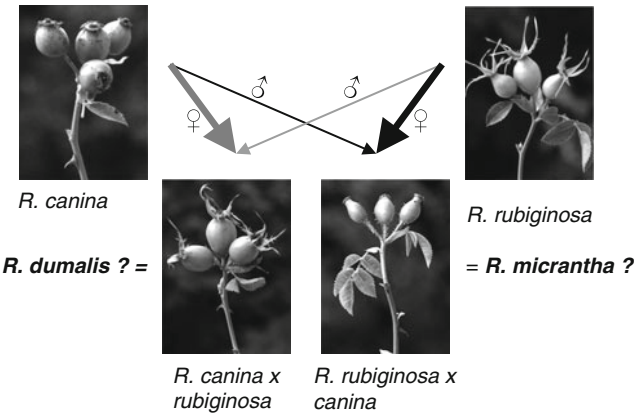


Fig. 3 Diagram of the crossing experiment (Wissemann and Hellwig 1997) between the two dogrose species *R. canina* (L-type: deciduous sepals during hip ripening) and *R. rubiginosa* (D-type: persistent sepals during hip ripening). Both parents were used as seed and pollen parent. *Arrows* symbolise the direction of the crosses. *Grey arrows*: *R. canina* was used as seed parent and *R. rubiginosa* was used as the pollen parent. *Black arrows*: *R. rubiginosa* was used as seed parent and *R. canina* as pollen parent. The *thickness* of the *arrows* symbolises the matroclinal inheritance due to the canina meiosis: The seed parent inherits 4/5 of the genome and the pollen parent only 1/5 of the genome. The outcomes of both crosses show the same type of sepal persistence as the pollen parent. The combination of the matroclinal vegetative characters (e.g. leaf surface) and the paternal type of sepal persistence is also found in already described species (*R. dumalis* and *R. micrantha*). Reproduction from Wissemann and Ritz (2007, Fig. 2).

4 Glandular Trichomes Matter: Rust Fungi on *Rosa*

In Rosaceae, rust fungi have long been recognised as important parasites. Co-evolution of rusts and Rosaceae seems so strong and evident that rust fungi can be used to determine phylogenetic relationships between certain hosts in Rosaceae (Savile 1979). El-Gazzar (1981) pointed out that more than 1,500 species from 49 genera of Rosaceae are susceptible to about 300 species out of 27 genera of the Uredinales, and that susceptibility to rust infection is strongly correlated with the chromosome base number of $x = 7$. The most important rust fungi on *Rosa* are *Phragmidium mucronatum* and *P. tuberculatum*. Both have been recorded in dog roses (Gäumann 1959; Scholler 1994; Brandenburger 1994). Comparable to the findings of Evans et al. (2000). who showed the formation of subspecific strains or races of *Phragmidium violaceum* (Schultz) Winter, a parasite on the blackberry (*Rubus* L., Rosaceae), we expected the two *Phragmidium* species on roses to evolve and radiate in the same manner. However, ever since Savile's publication (1979) in which the interaction between rust fungi and Rosaceae has been regarded as a co-evolutionary system, our findings have weakened this assumption (Ritz et al. 2005b). Host ranges of *P. mucronatum* and *P. tuberculatum* overlapped and their infection rates did not differ between *Rosa canina*, *R. corymbifera* Borkh. and *R. rubiginosa*. These three species served as examples for the variation of leaf trichomes and glands observed in dog roses which might be crucial for rust infection (Bahçecioğlu and Yildiz 2005; Valkama et al. 2005). Results showed that infection by *P. mucronatum* and *P. tuberculatum* did not significantly differ between species with glabrous and hairy leaves, *R. canina* and *R. corymbifera*, respectively. However, *R. rubiginosa*, developing hairy leaves with numerous odorous glands, was significantly less infected by both species (Ritz et al. 2005b). Despite their overlapping host ranges and their morphological similarity, both fungi are genetically only distantly related: *P. mucronatum* belongs to a clade of "rose rust sensu stricto", whereas *P. tuberculatum* is closely related to rusts living on *Rubus* and *Sanguisorba* L. and thus explored dog roses by a host jump. The lacking host specificity of rusts on dog roses might be explained by the hybrid bridge hypothesis (Floate and Whitham 1993). It predicts that hybridisation of hosts and thus the admixture of different genomes prevents the step-by-step process of co-evolution and co-speciation of hosts and parasites.

5 Evolution and Diversity of Plant–Pathogen–Insect Foodwebs on Dog Roses

Herbivorous insect species and their host plant species together comprise more than 50% of the macroscopic species (Strong et al. 1984). Thus, an understanding of the evolutionary driving forces as well as the ecological interactions is an important issue for our general understanding of biodiversity. During the last 30 years, the paradigm of interpreting the enigmatic diversity of insects changed

from co-evolution and co-speciation to a more individualistic view (e.g. Brandl et al. 1992; Schoonhoven et al. 1998).

Macro-ecological work of the last 20 years showed that the insect fauna associated with a particular plant species is a complex blend of generalists and specialists (Strong et al. 1984; Tscharntke and Greiler 1995; Schoonhoven et al. 1998; Brändle and Brandl 2001). The insect fauna on a particular host depends on the available species pool of phytophags, the distribution and abundance of the host, and the number of feeding niches provided by the host, as well as the host's taxonomic isolation and biochemical make-up (Strong et al. 1984; Lawton 1986; Tscharntke and Greiler 1995; Frenzel and Brandl 1998). Plant genotypes with different morphological traits may affect not only the abundance of single insect species but also the structure of associated herbivore communities (Maddox and Root 1987; Fritz and Price 1988). Whitham et al. (2003) showed that even single traits coded by few genes may have important effects on the community of exploiters (extended phenotype).

Rose bushes are characteristic features of European landscapes, in particular as components of hedges. Roses have been included into several attempts to understand α-diversity (e.g. Leather 1986). However, most of the available data on phytophages have not distinguished between the different rose species and thus ignored the bewildering diversity within that host taxon. As roses provide a fascinating example of an explosive radiation, roses are good candidates to study the effects of hybridisation as well as rapid radiation of hosts on herbivores across a flock of host plants. The available information on phytophages on roses shows that there are a number of generalists attacking roses (e.g. see Zwölfer et al. 1981, 1984), but also many specialists such as the cynipid wasp *Diplolepis rosae* L., the tephritid fly *Rhagoletis alternata* Fallén, and the tortricid moth *Notocelia roborana* Dennis and Schiffermüller. For example, Ferrari et al. (1997) sampled 6,000 ectophagous insect specimens from 32 rose stands around Göttingen. Eight out of the 10 tenthredinid wasps, 3 out of 19 Cicadina species, and 4 out of 76 beetle species were specialists on roses, whereas all the 20 bug species were less specialised.

For all our investigations, we selected three dog rose species (*Rosa canina* L., *R. corymbifera* Borkh. and *R. rubiginosa* L.), all members of the dog rose section *Caninae* (DC.) Ser. These three species are widely distributed and abundant in central Europe and often occur in the same habitats. They are supposed to have originated by allopolyploid hybridisation events (Ritz et al. 2005a; Wissemann 2002) and expanded their range to central and northern Europe after the last ice age (Zielinski 1986). Although closely related, they differ in several characters: *R. canina* is a glabrous rose, *R. corymbifera* has hairs on rhachis and abaxial leaf surface and *R. rubiginosa* has glandular trichomes on the lower leaf surface. Furthermore, the three rose species also differ in plant architecture (Wissemann et al. 2006) and phenology (Timmermann 1998). This leads to the question: how do these differences translate into the diversity of higher trophic levels? In the following, we compare (1) the community structure of these three dog rose species, (2) the densities of two consumer specialists on the rose species, and (3) the genetic

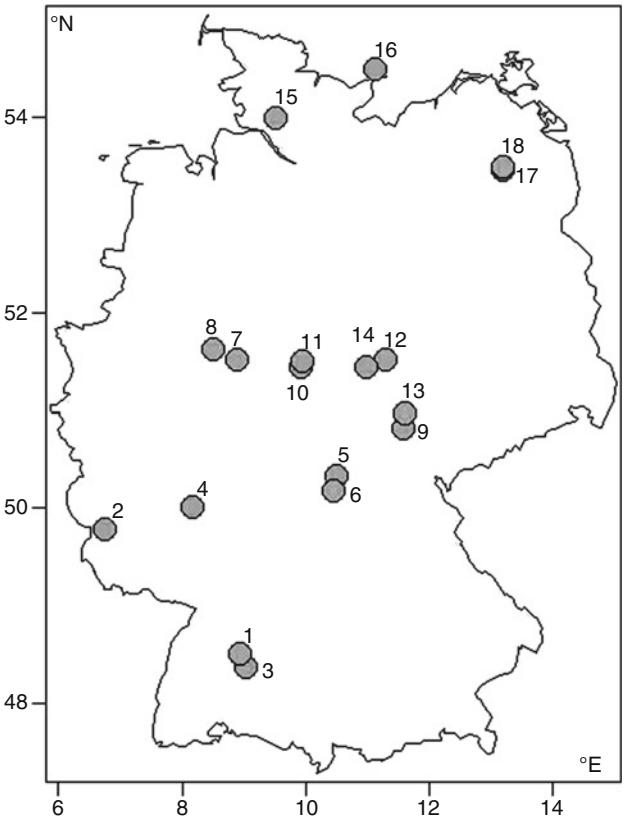


Fig. 4 Geographical location of the 18 study sites along a transect across Germany. At all study sites, the three dog rose species (*Rosa canina, R. corymbifera* and *R. rubiginosa*) occurred together. Community study was conducted at all 18 sample sites, density analyses of *Rhagoletis alternata* and *Diplolepis rosae* at 17 study sites (all except no. 10), and the *D. rosae* gall community was sampled on eight sites (nos. 1–3, 5–6, 9, 11, 18)

adaptation of one of these specialists to the three dog rose species. Along a gradient across Germany (Fig. 4), we sampled at 18 study sites where the three rose species occurred together in the same habitat.

5.1 Are Invertebrate Communities Affected by Leaf Trichome Traits of Hosts?

As already noted, the European dog roses differ in a variety of morphological traits, which may influence the interactions with associated food webs. In particular, dog

roses differ considerably across species in density and type of trichomes on the lower leaf surface. Trichomes are supposed to influence host choice of herbivores as well as of other invertebrates (Yencho and Tingey 1994; Zvereva et al. 1998; Ranger and Hower 2002). Although trichomes are often part of a defence system, they may, however, be beneficial for some herbivores (Eisner et al. 1998). Nevertheless, invertebrate communities may map the variation of trichomes across species (Andres and Connor 2003).

Insect communities were sampled using beating trays (diameter 70 cm; Stechmann et al. 1981) in May, June, July and August 2002 (Fricke, unpublished). Data were collected from 88 bushes of *R. rubiginosa*, 87 of *R. corymbifera* and 88 of *R. canina* (3–5 bushes per sampling site; Fig. 4). We sorted 75,937 individuals to insect orders (Table 1). Coleoptera and Heteroptera were identified to species to

Table 1 Basic information for the invertebrate groups sampled from the 263 shrubs of roses during May, June, and July 2002 across 18 sites in Germany (see Fig. 4). Taxa were sorted by the total number of individuals (*n*). The first five groups (names in *bold*) were used for detailed analyses of the abundances. *Mean* is average abundance across one subsample (5 subsamples per bush). Abundance was measured as the number of individuals divided by the subsamples. Correlations of abundances (means across dates of standardised transformed abundances for each bush, $n = 263$) with scores of the first two principal components (*PCA1* and *PCA2*) characterising plant architecture. Significant correlations in bold. The principal components analysis (PCA) was performed with six measured variables of each bush. Two components passed the Kaiser criterion. The first component represented the size (PCA 1; eigenvalue: 2.31, explained variance: 38.6%) and the second the form of an individual bush (PCA 2; eigenvalue: 1.31, explained variance: 21.8%)

Taxon	*n*	Mean	SD	PCA 1		PCA 2	
				r	p	r	p
Aphidina	19,656	4.97	12.44	−0.07	0.293	0.11	0.084
Collembola	19,140	4.97	8.21	−0.01	0.866	**0.16**	**<0.05**
Araneae	11,275	2.90	3.65	**−0.36**	**<0.001**	**0.19**	**<0.01**
Hymenoptera	5,842	1.50	2.32	**−0.23**	**<0.001**	**0.28**	**<0.001**
Coleoptera	3,714						
herbivorous	2,648	0.67	1.56	**−0.18**	**<0.01**	0.02	0.759
other	1,066	0.27	0.54	**−0.37**	**<0.001**	0.11	0.081
Thysanoptera	2,969	0.74	2.00	**−0.20**	**<0.001**	**0.18**	**<0.01**
Acari	2,642	0.68	1.55	0.02	0.780	0.01	0.902
Diptera	2,481	0.63	1.01	**−0.22**	**<0.001**	0.08	0.208
Auchenorrhyncha	2,259	0.59	1.81	0.02	0.796	−0.00	0.970
Heteroptera	1,504						
herbivorous	803	0.21	0.24	0.09	0.164	−0.09	0.155
carnivorous	701	0.18	0.31	0.03	0.598	0.06	0.339
Dermaptera	1,359	0.35	0.65	**−0.19**	**<0.01**	−0.04	0.549
Psocoptera	1,147	0.29	0.58	−0.06	0.315	0.09	0.154
Lepidoptera	814	0.21	0.38	−0.06	0.340	0.02	0.775
Gastropoda	337	0.09	0.28	0.09	0.142	0.03	0.646
Planipennia	311	0.08	0.18	−0.09	0.160	0.08	0.183
Opiliones	126	0.03	0.15	−0.06	0.333	−0.06	0.363
Orthoptera	59	0.02	0.07	−0.06	0.367	−0.06	0.370
Σ	75,937						

distinguish between phytophagous and non-phytophagous species. To characterise the architecture of the sampled host individuals, six variables of each bush were measured.

With a repeated measurement ANOVA, we found only minor differences in the abundance of common invertebrate groups (Aphidina, Collembola, Araneae, Hymenoptera and Coleoptera) between the three rose species (Tables 1 and 2). Ordinations using all major taxonomic units also showed few differences in the composition of the exploiter communities. Contrary to our expectations, the abundances of phytophagous invertebrates were higher on *R. rubiginosa* than on the other two rose species. This is remarkable, because feeding experiments showed a lower palatability of *R. rubiginosa* leaves using larvae of *Spodoptera littoralis* (Klinge 2005; Fig. 5). Nevertheless, the field data suggest that most of the phytophagous invertebrates are not negatively affected by glandular trichomes and these trichomes do not serve as a general defence against phytophages.

Apart from phytophagous invertebrates, dog roses suffer from infection by the rust fungi *Phragmidium* spec. which shows significant differences in density between the three rose species (Ritz et al. 2005b; Klinge 2005; Fig. 6). The species *R. corymbifera* and *R. canina* show higher infection rates than the glandular *R. rubiginosa* (see Chap. 5). With infection by this rust fungi, the lower leaf surface of the host plant is of special concern. The rust fungi infects plants by penetrating the stomata of the leaflets with the germination tubes of its uredospores. The trichomes of dog roses occur only on the lower leaf surface where the stomata are also located. Thus, the trichomes might serve as a defence against rust infections. This idea is not new and has already been proposed for other plant species by Bahçecioğlu and Yildiz (2005) and Valkama et al. (2005). Furthermore, in the glandular trichomes of *R. rubiginosa,* sesquiterpenes occur (Klinge, unpublished). These substances are known to inhibit the growth of fungi (e.g. Alvarez-Castellanos et al. 2001; Cakir et al. 2004). Overall, our data do not support the hypothesis that trichomes evolved as a defence strategy against invertebrates and especially against herbivores. However, our data are consistent with the hypotheses that trichomes are a defence strategy against rust fungi.

5.2 *Do* Rhagoletis alternata *and* Diplolepis rosae *Differ in Density Between the Three Rose Species?*

Two highly specialised consumer species of dog roses are the European rose-hip fly *Rhagoletis alternata* Fall. (Diptera, Tephritidae) and the rose gall wasp *Diplolepis rosae* L. (Hymenoptera, Cynipoidea). The fruit fly *Rh. alternata* infests the fleshy fruits of species from several *Rosa* sections (White 1988). Adults emerge in early summer and females oviposit into green hips marking the attacked hips by an oviposition-deterring pheromone (Bauer 1986). Larvae feed exclusively in the hypanthium and do not attack the seeds. Mature larvae leave the hips for pupation

Table 2 Repeated measures ANOVA (type I sums of squares) for the five most abundant invertebrate groups on roses (Table 1). The site was a random factor, while rose species and date were fixed factors. *Contrast I* was defined as *R. corymbifera* versus *R. canina*, *Contrast II* was defined as *R. rubiginosa* versus *R. corymbifera* and *R. canina*. In the part above the horizontal line, the means of each shrub over the dates were used, in the part below the line, all samples were used. Significant effects are marked $*p < 0.05$, $**p < 0{,}01$, $***p < 0.001$, and highlighted in bold

Source of variation	Aphidina		Collembola		Araneae		Hymenoptera		Phyto. Coleoptera	
	SS.	F	SS.	F	SS.	F	SS.	F	SS.	F
Foliage cover	0.33	3.0	5.99	**54.3*****	3.22	**86.3*****	1.54	**38.3*****	0.04	1.8
PCA 1	0.12	1.1	0.29	2.6	2.37	**63.5*****	0.50	**12.4*****	0.39	**16.0*****
PCA 2	0.42	**3.9***	0.13	1.2	0.29	**7.7****	0.76	**18.9*****	0.00	0.1
Site	14.35	**7.8*****	26.20	**14.0*****	12.72	**20.1*****	4.56	**6.7*****	4.87	**11.9*****
Rose species	4.69	**7.1****	0.23	0.4	0.21	1.7	0.17	1.0	0.00	0.0
Contrast I	0.11	0.6	0.25	1.2	0.00	0.0	0.01	0.4	0.00	0.0
Contrast II	4.60	**9.9****	0.01	0.0	0.21	3.4	0.16	1.2	0.00	0.0
Site × rose species	11.25	**3.0*****	10.17	**2.7*****	2.15	**1.7***	2.93	**2.1*****	2.28	**2.8*****
Site × contrast I	3.24	**1.8***	3.71	**2.1***	1.06	1.7	0.66	1.0	0.52	1.6
Site × contrast II	7.92	**4.0*****	6.19	**3.0*****	1.04	1.6	2.29	**3.4*****	1.76	**4.2*****
Residuals I	22.42		22.72		7.68		8.29		4.96	
Residuals I (contrast I)	14.20		14.49		5.09		5.28		2.59	
Residuals I (contrast II)	25.85		26.92		8.79		8.95		5.48	
Date	14.74	**11.1*****	31.18	**22.2*****	21.83	**87.7*****	5.68	**14.4*****	4.36	**17.8*****
Date × foliage cover	2.21	**15.1*****	1.44	**12.8*****	0.20	**5.1****	1.71	**35.0*****	0.67	**20.8*****
Date × PCA 1	0.17	1.2	0.03	0.3	0.04	0.9	0.74	**15.1*****	0.10	3.0
Date × PCA 2	0.02	0.2	0.36	**3.2***	0.22	**5.7****	0.14	2.8	0.12	**3.8***
Date × site	22.54	**9.0*****	23.90	**12.5*****	4.23	**6.3*****	6.72	**8.1*****	4.17	**7.6*****
Date × rose species	2.95	**5.2****	0.32	0.6	0.44	**4.8****	1.01	**4.8****	0.65	**5.1****
Date × contrast I	0.22	1.5	0.00	0.0	0.07	2.1	0.08	1.4	0.06	1.8
Date × contrast II	2.75	**6.5****	0.31	1.3	0.37	**6.5****	0.95	**6.0****	0.59	**6.2****
Date × site × rose species	9.65	**1.9*****	8.57	**2.2*****	1.56	1.2	3.62	**2.2*****	2.15	**2.0*****
Date × site × contrast I	2.54	1.0	4.38	**2.2*****	0.58	0.9	0.95	1.2	0.56	1.2
Date × site × contrast II	7.17	**2.9*****	4.17	**2.0****	0.96	1.4	2.67	**3.2*****	1.60	**2.9*****
Residuals II	30.27		23.23		8.19		10.09		6.63	
Residuals II (contrast I)	21.08		15.93		5.13		6.39		3.82	
Residuals II (contrast II)	32.95		27.64		8.87		11.10		7.25	
Total	136.14		154.77		65.35		38.37		31.39	

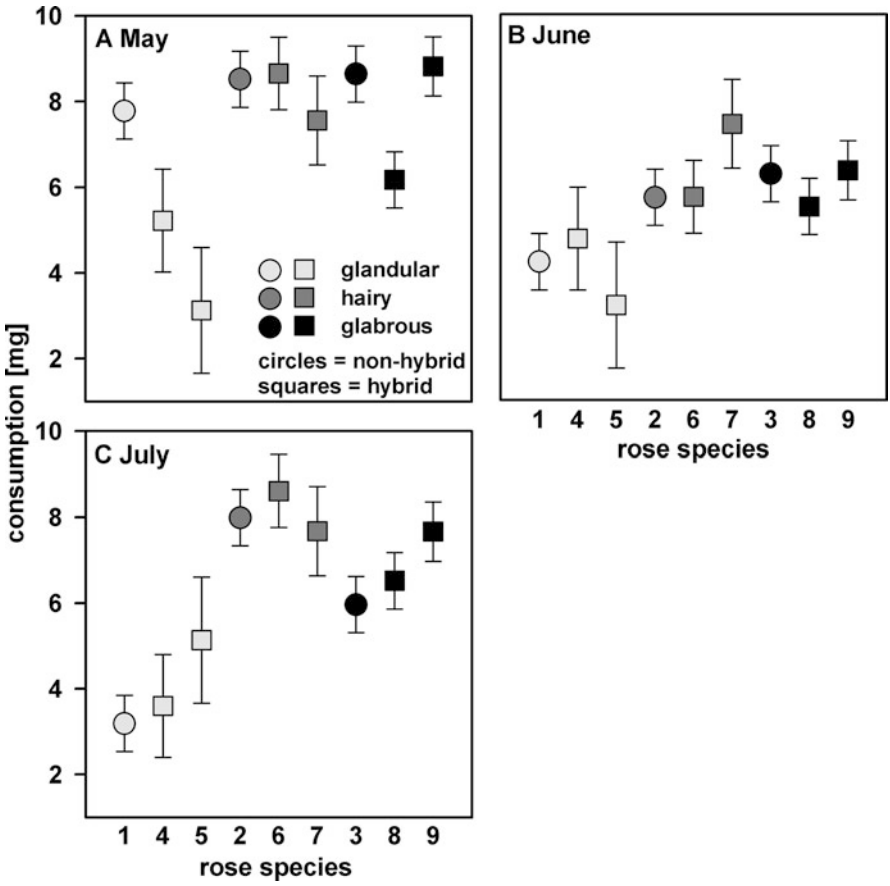


Fig. 5 *Spodoptera* leaf consumption across rose species and hybrids during 3 months. Corrected means ±1 SE from a Split-Plot-Model ANCOVA (Type I) with leaf consumption as response variable in relation to the two categorical factors (month of experiment and rose genotype) and the covariables (fresh weight of larvae and specific water content). Rose species: 1 *R. rubiginosa,* 2 *R. corymbifera*, 3 *R. canina*, 4 *R. rubiginosa* × *R. corymbifera*, 5 *R. rubiginosa* × *R. canina*, 6 *R. corymbifera* × *R. rubiginosa*, 7 *R. corymbifera* × *R. canina*, 8 *R. canina* × *R. rubiginosa*, 9 *R. canina* × *R. corymbifera*

and hibernate in the soil (Bush 1992). The percentage of infested hips per shrub is usually high, frequently reaching 100% (Bauer 1998). Overall, *Rh. alternata* has little impact on the sexual reproduction of roses and thus on the fitness of the hosts (Bauer 1998).

The holarctic cynipid wasp *D. rosae* is a univoltine gall maker (Adler 1877). In Europe, the conspicuous and multichambered galls have been found on *Rosa* species from several sections (Schröder 1967). In southern Sweden, Stille (1984) found that all the species on which *D. rosae* occur belonged to the section *Caninae*. Due to the physiological manipulation of the host plant, cynipid gall wasps are

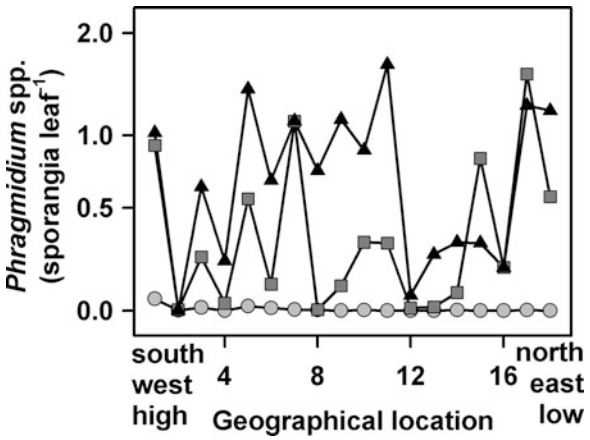


Fig. 6 Density of *Phragmidium spp.* across the three dog rose species (*grey circle* = *Rosa rubiginosa*, *dark-grey square* = *R. corymbifera*, *black triangle* = *R. canina*) and the geographical locations in the year 2002. Corrected means were calculated with general linear model analyses. *Geographical location* = ranking of the 18 sites from south to north, from west to east and from high to low altitudes based on results from a principal component analysis

closely adapted to their host plants (e.g. Crawley and Long 1995; Kato and Hijii 1997). The relationship between plants and gall-inducing insects are usually very specific, suggesting tight co-evolutionary processes (Hilker et al. 2002). Galls develop as a result of interactions between the inducing insect and plant, wherein the insect gain control and redirect the growth and physiology of attacked organs to the insects' advantage (Shorthouse et al. 2005). Both consumer species are known to attack all the three rose species. However, do the consumer species show differences in density between the host species? Klinge (2005) monitored the density of larvae of *Rh. alternata* and *D. rosae* galls for each rose species in September 2002 and 2003 on 3–5 randomly selected shrubs within each sample site (Fig. 4). To monitor the density of *Rh. alternata*, 50 hips from each shrub were collected haphazardly and the percentage of infested hips was used as an estimate of density. During the same sampling dates, rose shrubs were searched for galls of *D. rosae*. The total number of galls on each shrub was used as a measure of gall density.

Densities of the two consumer species varied between host plant species and geographical location (Fig. 7, Table 3). The highest densities of the two phytophages were found on the odorant *R. rubiginosa*. Although the density of *Rh. alternata* and *D. rosae* exhibited significant variations between sites, we found no general geographical trends. Two suites of factors may account for these differences: ecological and genetic factors. Plant phenology (Hodkinson 1997) and abundance of natural enemies (Koptur 1985) may be such possible factors or genetic variation of the consumer species may also trigger variation in density.

On the sexual reproduction of the roses, *Rh. alternata* had little impact. Even when larvae attacked all hips of a shrub, we did not find a negative impact of

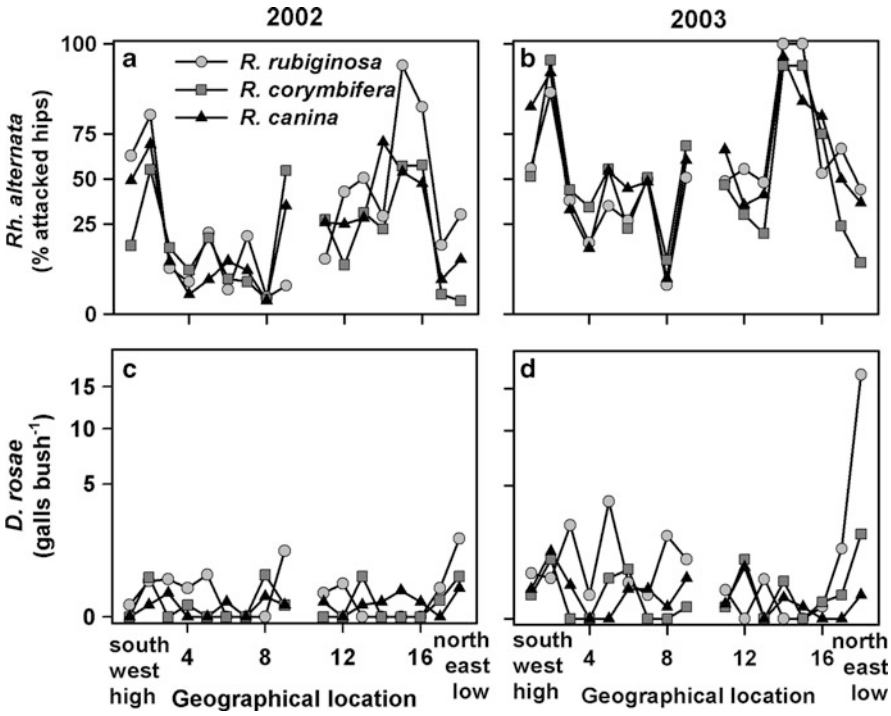


Fig. 7 Patterns of density across the three dog rose species and the geographical locations in the two study years 2002 and 2003 for (**a**, **b**) *Rhagoletis alternata* and (**c**, **d**) *Diplolepis rosae* (corrected means were calculated with general linear model analyses). Geographical location = ranking of the 18 sites from south to north, from west to east and from high to low altitudes based on results from a principal component analysis

Rh. alternata densities on reproduction (number of hips) and leaf cover in the following year. Furthermore, *Rh. alternata* density patterns across years were highly predictable: Host plants with a high level of attack in one year also showed a high level of attack in the following year ($r^2 = 0.45$, $P < 0.05$, $n = 212$). We interpret the predictable fruit production as further evidence that larvae of *Rh. alternata* have overall little impact on the fitness of roses.

In contrast to *Rh. alternata*, the gall-forming *D. rosae* manipulates the physiology of the rose shrubs to produce the gall and gall tissue (Bronner 1992; Bayer 1992; Bagatto et al. 1996; Harper et al. 2004). Although significant top-down effects of gall wasps on the population dynamics of the hosts seem to be rare (e.g. Stone et al. 2002), there is evidence that high cynipid densities can negatively affect host plant growth (e.g. Crawley and Long 1995; Kato and Hijii 1997). Due to the low density of *D. rosae* galls, with a mean density of 0.17 galls per bush, the roses did not show a negative response: The density of hips as well as leaf cover was independent of the number of galls in the previous year (hip density: $r^2 = 0.02$, P = n.s.; leaf cover: $r^2 = 0.02$, P = n.s.).

Table 3 Repeated measures ANOVA (type I sums of squares) for the densities of *Rhagoletis alternata* and *Diplolepis rosae*. The site was a random factor, while host species (*Rosa canina*, *R. corymbifera* and *R. rubiginosa*) and year were fixed factors. Differences in shrub characteristics were accounted for with the two principal components (*PC1* and *PC2*) derived in a PCA. PC1 characterises the size of a shrub (35% explained variance; variables with loading >0.6: height, diameter, diameter of the largest shoot) and PC2 the foliar density of a shrub (26% explained variance; loadings >0.6: density of leaflets and hips, leaf cover). *SS* Sum of square, *df* degrees of freedom, the *F* ratio was calculated with the appropriate error term; significant effects are marked $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and highlighted in bold

	Rh. alternata			*D. rosae*		
	SS	*df*	*F*	SS	*df*	*F*
PC1	0.16	1	2.24	0.59	1	1.24
PC2	0.20	1	2.71	16.93	1	**35.61*** **
Site (S)	38.73	16	**33.63*** **	34.33	16	**4.51*** **
Host (H)	1.15	2	**3.69***	12.86	2	**6.01****
S × H	4.98	32	**2.16*** **	34.21	32	**2.25*** **
Residuals	11.45	159		82.26	173	
Year (Y)	10.77	1	**48.09*** **	10.11	1	**18.14*** **
Y × PC1	0.18	1	**5.99***	0.02	1	0.06
Y × PC2	0.18	1	**6.21***	4.61	1	**17.45****
Y × S	3.58	16	**7.61*** **	8.92	16	**2.11****
Y × H	0.29	2	2.01	2.54	2	1.89
Y × S × H	2.28	32	**2.42*** **	21.53	32	**2.55*** **
Residuals	4.68	159		45.65	173	

5.3 *Does* Rhagoletis alternata *Form Host Races on the Three Dog Rose Species?*

Phytophagous insects may adapt to host plants, thereby forming host races as a first step during sympatric speciation. Host races are sympatric but genetically differentiated populations of exploiters that use different host species (Drès and Mallet 2002).

Vaupel et al. (2007) studied the population genetic structure of *Rh. alternata* along the geographic gradient and in relation to the three rose species. They collected larvae from 15 sites across Germany and from three valleys of Valais in Switzerland. They were able to score 9 allozyme loci (5 polymorphic). Populations from the three hosts did not differ in genetic variability. These results provide two further unexpected findings. Firstly, although they found significant genetic differentiation between populations from different host species, the differentiation was very low (0.9%) and cannot be interpreted as an indication for host races. A reason may be the permanent and ongoing hybridisation between rose species of the section *Caninae*. Secondly, they found surprisingly little geographic structure of genetic differentiation between populations of this fruit fly across central Europe. Additionally, analysis of amplified and sequenced fragments of the mitochondrial genes encoding cytochrome oxidase I (800 bp), cytochrome oxidase II (470 bp), and cytochrome *b* (450 bp) indicated that all individuals of *Rh. alternata* ($n = 21$)

from several sites in Europe shared the same haplotype (Kohnen et al 2009). This lack of genetic variation is unexpectedly low compared to data of other insect taxa ($p = 0.0016$, $n = 63$).

Three mutually non-exclusive reasons may explain these findings. Firstly, gene flow between populations of *Rh. alternata* is high. Secondly, the pattern of genetic differentiation is based on a recent expansion of the distributional range or a host shift of the fly. Thirdly, symbionts, such as *Wolbachia,* shape at least mtDNA evolution (Hurst and Jiggins 2005). During the initial phase of symbiont invasion, selective sweeps may reduce mtDNA diversity, thereby producing a genetic signal similar to that produced by a population bottleneck with subsequent expansion (Hurst and Jiggins 2005).

Because of the low gene flow estimated from allozyme data, *Rh. alternata* seems to be a good disperser (Leclaire and Brandl 1994; Vaupel et al. 2007). Even the Alps do not seem to be a geographical barrier for gene flow between populations. In part this is explained by the behaviour of the females which mark the hips with a pheromone after oviposition (Bauer 1986, 1998). Often a high proportion of hips (up to 100%) are infested. Females leave such localities and search for rose shrubs with a lower proportion of infested hips. Vaupel et al. (2007) found no isolation by distance ($r2 = 0.01$, $p = 0.19$), which may indicate that populations are not in an equilibrium between drift and gene flow (Hutchison and Tempelton 1999). The time to reach population genetic equilibrium increases with population size. The population size of tephritids is often very high (McPheron et al. 1988), and *Rh. alternata* is no exception. Low genetic differentiation and lack of isolation by distance points to a recent range expansion (Hutchison and Tempelton 1999). Such an expansion could be induced by colonisation events after the last ice ages or by very recent host-shift events. In both cases, a low number of founder individuals would lead to a population bottleneck. Lower levels of genetic variation would be expected (Harrison 1991). *Rh. alternata* is a specialist on members of the genus *Rosa* section *Caninae* and therefore dependent on the distribution of its host. These dog roses originated by hybridisation events during the last ice ages (Ritz et al. 2005a; Wissemann 2002) and recolonised Europe afterwards (Dingler 1907). Founder individuals of the fly species may have shifted to this new host, providing an explanation for the low genetic variability. Overall, the considerable gene flow between populations of *Rh. alternata* limited phenological differences between host species, and the ongoing hybridisation of hosts may prevent the formation of genetic differences between populations of exploiters on rose species.

6 How are the Differences Between the Three Closely Related Dog Rose Species Translated Into Higher Trophic Levels?

Due to the enclosed environment within the galls, gall-makers and their parasitoids provide a good opportunity to analyse tritrophic interactions. Gall characteristics like shape and toughness are plant-derived structures, but often regulated by insect

genes whereas the gall diameter, for example, is regulated by plant genotype (in *Salix lasiolepis*; Price and Clancy 1986). In turn, the galls' sizes and densities determine the success as well as composition of parasitoid communities (Brandl and Vidal 1987; Schlumprecht 1989; Weis 1983).

So far, we have shown certain differences in community structure and consumer density between the glandular rose *R. rubiginosa* and the other two rose species. The galls of *D. rosae* form the basis of a complex community of an inquiline and at least 12 species of parasitoids (Blair 1944; Redfern and Askew 1992). Besides *D. rosae*, the inquiline *Periclistus brandtii* R. (Hym. Cynipidae) and several parasitoid wasp species can be found within the galls (Redfern and Askew 1992). The gall-maker *D. rosae* is parasitised by at least five parasitoid species: *Orthopelma mediator* Thunb. (Hym. Ichneumonidae), *Torymus bedeguaris* L. (Hym. Torymidae), *Pteromalus bedeguaris* Thomson (Hym. Pteromalidae), *Glyphomerus stigma* Fabr. (Hym. Torymidae) and *Eupelmus urozonus* Dalman (Hym. Eupelmidae). The dominant one which is almost invariably present is *O. mediator* (Stille 1984). The inquilin *P. brandtii* utilises the galls to create its own chambers on the surface of the gall. The effect of *P. brandtii* attack on the gall is so far unknown, either it enlarges the gall or reduces the space otherwise available to *D. rosae*. The inquilin is also parasitised by *G. stigma* and *E. urozonus*, but additionally by *Caenacis inflexa* Ratzeburg (Hym. Pteromalidae) and *Eurytoma rosae* Nees (Hym. Eurytomidae).

We sampled all available *D. rosae* galls at eight of the sample sites (Fig. 4) and hatched the galls outside until all inhabitants emerged (Table 4). With a generalised linear model, the parasitism rate of *D. rosae* varied between sites as well as rose species and decreased with increasing gall volume (Klinge 2005; Kohnen et al. unpublished). Whereas the mean number of *D. rosae* galls was highest on *R. rubiginosa*, the mean parasitism rate was lowest on this rose species. But all two-way interactions between host species and site were also significant, pointing to complex effects of geography and rose species on the communities associated

Table 4 The inhabitants of *Diplolepis rosae* galls, based on rearings of $n = 299$ galls. Numbers of individuals are given for the gall-maker *D. rosae*, the inquilin *Periclistus brandtii* and the parasitoids [sum of individuals, mean ± standard error (back transformed values), the minimum and maximum]. Parasitoids of *D. rosae* are marked with *D* and parasitoids of *P. brandtii* with *P*

	Host	Sum of individuals	Mean	−SE	+SE	Maximum per gall
Diplolepis rosae		1,968	2.08	1.87	2.31	130
Periclistus brandtii		765	0.54	0.45	0.62	73
Orthopelma mediator	D	1,111	1.36	1.22	1.50	56
Glyphomerus stigma	D, P	751	1.14	1.03	1.26	28
Torymus bedeguaris	D	562	0.84	0.76	0.93	44
Pteromalus bedeguaris	D	377	0.67	0.61	0.74	24
Caenaxis inflexa	P	326	0.28	0.23	0.33	38
Eurytoma rosae	P	131	0.24	0.20	0.27	9
Eupelmus urozonus	D, P	105	0.18	0.15	0.21	20
Torymus rubi	D	32	0.06	0.05	0.08	4
Torymus spp.	?	16	0.03	0.02	0.04	3

with *D. rosae* galls. As mentioned above, *R. rubiginosa* is the only rose species we examined with glandular trichomes rich in secondary metabolites as sesquiterpenes. Host plant variation and differences in secondary metabolites often influence higher order interactions within insect communities (Eisenbach 1996; Fritz et al. 1997; Gange 1995; Marquis and Whelan 1996; Prezler and Boecklen 1994).

Beyond factors intrinsic to the host plant, local environmental conditions have also some influence on plant traits, e.g. resulting in different nutritive quality and thereby changing interactions with herbivores and their natural enemies (Moon and Stiling 2000; Price and Clancy 1986; Stiling and Rossi 1997). Even effects of genetic differences among and within host plant taxa may be modified by environmental conditions (Fritz et al. 1997). In the same way, Thompson (2005) suggests in his Geographic Mosaic Theory of Coevolution that interactions among species may be modified by environmental conditions. Thompson (2005) considers a dynamic process of varying species compositions, environmental conditions and subsequent variations in interactions. Our data show significant variations in community structures and consumer densities between geographical sites. Environmental variations among sites did not only alter abundance of *D. rosae* but also structure and success of higher trophic levels, the associated community of parasitoids (Klinge 2005; Kohnen et al. unpublished). During this study, it was investigated how the phenotypic plasticity of dog roses affects the next trophic levels. But interactions between the host plant species and herbivore communities changed depending not only on phenotypic differences between the host plant species but also on their geographical location.

7 Conclusion

The guiding question of our study was how the radiation and diversity of the hosts translate into the radiation and diversity of the higher trophic levels and what role interactions play in the whole system? We observed a major impact of the *Canina* breeding system on character evolution and thus influencing direct interaction between hosts and parasites. However, interactions between host plant species and herbivore communities changed depending not only on phenotypic differences between the host plant species but also on their geographical location. Thus, genetic diversity of dog roses is not translated into a host-specific radiation process of the parasites. We assume that the intensive reticulate evolution of dog roses via hybridisation prevents co-speciation.

Acknowledgements This study was enabled under the DFG-priority programme 1127: Radiationen – Genese Biologischer Diversität by financial support to Volker Wissemann (WI 2028/1), Franz Oberwinkler (OB 24/25), Teja Tscharntke (TS 45/16) and Roland Brandl (BR 1293/2). We are indebted to the DFG for providing us with the opportunity for an intensive 6-year cooperation project which, albeit under hard work pressure, established our scientific network, was great fun and formed friendships. We would like to thank our numerous collaborators who are too many to be named here individually. We are grateful to Konrad Bachmann (Gatersleben) and William Martin (Düsseldorf) for taking the initiative for the priority programme.

References

Adler H (1877) Beiträge zur Naturgeschichte der Cynipiden. Dt Ent Z 21:209–248
Alvarez-Castellanos PP, Bishop CD, Pasqual-Villalobos MJ (2001) Antifungal activity of the essential oil of flowerheads of garland chrysanthemum (*Chrysanthemum coronarium*) against agricultural pathogens. Phytochemistry 57:99–102
Andres MA, Connor EF (2003) The community-wide and guild specific effects of pubescence on the folivorous insects of manzanitas *Arctostaphylos* spp. Ecol Entomol 28:383–396
Bagatto G, Paquette C, Shorthouse JD (1996) Influence of galls of *Phanacis taraxaci* on carbon partitioning within common dandelion, *Taraxacum officinale*. Entomol Exp Appl 79:111–117
Bahçecioğlu Z, Yildiz B (2005) A study on the microfungi of Sivas Province. Turk J Bot 29:23–44
Bauer G (1986) Life history strategy of *Rhagoletis alternata* (Diptera:Tephritidae), a fruit fly operating in a "non-interactive" system. J Anim Ecol 55:785–794
Bauer G (1998) Structure and function of a non-interactive insect–plant system. Oecologia 115:154–160
Bayer MH (1992) Biochemical modification of the phenotype in cynipid galls. In: Williams MAJ (ed) Plant galls organisms, Interactions, Population. Clarendon, Oxford, pp 429–446
Blair KG (1944) A note on the economy of the rose bedeguar gall, *Rhodites rosae*, L. Proc Trans S Lond Entomol Nat Hist Soc 1943–44:55–59
Brandenburger W (1994) Die Verbreitung der in den westlichen Ländern der Bundesrepublik Deutschland beobachteten Rostpilze (Uredinales). Regensb Mykol Schr 3:1–381
Brandl R, Vidal S (1987) Ovipositor length in parasitoids and tentiform leaf mines - adaptations in eulophids (Hymenoptera, Chalcidoidea). Biol J Linn Soc 32:351–355
Brandl R, Mann W, Sprinzl M (1992) Estimation of the monocot-dicot age through tRNA sequences from the chloroplast. Proc R Soc Lond B 249:13–17
Brändle M, Brandl R (2001) Species richness of insects and mites on trees: expanding Southwood. J Anim Ecol 70:491–504
Bronner R (1992) The role of nutritive cells in the nutrition of cynipids and cecidomyiids. In: Shorthouse JD, Rohfritsch O (eds) Biology of insect-induced galls. Oxford University Press, New York, pp 118–140
Bruneau A, Starr JR, Joly S (2007) Phylogenetic relationships in the genus *Rosa*: new evidence from chloroplast DNA sequences and an appraisal of current knowledge. Syst Bot 32:366–378
Bush GL (1992) Host race formation and sympatric speciation in *Rhagoletis* fruit flies (Diptera: Tephritidae). Psyche 99:335–357
Cakir A, Kordali S, Zengin H, Izumi S, Hirata T (2004) Composition and antifungal activity of essential oils isolated from *Hypericum hyssopifolium* and *Hypericum heterophyllum*. Flav Frag J 19:62–68
Clay K (1989) Clavicipataceous fungal endophytes of grasses coevolution and the change from parasitism to mutualism. In: Pirozynski KA, Hawksworth DL (eds) Coevolution of fungi with plants and animals. Academic, London, pp 79–106
Crawley MJ, Long CR (1995) Alternate bearing, predator satiation and seedling recruitment in *Quercus robur*. J Ecol 83:683–696
Dingler H (1907) Versuch einer Erklärung gewisser Erscheinungen in der Ausbildung und Verbreitung der wilden Rosen. Mitt Naturwiss Ver Aschaffenburg 6:1–38
Doebley J (2004) The genetics of maize evolution. Ann Rev Genet 38:37–59
Drès M, Mallet J (2002) Host races in plant-feeding insects and their importance in sympatric speciation. Philos Trans R Soc Lond B 357:471–492
Eigner A, Wissemann V (1999) *Rosa* X*mangii*, eine neue intersektionelle Hybride charakterisiert durch morphologische und genetische Untersuchungen. Haussknechtia 7:35–40
Eisenbach J (1996) Three-trophic-level interactions in cattail hybrid zones. Oecologia 105: 258–265

Eisner T, Eisner M, Hoebeke ER (1998) When defense backfires: detrimental effect of a plant's protective trichomes on an insect beneficial to the plant. PNAS 95:4410–4414
El-Gazzar A (1981) Chromosome numbers and rust susceptibility as taxonomic criteria in Rosaceae. Plant Syst Evol 137:23–38
Evans KJ, Jones MK, Mahr FA, Roush RT (2000) DNA Phenotypes of the blackberry biological control agent, *Phragmidium violaceum*, in Australia. Australas Plant Pathol 29(4):249–254
Fagerlind F (1945) Die Bastarde der *canina*-Rosen, ihre syndese-und Formbildungsverhältnisse. Acta Horti Berg 14:7–37
Ferrari J, Kruess A, Tscharntke T (1997) Auswirkungen der Fragmentierung von Rosenbüschen auf deren Insektenlebensgemeinschaften (Effects of rosebush fragmentation on insect communities). Mitt Dtsch Ges Allg Angew Entomol (Bayreuth 1997) 11:87–90
Feuerhahn B, Spethmann W (1995) Kreuzungen bei Wildrosenarten. Gehölzforschung 3, Institut für Obstbau und Baumschule, Hannover
Floate KD, Whitham TG (1993) The "Hybrid Bridge" hypothesis: host shifting via plant hybrid swarms. Am Nat 141:651–662
Frenzel M, Brandl R (1998) Diversity and composition of phytophagous insect guilds on Brassicaceae. Oecologia 113:391–399
Fritz RS, McDonough SE, Rhoads AG (1997) Effects of plant hybridization on herbivore-parasitoid interactions. Oecologia 110:360–367
Fritz RS, Price PW (1988) Genetic variation among plants and insect community structure: willow and sawflies. Ecology 69:845–856
Gange AC (1995) Aphid performance in an Alder (*Alnus*) hybrid zone. Ecology 76:2074–2083
Gäumann E (1959) Rostpilze Mitteleuropas mit besonderer Berücksichtigung der Schweiz. Büchler, Bern
Gustafsson A (1944) The constitution of the *Rosa canina* Complex. Hereditas 30:405–428
Harper LJ, Schönrogge K, Lim KY, Francis P, Lichtenstein CP (2004) Cynipid galls: insect-induced modifications of plant development create novel plant organs. Plant Cell Environ 27:327–335
Harrison RG (1991) Molecular changes at speciation. Annu Rev Ecol Syst 22:281–308
Hilker M, Rohfritsch O, Meiners T (2002) The plant's response towards insect egg deposition. In: Hilkers M, Meiners T (eds) Chemoecology of insect eggs and egg deposition. Blackwell, Berlin, pp 205–233
Hodkinson ID (1997) Progressive restriction of host plant exploitation along a climatic gradient: the willow psyllid *Cacopsylla groenlandica* in Greenland. Ecol Entomol 22:47–54
Hurst GDD, Jiggins FM (2005) Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. Proc R Soc Lond B 272:1525–1534
Hutchison DW, Tempelton AR (1999) Correlation of pairwise genetic and geographic distance measures: inferring the relative influences of gene flow and drift on the distribution of genetic variability. Evolution 53:1898–1914
Joly S, Starr JR, Lewis WH, Bruenau A (2006) Polyploid and hybrid evolution in roses east of the Rocky Mountains. Am J Bot 9:412–425
Joly S, Bruenau A (2006) Incorporating allelic variation for reconstructing the evolutionary history of organisms from multiple genes: an example from Rosa in North America. Syst Biol 55:623–636
Kato K, Hijii N (1997) Effects of gall formation by *Dryocosmus kuriphilus* Yasumatsu (Hym., Cynipidae) on the growth of chestnut trees. J Appl Emtomol 121:9–15
Klášterská I, Natarajan AT (1974) Cytological studies of the genus *Rosa* with special reference to the section *Caninae*. Hereditas 76:97–108
Klášterská I (1969) Cytology and some chromosome numbers of Czechoslovak Roses I. Folia Geobot Phytotax, Praha 4:175–189
Klášterská I (1971) Une contribution au probleme de la reproduction sexuelle chez le roses de la section Caninae. Ann Univ et ARERS 9:140–44

Klinge K (2005) Pflanzen-Herbivore-Parasitoid Interaktionen auf Wildrosenarten und ihren Hybriden entlang eines geographischen Gradienten, Agroecology. PhD thesis, University of Göttingen, Germany

Kohnen A, Wissemann V, Brandl R (2009) No genetic differentiation of rose-infesting fruit flies *Rhagoletis alternata* and *Carpomya schineri* (Diptera: Tephritidae) across central Europe. Eur J Entomol 106:315–321

Koptur S (1985) Alternative defence against herbivores in Inga (Fabaceae: Mimosidae) over an elevational gradient. Ecology 66:1629–1650

Kovarik A, Werlemark G, Leitch AR, Souckova-Skalicka K, Lim YK, Khaitova L, Koulkalova B, Nybom H (2008) The asymmetric meiosis in pentaploid dog roses (*Rosa* sect. *Caninae*) is associated with a skewed distribution of rRNA gene families in the gametes. Heredity 101:359–367

Lawton JH (1986) Surface availability and insect community structure: effects of architecture and fractal dimension of plants. In: Juniper B, Southwood R (eds) Insects and the plant surface. Edward Arnold, London, pp 317–331

Leather SR (1986) Insect species richness of the British Rosaceae: the importance of host range, plant architecture, age of establishment, taxonomic isolation and species-area relationships. J Anim Ecol 55:841–860

Leclaire M, Brandl R (1994) Phenotypic plasticity and nutrition in a phytophagous insect: consequences of colonizing a new host. Oecologia 100:379–385

Lim KY, Werlemark G, Matyasek R, Bringloe JB, Sieber V, El Mokadem H, Meynet J, Hemming J, Leitch AR, Roberts AV (2005) Evolutionary implications of permanent odd polyploidy in the stable sexual, pentaploid of *Rosa canina* L. Heredity 94:501–506

Maddox GD, Root RB (1987) Resistance to 16 diverse species of herbivorous insects within a population of goldenrod; *Solidago altissima*: genetic variation and heritability. Oecologia 72:8–14

Marquis RJ, Whelan C (1996) Plant morphology, and recruitment of the third tropic level: Subtle and little-recognized defenses? Oikos 75:330–334

Matsumoto S, Kouchi M, Yabuki J, Kusunoki M, Ueda Y, Fukui H (1998) Phylogenetic analysis of the genus *Rosa* using the *mat*K sequence: molecular evidence for the narrow genetic background of modern roses. Sci Hort 77:73–82

McPheron BA, Smith DC, Berlocher SH (1988) Microgeographic genetic variation in the apple maggot *Rhagoletis pomonella*. Genetics 119:445–451

Moon DC, Stiling P (2000) Relative importance of abiotically induced direct and indirect effects on a salt-marsh herbivore. Ecology 81:470–481

Nybom H, Esselink GD, Werlemark G, Vosman B (2004) Microsatellite DNA marker inheritance indicates preferential pairing between two highly homologous genomes in polyploid and hemisexual dog roses. Heredity 92:139–150

Nybom H, Esselink GD, Werlemark G, Leus L, Vosman B (2006) Unique genomic configuration revealed by microsatellite DNA in polyploid dog roses. *Rosa* sect. Caninae. J Evol Biol 19:635–648

Pirozynski KA, Hawksworth DL (1989) Coevolution of fungi with plants and animals; introduction and overview. In: Pirozynski KA, Hawksworth DL (eds) Coevolution of fungi with plants and animals. Academic, London, pp 1–30

Prezler RW, Boecklen WJ (1994) A three-trophic-level analysis of the effects of plant hybridization on a leaf-mining moth. Oecologia 100:66–73

Price PW, Clancy KM (1986) Multiple effects of precipitation on *Salix lasiolepis* and populations of the stem-galling sawfly, *Euura lasiolepis*. Ecol Res 1:1–14

Ranger CM, Hower AA (2002) Glandular trichomes on perennial alfalfa affect host-selection behavior of *Empoasca fabae*. Entomol Exp Appl 105:71–81

Redfern M, Askew RR (1992) Plant galls. Naturalists' handbooks 17. Richmond, Slough, pp 1–99

Reichert H (1998) Beobachtungen und Versuche zur Fortpflanzung der Apfelrose, *Rosa villosa* L. (*R. pomifera* J. Herrmann). Delattinia 24:159–166

Ritz CM, Wissemann V (2003) Male correlated non-matroclinal character inheritance in reciprocal hybrids of *Rosa* section *Caninae* (DC) Ser. (Rosaceae). Plant Syst Evol 241:213–221
Ritz CM, Schmuths H, Wissemann V (2005a) Evolution by reticulation: European dog roses originated by multiple hybridization across the genus *Rosa*. J Hered 96:4–14
Ritz CM, Maier WFA, Oberwinkler F, Wissemann V (2005b) Different evolutionary histories of two *Phragmidium* species infecting the same dog rose hosts. Mycol Res 109:603–609
Ritz CM, Wissemann V (submitted) Microsatellite analyses of artificial and spontaneous dogrose hybrids reveal the hybridogenic origin of Rosa micrantha by the contribution of unreduced gametes
Roberts AV (1975) The nature and taxonomic significance of the system of inheritance in *Rosa nanothamnus* (Rosaceae). Bot J Linn Soc 71:59–66
Savile DBO (1979) Fungi as aids in higher plant classification. Bot Rev 45:377–503
Schlumprecht H (1989) Dispersal of the thistle gallfly *Urophora cardui* and its endoparasitoid *Eurytomas serratulae* (Hymenoptera, Eurytomidae). Ecol Entomol 14:341–348
Scholler M (1994) Die Erysiphales, Pucciniales und Ustilaginales der Vorpommerschen Boddenlandschaft. Regensb Mykol Schr 6:1–325
Schoonhoven LM, Jermy T, von Loon JJA (1998) Insect-plant biology. From physiology to evolution. Chapmann & Hall, London
Schröder D (1967) *Diplolepis* (=*Rhodites*) *rosae* (L.) (Hym.: Cynipidae) and a review of its parasite complex in Europe. Tech Bull Commonwealth Inst of Biol Control 9:93–131
Shorthouse JD, Wool D, Raman A (2005) Gall-inducing insects – nature's most sophisticated herbivores. Basic Appl Ecol 6:407–411
Stechmann D-H, Bauer G, Dreyer W, Heusinger G, Zwölfer H (1981) Die Erfassung der Entomofauna von Heckenpflanzen (Wildrose, Schlehe, Weißdorn) mit Hilfe der Klopfprobenmethode. Mitt Dtsch Ges Allge Angew Entomol 3:12–26
Stiling P, Rossi AM (1997) Experimental manipulations of top-down and bottom-up factors in a tri-trophic system. Ecology 78:1602–1606
Stille B (1984) The effect of host plant and parasitoids on the reproductive success of the parthenogenetic gall wasp *Diplolepis rosae* (Hymenoptera, Cynipidae). Oecologia 63:364–369
Stone GN, Schönrogge K, Atkinson RJ, Belldio D, Pujade-Villar J (2002) The population biology of oak gall wasps (Hymenoptera: Cynipidae). Annu Rev Entomol 47:633–668
Strong DR, Lawton JH, Southwood R (1984) Insects on plants. Community patterns and mechanisms. Blackwell, Oxford
Täckholm G (1920) On the cytology of the genus *Rosa*. A preliminary note. Svensk Bot Tidskr 14 (2/3):300–311
Täckholm G (1922) Zytologische Studien über die Gattung *Rosa*. Acta Hort Berg 7(3):97–381
Thompson JN (2005) The geographic mosaic of coevolution. University of Chicago press. Chicago. IL
Timmermann G (1998) Beobachtungen zur Phänologie der heimischen Wildrosen in Rottenburg am Neckar. Acta Rhodologica 1:7–14
Tscharntke T, Greiler HJ (1995) Insect communities, grasses, and grasslands. Annu Rev Entomol 40:535–558
Valkama E, Koricheva J, Salminen J-P, Helander M, Saloniemi I, Saikkonen K, Pihlaja K (2005) Leaf surface traits: overlooked determinants of birch resistance to herbivores and foliar microfungi? Trees 19:191–197
Vaupel A, Klinge K, Brändle M, Wissemann V, Tscharntke T, Brandl R (2007) Genetic differentiation between populations of the European rose hip fly *Rhagoletis alternata*. Biol J Linn Soc 90:619–625
Weis AE (1983) Patterns of parasitism by *Torymus capite* on hosts distributed in small patches. J Anim Ecol 52:867–877
Werlemark G, Uggla M, Nybom H (1999) Morphological and RAPD markers show a highly skewed distribution in a pair of reciprocal crosses between hemisexual dog rose species. Rosa sect. Caninae. Theor Appl Genet 98:557–563

Werlemark G, Nybom H (2001) Skewed distributionof morphological character scores and molecular markers in three interspecific crosses in *Rosa* sect. Caninae. Hereditas 134:1–13

White I (1988) Tephritid flies (Handbooks for the identification of British insects 10, part 5a). Royal Entomological Society, London

Whitham TG, Young WP, Martinsen GD, Gehring CA, Bailey JK, Lindroth RL, Woolbright S, Kuske CR (2003) Community and ecosystem genetics: a consequence of the extended phenotype. Ecology 84:559–573

Wissemann V, Hellwig FH (1997) Reproduction and hybridisation in the genus *Rosa*, section Caninae (Ser.) Rehd. Bot Acta 110:251–256

Wissemann V (1999) Genetic constitution of *Rosa* sect. *Caninae* (*R. canina, R. jundzillii*) and sect. *Gallicanae* (*R. gallica*). Angew Bot 73:191–196

Wissemann V (2000a) Epicuticular wax morphology and the taxonomy of *Rosa* (section *Caninae,* subsection *Rubiginosae*). Plant Syst Evol 221:107–112

Wissemann V (2000b) Molekulargenetische und morphologisch-anatomische Untersuchungen zur Evolution und Genomzusammensetzung von Wildrosen der Sektion *Caninae* (DC.) Ser. Bot Jahrb Syst 122(3):357–429

Wissemann V (2002) Molecular evidence for allopolyploid origin of the *Rosa canina*-complex (Rosaceae, Rosoideae). J Appl Bot 76:176–178

Wissemann V (2003) Conventional taxonomy of wild roses. In: Roberts A, Debener T, Gudin S (Hrsg): Encyclopedia of Rose science. Elsevier, London 111–117

Wissemann V, Ritz CM (2005) The genus Rosa (Rosoideae, Rosaceae) revised: moleculars analysis of nrITS-1 and *atpB-rbcl* intergenic spacer (IGS) versus conventional taxonomy. Bot J Linn Soc 147:275–290

Wissemann V, Ritz C (2007) Evolutionary patterns and processes in the genus *Rosa* (Rosaceae) and their implications for host-parasite co-evolution Pl. Syst Evol 266:79–90

Wissemann V, Gallenmüller F, Ritz C, Steinbrecher T, Speck T (2006) Inheritance of growth form and mechanical characters in reciprocal polyploid hybrids of *Rosa* section *Caninae* – implications for the ecological niche differentiation and radiation process of hybrid offspring. Trees-Structure Funct 20:340–347

Wissemann V, Riedel M, Riederer M (2007) Matroclinal inheritance of cuticular waxes in reciprocal hybrids of *Rosa* sect. *Caninae* (Rosaceae). Plant Syst Evol 263:181–190

Yencho GC, Tingey WM (1994) Glandular trichomes of *Solanum berthaultii* alter host preference of the colorado potatoe beetle, *Leptinotarsa decemlineata*. Entomol Exp Appl 70:217–225

Zielinski J (1986) Studia nad rodzajem *Rosa* L. - Systematyka sekcji *Caninae* DC. em Christ. Arboretum Kórnickie. Polska Akademia Nauk. Instytut Dendrologii. Panstwowe wydawnictwo Naukowe. Warszawa & Poznan 1986. Rocznik XXX:3–109

Zvereva EL, Kozlov MV, Niemelä P (1998) Effects of leaf pubescence in Salix borealis on host–plant choice and feeding behavior of the leaf beetle, *Melanosoma lapponica*. Entomol Exper Appl 89:297–303

Zwölfer H, Bauer G, Heusinger G (1981) Ökologische Funktionsanalyse von Feldhecken – Tierökologische Untersuchungen über Struktur und Funktion biozönotischer Komplexe. Schlußbericht an das Bayerische Landesamt für Umweltschutz, München

Zwölfer H, Bauer G, Heusinger G, Stechmann D (1984) Die tierökologische Bedeutung und Bewertung von Hecken. Beiheft 3, Teil 2, zu den Berichten der Akademie für Naturschutz und Landschaftspflege. Laufen

Speciation via Differential Host–Plant Use in the Tephritid Fly *Tephritis conura*

Jes Johannesen, Thorsten Diegisser, and Alfred Seitz

Abstract The close association between phytophagous insects and host plants and the possibility for specialization on new plants make phytophagous insects prime candidates for sympatric speciation via host-race evolution. In this chapter, we summarize results addressing host-race evolution in the tephritid fly *Tephritis conura* (Tephritidae) infesting *Cirsium heterophyllum* and *C. oleraceum* (Asteraceae). Host plant distributions in allopatry, sympatry and parapatry, and different infestation patterns enabled us to test geographic speciation scenarios, investigate adaptations, and address the importance of plant population history for diversification of *T. conura*.

1 Introduction

Phytophagous insects are immensely speciose, making up an estimated 25–40% of all animal species (Bush and Butlin 2004). This species diversity is thought to be driven largely by the inclusion of new host plants followed by adaptation to these plants (Bush 1975). The inclusion and adaptation to new host plants, however, has two outcomes with significantly different evolutionary implications. In the first case, an insect population may be pre-adapted and able to use the novel host without losing its ability to use old hosts. Such a "host-range expansion" (sensu Hare 1990) may happen either when no significant adaptations are required or if adaptations to the new host can evolve without fitness loss on the old host. In either case, the population will consist of polyphagous generalists and no host-related population

J. Johannesen (✉), T. Diegisser and A. Seitz
Institut für Zoologie, Abteilung Ökologie, Universität Mainz, Saarstrasse 21, 55099 Mainz, Germany
e-mail: jesjo@uni-mainz.de

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_12,

differentiation is expected to evolve. The second incidence occurs if populations cannot simultaneously adapt to the new and the old host. In this case, host–plant-associated fitness trade-offs may evolve (Rausher 1984; Via 1990). This so-called "host shift" may result in partially reproductively isolated insect populations called "host races" (Diehl and Bush 1984). Host races can be defined as two populations that differ consistently at one or more loci, have at least one adaptive trait that reduces gene flow, but still possess the ability for gene flow and reproduction (Berlocher and Feder 2002). Host races have been documented for a variety of insect groups (Bush 1969; Wood and Guttman 1982; Katakura and Hosogai 1994; Sezer and Butlin 1998; Via 1999; Emelianov et al. 2001; Diegisser et al. 2004, 2006a, b; Ohshima 2008), lending support for the notion that speciation via differential host use is common in phytophagous insects (Dres and Mallet 2002). In a study of insect species infesting *Solidago altissima* and *S. gigantea*, Stireman et al. (2005) found evidence for host-associated genetic divergence in four out of nine species.

Because the presence of both the traditional and the novel host plant are required in the initial phase of host shifts, speciation via host-race evolution is a prime candidate for sympatric speciation. The plausibility of sympatric speciation via host-shifts has been shown in empirical (Bush and Smith 1998; Feder et al. 1998; Via 1999) and theoretical studies (Rice 1984; Kawecki 1996, 1997). However, alternative scenarios are difficult to exclude completely. It is particularly difficult to rule out allopatric and/or parapatric phases during the speciation process (Coyne and Orr 2004). If the primary host is scarce or becomes extinct, the geographical context in which host shifts take place may have strong allopatric components. This can happen, for example, when geographic ranges increase. In the event of scarcity of the traditional host plant, an insect may be forced to colonize a new or suboptimal plant and this in turn may lead to specialization on different plants in isolation (Janz et al. 2006). Larson and Ekbom (1995) found that host shifts might be incidental if the proportion of poor host plants is large and the time for oviposition is short. These findings, moreover, suggest that newly colonized environments may raise the probability of diversification.

Aside from the debate of sympatric versus allopatric speciation, population history of the putative host plants themselves may also be of great relevance for diversification of insect populations. For example, genetic bottlenecks in plant populations may alter the genetic constitution of the plants by increasing inbreeding, which, in some instances, may enhance susceptibility to infestation (Carr and Eubanks 2002; Stephenson et al. 2004; Hull-Sanders and Eubanks 2005; Koslow and DeAngelis 2006). Thus, there is a strong geographic component affecting both plants and parasites and their interaction, and hence the mode of speciation. Perhaps this is why there is only a little evidence for strict co-phylogenies between plants and parasites but more for the importance of geography (Thompson 2005; but see Becerra and Venable, 1999). Elucidating the geographic scale of genetic diversification and of plant infestations between host races provides an opportunity to gain insights into the processes driving divergence.

Fruit flies (Tephritidae) form a speciose group of about 4,500 described species and subspecies (Norrbom 2004). Most species are specialists on one or a few plants (Aluja and Norrbom 2000). In some incidences, host–plant associations, more than morphology, delimit species (e.g., Kovac et al. 2006). The Tephritidae have several well-documented host races, including species in the genera *Eurosta* (Craig et al. 1993), *Rhagoletis* (Bush 1969; Feder et al. 1998) and *Tephritis* (Diegisser et al. 2004, 2006a, b). The Tephritidae are thus prime targets for speciation via host-race evolution. However, several studies either have found no or ambiguous evidence for host races (Leclaire and Brandl 1994; Schwarz et al. 2003; Stireman et al. 2005; Vaupel et al. 2007). To understand the importance of speciation via host-race evolution in phytophagous insects, knowledge of the relative frequency of host-range expansions (phenotypic plasticity) and host shifts (specialization) and under what conditions the two differ is important.

The univoltine tephritid *T. conura* (Loew 1844) infests several thistles of the genus *Cirsium* (Asteraceae) (Zwölfer 1988; Romstöck-Völkl 1997). Interestingly, *T. conura* does not attack every host over its entire distribution range, and mixed stands of different host species exist where only one host is attacked (Romstöck-Völkl 1997). Many *Cirsium* species are largely allopatric in their distribution while many also exhibit contact zones and areas of true sympatry. Two species, *C. heterophyllum* (L.) Hill. (melancholy thistle) and *C. oleraceum* (L.) Scop. (cabbage thistle), harbor host races of *T. conura* (Seitz and Komma 1984; Komma 1990; Romstöck-Völkl 1997; Diegisser et al. 2006a, b). The two plants are found in sympatry, parapatry and allopatry in different parts of their distribution ranges. In this chapter, we summarize research on the evolution of *T. conura* host races using the geographic distributions to address the geographic context and the direction of host-race evolution, investigate adaptations, and address the importance of plant population history in diversification of *T. conura*.

2 Natural History of the *T. conura – Cirsium* System

2.1 T. conura

T. conura infests several species of the genus *Cirsium*. The two most important host plants, *C. oleraceum* and *C. heterophyllum*, are attacked across their wide distributions from western Europe to the Ural Mountains in Russia. Three host species, *C. acaule*, *C. spinossisimum* and *C. eristhales,* have distribution centres in the region of the European Alps (Hegi, 1987) and are here locally attacked. In other incidences, *T. conura* probably exploits *Cirsium* species on a temporary basis only, but exact data are missing. In one extreme case, the host *C. palustre* is infested only in northern Britain but not in continental Europe in areas of sympatry with the two main hosts (Romstöck and Arnold 1987; Johannesen et al. 2008). The general

infestation pattern of *T. conura* suggests that it has recently expanded its host range on plants associated with the European Alps (J. Johannesen, unpublished data). Seitz and Komma (1984) and Komma (1990) showed that flies utilizing *C. oleraceum* and *C. heterophyllum* in the German Fichtel Mountains differed in allele frequencies at the allozyme locus hexokinase (*Hex*), suggesting genetic host races of *T. conura*. Flies infesting other *Cirsium* species can be divided into the *C. oleraceum* and *C. heterophyllum* host-race groups (Romstöck-Völkl 1997; Johannesen et al., unpublished results).

Mating in *T. conura* is associated with the host plants. Both host races lay eggs into small apical buds. Flies infesting *C. heterophyllum* insert eggs through the top of the freely exposed flower heads. By contrast, buds of *C. oleraceum* are enveloped by small leaflets, which must be pierced by the fly's ovipositor to lay eggs in the flower head (Eschenbacher 1982; Romstöck 1982). Within the developing flower heads, first instar larvae feed on the florets while second and third instars use the receptacle area. Pupation starts roughly 3 weeks after oviposition and imagos emerge 3 weeks after pupation. Hence, adults hibernate the winter. In spring, flies can be found on host plants before bud development, re-emerging from unknown places. Winter hibernation is required for flies to mature sexually (Diegisser 2005). Adult hibernation means that adults of the two host races have the potential to meet at sympatric sites at the onset of spring (Fig. 1).

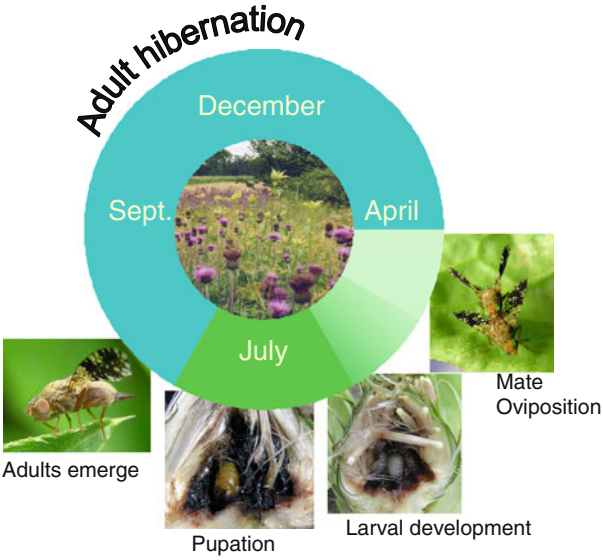


Fig. 1 Life cycle of *T. conura*. Flies for analysis were sampled during the pupae stage by collecting infested flowers. All flies used in analyses emerged in the laboratory. Flies for genetic analyses were frozen immediately upon emergence. Flies for behavioral studies were separated by sex, removed from the host plants, and exposed to winter dormancy for approximately 2 months. This procedure excludes imprinting by host plant and mate

2.2 The Host Plants

In this section, we focus on host-race divergence of *T. conura* associated with *C. heterophyllum and C. oleraceum*. The two plants are the most widely distributed of the *Cirsium* species known to be infested. Due to different habitat requirements, they are largely allopatrically distributed in Europe (Hegi 1987). The distribution of *C. oleraceum* is more or less continuous from eastern France to Russia, from sea level to approximately 1,200 m (Fig. 2). The southern distribution limit is northern Italy and the northern Balkans; in the north, it is found in southern Scandinavia and the West Siberian Lowlands. It is absent on the British Isles. *C. heterophyllum* is associated with northern boreal and subalpine to alpine environments. It is distributed contiguously in central Scandinavia and Finland, across the Baltic States to Siberia. In Central Europe and Britain it is restricted to higher altitude ranges, e.g., in the Alps, Carpathians, Ore Mountains and the Bavarian Fichtel Mountains, the Tatra Mountains and northern British highlands (Fig. 2). In these mountain ranges (except in Britain), the two plants may be found parapatrically with *C. heterophyllum* at high altitudes and *C. oleraceum* at lower altitudes, with range overlap at intermediate altitudes. The two plants occur in true sympatry (i.e., syntopic) in southern Sweden and parts of Russia.

The main flowering period of *C. heterophyllum* is May–June but extends into August at very high altitudes. The main flowering period of *C. oleraceum* is July–August. At syntopic sites, *C. heterophyllum* starts producing buds 2–3 weeks earlier than *C. oleraceum*. Due to the geographic and temporal separation,

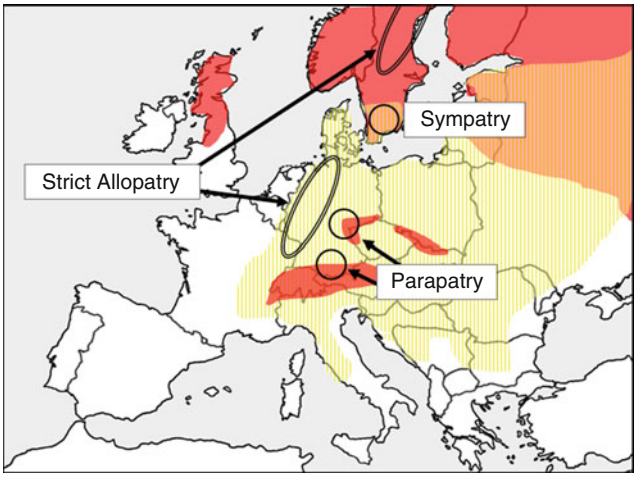


Fig. 2 Main distribution areas of *C. heterophyllum* (*red*) and *C. oleraceum* (*yellow*) and their common occurrence (*orange*) in Europe. *Circles* show regions used for analysis of host races where plants occur in strict allopatry (German lowlands and northern Sweden), parapaptry (altitude gradients in the Alps and Fichtel Moutains) and sympatry (southern Sweden). At syntopic sites, phenology differs by 2–3 weeks, *C. heterophyllum* being first

the two host plants are effectively only syntopic for oviposition by *T. conura* within a short time span when late buds of *C. heterophyllum* and early buds of *C. oleraceum* are available.

3 Geographic Speciation

Knowledge of the geographic component of genetic differentiation between incipient species provides an opportunity for studying diversification processes because speciation scenarios in distinct geographic settings are based on different genetic model assumptions. For example, the predominant mode of speciation is traditionally assumed to happen in geographically isolated populations (Mayr 1963). Although environmental adaptation may occur within such isolates, the spread of alleles responsible for reproductive incompatibility (i.e., speciation) is a passive consequence of separation, and in this sense speciation is non-adaptive (Turelli et al. 2001; Presgraves et al. 2003). In contrast, speciation within a shared, sympatric area assumes an adaptational process by restricting gene flow between two populations via disruptive selection against intermediate forms (Kawecki 1997; Dieckmann and Doebeli 1999). Because sympatric speciation requires linkage between fitness and reproduction "genes" it has been highly debated (Coyne and Orr 2004).

The debate today is not whether sympatric speciation is possible but rather one of its relative importance (Mallet 2001) and of the definition of "isolation" (Fitzpatrick et al. 2008). In phytophagous insects, for example, different host–plant phenologies may make sympatric insect populations effectively allopatric in time (Wood and Guttman 1982). This may cause restricted gene flow in sympatry even in the absence of selection (Butlin 1990). If habitat selection is strong, adaptive divergence in any sympatric area with environmental distinct habitats can potentially reduce gene flow, which further may enhance divergence (Mallet 2001). A question regarding sympatric speciation is also whether it should be thought of as a process strictly occurring among populations with identical starting conditions. Divergence may begin in allopatry and reinforcement selection in sympatry could take over later (Kondrashov et al. 1998; Feder et al. 2003; Michel et al. 2007). In the now classic comparative study of species-pairs in *Drosophila*, Coyne and Orr (1989, 1997) found that sympatric species-pairs tend to show more prezygotic isolation than allopatric pairs at low genetic distances, implying that reproductive isolation occurs by some sort of reinforcement and more rapidly in sympatry than in allopatry. However, these studies cannot completely rule out allopatric phases during speciation. The difficulty of separating allopatric from sympatric speciation events is particularly pertinent to speciation via host-race evolution, which will often take place in open environments (see Coyne and Orr 2004 for "requirements" for sympatric speciation; Fitzpatrick et al. 2008) such as those experienced by the *T. conura–Cirsium* system where geographic range alterations during the ice ages may initiate diversification in allopatry but be completed in sympatry. For example,

genetic variation promoting sympatric host-race formation in *Rhagoletis pomonella* may have had an allopatric source (Feder et al. 2003).

In the study of *T. conura,* we used the unique combination of sympatric, parapatric and allopatric distributions of *C. heterophyllum* and *C. oleraceum* to address the geographic context (this subchapter) and processes (Sect. 4) involved in host-race evolution. The analyses of the geographic context of host-race evolution were based on the distribution of mitochondrial DNA (mtDNA) variation and on coalescence theory predictions, where the distribution of shared and/or unique polymorphisms may specify distinct geographic histories (see Berlocher 1998; Berlocher and Feder 2002). Furthermore, the direction of host shifts can be inferred by amounts and geographic patterns of variation, and allele phylogenies of the host races (Harrison 1991).

Sixty-one *T. conura* populations (33 *C. heterophyllum* sites, $N_{ind} = 128$; 28 *C. oleraceum* sites, $N_{ind} = 113$) were analyzed for mtDNA variation from the three geographic settings (Fig. 2), together with flies from Scotland, Croatia, Finland, and Russia. The host races differed significantly in the distribution and genealogical diversity of mtDNA variation. Flies infesting *C. heterophyllum* were more diverse and geographically structured than flies infesting *C. oleraceum* (mean number of haplotypes = 2.27 vs 1.67, $P < 0.01$; nucleotide diversity (0.00137 vs 0.00037, $P < 0.0001$). Flies infesting *C. heterophyllum* had several common haplotypes that were regionally structured (Fig. 3). By contrast, flies infesting *C. oleraceum* had a genealogically star-like mtDNA network with one dominant haplotype (H1) and were not structured geographically. The haplotype H1 was common to both host races. Assuming that high genetic diversity can be interpreted in terms of the original host, and because the predominant *C. oleraceum* haplotype H1 was located within the *C. heterophyllum* network and there was no long branch from *C. heterophyllum* populations to *C. oleraceum* populations, we hypothesize that the host shift happened relatively recently from *C. heterophyllum* to *C. oleraceum.*

The direction and age of the host shift was supported by demographic analyses of population equilibrium using Tajima's *D* test (Tajima 1989) and Fu's F_s test (Fu 1997) – after ruling out selection on mtDNA, and using mismatch distributions (Harpending et al. 1998). Population equilibrium was rejected for *C. oleraceum* in all three geographic settings and over all populations; highly significant negative F_s values indicated an excess of recent mutations and supported a demographic expansion of the *C. oleraceum*-infesting populations. By contrast, *T. conura* infesting *C. heterophyllum* did not show significant deviations from equilibrium in any geographic setting. Looking at the mismatch distributions, neither host-race differed significantly from a sudden expansion model but the mismatch did differ significantly. *C. heterophyllum* flies showed a unimodal, left-truncated distribution, while the mismatch for flies infesting *C. oleraceum* was L-shaped due to very low molecular diversity. The demographic analyses thus support a recent and rapid demographic expansion of *C. oleraceum* flies in all regions and across its Eurasian geographic range.

Flies infesting *C. oleraceum* were most diverse in the European Alps. Outside this area, populations became progressively less polymorphic away from the Alps.

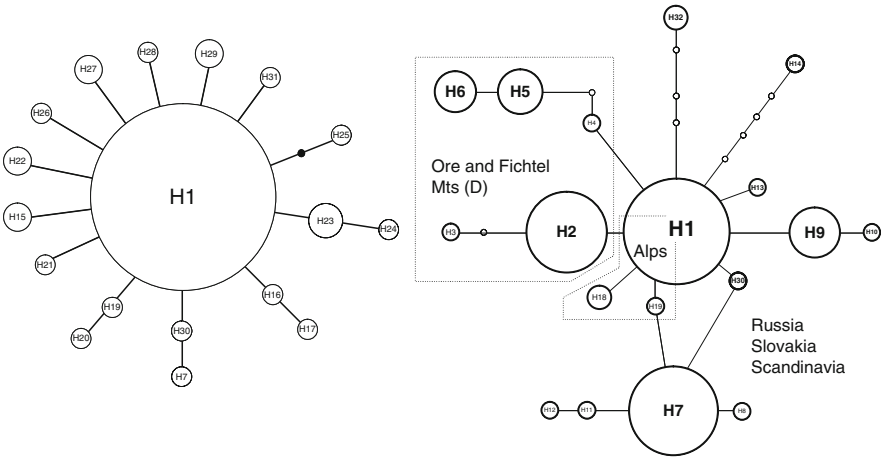


Fig. 3 Gene trees of mtDNA haplotypes for host races of *T. conura* infesting *C. heterophyllum* and *C. oleraceum*. The host races shared the common haplotype H1, otherwise there was little overlap. *Circle sizes* are proportional to haplotype frequencies. Populations of *T. conura* infesting *C. heterophyllum* were genetically diverse and regionally structured. Note also that Alpine populations of flies infesting *C. heterophyllum* were genetically little diverse (compare with Fig. 5). Populations of *T. conura* infesting *C. oleraceum* had a star-like gene tree and showed signs of recent population expansion with no regional structure. The main haplotype H1 was distributed across the entire sampling range of *T. conura* infesting *C. oleraceum* including Germany, Croatia, Scandinavia and Russia (figure redrawn from Diegisser et al. 2006a)

Assuming again that genetic diversity can be interpreted in terms of centres of origin, the European Alps or their vicinity may have served as the geographical origin of flies infesting *C. oleraceum*. Conversely, the level of mtDNA diversity for flies infesting *C. heterophyllum* in the Alps and in western Europe was much reduced relative to eastern populations (Fig. 3), suggesting that the geographic origin of *T. conura* lay towards Russia where *C. heterophyllum* has a wide contiguous distribution (Diegisser et al. 2006a).

The mtDNA diversity pattern for flies infesting *C. oleraceum* had both sympatric and allopatric components: species evolving in sympatry should share alleles and newly colonized allopatric populations should lose alleles (Berlocher 1998), and early stages of allopatric speciation should leave an imprint of reduced number and increased similarity of haplotypes (Berlocher and Feder 2002). Common to both scenarios is the loss of diversity. In the first case, it is due to colonization during range expansion, in the second case, due to the bottleneck effect in isolation. Combined, these predictions fit peripatric diversification, i.e., diversification in a small population separated from the main distribution range. Under a peripatric speciation scenario, populations infesting the new host should be genetically less diverse, narrowly distributed, and haplotypes should be nested within the haplotypes of the (paraphyletic) ancestral host (Harrison 1991, 1998).

Thus, the most parsimonious geographic mode of host-race formation was peripatric divergence of flies infesting *C. oleraceum*. The distribution of mtDNA haplotypes supported neither on-going sympatric host-race divergence in present-day contact areas nor an old allopatric division. Subdivision of Fichtel Mountains and European Alps populations of the host race infesting *C. heterophyllum*, and the existence of private haplotypes in the derived host race infesting *C. oleraceum* make it unlikely that the host shift occurred in historical times as proposed for other host-race systems (Bush 1969; Shirai and Morimoto 1999; Groman and Pellmyr 2002), but rather that host-race divergence is as old, or even older, than the last glacial cycle. Host races thus evolved before the present-day distributions. The findings for *T. conura* draw attention to the notion that geographic speciation scenarios need not be mutually exclusive but act in combination (Michel et al. 2007; Xie et al. 2007).

4 Gene Flow and Signs of Selection

The distribution of mtDNA variation supported diversification of host races before present-day plant distributions and suggested limited (female) gene flow between host races. However, shared mtDNA variation in the Alps and renewed secondary contact between host races today may restore gene flow. How stable are *T. conura* host races in areas of contact? We addressed this question with biparental inherited allozyme loci using two approaches. Forty *T. conura* populations (19 *C. heterophyllum* sites, 21 *C. oleraceum* sites) from the three geographic settings were analyzed.

In the first approach, we analyzed whether genetic differentiation between host races living in sympatry and parapatry differs from populations living in allopatry (Diegisser et al. 2006b). In these analyses, we also assessed linkage disequilibria at host-race related loci. Linkage might be interpreted in terms of oviposition on wrong host plants and/or hybridization among host races. The distribution of genetic variation was analyzed within and between each of the three geographic settings for each host race, while hierarchical analyses of variance determined how much of the total variance was attributable to host–plant affiliation and host–plant independent variance. Host races were sampled on pure parental host plants. Based on the geographic–genetic variances, several inferences about gene flow and the integrity of host races can be made. If host-races differ more in allopatry than in sympatry and/or parapatry, this might indicate that the original diversification was a passive consequence of either genetic drift or selection in isolation and that host races are genetically and behaviorally compatible due to on-going gene flow. Host races in contact areas may become genetically more similar due to hybrid matings and/or oviposition into the wrong host plant. In contrast, a pattern consistent with selection against hybridization and/or oviposition on the wrong host plant is indicated when differentiation among host-races is higher in contact areas than in allopatry. If allozyme loci are linked to traits which reduce maladaptive events

in contact zones, e.g., wrong host choice and/or mating, they should be more highly differentiated in contact areas. A third pattern of differentiation reflects a uniform level in all geographic settings. Uniformity can happen either when both the processes of gene flow and selection against maladaptive hybridization/wrong host choice occur but counterbalance each other, or, alternatively, genes may be so strongly restricted that allele frequencies are constant irrespective of geographic setting. The latter suggests geographically stable, reproductively isolated host races.

The comparative geographic analysis showed that *T. conura* host races had diagnostic, albeit not species-specific, alleles at the loci *Hex* and *PepD*, and significant differences at three other loci (Diegisser et al. 2006b). Host-related differentiation was not significantly higher in sympatry/parapatry than in allopatry when analyzed over all loci. However, host-related differentiation at the diagnostic locus *Hex* was significantly higher in sympatry/parapatry than between sympatric/parapatric and allopatric regions ($P = 0.008$). The difference at *Hex* was found in the parapatric Bavarian region as well as in the sympatric Swedish region. This pattern resembles a pattern expected under reinforcement selection at *Hex* against maladaptive events. Flies infesting *C. oleraceum* in contact areas showed significant linkage at the locus combination *Hex*/*PepD* ($P = 0.01$) caused by an excess of the F1 equivalent genotype *Hex 100*/*107*-*PepD 92*/*100*. By contrast, there was no evidence for linkage among *Hex* and *PepD* in allopatric populations infesting *C. oleraceum* ($P = 0.82$) nor in any geographic setting for flies on *C. heterophyllum*. The low but significant excess of F1 equivalents (4 expected, 8 observed) provided evidence for restricted gene flow in contact areas involving the derived host *C. oleraceum* as mating site/host. Nevertheless, the constant differentiation across geographic settings show that *T. conura* host races are discrete, experiencing fine-tuning selection at most, and that the major diversification process took place before today's geographical settings were established. Gene flow via hybrid F1 may explain fine-tuning selection at *Hex* related to pure parental plants.

The second approach for estimating host race integrity was an analysis of gene flow in a syntopic population with both host plants and their hybrids (*C. oleraceum* x *C. heterophyllum*). Hybrid plants constitute an intermediate environment and may act as a bridge (vector) for gene flow (Diegisser et al. 2007). Indeed, the frequency of *T. conura* double heterozygote F1 equivalents from hybrid plants significantly exceeded expectation had mating taken place only within host races (F1 from hybrid plants = 0.062, *C. heterophyllum* = 0.012, *C. oleraceum* = 0.017; T. Diegisser, unpublished data). The rate of F1 equivalents was also much higher than in the sympatric/parapatric populations where only parental host plants but no hybrid plants occurred. Interestingly, the frequency of the *C. heterophyllum* associated *Hex 100* allele was higher in flies infesting pure *C. oleraceum* than on average in other sympatric/parapatric populations (0.34 vs 0.19). This result contradicts the global analyses where *greater* diversification between host races in contact populations was found, and it is in disagreement with the reinforcement-type selection advocated above. These apparently contradictory results are important for two reasons. First, they indirectly prove that mating is genetically associated to host plant

recognition (e.g., chemical cues) and not mate recognition. In the global analysis, sympatric sites only had pure parental plants, on which oviposition is selected against. Second, the results suggest that genes may leak between the host-races, which are not reproductively incompatible. Both findings agree with Bush's (1969) original concept of host–plant mediated diversification. Whether this hybrid-plant-mediated gene flow is relevant for the "hybrid bridge" hypothesis (Floate and Whitman 1993) is unsure. The "hybrid bridge" hypothesis states that hybrid plants may raise the likelihood of insects shifting between the parental plants. There has only been a little evidence for insect host-shifts mediated by introgressive plant genomes (Pilson 1999). For the evidence presented here, gene flow via the hybrid plants may simply be a secondary consequence of mate localization, and may actually act opposite to the "hybrid bridge" by increasing gene flow between host races. We can only speculate about the frequency of gene flow via hybrid plants, but considering the global constancy of allele frequency differences, it cannot be common, otherwise selection, as indicated at *Hex*, is strong enough to keep host races genetically separated.

5 Host-Race Adaptations

Several traits may, alone or in combination, adapt insects to new hosts and assure discreteness of host races. Four major types of adaptations can be identified. First and most important is plant recognition including the willingness of females to use plants as an oviposition substrate (Bernays and Chapman 1994; Wood et al. 1999; Gassmann et al. 2006). Second, insects may need to adapt physiologically to host chemistry. Plants differ in secondary metabolites (Roitberg and Isman 1992) and nutritional value (Häggström and Larsson 1995). Host-dependent larval performance has been demonstrated in numerous studies (Via 1991; Craig et al. 1993, 1997; Katakura and Hosogai, 1994), indicating that physiological adaptations play an important role in the course of a host shift. Third, adaptation to plant phenologies assures optimal timing for oviposition. Plant phenologies may play a pivotal role in keeping diverging populations separate. Indeed, all reported tephritid host races have allochronic timing to plant phenologies (Horner et al. 1999; Diegisser et al. 2004, 2006b; Dambroski and Feder 2007). Lastly, once the parasite has adapted physiologically and behaviorally to the novel host, it may adapt morphologically to the plant. However, morphological differences, in particular, often have a non-hereditary background due to non-adaptive phenotypic plasticity. Differences in nutritional value (Häggström and Larsson 1995) and amount of secondary metabolites (Roitberg and Isman 1992) may affect body-size differences (Leclaire and Brandl 1994; Stoyenoff et al. 1994; Lill and Marquis 2001; Blair et al. 2005) and shape variation (Gillham and Claridge 1994).

The study of specific adaptations of *T. conura* host races to *C. heterophyllum* and *C. oleraceum* has so far been hampered by not getting both host plants to flower in

the laboratory and simultaneously having naïve flies of both host races (which must survive a hibernation phase). This means that reciprocal experiments to evaluate performance and hereditary differences can only be partially performed. Nevertheless, several direct and indirect results show that *T. conura* host races have rapidly evolved a suit of behavioral and morphological differences.

5.1 Plant Recognition and Willingness to Mate

Plant recognition in *T. conura* was analyzed as attraction to plants and as the willingness to mate on alternative plants. In these tests, naïve flies with no experience of host plants after emergence were used to assure that behavioral differences were not biased by imprinting. Flies were kept at 4°C for 2 months to simulate winter dormancy, which is required for sexual maturity (Diegisser 2005). Plant attraction tests were performed in cages with both host plants, with only *C. heterophyllum* or only *C. oleraceum*, while tests studying willingness to mate had additional test cages with three other *Cirsium* species and control cages with no plants. These latter tests included males and females of both host races. The first experiment, attraction to plants, showed that host races were significantly attracted to their own host plants (Table 1). In the second experiment, willingness to mate, dormant flies from *C. heterophyllum* were ready to mate as soon as the temperature was raised from 4°C to room temperature, and was independent of host plant (Fig. 4). By contrast, flies infesting *C. oleraceum* needed 2–3 weeks to become sexually active. Hence, flies infesting *C. heterophyllum*, who did not need the host plant for mating, finished mating before both sexes of flies from *C. oleraceum* became sexually active. Due to the lag-phase of flies infesting *C. oleraceum*, only a few pure matings of these flies were observed, but those that did occur took place in the presence of *C. oleraceum* (Fig. 4b). In a third preliminary experiment, we tested whether flies infesting *C. oleraceum* matured sexually after contact with the host plant. Again, mating commenced after a lag-phase (ca. 1 week) and only in the presence of *C. oleraceum*. The lag-phase differed slightly between the two experiments but was present in both trials. The different onset of mating between the host

Table 1 Host plant attraction tests for *T. conura* host races infesting *C. heterophyllum* and *C. oleraceum* (Diegisser 2005). Host races and sexes were tested separately. Each trial exposed flies to both plants simultaneously

			Number of visits on		
Origin	Sex	*n*	*C. heterophyllum*	*C. oleraceum*	*P*
C. heterophyllum	Male	50	101	32	<0.001
	Female	50	96	39	<0.001
	Total	100	197	71	<0.001
C. oleraceum	Male	50	86	124	<0.01
	Female	50	45	97	<0.001
	Total	100	131	221	<0.001

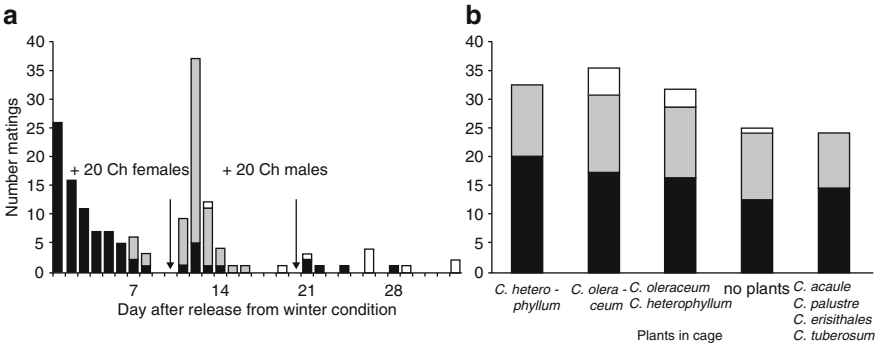


Fig. 4 Mating preference tests for *T. conura* host races infesting *C. heterophyllum* and *C. oleraceum*. The preference tests were performed in four cages each with 20 males and females of each host race. Flies were allowed to mate only once and all mating pairs were immediately removed from the experiment. Host-race-specific mating between flies infesting *C. heterophyllum* (*black bars*) started immediately (**a**) and was independent of host plant (**b**). At day ten, 20 new females of the *C. heterophyllum*-host race were introduced into each cage, which resulted in hybrid pairs between males originating from *C. oleraceum* and females originating from *C. heterophyllum* (*gray bars*) (**a**). These matings were independent of host plant (**b**). At day twenty, 20 new males of the *C. heterophyllum*-host race were introduced into each cage. This did not result in hybrid pairs. Host-race-specific mating between flies infesting *C. oleraceum* (*white bars*) commenced after a lag phase of 2–3 weeks and was only, with one exception, recorded in the presence of *C. oleraceum* (**a, b**)

races coincides with allochronic timing to host–plant phenologies in nature and suggests a genetic component for mating time. This component is probably not directly linked to temperature as no altitude or latitude allele frequency gradients have been found *within* host races (Diegisser et al. 2006b). This result differs from findings for the apple maggot fly *R. pomonella* where larval eclosure is temperature related and where latitudinal host-race clines are observed (Feder et al. 1997; Dambroski and Feder 2007). *R. pomonella* emerge in spring which allows selection on diapause length while *T. conura* emerge in summer, surviving the winter as adults.

In summary, plant attraction and mating tests showed that both host races were attracted to their own hosts, but only the derived host race, i.e., flies infesting *C. oleraceum*, required its host plant to become sexually active. This asymmetry differs slightly from reciprocal behaviour assumed by theoretical host–plant-related speciation models.

5.2 *Survival on* C. oleraceum

As mentioned above, genetic analyses of the host races indicated that *C. oleraceum* is the derived host plant. In an egg-laying experiment, we tested whether the shift to the derived *C. oleraceum* involved physiological adaptations by studying oviposition acceptance and survival of the two host races on this plant. Wild-caught gravid

females of both host races were offered *C. oleraceum* plants for oviposition. The number of successful females that (1) accepted buds, (2) successfully produced larvae, (3) successfully produced pupae (survival from larva to pupae), and (4) produced adults (survival from pupa to adult) were recorded (Diegisser et al. 2008). *T. conura* originating from *C. oleraceum* produced adults in 75% of all egg-laying trials in contrast to only 6.6% in *T. conura* originating from *C. heterophyllum*. The low performance of the latter was determined by lack of plant acceptance and by high larval mortality. By contrast, hatching of at least one larva per batch and survival of pupae were not affected (Diegisser et al. 2008). The former two parameters involve stages not directly involved in interactions with the host plant, whereas significant mortality differences during the larval stage imply different responses to plant physiology. This experiment also showed that females, if forced, will lay eggs on a suboptimal plant, and that the ancestral host race (flies infesting *C. heterophyllum*) exhibits genetic variance for physiological adaptation to a novel host.

5.3 Adaptation of Ovipositor Length

The host plants *C. heterophyllum* and *C. oleraceum* differ significantly in flower-head size. The ovipositor of *T. conura* must be long enough to reach the cavity within the flower head. Studies by Zwölfer and Romstöck-Völkl (1991) at one site in the European Alps showed that flies parasitizing *C. heterophyllum* have significantly longer oviscarps than flies associated with *C. oleraceum.* However, these studies could not rule out phenotypic host-induced and/or geographic causes for the difference. Because *C. oleraceum* is largely restricted to altitudes below ca. 1,200 m while *C. heterophyllum* is found from ca. 1,100 m to above 2,000 m, the potential for altitude affecting body size exists (Stalker and Carson 1948; Bitner-Mathé and Klaczko 1999; Dahlgaard et al. 2001). Because we have not yet been able to rear flies in the laboratory, we used two indirect approaches to evaluate if ovipositor length is genetically determined and thus adapted to inflorescence size. The analyses were based on the ratio ovipositor/wing length. The ratio removes host-related ovipositor differences caused by body size. Wing length was used as a substitute for body size.

We compared flies from each host plant from geographically separated sympatric sites (southern Sweden and Bavarian Fichtelgebirge), and at one syntopic site in the German Ore Mountains (Erzgebirge), we studied flies emerging from pure parental plants and hybrids plants (*C. heterophyllum* x *C. oleraceum*), and hybrid flies emerging from the hybrid plants. Because the larval environment within hybrid flower-heads is the same for parental and hybrid flies, the emerging flies can be used to infer the underlying cause of morphological differentiation. If host races differ when developing in their original host but not when developing in the same environment, differences can be assigned to phenotypic plasticity. A genetic basis of morphological divergence is supported when host races do not react to the ambient environment (i.e., original host or hybrid plant). A combination of

host-independent morphology *and* intermediate hybrid fly morphology provides strong evidence for a genetic basis and adaptations to the host.

The results showed that pure host races had significantly different ovipositor lengths, which were independent of both geographic location (i.e., temperature) and host environment (plant). Hybrid flies were intermediate, proving that ovipositor length is genetically determined in *T. conura*. Interestingly, this result contrasts with what has happened in host races of the congeneric *T. bardanae* infesting *Arctium tomentosum* and *A. minus*. In *T. bardanae*, ovipositor length has adapted to inflorescence size differences by adjusting body size (Diegisser et al. 2004). The latter is common among host races (Emelianov et al. 1995; Carroll et al. 1997) and other phytophagous insects. For example, in acorn beetles (*Curculio*), the rostrum, which is used for excavating an oviposition site in plant structures, increases proportionately to body size, and suggests seed size is an important selective agent in speciation (Zwölfer 1975; Hughes and Vogler 2004). The different solutions for increasing ovipositor length in the sister species *T. bardanae* and *T. conura* suggest different selection pressures on two related species.

6 Does Plant History Matter in Host-Race Evolution?

The study of adaptation by phytophagous insects to alternative host plants has traditionally focused on finding parameters that make it possible for an insect to survive and evolve on different hosts. But the question can also be asked in reverse, namely, what makes plants liable to attack? Why do some plant species become infested and others not, and some species only locally? Liability for infestation will be influenced by genetic variance among host plants (Fritz 1995; Cronin and Abrahamson 1999; Nuismer and Thompson 2001). For *T. conura*, this is observed directly in the population of hybrid *C. heterophyllum* x *C. oleraceum* where both host races used hybrid plants but not the alternative host plant. Such liability differences indicate the existence of host-range hierarchies and different infestation levels when host plants occur over wide geographical areas (Thompson 2005). A related question to liability per se is if it is possible to make inferences about host-race formation from the population history of the host plants (Despres et al. 2002). Do the two coincide; are patterns congruent?

This topic is still in its infancy in the *T. conura–Cirsium* system but population genetic analyses of *C. heterophyllum* and *C. oleraceum* (Johannesen and Lampei, unpublished results), and an investigation of a recent host range expansion by *T. conura* from *C. heterophyllum* to *C. palustre* in northern Britain (Johannesen et al. 2008), suggest that plant history and/or constitution may play an important, if not decisive, role.

Regarding first *C. heterophyllum* and *C. oleraceum*, their genetic structures and population histories were congruent with the global pattern of mtDNA variation found in *T. conura* host races: *C. heterophyllum* populations are highly structured and divided in an eastern and western lineage, while *C. oleraceum* shows little

structure across Europe and Russia. The plant histories suggest a Russian or eastern European origin of the association, followed by an expansion of *T. conura* into western Europe. A decisive difference, however, is that western European populations of *C. heterophyllum* show signs of hybridization or gene duplication (seen as putative heterozygotic ITS and ETS sequences; Johannesen and Lampei, unpublished results). The flies infesting these putative hybrid *C. heterophyllum* in the Alps, and all other infested *Cirsium* in this region, are nearly monomorphic for the haplotype H1 (Fig. 5) (Diegisser et al. 2006a; Johannesen et al., unpublished results). The combination of having one main haplotype in *T. conura* and altered genetics of *C. heterophyllum* in western Europe suggest that *C. heterophyllum* in western Europe itself has been colonized only recently. One can speculate that the genetic change of *C. heterophyllum* mediated the ability of *T. conura* to diversify to other plants altogether. If this is correct, it suggests that area alterations of western *C. heterophyllum* populations allowed host range expansion on previously uninfested plants such as *C. oleraceum*. Interestingly, we did not observe infestation of a west alpine population of *C. heterophyllum* that showed no signs of introgression and which was genetically most related to *C. monspessulanum* (Johannesen et al., unpublished results).

The second example of possible influence of plant population history on host range expansion is the infestation of *C. palustre* in northern Britain by

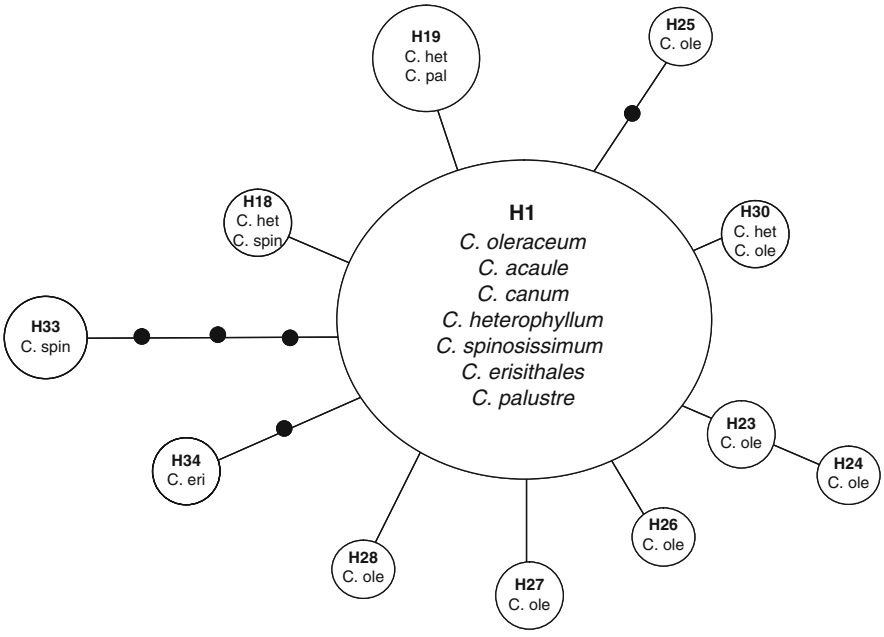


Fig. 5 Haplotypes found in *T. conura* infesting *Cirsium* species in the European Alps showed a star-like gene tree with little diversity. The common haplotype H1 was found in *T. conura* emerging from all *Cirsium* speices

C. heterophyllum flies (Johannesen et al. 2008; Diegisser et al. 2009). *C. palustre* is neither infested in southern Britain where *C. heterophyllum* is absent nor in continental Europe where it occurs sympatrically with infested *C. heterophyllum* and *C. oleraceum*. Genetic analyses using microsatellites showed that non-infested and infested British *C. palustre* are closely related but also that infested *C. palustre* are genetically very impoverished. Loss of genetic diversity was most likely caused by bottlenecks during range expansion.

For both the host shift of flies infesting *C. heterophyllum* and *C. oleraceum* and the host range expansion of flies infesting both *C. heterophyllum* and *C. palustre* in northern Britain, local genetic peculiarities of host plants and population genetic histories of *T. conura* coincide. These patterns suggest that origins of infestations are influenced by the population history and/or genetic constitution of the plants. To verify these assumptions, we need experimental data of attraction and performance of flies to genetic lineages of the various host plants and quantification of secondary metabolites of these genetic lineages. The combination of plant and parasite histories may be a future guideline to elucidate speciation processes in phytophagous insects in further detail.

7 Conclusions

The tephritid fly *T. conura* exhibits two host races infesting *C. heterophyllum* and *C. oleraceum*, respectively. Based on geographic distributions of genetic variation in *T. conura* and its host plants, the host shift most likely occurred from *C. heterophyllum* to *C. oleraceum* and via peripatric isolation. The host shift probably occurred rapidly but before the current host–plant distributions. The acquisition of a novel host plant resulted in a rapid suit of new adaptations. These include adaptations to plant phenology, plant attraction and physiology as well as morphological adjustment of the ovipositor to flower-head morphology. The behavioral changes are most pronounced in the derived host race, i.e., flies infesting *C. oleraceum*. The results also suggest that the geographic distributions of the host plants rather than co-phylogenetic evolution between fly and plants played a role in diversification of the host races. This implies that changing geographic conditions may have been prerequisite for the initial diversification. Hence, sympatric and allopatric speciation are not mutually exclusive but should probably, in open systems such as the *T. conura*–*Cirsium* system, be thought of as a continuum of events.

Acknowledgements This study greatly profited from investigations by Christian Tritsch, Christian Lampei, Karoline Köhler and Ariane Grastat. We thank Dagmar Klebsch and Susanne Maus for practical work in the laboratory. Harald Schneider and an anonymous reviewer for made helpful suggestions on an earlier MS draft. The study was supported by the Deutsche Forschungsgemeinschaft (DFG Se506/10-1/3 to A.S. and J.J.

References

Aluja M, Norrbom AL (2000) Fruit flies (Tephritidae): phylogeny and evolution of behaviour. CRC Press, Boca Raton

Becerra JX, Venable DL (1999) Macroevolution of insect-plant associations: the relevance of host biogrography to host affiliation. Proc Natl Acad Sci USA 96:12626–12631

Berlocher SH (1998) Can sympatric speciation via host or habitat shift be proven from phylogenetic and biogeographic evidence? In: Howard DJ, Berlocher SH (eds) Endless Forms: Species and Speciation, Oxford University Press, New York, pp 99–113

Berlocher SH, Feder JL (2002) Sympatric speciation in phytophagous insects: moving beyond controversy? Annu Rev Entomol 47:773–815

Bernays EA, Chapman RF (1994) Host-plant selection by phytophagous insects. Chapman and Hall, New York

Bitner-Mathé BC, Klaczko LB (1999) Size and shape heritability in natural populations of *Drosophila mediopunctata*: temporal and microgeographical variation. Genetica 105:35–42

Blair CP, Abrahamson WG, Jackman JA, Tyrrell L (2005) Cryptic speciation and host-race formation in a purportedly generalist tumbling flower beetle. Evolution 59:304–316

Bush GL (1969) Sympatric host-race formation and speciation in frugivorous flies of the genus *Rhagoletis* (Diptera, Tephritidae). Evolution 23:237–251

Bush GL (1975) Modes of animal speciation. Annu Rev Ecol Syst 6:339–364

Bush GL, Butlin RK (2004) Sympatric speciation in insects. In: Dieckmann U, Doebeli M, Metz JAJ, Tautz D (eds) Adaptive speciation. Cambridge University Press, pp 229–248

Bush GL, Smith JJ (1998) The genetics and ecology of sympatric speciation: a case study. Res Pop Ecol 40:175–187

Butlin RK (1990) Divergence in emergence time of host-races due to differential gene flow. Heredity 65:47–50

Carr DE, Eubanks MD (2002) Inbreeding alters resistance to insect herbivory and host plant quality in *Mimulus guttatus* (Scrophulariaceae). Evolution 56:22–30

Carroll SP, Dingle H, Klassen SP (1997) Genetic differentiation of fitness-associated traits among rapidly evolving populations of the soapberry bug. Evolution 51:1182–1188

Craig,TP, Horner JD, Itami JK (1997) Hybridization studies on the host races of Eurosta solidaginis: Implications for sympatric speciation. Evolution 51:1552–1560

Craig TP, Itami JK, Abrahamson WG, Horner JD (1993) Behavioral evidence for host-race formation in *Eurosta solidaginis*. Evolution 47:1696–1710

Cronin JT, Abrahamson WG (1999) Host-plant genotype and their herbivores influence goldenrod stem galler preference and performance. Oecologia 121:392–404

Coyne JA, Orr HA (1989) Patterns of speciation in *Drosophila*. Evolution 43:362–381

Coyne JA, Orr HA (1997) "Patterns of speciation in *Drosophila*" revisited. Evolution 51:295–303

Coyne JA, Orr HA (2004) Speciation. Sinauer, Sunderland

Dahlgaard J, Hasson E, Loeschke V (2001) Behavioral differentiation in oviposition activity in *Drosophila buzzatii* from highland and lowland populations in Argentina: plasticity or thermal adaptation? Evolution 55:738–747

Dambroski HR, Feder JL (2007) Host plant and latitude-related diapause variation in *Rhagoletis pomonella*: a test for multifaceted life history adaptation on different stages of diapause development. J Evol Biol 20:2101–2112

Despres L, Loriot S, Gaueul M (2002) Geographic pattern of genetic variation in the European globeflower *Trollius europaeus* L. (Ranunculaceae) inferred from amplified fragment length polymorphism markers. Mol Ecol 11:2337–2347

Dieckmann U, Doebeli M (1999) On the origin of species by sympatric speciation. Nature 400:354–357

Diegisser T, Johannesen J, Lehr C, Seitz A (2004) Genetic and morphological differentiation in *Tephritis bardanae* (Diptera: Tephritidae): evidence for host-race formation. J Evol Biol 17:83–93

Diegisser T (2005) Artbildung via Wirtsrassen bei Tephritis conura (Diptera: Tephritidae). PhD Dissertation, University of Mainz, Germany

Diegisser T, Seitz A, Johannesen J (2006a) Phylogeographic patterns of host-race evolution in *Tephritis conura* (Diptera: Tephritidae). Mol Ecol 15:681–694

Diegisser T, Johannesen J, Seitz A (2006b) The role of geographic setting on the diversification process among *Tephritis conura* (Tephritidae) host-races. Heredity 96:410–418

Diegisser T, Seitz A, Johannesen J (2007) Morphological adaptation in host races of *Tephritis conura* (Diptera: Tephritidae). Entomol Exp Appl 122:155–164

Diegisser T, Johannesen J, Seitz A (2008) Performance of *Tephritis conura* host-races (Diptera: Tephritidae) on a derived host plant (*Cirsium oleraceum*): implications for the original host shift. J Insect Sci 8:66

Diegisser T, Tritsch C, Seitz A, Johannesen J (2009) Infestation of the marsh thistle *Cirsium palustre* by *Tephritis conura* (Diptera: Tephritidae) in northern Britain – host-range expansion or host shift? Genetica 137:87–97

Diehl SR, Bush GL (1984) An evolutionary and applied perspective of insect biotypes. Annu Rev Entomol 29:471–504

Dres M, Mallet J (2002) Host races in plant-feeding insects and their importance in sympatric speciation. Philos Trans R Soc Lond B 357:471–492

Emelianov I, Mallet J, Baltensweiler W (1995) Genetic differentiation in *Zeiraphera diniana* (Lepidoptera: Tortricidae, the larch budmoth): polymorphism, host races or sibling species? Heredity 75:416–424

Emelianov I, Dres M, Baltensweiler W, Mallet J (2001) Host-induced assortative mating in host races of the larch budmoth. Evolution 55:2002–2010

Eschenbacher H (1982) Untersuchungen über den Insektenkomplex in den Blütenköpfen der Kohldistel, *Cirsium oleraceum* L. (Compositae). Diploma thesis, University of Bayreuth, Germany

Feder JL (1995) The effects of parasitoids on sympatric host races of *Rhagoletis pomonella* (Diptera, Tephritidae). Ecology 76:801–813

Feder JL, Stolz U, Lewis KM, Perry W, Roethele JB, Rogers A (1997) The effects of winter length on the genetics of apple and hawthorn races of *Rhagoletis pomonella* (Diptera : Tephritidae). Evolution 51:1862–1876

Feder JL, Berlocher SH, Opp SB (1998) Sympatric host-race formation and speciation in *Rhagoletis* (Diptera: Tephritidae): a tale of two species for Charles D. In: Mopper S, Strauss SY (eds) Genetic structure and local adaptation in natural insect populations. Effects of ecology, life history, and behavior. Chapman and Hall, New York, pp 408–434

Feder JL, Berlocher SH, Roethele JB, Dambroski H, Smith JJ, Perry WL, Gavrilovic V, Filchak KE, Rull J, Aluja M (2003) Allopatric genetic origins for sympatric host-plant shifts and race formation in *Rhagoletis*. Proc Natl Acad Sci USA 100:10314–10319

Fitzpatrick BM, Fordyce JA, Gavrilets S (2008) What, if anything, is sympatric speciation? J Evol Biol 21:1452–1459

Floate K, Whitman TG (1993) The "hybrid bridge" hypothesis: host shifting via plant hybrid swarms. Am Nat 141:651–662

Fritz RS (1995) Direct and indirect effects of genetic variation on enemy impact. Ecol Entomol 20:18–26

Fu YX (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147:915–925

Gassmann AJ, Levy A, Tran T, Futuyma DJ (2006) Adaptations of an insect to a novel host plant: a phylogenetic approach. Funct Ecol 20:478–485

Gillham MC, Claridge MF (1994) A multivariate approach to host-plant associated morphological variation in the polyphagous leafhopper, *Alnetoidia-Alneti* (Dahlbom). Biol J Linn Soc 53:127–151

Groman JD, Pellmyr O (2002) Rapid evolution and specialization following host colonization in a yucca moth. J Evol Biol 13:223–236

Häggström H, Larsson S (1995) Slow larval growth on a suboptimal willow results in high predation mortality in the leaf beetle *Galerucella lineola*. Oecologia 104:308–315

Hare JD (1990) Ecology and management of the Colorado potato beetle. Annu Rev Entomol 35:81–100

Harpending HC, Batzer MA, Gurven M, Jorde LB, Rogers AR, Sherry ST (1998) Genetic traces of ancient demography. Proc Natl Acad Sci USA 95:1961–1967

Harrison RG (1991) Molecular changes at speciation. Annu Rev Ecol Syst 22:281–308

Hegi G (1987) DCCLXXIV *Cirsium*. In: Wagenitz G (ed) Illustrierte Flora Mitteleuropas, Band 6 Teil IV, Compositae II: Matricaria-Hieracium, 2nd edn. Paul Parey, Berlin-Hamburg, pp 866–916

Harrison RG (1998) Linking evolutionary pattern and process: the relevance of species concepts for the study of speciation. In: Howard DJ, Berlocher SH (eds) Endless forms: species and speciation. Oxford University Press, New York, pp 19–31

Horner JD, Craig TP, Itami JK (1999) The influence of oviposition phenology on survival in host races of *Eurosta solidaginis*. Entomol Exp Appl 93:121–129

Hughes J, Vogler AP (2004) Ecomorphological adaptation of acorn weevils to their oviposition site. Evolution 58:1971–1983

Hull-Sanders HM, Eubanks MD (2005) Plant defense theory provides insight into interactions involving inbred plants and insect herbivores. Ecology 86:897–904

Janz N, Nylin S, Wahlberg N (2006) Diversity begets diversity: host expansions and the diversity of plant feeding insects. BMC Evol Biol 6:4

Johannesen J, Tritsch C, Seitz A, Diegisser T (2008) Genetic structure of *Cirsium palustre* (Asteraceae) and its role in host diversification of *Tephritis conura* (Diptera: Tephritidae). Biol J Linn Soc 95:221–232

Katakura H, Hosogai T (1994) Performance of hybrid ladybird beetles (*Epilachna* ssp.) on the host plants of parental species. Entomol Exp Appl 71:81–84

Kawecki TJ (1996) Sympatric speciation driven by beneficial mutations. Proc R Soc Lond B 263:1515–1520

Kawecki TJ (1997) Sympatric speciation by habitat specialization driven by deleterious mutations. Evolution 51:1751–1763

Komma M (1990) Der Pflanzenparasit *Tephritis conura* und die Wirtsgattung *Cirsium*. PhD Thesis, University of Bayreuth, Germany

Kondrashov AS, Yampolsky LV, Shabalina SA (1998) On the sympatric origin of species by means of natural selection. In: Howard DJ, Berlocher SH (eds) Endless forms: species and speciation. Oxford University Press, New York, pp 90–98

Koslow JM, DeAngelis DL (2006) Host mating system and the prevalence of disease in a plant population. Proc R Soc Lond B 273:1825–1831

Kovac D, Dohm P, Freidberg A, Norrbom AL (2006) Catalog and revised classification of the Gastrozonin (Diptera: Tephritidae: Dacinae). In: Freidberg A (ed) Biotaxonomy of Tephritoidae. Isr J Entomol 35–36:163–196

Larson S, Ekbom B (1995) Oviposition mistakes in herbivorous insects: confusion or a step towards a new host species. Oikos 72:155–160

Leclaire M, Brandl R (1994) Phenotypic plasticity and nutrition in a phytophagous insect - consequences of colonizing a new host. Oecologia 100:379–385

Lill JT, Marquis RJ (2001) The effects of leaf quality on herbivore performance and attack from natural enemies. Oecologia 126:418–428

Mayr E (1963) Animal Species and Evolution. Belknap Press, Cambridge, MA

Mallet J (2001) The speciation revolution. J Evol Biol 14:887–888

Michel AP, Rull J, Aluja M, Feder JL (2007) The genetic structure of hawthorn-infesting *Rhagoletis pomonella* populations in Mexico: implications for sympatric host race formation. Mol Ecol 16:2867–2878

Norrbom AL (2004) The Diptera site. Fruit fly (Diptera: Tephritidae) classification & diversity, http://www.sel.barc.usda.gov/diptera/tephriti/TephClas.htm. Assessed 17.05.2010

Nuismer SL, Thompson JN (2001) Plant polyploidy and non-uniform effects on insect herbivores. Proc R Soc Lond B 268:1937–1940

Ohshima I (2008) Host race formation in the leaf-mining moth *Acrocercops transecta* (Lepidoptera: Gracillariidae). Biol J Linn Soc 93:135–145

Presgraves DC, Balagopalan L, Abmayr SM, Orr HA (2003) Adaptive evolution drives divergence of a hybrid inviability gene between two species of *Drosophila*. Nature 423:715–719

Pilson D (1999) Plant hybrid zones and insect host range expansion. Ecology 80:407–415

Rausher MD (1984) Tradeoffs in performance on different hosts: evidence from within- and between-site variation in the beetle *Deloyala guttata*. Evolution 38:582–595

Rice WR (1984) Disruptive selection of habitat preference and the evolution of reproductive isolation: a simulation study. Evolution 38:1251–1260

Roitberg BD, Isman MB (1992) Insect chemical ecology: an evolutionary approach. Chapman and Hall, New York

Romstöck M (1982) Untersuchungen über den Insektenkomplex in den Blütenköpfen von *Cirsium heterophyllum* (Caedueae). MSc thesis, Bayreuth University, Bayreuth, Germany

Romstöck-Völkl M (1997) Host-race formation in *Tephritis conura*: determinants from three trophic levels. Ecol Stud 130:21–38

Romstöck M, Arnold H (1987) Populationsökologie und Wirtswahl bei *Tephritis conura* Loew-Biotypen (Dipt.: Tephritidae). Zool Anz 219:83–120

Seitz A, Komma M (1984) Genetic polymorphism and its ecological background in Tephritid populations (Diptera: Tephritidae). In: Wöhrmann K, Loeschcke V (eds) Population biology and evolution. Springer, Berlin, pp 143–158

Schwarz D, McPheron B, Hartl GB, Boller EF, Hoffmeister TS (2003) A second case of genetic host races in *Rhagoletis*? A population genetic comparison of sympatric host populations in the European cherry fruit fly *Rhagoletis cerasi*. Entomol Exp Appl 108:11–17

Sezer M, Butlin RK (1998) The genetic basis of host plant adaptation in the brown planthopper (*Nilaparvata lugens*). Heredity 80:499–508

Shirai Y, Morimoto N (1999) A host shift from wild blue cohosh to cultivated potato by the phytophagous ladybird beetle, *Epilachna yasutomii* (Coleoptera, Coccinellidae). Res Pop Ecol 41:161–167

Stalker HD, Carson HL (1948) An altitudinal transect of *Drosophila robusta* Sturtevant. Evolution 2:295–305

Stephenson AG, Leyshon B, Travers SE, Hayes CN, Winsor JA (2004) Interrelationships among inbreeding, herbivory, and disease on reproduction in a wild gourd. Ecology 85:3023–3034

Stireman JO III, Nason JD, Heard SB (2005) Host-associated genetic differentiation in phytophagous insects: general phenomenon or isolated exceptions? Evidence from a goldenrod-insect community. Evolution 59:2573–2587

Stoyenoff JL, Witter JA, Montgomery ME, Chilcote CA (1994) Effects of host switching on gypsy moth (*Lymantria dispar* (L.)) under field conditions. Oecologia 97:143–157

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595

Thompson JN (2005) The geographic mosaic of coevolution. University of Chicago Press, Chicago

Turelli M, Barton NH, Coyne JA (2001) Theory and speciation. Trends Ecol Evol 16:330–343

Via S (1990) Ecological genetics and host adaptation in herbivorous insects: the experimental study of evolution in natural and agricultural systems. Annu Rev Entomol 35:421–446

Via S (1991) Specialized host plant performance of pea aphid clones is not altered by experience. Ecology 72:1420–1427

Via S (1999) Reproductive isolation between sympatric races of pea aphids. I. Gene flow restriction and habitat choice. Evolution 53:1446–1457

Via S (2001) Sympatric speciation in animals: the ugly duckling grows up. Trends Ecol Evol 16:381–390

Vaupel A, Klinge K, Brändle M, Wissemann V, Tscharntke T, Brandl R (2007) Genetic differentiation between populations of the European rose hip fly *Rhagoletis alternate*. Biol J Linn Soc 90:619–625

Wood TK, Guttman SI (1982) Ecological and behavioural basis for reproductive isolation in the sympatric *Enchenopa binotata* complex. Evolution 36:233–242

Wood TK, Tilmon KJ, Shantz AB, Harris CK, Pesek J (1999) The role of host-plant fidelity in initiating insect race formation. Evol Ecol Res 1:317–332

Xie XF, Rull J, Michel AP, Velez S, Forbes AA, Lobo NF, Aluja M, Feder JL (2007) Hawthorn-infesting populations of *Rhagoletis pomonella* in Mexico and speciation mode plurality. Evolution 61:1091–1105

Zwölfer H (1975) Rüsselkäfer und ihre Umwelt – ein Kapitel Ökologie. Stuttg Beitr Naturk 3:19–31

Zwölfer H (1988) Evolutionary and ecological relationships among the insect fauna of thistles. Annu Rev Entomol 33:103–122

Zwölfer H, Romstöck-Völkl M (1991) Biotypes and the evolution of niches in phytophagous insects on Cardueae hosts. In: Price PW, Lewinsohn TM, Fernandes GW, Woodruff WB (eds) Plant-animal interactions: evolutionary ecology in tropical and temperate regions. Wiley, New York, pp 487–507

Part III
Approaches in Zoology

Solar Powered Seaslugs (Opisthobranchia, Gastropoda, Mollusca): Incorporation of Photosynthetic Units: A Key Character Enhancing Radiation?

Heike Wägele, Michael J. Raupach, Ingo Burghardt, Yvonne Grzymbowski, and Katharina Händeler

Abstract Incorporation of photosynthetic units has been investigated under the assumption of them representing a key character that enhanced speciation and led to an adaptive radiation. Two independent opisthobranch systems were chosen to test this hypothesis: the nudibranch genus *Phyllodesmium* living in mutualistic symbiotic relationship with the dinoflagellate *Symbiodinium*, and the taxon Sacoglossa (herbivorous seaslugs) sequestering and partly incorporating healthy chloroplasts from chlorophytes. Photosynthetic activity of various members of both taxa was measured under starving conditions and efficiency of incorporation studied by analysing yield values of photosynthesis. For both systems, certain trends were observed: clades exhibiting a functional incorporation of photosynthetic units (i.e., these units were not digested after sequestration) are about five times more species rich than their sister taxon. When comparing the species, observed efficiency differed. Furthermore, morphological adaptations to higher efficiencies could be identified in long-term retention forms (e.g., *Phyllodesmium longicirrum* with a highly branched digestive gland in the dorsal appendages, and sacoglossan *Plakobranchus ocellatus* with special ridges on the dorsal side, housing most of the incorporated chloroplasts). Nevertheless, other factors driving evolution of these clades cannot be excluded; they might even be linked with the investigated key character. These are, e.g., food switch and/or incorporation of secondary metabolites for defense.

H. Wägele (✉), M.J. Raupach, Y. Grzymbowski, and K. Händeler
Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany
e-mail: hwaegele@evolution.uni-bonn.de

I. Burghardt
Australian Institute of Marine Science, PMB No. 3, Townsville QLD 4810, Australia
University of Technology Sydney, Climate Change Cluster, PO Box 123, Sydney, NSW 2007, Australia

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_13,

1 Introduction

Adaptive radiation is defined as a "rise in the rate of appearance of new species and a concurrent increase in ecological and phenotypic diversity" (Schluter 2000: p.10). Adaptive radiation can be driven by features, such as the absence of competing taxa in an adaptive zone. Such a zone can be entered when a novel trait that helps to exploit resources is not or little used before (Schluter 2000). The gastropod taxon Opisthobranchia, with about 6,000 described species, is known for some remarkable traits which might be interpreted as key characters (Wägele 2004; Wägele and Klussmann-Kolb 2005). One unique character is the ability to incorporate functional cnidocysts from cnidarian prey to use in own defense (Edmunds 1966; Kälker and Schmekel 1976; Aguado and Marin 2007). Another widespread feature is the sequestration of chemical compounds from prey, their biotransformation and deployment in defense (see Cimino and Gavagnin 2006; Wägele et al. 2006).

In this study, we investigated the acquisition of photosynthetic units (unicellular dinoflagellate *Symbiodinium* or chloroplasts) within the Opisthobranchia in two distantly related taxa to test the premise that the incorporation of photosynthetic units may be a key character that enhanced speciation rate and influenced adaptive radiation. The analysis of two independent systems possessing the envisaged character can test the hypothesis that this character was in fact a key innovation.

The first system comprises the genus *Phyllodesmium* (Facelinidae, Aeolidoidea, Nudibranchia), currently including 30 species (Fig. 1a–l). Distribution is known from the whole Indo-Pacific including temperate and tropical areas. These slugs feed on specific members of the Octocorallia (Anthozoa, Cnidaria) and incorporate the dinoflagellate *Symbiodinium* spp. (often called zooxanthellae) of their sequestered prey organisms, and then live in a mutualistic symbiotic relationship with these protists (Rudman 1981, 1987, 1991; Burghardt and Wägele 2004; Burghardt et al. 2005, 2008a, b; Burghardt and Gosliner 2006; Burghardt and Wägele 2006).

The second system is the taxon Sacoglossa with about 300 known species (Fig. 2a–l). These slugs feed on algae mainly belonging to the Chlorophyta (Jensen 1993a, b, 1997; see Händeler and Wägele 2007; Händeler et al. 2009). A few members are known to incorporate chloroplasts and exploit photosynthetic products from the still functioning chloroplasts within the slugs (Greene 1970; Trench 1973; Trench et al. 1973a, b; Clark et al. 1981; Rumpho et al. 2000, 2001; Evertsen et al. 2007).

Both taxa incorporate the photosynthetic units within the digestive glandular cells of their digestive system (Fig. 3a, b, d, e). However, *Phyllodesmium* incorporates fully functional organisms, while only cell organelles (chloroplasts) with reduced genomic equipment for self sustenance is sequestered and housed in the digestive gland of sacoglossan members. In both systems, slugs are assumed to benefit from the incorporation of functional photosynthetic units (PUs: zooxanthellae or chloroplasts) by exploiting photosynthetic products over variable time.

Several advantages may be derived by incorporating PUs. First, the slugs attain the same color as their food organisms and therefore become camouflaged.

Fig. 1 *Phyllodesmium* species: (**a**) *P. lembehensis*, (**b**) *P. koehleri*, (**c**) *P. kabiranum*, (**d**) *P. rudmani*, (**e**) *P. lizardensis*, (**f**) *P. jakobsenae*, (**g**) *P. briareum*, (**h**) *P. colemani*, (**i**) *P. longicirrum*, (**k**) *P. lizardensis* (*arrow*) on its food, a softcoral of the genus *Heteroxenia*, (**l**) juvenile *P. briareum* (*arrow*) next to two polyps of its food, the soft coral *Briareum violacea*

This renders detection by visual predators like fish or crabs more difficult (Figs. 1k, l, and 2k, l). Second, utilizing photosynthetic products may permit specialization on prey which is rarer or of lower energetic content. This aspect is important since many opisthobranch taxa are highly specialized on certain food species, and only a few show a wider range in their prey spectrum. Hence, an independent energy supply may allow members of some species to prolong time periods in search of new and rare food. Third, a self-contained energy supply may sustain slugs

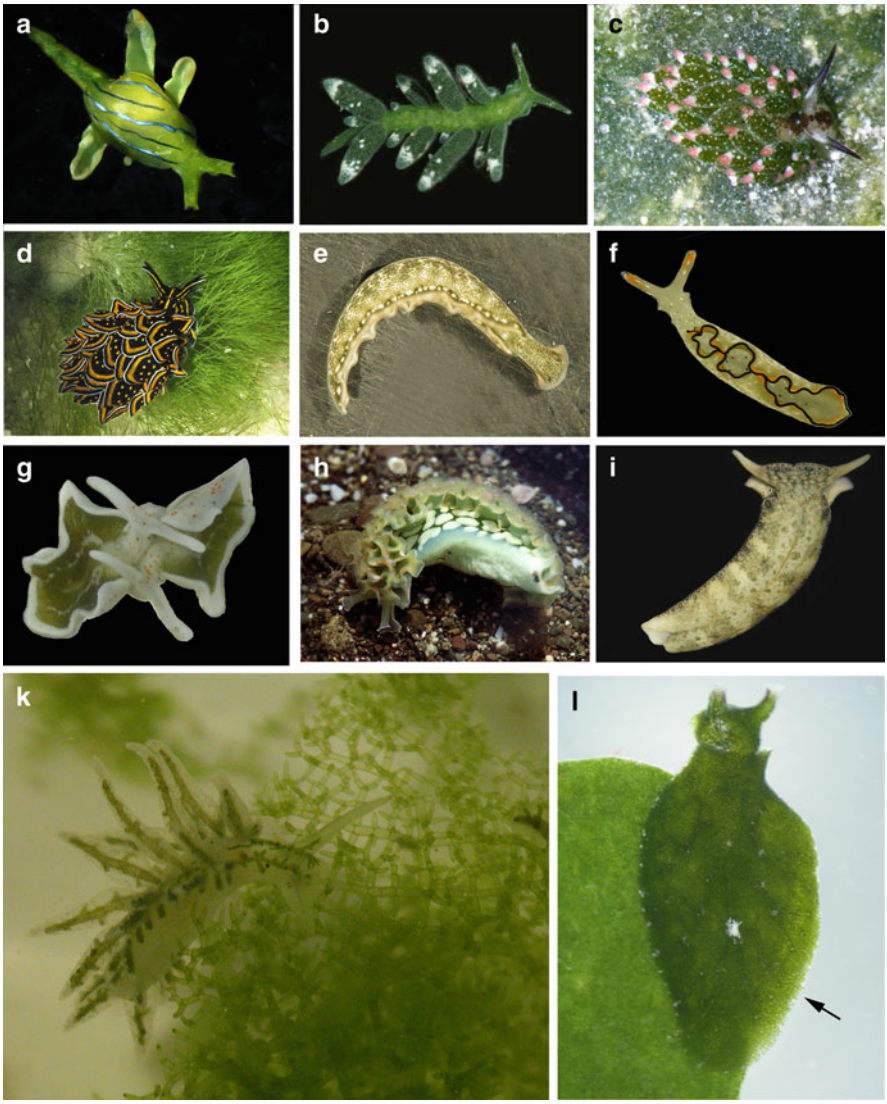


Fig. 2 Sacoglossan species: (**a**) *Lobiger viridis*, (**b**) *Ercolania kencolesi*, (**c**) *Costasiella kuroshimae*, (**d**) *Cyerce nigricans*, (**e**) *Thuridilla carlsoni*, (**f**) *Elysia ornata*, (**g**) two *Elysia timida* specimens in mating position, (**h**) *Elysia crispata*, (**i**) *Plakobranchus ocellatus*, (**K**) *Ercolania* sp on its chlorophyte food *Avrainvillea*, (**l**) *Bosellia mimetica* on its chlorophyte food *Halimeda tuna*. *Arrow* indicates the part which is shown enlarged in Fig. 3g

searching for mating partners. Opisthobranchs are usually not very abundant; therefore, searching for adequate partners might take a long time. Finally, a surplus of energy produced by sequestered PUs could enable an increase in fecundity and fitness (Burghardt and Wägele, unpublished results).

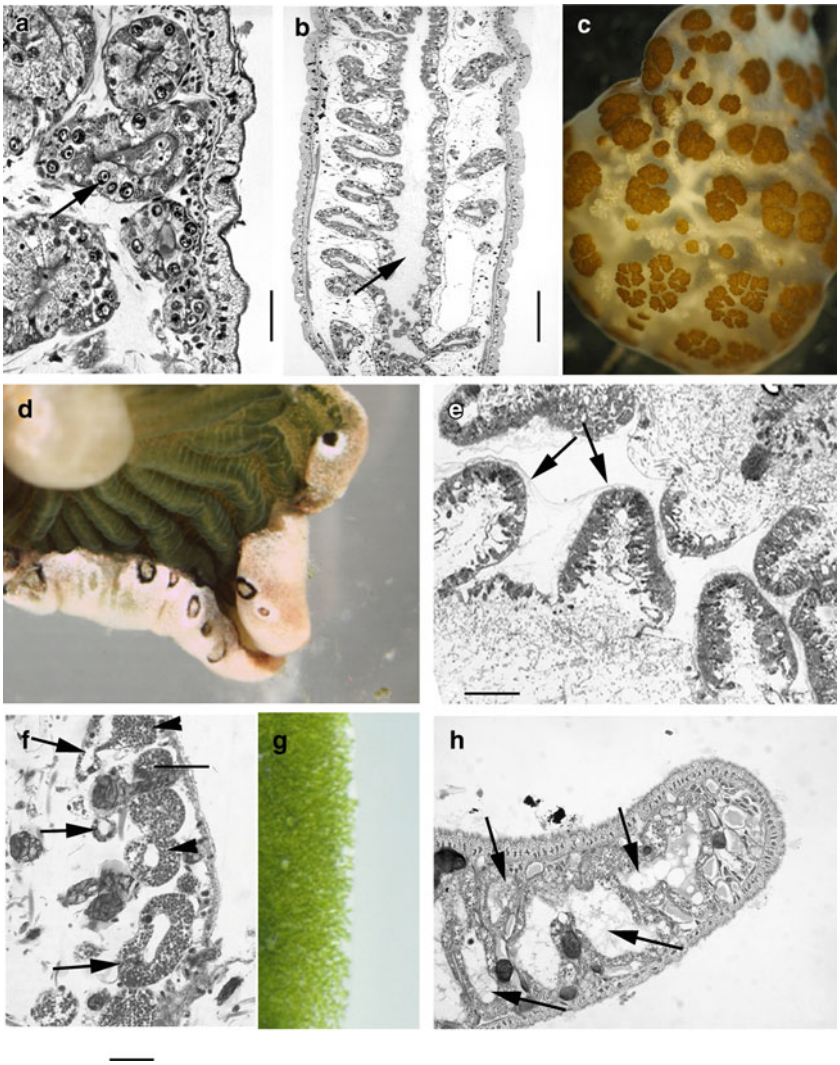


Fig. 3 Branching patterns of the digestive gland and incorporation of PUs (**a–c**) *Phyllodesmium*, (**d–h**) Sacoglossa: (**b**) zooxanthellae (*arrows*) within cells of the digestive glandular branches in *Phyllodesmium briareum*; (**b**) Longitudinal section of ceras of *P. lizardensis* with digestive glandular branches and central canal (*arrow*); (**c**) General view of ceras of *P. longicirrum*. Note the white branches inside the ceras and the brown spots on the outer part, representing the areas, where zooxanthellae are stored. (**d**) Posterior part of *Plakobranchus ocellatus* with parapodia opened. Note the green ridges, which contain the chloroplasts. (**e**) Transversial section through parapodia with several ridges (*arrows*). (**f**) Histological section through one of these ridges, showning details of the digestive glandular tubules (*arrows*), and the accumulation of chloroplasts in the outer parts of the tubules (*arrowheads*). (**g**) Detail of the lateral parapodia of *Bosellia mimetica* (see Fig. 2l, *arrow*) showing the highly branched tubular system of the digestive gland. (**h**) Longitudinal section of a ceras of *Ercolania kencolesi* showing the tubules of the digestive gland (*arrows*). *Scale bars* (**a**) 50 μm, (**b**) 250 μm, (**e**) 200 μm, (**f**) 25 μm, (**g**) 200 μm

We tested several hypotheses to address the main question, whether incorporation of PUs enhanced speciation and enabled adaptive radiation:

1. Speciation rate increased as consequence of the incorporation of functional photosynthetic units.
2. Derived species exhibit a higher efficiency in their photosynthetic activity during starving conditions. We assume that sequestration of zooxanthellae or chloroplasts usually results in their digestion for direct extraction of energy content. In the systems investigated here, PUs were not digested any more by many members but kept in the digestive gland of the slugs for a certain period of time, thus enhancing cryptic appearance on their preferred food organism. The non- or delayed digestion gave rise to the usage of photosynthetic activity of zooxanthellae/chloroplasts earlier in the evolution of this group and eventually resulted in the cultivation of PUs within the digestive system.
3. The branching of the digestive gland is correlated with the efficiency of the photosynthetic activity of the slug/zooxanthellae or slug/chloroplast system. Usually, the digestive gland within Opisthobranchia is a more or less globular structure, internally highly folded or tube-like to increase the surface for secretion of enzymes and for phagocytosis of nutritional items. When zooxanthellae or chloroplasts are exploited for their photosynthetic products, an optimization of housing the PUs is likely. This can be achieved by extending digestive glandular branches into the periphery of the slug's body and by forming special areas in these parts of the body, which allows an optimal exposure to light and minimizes shading of the PUs.

2 Materials and Methods

Specimens of the genus *Phyllodesmium* were mainly collected in tropical areas (see Burghardt and Wägele 2004; Burghardt and Gosliner 2006; Burghardt et al. 2008a, b), while animals of the Sacoglossa were collected in the Mediterranean Sea, the Caribbean Sea and around Australia (see Händeler et al. 2009). Measurements of the photosynthetic performance in sea slugs were performed with a Pulse Amplitude Modulated Fluorometer (Diving PAM; Walz, Germany). This method has been established for slugs (Wägele and Johnsen 2001; Burghardt and Wägele 2006). Photosynthetic activity is measured as a relative yield value, with high values indicating functional photosynthesis due to healthy PUs. For investigating efficiency of photosynthesis over the long term, animals collected in the field were brought back to the laboratory and kept in small aquaria under controlled conditions (temperature and salinity according to their origin) and usually in natural day and night rhythm. The aquaria were not exposed to direct sunlight but shaded by a roof of plastic or similar construction. Therefore, irradiance during daylight was reduced to maximum values of about 150–200 μmol quanta m^{-2} s^{-1}. All measurements of photosynthesis were performed in the early night hours after allowing the animals to acclimate to darkness. In consequence, the first measurement session usually

started several hours after collecting the animals and were continued in a regular 24-h rhythm. Each specimen was usually measured three times per session.

3 Results

3.1 Phyllodesmium

Out of the 30 known species of *Phyllodesmium*, 14 different species have been investigated for photosynthetic activity, with some of them retaining activity for 5 months or more (*P. longicirrum*; see Burghardt et al. 2008b). Figure 4 shows a current hypothesis of the phylogenetic relationship of these 14 *Phyllodesmium* species and their ability to incorporate zooxanthellae. The cladogram represents a schematic illustration of a Bayesian analysis (16S and CO1 genfragments) with the sea slugs *Austraeolis ornatus* and *Dondice banyulensis* as outgroup taxa. This choice is based on unpublished results of Wägele and Bleidissel. Both outgroup species belong to the same family as *Phyllodesmium*, the Facelinidae, but they do not house zooxanthellae. Two distinct clades were recovered in the tree reconstruction, as well as in NeigborNet analyses. But, contrary to tree reconstruction (shown here), the NeigborNet analyses came out with *P. poindimiei* as the species with the highest affinity to the outgroups. This incongruence is reflected in the low posterior probability of this clade (see Fig. 4). The investigation of photosynthetic activity revealed that, except for *P. poindimiei* (the most basal arranged taxon in the red clade) and the non-xanthellate outgroup taxa *Austraeolis ornata* and *Dondice banyulensis*, all investigated *Phyllodesmium* species exhibited photosynthetic activity when freshly captured (indicated by a green square in front of the species name in Fig. 4). This is in accordance with the presence or absence of zooxanthellae in the prey organisms. *Austraeolis ornata* and *Dondice banyulensis* are mentioned as feeding on non-xanthellate hydrozoans (see Rudman 2001; McDonald and Nybakken 2009) and *Phyllodesmium poindimiei* on *Carijoa*, a clavulariid octocoral not known to be xanthellate (Fabricius and Alderslade 2001). *Phyllodesmium rudmani*, *P. jakobsenae*, *P. lizardensis*, *P. hyalinum*, *P. kabiranum*, *P. lembehensis* and *P. crypticum* feed on members of the zooxanthellate Xeniidae, whereas all other analyzed *Phyllodesmium* species feed on zooxanthellate members of different octocoral families (Fig. 4)

Long-term investigations of photosynthetic activity of various *Phyllodesmium* species revealed distinct differences in efficiency, although a statistical approach was not possible due to the rareness of many species and the difficulties in performing long-term measurements under tropical conditions without adequate infrastructure. Figure 5a, b show photosynthetic performance of different species under starving conditions (pooled data from several individuals). *P. longicirrum* shows the longest retention ability compared to all other *Phyllodesmium* species measured. Figure 5a shows this species in comparison with two species from the blue clade and Fig. 5b from the red clade. Comparing the species within the clades,

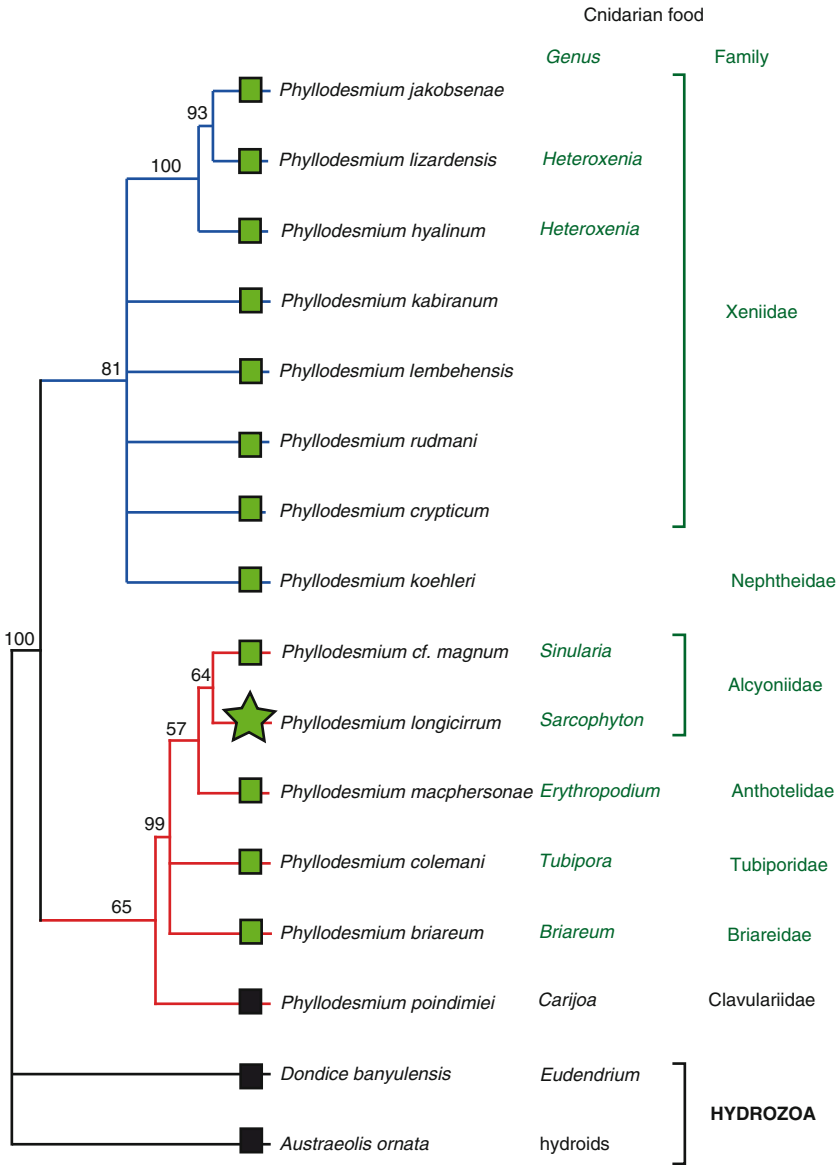


Fig. 4 Phylogeny and evolutionary traits of *Phyllodesmium* species. Cladogram based on a Baysian analysis of 16S rDNA with several specimens per species. *Values* indicate posterior probabilities. Presence of zooxanthellae in the slugs, therefore presence of photosynthesis, is indicated with a *green*, absence with a *black square*. The food organisms, on which the species can be found, are indicated. Two monophyletic clades can be observed. Members of the blue clade mainly feed on zooxanthellate soft coral species of Xeniidae (except *P. koehleri*). Members of the red clade feed on various members of different soft coral families. *P. longicirrum* as the best performing solar powered slug is highlighted with a *green star*. Those species which show no photosynthesis are feeding on non-xanthellate species

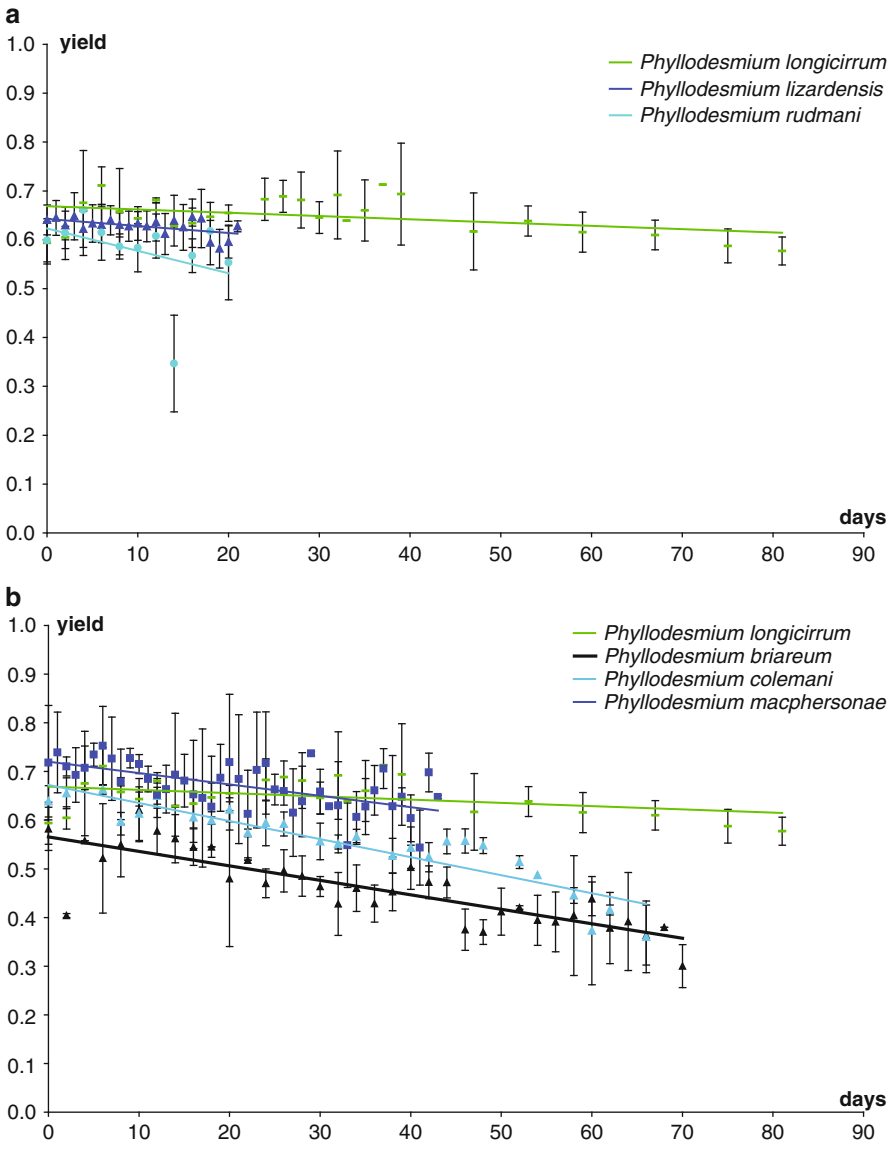


Fig. 5 Photosynthetic activity of various *Phyllodesmium* species in comparison. Yield values are plotted against starvation time. (**a**) Yield versus time plots of photosynthesis of two Xeniidae feeders, *P. lizardensis* and *P. rudmani*, in comparison with *P. longicirrum* as the best performing one. Note that *P. rudmani* is less effective in photosynthetic performance after 20 days than *P. lizardensis*. (**b**) Yield versus time plots of photosynthesis of three *Phyllodesmium* species feeding on different kinds of soft corals (see Fig. 4). Note the lowest efficiency in *P. briareum*

P. lizardensis shows a less distinct decline than the closely related *P. rudmani*. All members of the red clade also show a distinct decline but efficiency started on different levels. When mapping these results on the cladogram, it seems that a more derived species, namely *P. longicirrum,* exhibits the best performing photosynthetic activity, here indicated with a star, while more basal species like *P. briareum* and *P. colemani* of the same clade exhibit lower efficiencies. The arrangement of the species within the blue clade, which comprise mainly xeniid feeders, into good or less efficient performing photosynthetic species, is not evident due to the lack of long-term data. Furthermore, tree reconstruction resulted in polytomies and NeighborNet analyses indicated conflict in the data, which makes a conclusion with regard to evolution of efficiency vague at this state. However, none of the xeniid feeders shows the efficiency of *P. longicirrum*. In the clade of xeniid feeders, *P. koehleri* stands apart due to its differing food prey (Nephteidae; see Burghardt et al. 2008a). But the position of this species is still a subject of discussion as it is shown in the low posterior probability value in the cladogram (Fig. 4).

A comparison of branching patterns in total mounts of dorsal appendages (cerata), as well as in histological investigations, shows considerable differences between investigated species (Fig. 3a–c). For example, *P. longicirrum* exhibits high photosynthetic yield values for more than 5 months and shows a much higher branching digestive glandular pattern (Fig. 3c) than those with lower efficiencies (e.g., *P. briareum*, *P. lizardensis*, Fig. 3a, b). Preliminary morphometric investigation of digestive glandular epithelia and zooxanthellae in *P. longicirrum* in comparison to non-xanthellate species of various aeolids (*Cuthona caerulea*, *Facelina rubrovittata*) revealed a much smaller total volume of the digestive glandular epithelium within the whole appendage compared to non-xanthellate species with a ratio of 25% for the former and 50% for the latter (Wägele and Krüger, unpublished data). This is surprising at first sight. Probably, the digestive glandular surface has to be larger in species which have to feed continuously, while zooxanthellate species that perform photosynthesis have reduced the secretionary epidermis in favor of a highly and more effective branched system with less surface. On the other hand, the total cerata volume compared to the rest of the body is much more increased, indicating the importance of the cerata for storing zooxanthellae. Figure 3 shows a branching system with only primary and secondary branches in *P. lizardensis* (Fig. 3b), while *P. longicirrum* (Fig. 3c) possesses a highly ramifying digestive gland within the dorsal appendages. Extensive morphometric measurements have to follow in the future to reveal more detailed results on this feature.

4 Sacoglossa

Of the 300 known sacoglossan species, more than 60 selected species have been included in a phylogenetic analysis based on mitochondrial 16S rDNA, CO1 and the nuclear 28S rDNA gene (Händeler et al. 2009). Figure 6 shows a Baysian analysis with terminal branches united on species level. In congruence with

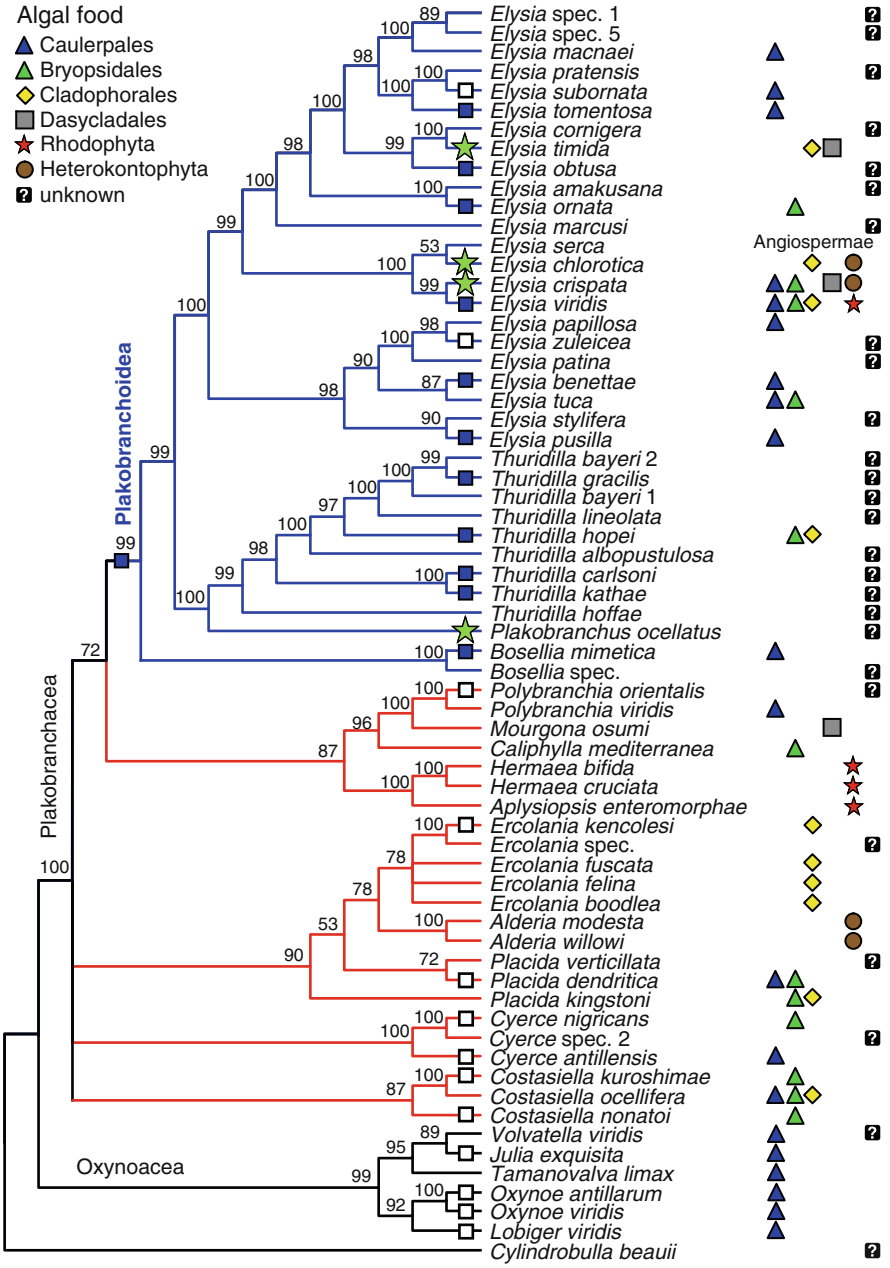


Fig. 6 Phylogeny and evolutionary traits of the Sacoglossa. The cladogram originally based on a Baysian analysis of 16S rDNA, CO1 and 28S rDNA sequences with several specimens per species (after Händeler et al. 2009). *Values* indicate posterior probabilities. Presence of photosynthetic activity within the first few days is indicated with a *blue square* (yield values above 0.4), and those with no photosynthesis from the very beginning with an *empty square*. The *green stars* indicate long-term retention forms. Known algal food sources are indicated behind the species names

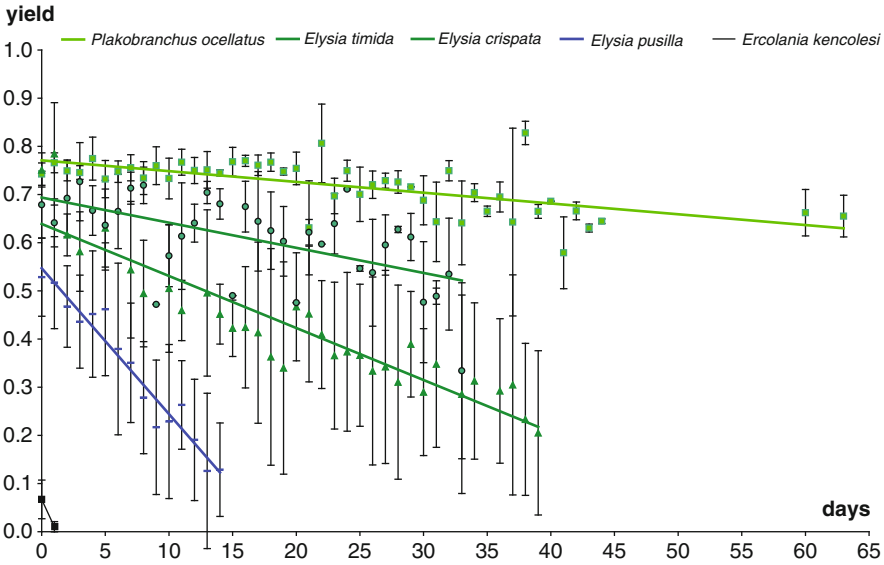


Fig. 7 Photosynthetic activity of various Sacoglossan species in comparison. Yield values are plotted against starvation time (after Händeler et al. 2009). The three long-term retention forms are indicated in *green colors*. Only one species is plotted in *blue* representing a large group of species which do not digest chloroplasts in the very beginning and therefore show a high yield value during the very first days. But chloroplasts degrade after a few days and yield values decrease down to zero. One graph in *black* signifies the group which digests chloroplasts at once and yield values are never higher than around 0.2 or even lower

morphological data, two of the major taxa (Oxynoacea: black clade; Plakobranchacea: blue clade) are recognized as monophyla, but the molecular data indicate the partly non-resolved Limapontioidea (red clades) as a paraphyletic taxon. Photosynthesis measurements under starvation conditions have been performed in 28 species; selected examples are plotted in Fig. 7. Data of several specimens per species are pooled and standard deviations given. Trend lines show different efficiencies across species (after Händeler et al. 2009). The monophyletic Oxynoacea and paraphyletic Limapontioidea never exhibited yield values higher than 0.2–0.3, despite collecting them directly from their preferred food and measuring them a few hours later (black graph in Fig. 7, and black squares in Fig. 6). In these species, chloroplasts are digested directly after sequestration. However, PAM measurements in low-performing sacoglossans are difficult, with a much higher rate of errors. Therefore, results for some species have to be considered as preliminary.

Many specimens showed a high yield value of 0.4–0.7 in the beginning, indicating a photosynthetic activity of the sequestered chloroplasts, but activity decreased to zero after 10–20 days. The blue graph in Fig. 7 of *Elysia pusilla* exemplifies this short-term retention. Further examples are indicated with blue squares in Fig. 6. These yield values indicate that chloroplasts remain in functional condition for a couple of days before degradation and/or digestion occurs (Händeler et al. 2009).

The few available values for *Elysia subornata* and *E. zuleicea* lay below 0.4 and might indicate already a certain period of starvation time. During our studies only three species turned out to exhibit long term retention of functional chloroplasts: (1) *Plakobranchus ocellatus* from the Indopacific, with a slight decline of yield values during 2 months of measurement, (2) *Elysia timida* from the Mediterranean Sea, with a steady decrease of about 0.2 within 5 weeks, and (3) *Elysia crispata* from the Caribbean, with a decrease of 0.4 within almost 6 weeks (green graphs in Fig. 7, and green stars in Fig. 6). Chloroplasts are maintained in a healthy condition within these three slug species for a very long period in comparison to other sacoglossan species, demonstrating functional kleptoplasty. It has to be mentioned here that data are pooled from various specimens, and it is not known when and which algal type has been consumed prior to analyses (see also Händeler et al. 2009). In consequence, intraspecific variations of the yield values in species with a broader food spectrum (e.g., *Elysia crispata*; Fig. 6) can result from consumption of different algal species, which may vary in the extent to which their chloroplasts can be maintained in the slugs. Functional kleptoplasty for more than 8 months has been described for *Elysia chlorotica* (Rumpho et al. 2000, 2001). Therefore, this species represents another long-term retention form (indicated by a green asterisk in Fig. 6). Both groups (long-term and short-term retention) represent the monophyletic taxon Plakobranchacea. However, the four identified long-term retention species do not form a monophyletic group.

Within shelled Oxynoacea, the morphology of the digestive gland is rather simple, forming a compact mass. In contrast to this, all other sacoglossans have branched digestive glands with tiny tubules, and, in the case of many Plakobranchacea, those tubules directly lead to the surface beneath the epidermis. This can be observed, for example, in *Bosellia mimetica*, which exhibits a high photosynthetic activity at least during the first few days (Fig. 2 and 3g), but also in those species with a long-term retention of chloroplasts (e.g., *Elysia crispata*). *Plakobranchus ocellatus* exhibits a morphological feature not observed in any other species so far: the dorsal part of the body forms ridges which are filled with tiny digestive glandular tubules (Fig. 3d, e) and in which the predominant part of the chloroplasts is stored in enlarged tubules of the digestive gland (Fig. 3f). These structures are usually hidden by the folded parapodia. Nevertheless, the presence of a highly folded digestive gland within species, which digest chloroplasts directly (e.g., *Ercolania kencolesi*; Fig. 3h; see Grzymbowski et al. 2007), indicates that complex branching patterns within the Sacoglossa are not correlated with efficiency in photosynthesis.

5 Discussion

When comparing the two studied systems, we have to keep in mind that they differ in essential features. Whereas *Phyllodesmium* incorporates a whole self-sustaining organism, namely *Symbiodinium* spp, sacoglossans only incorporate an organelle,

which is not able to survive on its own. Nevertheless, similarities in the two systems can be observed.

First, the incorporation of photosynthetic units is not present in all members of the analyzed clades (*Phyllodesmium* as well as Sacoglossa). Those species branching off earlier do not exhibit incorporation due to lack of PUs in the prey organism (e.g., *Phyllodesmium poindimiei*) or digestion of PUs (mainly members of the Oxynoacea and limapontioidean clades). Nevertheless, higher speciation rates are likely associated with acquired photosynthetic ability in both systems, since taxa with PUs are at least five times as species-rich compared to their putative sister taxon. In the case of *Phyllodesmium*, the outgroup genera *Austraeolis* and *Dondice* comprise two to three species each. These numbers are similar to nearly all other facelinid genera, while *Phyllodesmium* includes about 30 species. Within the Sacoglossa, the putative sister taxon comprises the family Hermaeidae and part of the Polybranchiidae (Fig. 6) with about 25 species, whereas the Plakobranchoidea include more than 130 species. Nevertheless, current data are still limited, and statistical approaches, as lineages-through-time plots (Nei 1992), cannot be applied, because this method is based on complete trees including all species, which are not present for either *Phyllodesmium* or Sacoglossa.

Second, there is some evidence that derived *Phyllodesmium* species (here *Phyllodesmium longicirrum*) show a more efficient photosynthesis than more basal, and therefore older, lineages. In contrast to this, sacoglossans classified as long-term retention forms do not form a monophylum and do not represent highly derived taxa in the cladogram (see position of *Plakobranchus ocellatus* in Fig. 6). Nevertheless, the stemline of the Plakobranchoidea has evolved a trait that results in the loss of direct digestion of sequestered chloroplasts, and hence is an important step towards long-term retention.

The branching pattern of the digestive gland correlates with the presence of zooxanthellae and their photosynthetic efficiency at least in zooxanthellate *Phyllodesmium* species. Species with lower photosynthetic efficiency have a less ramified digestive glandular system within the dorsal appendages than the highly efficient *P. longicirrum*. In the latter, zooxanthellae are concentrated at the outer parts of the digestive glandular branches, whereas in *P. briareum* no such concentration can be observed (compare Fig. 3a and c). So far, our results on branching patterns correlating with efficiency of photosynthesis confirm the assumptions described by Rudman (1981). Within the Sacoglossa, this aspect is more difficult to evaluate due to the highly variable morphology of different species, depending on the presence of dorsal appendages or parapodia (compare Fig. 2b and f). Even species with no photosynthetic activity and direct digestion of chloroplasts might exhibit a branched or folded digestive gland. Those animals appear green-colored (e.g., *Ercolania kencolesi*), and hence are cryptic in their natural habitat on the algae. On the other hand, the ridges on the dorsal side of *Plakobranchus ocellatus* with an accumulation of chloroplasts in digestive glandular branches certainly represent an adaptive morphological trait, allowing an optimal exposure of the chloroplast sites to the sunlight. Furthermore, the presence of parapodia, which cover these ridges mainly during bright daylight and which open in dim light, indicate behavioral adaptations according to irradiances.

A similar behavior is also observed in *Elysia timida*, a species which shows long-term retention of chloroplasts, but has no special morphological adaptations (Rahat and Monselise 1979; Casalduero and Munain 2008).

To date, we have no information about the number of different clades of *Symbiodinium* that are incorporated in the different species of *Phyllodesmium*. Of course, the composition in the slugs is dependant on the composition of zooxanthellae in the food source. While a latitudinal variability in symbiont specifity was shown for scleractinian corals (e.g., Rodriguez-Lanetty et al. 2001), other studies show a bathymetric variability in symbiont diversity (Iglesias-Prieto et al. 2004; Frade et al. 2008). But this still has to be investigated for most zooxanthellate slugs. Loh et al. (2006) have shown for *Pteraeolidia ianthina*, another member of the slug taxon Facelinidae, that the composition of *Symbiodinium* clades depends on locality, and the housed clades reflect the environmental conditions. The heterogeneity of zooxanthellae within this species might be correlated to the different food sources and hence does not reflect active selection of certain clades. Unfortunately, information on food organisms is very limited for this species. All *Phyllodesmium* species show a close affinity to a certain coral species and do not switch even to closely related species. Unpublished data of K. Stemmer indicate similar zooxanthellae clade composition in *Phyllodesmium lizardensis* compared to its unidentified host species *Heteroxenia* sp.

The origin of chloroplasts and the heterogeneity of chloroplast composition on an intraspecific and/or intra-individual level of the sacoglossans, as well as the factors which affect the time of chloroplast retention and efficiency of photosynthesis, are hardly understood (see, e.g., Curtis et al. 2005; Casalduero and Munain 2008; Vieira et al. 2009). Members of the Sacoglossa are generally considered as food specialists feeding on one certain algal species. Nevertheless, species exhibiting a more efficient photosynthesis feed on a variety of algal species, and the role of sequestration of chloroplasts from heterokontophytes (e.g., in *Elysia chlorotica*, *E. crispata*) seems to be of special interest (Rumpho et al. 2000; Curtis et al. 2005; Pierce et al. 2006; Händeler and Wägele 2007) (see Fig. 6). Unfortunately, the food of *Plakobranchus ocellatus*, representing one of the most efficient species in performing photosynthesis, is currently unknown. Preliminary feeding experiments with *Elysia crispata* by offering certain algal species and measuring photosynthetic activity showed that some of the consumed algae do not contribute to photosynthesis: the photosynthetic activity decreased after consumption of *Halimeda opuntia*, whereas the chloroplasts of *Caulerpa verticillata* were not digested and yield values remained high (Grzymbowski and Wägele, unpublished data). Nevertheless, this observation has to be investigated in detail. Trench et al. (1973a, b) suspected that the "robustness" of the chloroplast's outer membranes in siphonaceous algae was a prerequisite for retention in sea slugs. A recent study (Händeler et al. 2010) identified at least three different types of chloroplasts in the digestive system of an undescribed *Elysia* species by analyzing the chloroplast gene *tufA*. Using such molecular techniques, the species identity of chloroplasts that are responsible for photosynthesis and retained for many weeks may be identified. These analyses are essential for understanding the evolution of solar power in sacoglossans.

6 Can We Consider the Incorporation of Photosynthetic Units (PUs) an Adaptive Radiation?

This question is still difficult to answer due to many questions that arose during the studies. It seems likely that, when the ability has evolved to incorporate and not digest photosynthetic units any more, no reversion to the plesiomorphic condition can be observed within the investigated taxa. Ambiguous results, like those for *Elysia zuleicea* and *E. subornata*, have to be re-investigated. According to the results presented here, evolution of solar power has occurred once (NeighborNet Analysis) or twice (tree reconstruction) in *Phyllodesmium*, depending on the ambiguous position of the non-xanthellate *P. poindimiei*. Ancestral character state analysis clearly demonstrated the evolution of non-digestion of chloroplasts, the prerequisite for long-term retention, as a single event within Sacoglossa (Händeler et al. 2009).

The high number of species in those clades with incorporation of PUs in comparison to other, closely related taxa, including putative sister taxa, suggests a higher speciation rate. In consequence, one prerequisite is fulfilled to define a key character (Schluter 2000). But we have no idea about extinction rates in the different clades, and these of course have an impact on the net rate of speciation (Skelton 1993). In the genus *Phyllodesmium*, we see morphological adaptations evolving within the clade that allow a better storage and exposure of the incorporated zooxanthellae. According to the current results, two distinct clades with members exhibiting various phenotypes have evolved: one clade with several species on different soft coral families, and a second with species that live and feed exclusively on the family Xeniidae. The "mini-radiation" on Xeniidae might have been triggered by other factors, e.g., secondary metabolites, which are also incorporated by the slugs (Affeld et al. 2009).

In the case of the Sacoglossa, branching of the digestive gland is a pre-adaptation that evolved before incorporation (i.e., non-digestion) of chloroplasts. Many species of the Oxynoacea are green without incorporation of chloroplasts. Here, the pigment is not associated with the digestive gland but distributed throughout the body, even the shell. Therefore, de novo biosynthesis by the slug seems likely, a process that certainly incurs energy costs. Becoming green and cryptic by retaining ingested chlorophyll for some time might be a less costly strategy. However, long-term retention of chloroplasts is only observed in four species. Therefore, the incorporation of PUs plus the advantage to exploit photosynthetic products is not a common feature of the Sacoglossa and is restricted to these few species. Trench et al. (1973a, b) investigated photosynthetic carbon fixation by chloroplasts in *Codium* and compared these results with carbon fixation in *Elysia viridis*, a species that is only a short-term retention form. More than 30% of the fixed carbon is incorporated in galactose produced by the slug and this percentage is comparable to fixed carbon release in *Codium*. In contrast, only about 2% of the fixed carbon is released to the medium by isolated chloroplasts. Hence, not digesting chloroplasts at once, but using them until they degrade, constitutes an advantage for these slugs. Unfortunately, no similar studies have been performed for other species.

According to phylogenetic results, the Plakobranchoidea are monophyletic. PAM measurements have shown that the ancestor of this clade has lost the ability to digest chloroplasts or/and switched to algal food organisms, which allow a retention of chloroplasts until their degradation. Even if we do not see an adaptation in morphology or other life traits for this group in general, we can say that speciation rate increased and finally led to evolution of long-term retention in at least four not closely related species. Interestingly, a horizontal gene transfer from the algal genome of a heterokontophyte to the genome of *Elysia chlorotica* is described (Pierce et al. 2007; Rumpho et al. 2008). *E. chlorotica* is known to survive 8 months without feeding, and metabolism is sustained by photosynthetic products of the incorporated chloroplasts. Chloroplasts have only a small percentage of the necessary genes to perform photosynthesis; a horizontal gene transfer seems to be a likely explanation for the possibility to perform photosynthesis. We now have to ask, did such a gene transfer happen in the early evolution of the Sacoglossa, followed by the independent activation of transferred genes in the lineages capable of long-term chloroplast retention?

7 Conclusion

Our final conclusion is that it was not the evolution of one character alone, but the combination and joined evolution of several traits which enhanced speciation. "...indeed it seems only reasonable that a diversity of mechanisms should exist in nature, given the huge variation among organisms..." (Skelton 1993: p.54). In the genus *Phyllodesmium*, an additional "key innovation" might have been the switch to other food organisms that offered an alternative composition of natural products and rendered the slugs better defended. This would explain the additional radiation on the soft coral taxon Xeniidae. Within Sacoglossa, a switch from ancestral food *Caulerpa* to other algal food might have facilitated penetration of the algal cell wall (Jensen 1997), or allowed a better usage of natural products in the algae (Cimino et al. 1999), or allowed a more cryptic appearance, because chloroplasts were not destroyed during sequestration. Hence, incorporation of photosynthetic active chloroplasts may have only been a byproduct. The postulated gene transfer for functional retention of chloroplasts can also be hypothesized as a key character which finally might have led to long-term retention in few taxa. All these discussed changes of lifestyle, induced by an alternative use of environmental options, can be interpreted as adaptations and hence lead to an adaptive radiation in the sense of Schluter (2000).

8 Summary

Incorporation of photosynthetic units was apparently a character with an important impact on the evolution of the genus *Phyllodesmium* and the taxon Sacoglossa. In both systems, it has been shown that speciation rates are higher in those clades

exhibiting this trait. Nevertheless, the incorporation of photosynthetic units probably went in parallel with a food switch and/or change of defensive strategies. In consequence, the separation of these different traits and naming them as the only key character that enhanced speciation would reduce our observation of evolutionary processes to simple events. Contrary to radiations induced by geographical events, the speciation in complex habitats already inhabited by relatives probably needs more cues that certainly are closely linked and therefore should not be considered as separate processes.

Acknowledgments We would like to express our sincere thanks to the organizer of the "Schwerpunktprogramm" SPP 1127, Bill Martin (Düsseldorf) and Konrad Bachmann (Jena). Without their initiative, this project would probably not have taken place. Thank you also to Roswitha Schönwitz from the German Science Foundation (Bonn) and the reviewers of this programme. We wish to thank Kristina Stemmer (Bochum) for providing information on Xeniidae and Nils Anthes (Tübingen) for providing the slide of *P. longicirrum*. Thanks to Patrick Krug (Los Angeles) for sharing sacoglossan sequences and especially for the discussions and his help in improving the manuscript. This project was funded by the German Science Foundation (Wa 618/8-1 to 3).

References

Affeld S, Kehraus S, Wägele H, König GM (2009) Dietary derived sesquiterpenes from *Phyllodesmium lizardensis*. J Nat Prod 72:298–300

Aguado F, Marin A (2007) Warning coloration associated with nematocyst-based defences in aeolidioidean nudibranchs. J Molluscan Stud 73:23–38

Burghardt I, Wägele H (2004) A new solar powered speceis of the genus *Phyllodesmium* Ehrenberg, 1831 (Mollusca: Nudibranchia: Aeolidoidea) from Indonesia with analysis of its photosynthetic activity and notes on biology. Zootaxa 596:1–18

Burghardt I, Evertson J, Johnsen G, Wägele H (2005) Solar powered seaslugs -mutualistic symbiosis of aeolid Nudibranchia (Mollusca, Gastropoda, Opisthobranchia) with *Symbiodinium*. Symbiosis 38:227–250

Burghardt I, Gosliner TM (2006) *Phyllodesmium rudmani* (Mollusca: Nudibranchia: Aeolidoidea), a new solar powered species from the Indo-West Pacific with data on its symbiosis with zooxanthellae. Zootaxa 1308:31–47

Burghardt I, Wägele H (2006) Interspecific differences in the efficiency and photosynthetic characteristics of the symbiosis of "solarpowered" Nudibranchia (Mollusca: Gastropoda) with zooxanthellae. Rec West Austr Mus Suppl 69:1–9

Burghardt I, Schrödl M, Wägele H (2008a) Three new solar-powered species of the genus *Phyllodesmium* Ehrenberg, 1831 (Mollusca: Nudibranchia: Aeolidoidea) from the tropical Indo-Pacific, with analysis of their photosynthetic activity and notes on biology. J Molluscan Stud 74:277–292

Burghardt I, Stemmer K, Wägele H (2008b) Symbiosis between *Symbiodinium* (Dinophyceae) and various taxa of Nudibranchia (Mollusca: Gastropoda), with analyses of long-term retention. Org Div Evol 8:66–76

Casalduero FG, Munain C (2008) The role of kleptoplasts in the survival rates of *Elysia timida* (Risso, 1818): (Sacoglossa: Opisthobranchia) during periods of food shortage. J Exp Mar Biol Ecol 357:181–187

Cimino G, Fontana A, Gavagnin M (1999) Marine opisthobranch molluscs: chemistry and ecology in sacoglossans and dorids. Curr Org Chem 3:327–372

Cimino G, Gavagnin M (2006) Molluscs from chemo-ecological study to biotechnological application. Springer, Berlin

Clark KB, Jensen KR, Stirts HM, Fermin C (1981) Chloroplast symbiosis in a non-Elysiid mollusc, *Costasiella lilianae* Marcus (Hermaeidae: Ascoglossa (=Sacoglossa): effects of temperature, light intensity, and starvation on carbon fixation rate. Biol Bull 160:42–54

Curtis NE, Massey SE, Schwartz JA, Maugel TK, Pierce SK (2005) The intracellular, functional chloroplasts in adult sea slugs (*Elysia crispata*) come from several algal species, and are also different from those in juvenile slugs. Microsc Microanal 11:1194–1195

Edmunds M (1966) Protective mechanisms in the Eolidacea (Mollusca Nudibranchia). J Linn Soc (Zool) 47:27–70

Evertsen J, Burghardt I, Johnsen G, Wägele H (2007) Retention of functional chloroplasts in some sacoglossans from the Indo-Pacific and Mediterranean. Mar Biol 151:2159–2166

Fabricius K, Alderslade P (2001) Soft corals and Sea fans; a comprehensive guide to the triopical shallow water genera of the central-west Pacific, the Indian Ocean and the Red Sea. Australian Institute of Marine Science, Townsville

Frade PR, de Jongh F, Vemeulen F, van Bleijswijk J, Bak RPM (2008) Variation in symbiont distribution between closely related coral species over large depth ranges. Mol Ecol 17:691–703

Greene RW (1970) Symbiosis in sacoglossan opisthobranchs: symbiosis with algal chloroplasts. Malacologia 10:357–368

Grzymbowski Y, Stemmer K, Wägele H (2007) On a new *Ercolania* Trinchese, 1872 (OPisthobranchia, Sacoglossa, Limapontiidae) living within *Boergensenia* Feldmann, 1950 (Cladophorales), with notes on anatomy, histology and biology. Zootaxa 1577:3–16

Händeler K, Wägele H (2007) Preliminary study on molecular phylogeny of Sacoglossa and a compilation of their food organisms. Bonn Zool Beitr 3:231–254

Händeler K, Grzymbowski YP, Krug PJ, Wägele H (2009) Functional chloroplasts in metazoan cells – a unique evolutionary strategy in animal life. Front Zool 6:28

Händeler K, Wägele H, Wahrmund U, Rüdinger M, Knoop V (2010) Slugs' last meals: Kleptoplastids in Sacoglossa (Opisthobranchia, Gastropoda): molecular identification of sequestered chloroplasts from different algal origins. Mol Ecol Res doi: 10.1111/j.1755-0998.2010.02853.x

Iglesias-Prieto R, Beltran VH, Lajeuness TC, Reyes-Bonilla H, Thome PE (2004) Different algal symbionts explain the vertical distribution of dominant reef corals in the eastern Pacific. Proc R Soc Lond B 271:1757–1763

Jensen KR (1993a) Evolution of buccal apparatus and diet radiation in the Sacoglossa (Opisthobranchia). Boll Malacol 29:147–172

Jensen KR (1993b) Morphological adaptations and plasticity of radular teeth of the Sacoglossa (=Ascoglossa) (Mollusca: Opisthobranchia) in relation to their food plants. Biol J Linn Soc 48:135–155

Jensen KR (1997) Evolution of the Sacoglossa (Mollusca, Opisthobranchia) and the ecological associations with their food plants. Evol Ecol 11:301–335

Kälker H, Schmekel L (1976) Bau und Funktion des Cnidosacks der Aeolidoidea (Gastropoda Nudibranchia). Zoomorphologie 86:41–60

Loh WKW, Cowlishaw M, Wilson N (2006) Diversity of *Symbiodinium* dinoflagellate symbionts from the Indo-Pacific sea slug *Pteraeolidia ianthina* (Gastropoda: Mollusca). Mar Ecol Progr Ser 320:177–184

McDonald GR, Nybakken JW (2009) A list of the worldwide food habits of Nudibranchs. http://people.ucsc.edu/~mcduck/nudifood.htm. Cited 6 Feb 2009 http://people.ucsc.edu/~mcduck/nudifood.htm last access 20 May 2009

Nei M (1992) Genetic distance between populations. Am Nat 106:283–292

Pierce SK, Curtis NE, Massey SE, Bass AL, Karl SA, Finney CM (2006) A morphological and molecular comparison between *Elysia crispata* and a new species of kleptoplastic sacoglossan sea slug (Gastropoda: Opisthobranchia) from the Florida Keys, USA. Molluscan Res 26:23–38

Pierce SK, Curtis NE, Hanten JJ, Boerner SL, Schwartz JA (2007) Transfer, integration and expression of functional nuclear genes between multicellular species. Symbiosis 43:57–64

Rahat M, Monselise EBI (1979) Photobiology of the chloroplast hosting mollusc *Elysia timida* (Opisthobranchia). J Exp Biol 79:225–233

Rodriguez-Lanetty M, Loh W, Carter D, Hoegh-Guldberg O (2001) Latitudinal variability in symbiont specifity within the widespread scleractinian coral *Plesiastrea versipora*. Mar Biol 138:1175–1181

Rudman WB (1981) The anatomy and biology of alcyonarian feeding aeolid opisthobranch molluscs and their development of symbiosis with zooxanthellae. Zool J Linn Soc 72:219–262

Rudman WB (1987) Solar-powered animals. Nat Hist 10:50–52

Rudman WB (1991) Further studies on the taxonomy and biology of the octocoral-feeding genus *Phyllodesmium* Ehrenberg, 1831 (Nudibranchia: Aeolidoidea). J Molluscan Stud 57:167–203

Rudman WB 2001 (Nov 26). Comment *on Austraeolis ornata* from Sydney by Mairi Prisk. In: Sea slug forum. Australian museum, Sydney. http://www.seaslugforum.net/find.cfm?id=5726 Accessed 30 May 2009

Rumpho ME, Summer EJ, Green BJ, Fox TC, Manhart JR (2001) Mollusc/algal chloroplast symbiosis: how can isolated chloroplasts continue to function for months in the cytosol of a sea slug in the absence of an algal nucleus? Zoology 104:303–312

Rumpho ME, Summer EJ, Manhart JR (2000) Solar-powered Sea slugs. Mollusc/algal chloroplast symbiosis. Plant Physiol 123:29–38

Rumpho ME, Worful JM, Lee J, Kannan K, Tyler MS, Bhattacharya D, Moustafa A, Manhart JR (2008) Horizontal gene transfer of the algal nuclear gene psbO to the photosynthetic sea slug Elysia chlorotica. Proc Natl Acad Sci USA 105:17867–17871

Schluter D (2000) The ecology of adaptive radiation. In: Oxford series in ecology and evolution. Oxford University Press, Oxford, pp 1–288

Skelton PW (1993) Adaptive radiation definition and diagnostic tests. In: Lees DE, Edwards D (eds) Evolutionary patterns and processes. Academic, New York, pp 45–58

Trench RK (1973) Further studies on the mucoploysaccharide secreted by the pedal gland of the marine slug *Tridachia crispata* (Opisthobranchia, Sacoglossa). Bull Mar Sci 23:299–312

Trench RK, Boyle JE, Smith DC (1973a) The association between chloroplasts of *Codium fragile* and the mollusc *Elysia viridis* I. Characteristics of isolated *Codium* chloroplasts. Proc R Soc Lond B 184:51–61

Trench RK, Boyle JE, Smith DC (1973b) The association between chloroplasts of *Codium fragile* and the mollusc *Elysia viridis* II. Chloroplast ultrastructure and photosynthetic carbon fixation in *E. viridis*. Proc R Soc Lond B 184:63–81

Vieira S, Calado R, Coelho H, Serôdio J (2009) Effects of light exposure on the retention of kleptoplastic photosynthetic activity in the sacoglossan mollusc *Elysia viridis*. Mar Biol 156:1007–1020

Wägele H (2004) Potential key characters in Opisthobranchia (Gastropoda, Mollusca) enhancing adaptive radiation. Org Div Evol 4:175–188

Wägele H, Ballesteros M, Avila C (2006) Defensive glandular structures in opisthobranch molluscs - from histology to ecology. Oceanogr Mar Biol Annu Rev 44:197–276

Wägele H, Johnsen G (2001) Observations on the histology and photosynthetic performance of "solar-powered" opisthobranchs (Mollusca, Gastropoda; Opisthobranchia) containing symbiotic chloroplasts or zooxanthellae. Org Divers Evol 1:193–210

Wägele H, Klussmann-Kolb A (2005) Opisthobranchia (Mollusca, Gastropoda) - more than just slimy slugs. Shell reduction and its implications on defence and foraging. Front Zool 2:1–18

Are Cuticular Hydrocarbons Involved in Speciation of Fungus-Growing Termites (Isoptera: Macrotermitinae)?

Andreas Marten, Manfred Kaib, and Roland Brandl

Abstract Although termites are keystone species of tropical ecosystems, little is known about factors and processes involved during diversification. A prerequisite of all speciation processes is the isolation of lineages. We investigated the potential role of cuticular hydrocarbons for behavioral isolation in termites. The hydrocarbon composition on the cuticle of inquilines matches the composition of the host termite, suggesting that hydrocarbons provide cues for nestmate recognition and, therefore, also have the potential to be involved in species recognition. We studied the variation of cuticular hydrocarbons within and between species of the genus *Macrotermes* and behavioral responses to these variations. Our results indicate that cuticular hydrocarbons are at least one factor involved in nestmate recognition and might act as a defense strategy against inquilines. However, they do not play a major role during speciation events of higher termites; the situation in lower termites probably differs.

1 Introduction

Termites are keystone species in many tropical ecosystems. They contribute to ecosystem processes such as litter decomposition, and are, therefore, an important part of the carbon and nitrogen cycles (e.g., Wood and Sands 1978; Holt and Lepage 2000; Sugimoto et al. 2000; Donovan et al. 2001). Besides their ecological

A. Marten and R. Brandl (✉)
Animal Ecology, Department of Ecology, Faculty of Biology, University of Marburg, Karl-von-Frisch Str. 8, 35032 Marburg, Germany
e-mail: brandlr@staff.uni-marburg.de

M. Kaib
Department of Animal Physiology, Faculty of Biology, University of Bayreuth, Universitätsstr. 30, 95440 Bayreuth, Germany
e-mail: manfred.kaib@uni-bayreuth.de

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_14,

importance, termites attract the interest of researchers for two other reasons. First, the eusocial lifestyle of termites, with extensive division of labor between castes, makes termites a promising target for studying the evolution of eusociality in comparison to ants and bees. In contrast to social hymenopterans, termites are diplo-diploid, and reproductive females (queens) and males (kings) occur in each colony. Thus, a comparison of the social systems of Hymenoptera and Isoptera could reveal insights into the general processes behind the evolution of eusociality (Husseneder et al. 1998). Secondly, some termites are pests that attack crops, food stocks, and houses, causing extensive economical loss (e.g., Su and Scheffrahn 2000).

Termites (Isoptera) comprise over 280 genera with more than 2,600 known species. However, each year more than 20 new species are described (Eggleton 1999). Termites are morphologically rather uniform, and thus only a few traditional taxonomic characters are available for taxonomic and phylogenetic studies, and cryptic species seem to be common in termites (e.g., Page et al. 2002; Copren et al. 2005; Brandl et al. 2007; Marten et al. 2009). Only the soldier castes show a diversity of morphological structures, mostly adaptations for defense (Kaib 1985). In addition to this morphological diversity, soldiers use a considerable diverse set of chemical substances for defense that characterize certain major lineages (Prestwich 1983).

Termites are blind and silent. Therefore, the only ways to communicate are tactile and chemical signals. The low diversity of pheromones compared to ants (Pasteels and Bordereau 1998; Kaib 2000) initiated the search for alternative substances which might be involved in the communication between individuals or species during nuptial flights. Many insects show considerable variation in the chemical composition of the epicuticle, in particular hydrocarbons, within and between species (Lockey 1991; Howard 1993). These cuticular hydrocarbons have become a focus of entomological research during the last two decades, mainly for two reasons. First, hydrocarbon compositions are assumed to be species-specific and therefore may be used as characters in chemotaxonomy (e.g., Carlson and Service 1980; Bagnères et al. 1991; Estrada-Pena et al. 1994; Espelie et al. 1996; Brown et al. 1997; Steiner et al. 2002; Raboudi et al. 2005; Ye et al. 2007; see also the comprehensive review by Lockey 1988). Secondly, cuticular hydrocarbons may be involved in nestmate recognition in social insects and therefore important issues for the maintenance of the social system (e.g., Howard et al. 1980; Smith and Breed 1995; Takahashi and Gassa 1995; Clément and Bagnères 1998; Singer 1998; Lahav et al. 1999; Ruther et al. 2002; Howard and Blomquist 2005).

Speciation always requires mechanisms of reproductive isolation. These could be prezygotic mechanisms of geographical, ecological, temporal, or behavioral isolation, or zygotic mechanisms, e.g., hybrid sterility (Bouillon 1981). In termites, isolating mechanisms between sympatric species occur during the nuptial flights of the alates and the subsequent pairings and may involve different swarming times (e.g., Wood 1981; Haverty et al. 2003), differences in sexual attractants (e.g., Peppuy et al. 2004), differences in pairing behavior (Wood 1981), or aggression between lineages as responses to differences in their recognition cues. In many

insect taxa, reproductive isolation is based on the variation of pheromones (e.g., reviewed in Howard and Blomquist 1982; Howard 1993). However, there is only a very low number of known sex pheromones in termites that are used, moreover, for other purposes (e.g., trail following) and that are not species-specific (Billen and Morgan 1998; Pasteels and Bordereau 1998; but see Peppuy et al. 2004). As termites may use hydrocarbons to define the "gestalt" of a colony and species, differences in hydrocarbon composition within and between populations may therefore provide additional cues for the isolation of lineages by triggering agonistic behavior during pairings between different species.

We investigated the potential of hydrocarbons for isolation of lineages in several species of the higher termite genus *Macrotermes* (Termitidae) in East and West Africa (Fig. 1) using methods ranging from chemical and behavioral to phylogenetic analyses. The genus *Macrotermes* belongs to the subfamily Macrotermitinae, which cultivate fungi of the genus *Termitomyces* (Lyophyllaceae). Colonies of *Macrotermes* are closed societies without any exchange of individuals between colonies. The reproductives are found in a special compartment within the mound, the so-called "royal chamber," where they are trapped. Sometimes, several non-related reproductives co-occur within one chamber (Hacker et al. 2005).

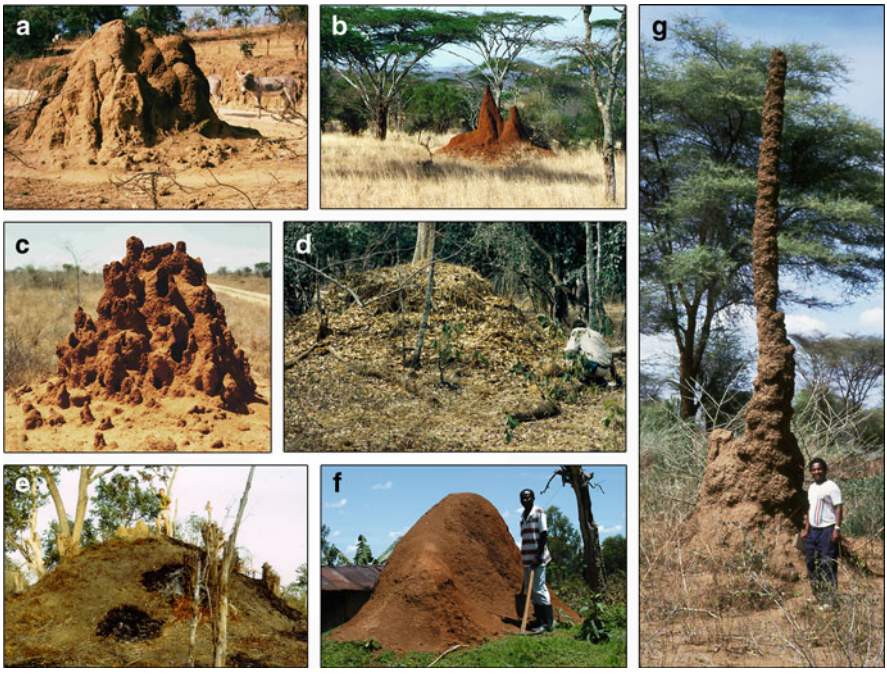


Fig. 1 Typical mounds of the investigated *Macrotermes* species. (**a**) *M. bellicosus*, Kapenguria, Kenya, (**b**) *M. michaelseni*, Kajiado, Kenya, (**c**) *M. subhyalinus*, Magadi, Kenya, (**d**) *M. subhyalinus*, Ivory Coast, (**e**) *M. falciger*, Lunga-Lunga, Kenya, (**f**) *M. herus*, Kakamega, Kenya, (**g**) *M. jeanneli*, Kerio Valley, Kenya

To maintain the colony as a functional unit, social insects need a consistent system to distinguish nestmates from non-nestmates or other invaders. Therefore, we first provide evidence that hydrocarbons may be involved in nestmate recognition.

2 Cuticular Hydrocarbons and Nestmate Recognition

Nestmate recognition is important for the integrity of a colony and for territorial behavior (Hamilton 1964; Clément and Bagnères 1998). During encounters between two individuals, each of these individuals searches for labels on the partner and compares these labels with an innate or learned template (Shorey 1973). If labels and template fit together, the two individuals will recognize each other as members of the same colony or unit (Fig. 2). Labels and templates might be influenced by genetic factors as well as by the environment. As explained above, cuticular hydrocarbons are prime candidates for the major labels of species and nestmate recognition in termites, but only if the hydrocarbon composition consistently varies between colonies and species.

Inquilines, mostly other insect species living in a colony, are invaders, which need to crack the defense system of the host colony. If cuticular hydrocarbons are colony-specific and involved in the recognition of nestmates, we expected the termite hosts and their inquilines to have almost identical profiles (see Howard et al. 1980, 1982). Therefore, we analyzed *Termitobia herus*, a staphylinid beetle with a physogastric abdomen like that of a termite queen (Fig. 3c), which lives in the termite nests of *Macrotermes herus* of East Africa (a number of cryptic species may fall under that name; see Brandl et al. 2007; Marten et al. 2009). The beetles are often found near the royal chamber and are fed by termite workers (see Kistner

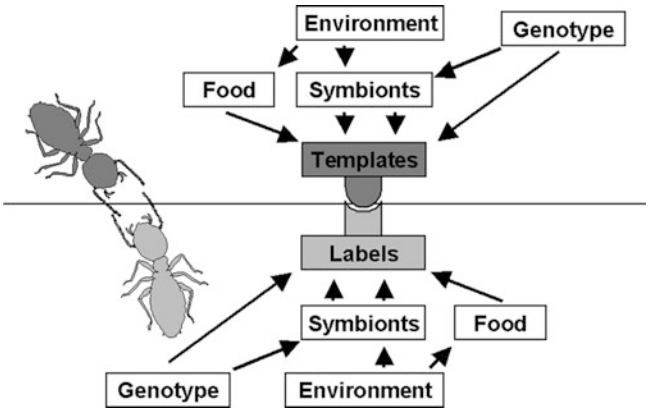


Fig. 2 Nestmate recognition in termites. Each individual has a label or a set of labels as recognition cue. These labels are compared with templates to test the status of another individual as a nestmate or a stranger. Labels and templates could be influenced by genetic and environmental factors

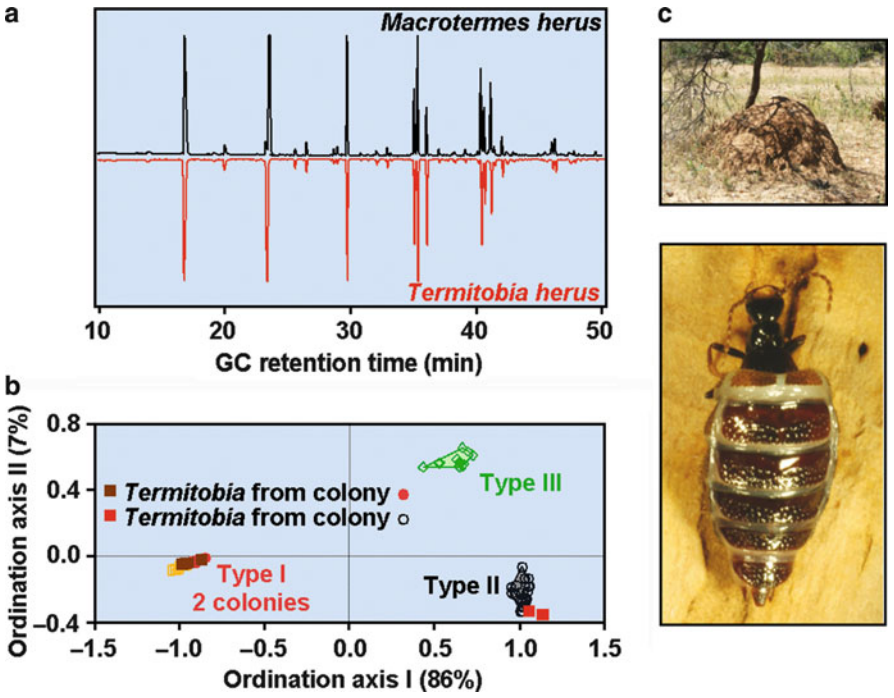


Fig. 3 Comparison of the cuticular hydrocarbon composition of the termitophile beetle *Termitobia herus* and its termite host *Macrotermes herus*. (**a**) Hydrocarbon profiles obtained by gas chromatography of termites and beetles from the same colony. (**b**) Ordination plot (correspondence analysis; ter Braak and Smilauer 2002) of cuticular hydrocarbon profiles from 62 major workers of four termite colonies and six beetles from two of these colonies. Three hydrocarbon phenotypes were observed among the four termite colonies, i.e., two colonies had the Type I phenotype. (**c**) Termite mound of *M. herus* (*top*) and the staphylinid beetle *T. herus* (*bottom*) with a physogastric abdomen

2001). As far as we know, they provide no benefits to the termites. Indeed, the same cuticular hydrocarbon patterns were found for hosts and inquilines (Fig. 3; Kaib et al. 2004b). Similar results were obtained for the dampwood termite *Schedorhinotermes lamanianus* (Rhinotermitidae) and its termitophile tineid moth *Paraclystis integer* (data not shown; see Brandl et al. 1996 for more information on this termite–inquiline system). It is not clear whether the inquilines mimic the hydrocarbon profiles actively by synthesizing their own hydrocarbons (see Howard et al. 1980) or whether they receive the hydrocarbons passively from the termites (see Vander Meer and Wojcik 1982).

Hydrocarbons also vary somewhat between castes. In *M. herus*, we found subtle differences in the cuticular hydrocarbon profiles between castes. Whereas the profiles of the worker castes, the minor soldiers, and the nymphs overlap, the major soldiers and the reproductives show separate distinct patterns (Fig. 4). Remarkably, the composition of the cuticular hydrocarbons of the termitophile beetles matches

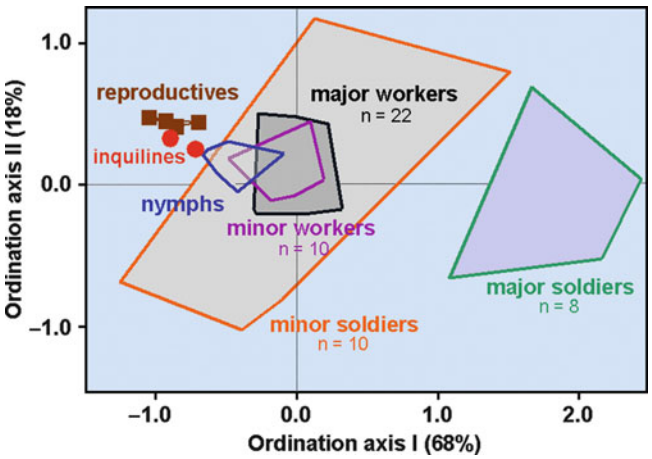


Fig. 4 Ordination plot of cuticular hydrocarbon profiles from different castes of one colony of *Macrotermes herus* and two *Termitobia herus* beetles (inquilines) of the same colony. Ordination is based on correspondence analysis (ter Braak and Smilauer 2002)

the profile of the reproductives. Workers may confuse beetles with queens when supplying food, providing them with the same food as the reproductives. The physogastric abdomen of the beetles may therefore be a consequence of this confusion (Kistner 2001). Overall, these findings provide evidence that cuticular hydrocarbons provide the labels for recognition processes at a variety of hierarchical levels: between colonies ("gestalt" of a colony) and within colonies between castes.

Speciation in sexual organisms always implies some mechanisms of recognition among members of the same species, or some mechanisms of isolation from members of other species, that involve reproduction (Bouillon 1981; Turelli et al. 2001). In termites, hydrocarbons may therefore also play a role for the isolation of reproductives. However, it is very difficult to study nuptial flights and, in particular, the interactions occurring between flying sexuals, called alates. Furthermore, pairings between alates above-ground are triggered by the pressure of predators and therefore dealates bury themselves as quickly as possible in the soil. But in the first stage of colony formation below the surface, many important interactions occur (see, e.g., Brandl et al. 2001, 2004) which are difficult to observe. This cryptic lifestyle makes observational as well as experimental studies on reproductive isolation difficult, and evolutionary and ecological studies in termites lag behind the progress in other groups. Nevertheless, our investigations tried to infer the potential role of hydrocarbons in species recognition during nuptial flights by evaluating a number of corollaries of the hypothesis that cuticular hydrocarbons may be involved in reproductive isolation:

(1) There must be sufficient variability in the composition of cuticular hydrocarbons among colonies, populations, and species to define the "gestalt" of a colony, population, or species.

(2) Cuticular hydrocarbons should be heritable, and the environment should have only limited influence on the composition.
(3) Termites should respond to differences in the composition of cuticular hydrocarbons by agonistic behavior.
(4) Differences in the composition of cuticular hydrocarbons between colonies should be correlated to genetic differentiation.

3 Variation of Cuticular Hydrocarbons Within and Between Termite Species

The hydrocarbons found in the cuticle of most examined insects typically consist of odd-numbered saturated, unsaturated, and branched aliphatic compounds with a chain length between C_{21} and C_{37} (see Lockey 1988); the chain length in some desert species might exceed well over C_{41} (e.g., Akino 2006). These hydrocarbons are synthesized with fatty acids as precursors in modified epidermis cells (oenocytes; reviewed by Howard and Blomquist 1982; Lockey 1988; Blomquist et al. 1998). One main function of hydrocarbons and other lipophilic substances in the cuticle is proposed to be the reduction of water loss (reviewed by Lockey 1988). Other functions included protection against UV radiation, toxins, and pathogenic bacteria or fungi. We have analyzed which cuticular hydrocarbons vary between species, within species but between different colonies, and between castes within a colony. We extracted hydrocarbons from the cuticle of major workers with *n*-hexane and evaluated the hydrocarbon profiles using gas chromatography. Peaks from different chromatograms were classified by comparison with peaks of an *n*-alkane series from eicosane to hexatriacontane. Individual hydrocarbons were identified by coupled gas chromatography/mass spectrometry (for detailed methods, see Kaib et al. 2002). For these analyses, we used the relative intensities of 135 gas chromatographic peaks. In some rare cases, peaks assigned to one retention time may have included more than one compound.

The cuticular hydrocarbons of 62 colonies of 7 *Macrotermes* taxa (*M. bellicosus*, *M. falciger*, *M. herus*, *M. jeanneli*, *M. michaelseni*, *M. subhyalinus* West Africa, *M. subhyalinus* East Africa) contained a mixture of saturated (*n*-alkanes), unsaturated (alkenes, alkadienes, and alkatrienes), and branched (methyl- or dimethylalkanes) hydrocarbons (Fig. 5b). Compounds with an odd-numbered chain length clearly dominated the profiles. Within a colony, the compositions of cuticular hydrocarbons between individuals were similar (see also Bagine et al. 1994). Furthermore, hydrocarbon profiles of a colony seem to be stable between years with different climatic conditions (Kaib et al. 2002). In contrast, the composition of cuticular hydrocarbons of different colonies of a specific species varied considerably; mainly, it was the unsaturated, short-chain hydrocarbons which differed (Fig. 5a, c). Different species, on the other hand, differed mainly in long-chain

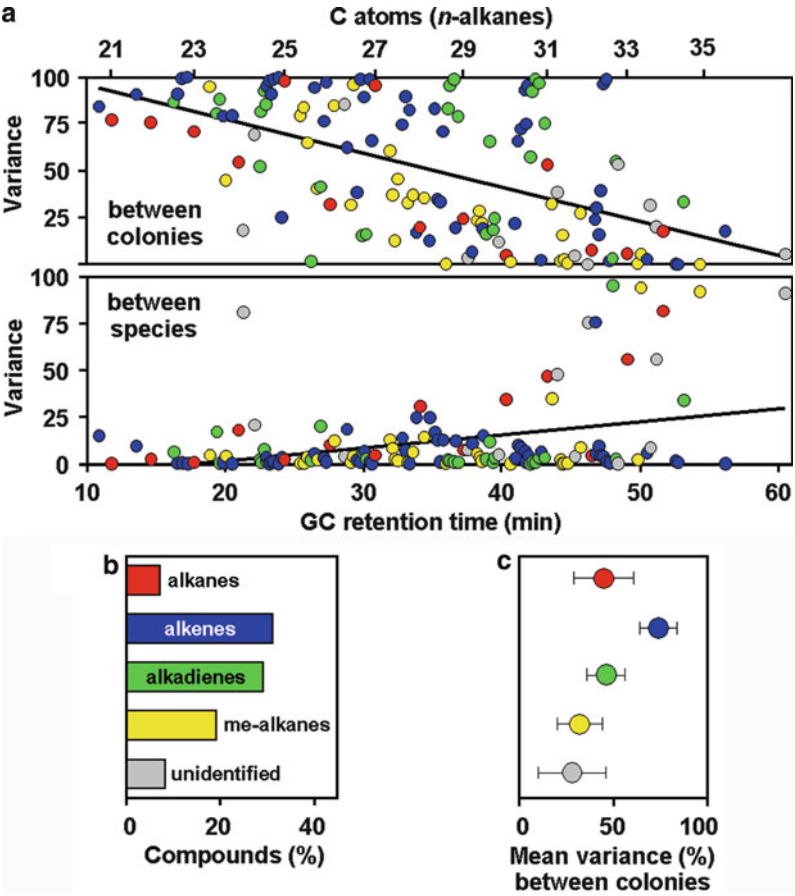


Fig. 5 Variability of cuticular hydrocarbons between colonies and species of 7 *Macrotermes* species (62 colonies, 357 individuals, 135 GC hydrocarbon peaks). (**a**) Results of a nested analysis of variance by restricted maximum likelihood, with individuals nested within colonies and colonies nested within species. Using this hierarchy, we estimated the relative variance components of each compound between individuals (not shown), between colonies (*top plot*) and between species (*bottom plot*). The *plotted regression lines* are for illustrative reasons only. (**b**) Relative amounts of the different classes of hydrocarbons. (**c**) Variability of the different hydrocarbon classes between colonies

hydrocarbons (Fig. 5a). Such long-chain hydrocarbons may be an important factor in preventing desiccation, and the variation in these hydrocarbons between species might reflect differences in their microclimatic preferences (see below, and Pomeroy 1978). For example, compared with other *Macrotermes* species, the cuticle of *M. jeanneli* and East African *M. subhyalinus* contains a larger amount of hydrocarbons with long backbone chains ($>C_{29}$). Indeed, both species build

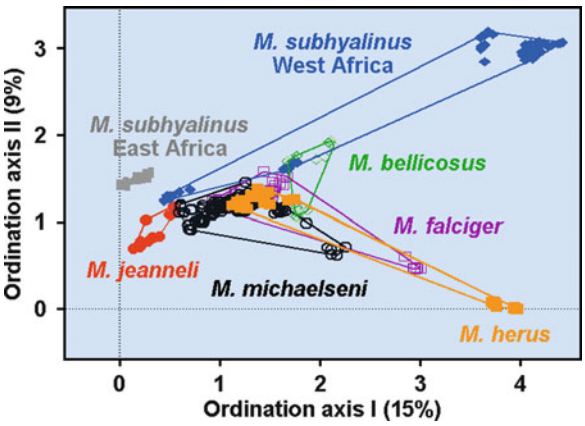


Fig. 6 Ordination plot of all cuticular hydrocarbon profiles of 7 *Macrotermes* species (62 colonies, 357 individuals, 135 GC hydrocarbon peaks) based on correspondence analysis (ter Braak and Smilauer 2002). Different species are indicated by different *colors* and *symbols*. All individuals of the same species are surrounded by an *envelope*

mounds with an open ventilation system (Fig. 1c, g) and occur predominantly in arid areas that require protection against water loss (see also below).

The variation in cuticular hydrocarbon profiles within species is often much higher than the variation between species (Figs. 5a and 6). For several species, we found distinct differences in sympatric colonies that clearly differed in their cuticular hydrocarbon profiles, and these differences were much larger than differences between species. In the following, we call these distinct types of hydrocarbon composition "phenotypes". One well-analyzed example is *M. falciger* (Kaib et al. 2002). Among colonies within an area of a few hectares in the Shimba Hills National Reserve (Kwale District, Coast Province, Kenya), we found three phenotypes that differed in their qualitative and quantitative composition (in particular, unsaturated hydrocarbons). Within phenotypes, it was not always possible to distinguish colonies unambiguously by their hydrocarbon profiles. In another study on *M. subhyalinus* in the Comoë National Park (Ivory Coast), we found four phenotypes among ten colonies, again on a small spatial scale (Fig. 7c; Kaib et al. 2004a). The phenotypes were characterized by alkenes of different chain length and with different positions of the double bond and by the relative amounts of alkanes and alkenes (Fig. 7a, b). Again, phenotypes showed considerable differences in their qualitative and quantitative composition. Such phenotypes were also found in other *Macrotermes* species (Fig. 6).

In summary, we found considerable variation of hydrocarbons within and between species. In a number of species, we found distinct and discrete phenotypes, which differed in their qualitative and quantitative composition of compounds. Phenotypes occur sympatrically and therefore these phenotypes may represent cryptic species.

a

Type	% of total hydrocarbons -ane	-ene	-diene	me-	unknown
I	65.17	23.63	8.85	0.00	0.00
II	29.66	62.82	2.48	0.00	0.00
III	39.83	58.19	0.00	0.95	0.38
IV	51.21	45.89	0.61	0.81	0.15

b

	% of total alkenes 5-ene	7-ene	9-ene	11-ene
I	72.96	4.57	8.72	13.29
II	0.00	19.44	41.71	2.18
III	0.00	10.10	89.71	0.19
IV	0.00	0.02	2.57	95.53

c

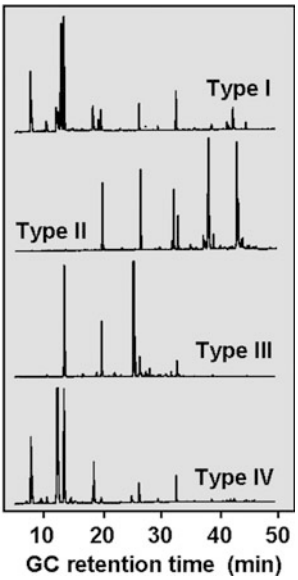


Fig. 7 Composition of cuticular hydrocarbons of four phenotypes of *Macrotermes subhyalinus* collected in West Africa (see Kaib et al. 2004a). (**a**) Relative amounts of alkanes, alkenes, alkadienes, and methyl-branched alkanes. (**b**) Relative amounts of different classes of alkenes. (**c**) Examples of hydrocarbon profiles of the different phenotypes obtained by gas chromatography

4 Heritability of Cuticular Hydrocarbons and Environmental Variation

A genetic control of hydrocarbon composition has been shown for the fly genus *Drosophila* (e.g., Coyne 1996; Ferveur and Jallon 1996; Dallerac et al. 2000; Takahashi et al. 2001). For termites, there are a few studies that suggest a genetic basis of recognition cues based on a comparison of agonistic behavior between colonies and genetic relatedness (*Microcerotermes*: Adams 1991; *Schedorhinotermes*: e.g., Husseneder et al. 1998). However, the chemical composition of the recognition cues themselves was not analyzed in these studies. In a later study by Dronnet et al. (2006) on the lower termite *Reticulitermes santonensis*, a strong positive correlation between chemical distances of hydrocarbon profiles and genetic distances obtained from microsatellite data was observed. During our own studies of *M. subhyalinus* collected in West Africa (Kaib et al. 2004a), we found that cuticular hydrocarbon differences between colonies are related to morphometric differences as well as to genetic dissimilarity, estimated from amplified fragment-length polymorphism (AFLP) fingerprints (Fig. 8). These are clear indications of a genetic component for the variation of cuticular hydrocarbons within species.

The epicuticle with its cuticular hydrocarbons is at the interface between the organism and the environment. Hydrocarbons protect against environmental

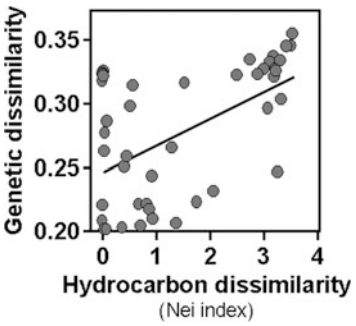


Fig. 8 Positive relationship ($r = 0.54$, $P = 0.039$; Mantel test) between genetic dissimilarity (based on AFLP data, estimated by Jaccard index) and differences in the composition of cuticular hydrocarbons (estimated by Nei distances, described by Kaib et al. 1991) based on pairwise comparisons between colonies of West African *Macrotermes subhyalinus* (for more details, see Kaib et al. 2004a)

influences. Consequently, the environment should affect the composition of hydrocarbons to a certain degree. This might occur in two possible ways. First, different hydrocarbons might be synthesized or the relative amounts of specific hydrocarbons might be adjusted according to the environmental conditions. Secondly, in social insects, where trophallaxis and allo-grooming between members of a colony frequently take place, substances acquired from the environment might affect the hydrocarbon composition. One example of the latter scenario is found with the Argentine ant *Linepithema humile*, in which cuticular hydrocarbon composition is dominated by compounds acquired from prey (Liang and Silverman 2000). In the lower termite *Zootermopsis nevadensis*, gut microorganisms supply precursors (e.g., propionate) for methyl-branched hydrocarbon biosynthesis (Guo et al. 1991). Macrotermitinae cultivate fungi and harbor a diverse microbiota in their gut (e.g., Darlington 1994; Brune 1998). Substances acquired from different strains of fungi or from gut symbionts may become either directly integrated into the colony-specific odor or incorporated into the pathways of hydrocarbon biosynthesis. To our knowledge, a relationship between cuticular hydrocarbon composition and cultivated fungal strains or gut microbiota in higher termites has never been tested.

As noted above, surface lipids such as cuticular hydrocarbons have an important function in the water balance of insects (e.g., Hardley 1978). It is thought that the waterproofing ability of hydrocarbons depends on their biophysical properties, mainly the melting temperature. The melting temperature increases in an almost linear function with elongation of the hydrocarbon chain (Gibbs and Pomonis 1995). However, in unsaturated and branched hydrocarbons, the position of the double bond or the methyl group also affects the melting temperature. Nevertheless, a correlation between mean chain lengths of cuticular hydrocarbons of the desert fruit fly *Drosophila mojavensis* and ambient temperature has been reported (Gibbs et al. 1998).

In East Africa, the genus *Macrotermes* tolerates a steep gradient in climatic conditions ranging from humid forest regions to hot semi-arid areas. When the cuticular hydrocarbon profile of a forest species (e.g., *M. herus*) is compared with that of *M. subhyalinus* from an area with less than 500 mm annual rainfall,

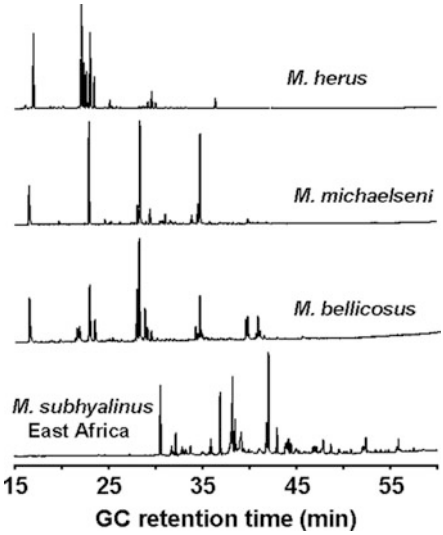


Fig. 9 Cuticular hydrocarbon profiles of different *Macrotermes* species from Kenya, occupying different climates from humid (*top*) to arid climates (*bottom*). The retention times in the gas chromatographs are a surrogate for the chain length of the hydrocarbons

a remarkable increase in the hydrocarbon backbone chain length in parallel to an increase in the ambient temperature and a decrease in the annual rainfall becomes apparent (Fig. 9). In keeping with this observation, the cuticular hydrocarbon chains of the species *M. bellicosus* and *M. michaelseni*, which inhabit intermediary climates, are of intermediate length (Fig. 9). Furthermore, we investigated the variation of cuticular hydrocarbons between colonies of *M. subhyalinus* (East Africa) along an environmental gradient [according to Brandl et al. 2007, and according to the different architecture of the mounds (see Fig. 1c, d), *M. subhyalinus* from East Africa is also a cryptic species when compared to the West African *M. subhyalinus*]. The investigated transect started near Nairobi (Kenya) at ca. 1,530 m asl, with a moderately humid climate, and ran across a steep escarpment to the bottom of the Rift Valley at Lake Magadi (ca. 640 m asl), with a hot, semi-arid climate. We found no distinct phenotypes in this species, but did find a continuous change in the hydrocarbon composition along the climatic gradient (Fig. 10a). Despite this continuous change in the hydrocarbon composition along the climatic gradient, only the concentration of 4,22-hentriacontadiene strongly increased as the altitude decreased (Fig. 10b). In the dry and hot arid areas at low altitudes, the concentration of this alkadiene reaches almost 20% of the total hydrocarbon fraction. The physiological implications of the observed geographic changes in the composition of hydrocarbons deserves further investigations.

In summary, there is limited evidence that the hydrocarbon composition is heritable. Furthermore, there is some evidence that the composition of hydrocarbons is influenced by the environment on evolutionary (differences between species according to climate of the distributional range) and ecological time scales (e.g., food). However, climate and environmental factors vary in a more or less continuous way and therefore cannot explain the occurrence of discrete hydrocarbon phenotypes.

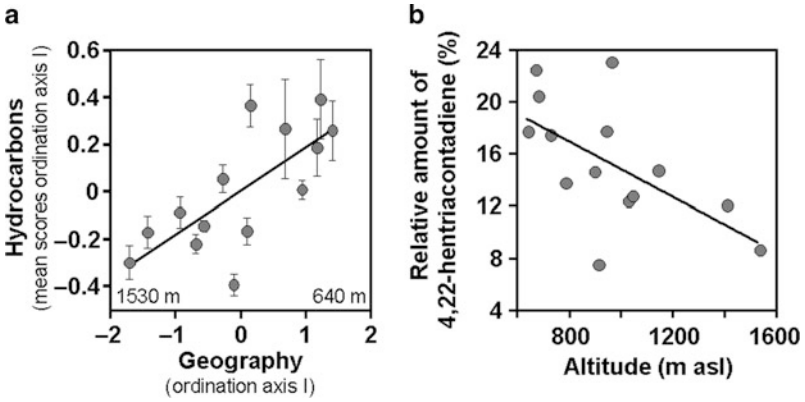


Fig. 10 Variation of cuticular hydrocarbons of *Macrotermes subhyalinus* along a transect in Kenya. The geographic gradient is an altitudinal and climatic gradient. Upper regions are characterized by moderate temperatures and a more humid climate (e.g., 1,530 m asl, 900 mm annual rainfall), whereas the lower regions in the Rift Valley are hot and dry (e.g., 640 m asl, 400 mm annual rainfall). (**a**) Composition of cuticular hydrocarbons along the gradient ($r = 0.72$, $P = 0.003$). Scores for geography (latitude, longitude, altitude) and hydrocarbon profiles were extracted from the first axes of ordination analyses. (**b**) Relationship between the relative abundance of 4,22-hentriacontadiene in the mixture of hydrocarbons and altitude ($r = 0.97, P < 0.001$)

5 Cuticular Hydrocarbons and Agonistic Behavior

It is generally accepted that kin recognition in social insects is based on chemical signals. In Hymenoptera, cuticular hydrocarbons play an important role in recognition (e.g., Clément et al. 1987; Page et al. 1991; Vander Meer and Morel 1998; Lucas et al. 2005). However, some studies with ants (Obin 1986; Kaib et al. 1993) and termites (e.g., Su and Haverty 1991) found no evidence that cuticular hydrocarbons are involved in nestmate recognition. In laboratory experiments, intercolonial aggression in the Formosan subterranean termite *Coptotermes formosanus* depended on the rearing conditions rather than on cuticular hydrocarbons (Florane et al. 2004; Pan et al. 2006). The authors suggested that diet, soil microorganisms, or volatile components in soil provide the cues for nestmate recognition. Other experiments suggested that intestinal microbes play a role in nestmate recognition (*Reticulitermes*: Matsuura 2001; *Coptotermes*: Wei et al. 2007; *Hodotermes*: Kirchner and Minkley 2003). Agonistic behavior changed when termites were treated with antibiotics to manipulate the microbial gut community. The authors argued that volatile compounds produced by intestinal microbes may be responsible for a colony-specific odor. However, the behavioral responses should be interpreted with caution because antibiotic treatments could induce a number of unexpected side effects.

There are numerous studies on aggression within and between castes, colonies, and species of termites (e.g., Kaib and Brandl 1992; Husseneder et al. 1998; reviews by Thorne and Haverty 1991; Clément and Bagnères 1998). But only in recent years

has agonistic behavior been related to differences in hydrocarbon profiles (see Haverty and Thorne 1989 or Bagnères et al. 1991 for very early studies). In a bioassay, Haverty et al. (1999a) paired groups of workers from different colonies of *Reticulitermes* from North Carolina in petri dishes and after 24 h correlated agonistic behavior and mortality with cuticular hydrocarbon phenotypes. Forced encounters between workers of different phenotypes resulted in a higher level of immediate aggression and a higher mortality compared to intra-phenotype pairings. However, although the level of immediate aggression was lower in intra-phenotype pairings, a comparatively high number of inter-colonial encounters between workers of the same phenotype also resulted in high levels of aggression and mortality. This suggests either that some variation of cuticular hydrocarbons within phenotypes might also be responsible for agonistic behavior or that, in addition to cuticular hydrocarbons, other endogenous or exogenous cues are involved in triggering the agonistic behavior between workers of different colonies (Shelton and Grace 1996).

During our project, we carried out arena experiments on the agonistic behavior between nestmates, conspecific non-nestmates, and different species with differences in their cuticular hydrocarbon profiles. In these bioassays, groups of major workers from the same colony or from two different colonies were paired in petri dishes, and the subsequent agonistic behavior was quantified (for methods, see Kaib et al. 2002). We used major workers because they perform most of the foraging activities in the genus *Macrotermes* and hence are most likely to interact with competitors or invaders. Although somewhat artificial experimental conditions, the results correlated between castes and corresponded to field observations (Jmhasly and Leuthold 1999). Our behavioral tests revealed a clear and consistent behavioral response to differences in hydrocarbon profiles: (1) no alarm behavior or mortality between workers from the same colony was observed; (2) in contrast, during encounters of different species, aggression and mortality were frequently observed; (3) the level of agonistic behavior was correlated to differences between the hydrocarbon profiles (Fig. 11a); and (4) the intensity of agonistic interactions between conspecific non-nestmates increased with dissimilarity in the composition of the cuticular hydrocarbons (Fig. 11b) (*M. falciger*: Kaib et al. 2002; *M. subhyalinus*: Kaib et al. 2004a). In *M. subhyalinus* from West Africa, variation in hydrocarbon composition contributes much more to aggressive behavior between groups of termites than genetic relatedness or morphological similarity (Fig. 11c). No single compound in the hydrocarbon profiles was sufficient to explain variation in aggression. Statistical pattern searching suggested that the triggers of aggression were minor, short-chain, unsaturated hydrocarbons of the hydrocarbon bouquet.

However, during our studies, we demonstrated that the behavioral response to hydrocarbon differences depends on the spatial context. For example, in *M. falciger*, a so-called "neighbor-stranger effect" or "dear-enemy phenomenon" was observed (Kaib et al. 2002). As described above, colonies differed in their cuticular hydrocarbon composition, and three phenotypes occurred on a scale of less than 200 m. During the arena experiments, the proximity of colonies contributed much more to the alarm behavior between members of different colonies than the

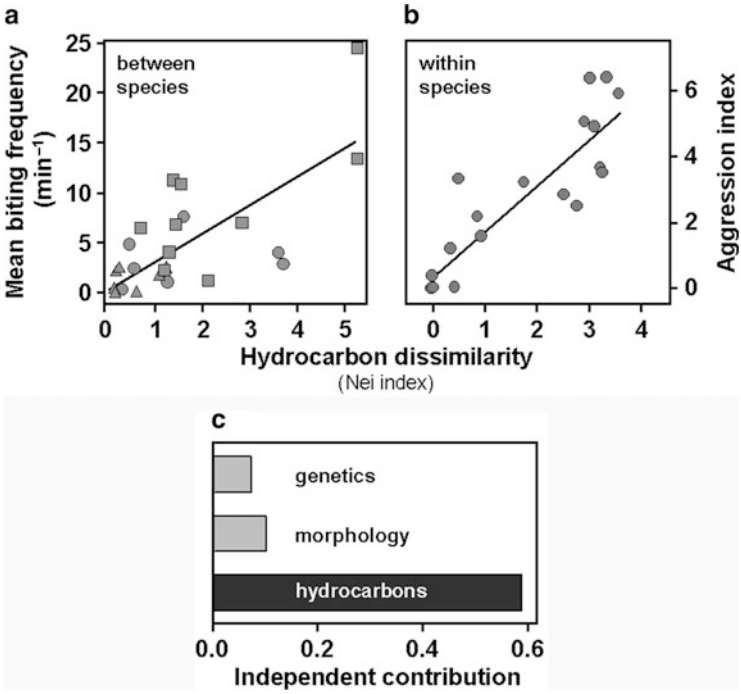


Fig. 11 Positive relationships between differences in the composition of cuticular hydrocarbons (estimated by Nei distances, described by Kaib et al. 1991) and agonistic behavior. (**a**) Aggressive behavior between species of *Macrotermes* correlated to hydrocarbon differences ($r = 0.78$, $P < 0.001$). Different *symbols* indicate different field seasons. (**b**) Aggression between colonies of West African *M. subhyalinus* correlated to hydrocarbon differences ($r = 0.89$, $P = 0.002$, Mantel test; for details, see Kaib et al. 2004a). (**c**) Independent contributions (estimated by hierarchical partitioning, Chevan and Sutherland 1991) of genetic dissimilarity, morphological distance, and differences in the composition of cuticular hydrocarbons to the variance of agonistic behavior between these colonies

hydrocarbon composition. Territorial residents apparently discriminated between neighbors and strangers and were less aggressive against direct neighbors even if the neighbors belonged to different hydrocarbon phenotypes. However, the opposite phenomenon was observed with *Nasutitermes corniger* (Dunn and Messier 1999). The reasons for these contradictory results are not clear, but they underline the importance of the spatial context and the environment for the understanding of behavioral interactions and competition between colonies (see also Kaib and Brandl 1992). However, the ability to discriminate neighbors from strangers independent of their chemical phenotype suggests that termites cannot only differentiate between nestmates and non-nestmates but also between different categories of non-nestmates. Learning or habituation may account for neighbor recognition, as suggested for carpenter ants (Carlin and Hölldobler 1986).

In summary, our arena experiments showed that differences in cuticular hydrocarbon composition often induce inter-colonial and inter-specific aggression and seem to play a role in the discrimination of non-nestmates. However, direct evidence for the use of cuticular hydrocarbons as colony and species labels is still lacking because one cannot rule out the possibility that the composition of cuticular hydrocarbons is correlated to other cues. Labeling lures with synthetic or extracted hydrocarbon blends may solve this problem. A first experiment in this direction with a focus on inter-specific recognition has already been made by Bagnères et al. (1991), who showed that a lure covered with cuticular extract from one *Reticulitermes* species initiated aggressive behavior in another (see Takahashi and Gassa 1995 for similar results). To test whether agonistic behavior as a response to different compositions of cuticular hydrocarbons may act as isolation mechanism during pairings, it would be better to use alates in such arena experiments. However, there is no practical alternative to the use of workers, because mature alates only occur in the nests shortly before nuptial flights. Furthermore, it has not been possible to directly observe aggressive behavior between alates during nuptial flights. Nevertheless, there is some indirect evidence for high levels of aggression between reproductives; at least during the first steps of colony foundation, reproductives are often mutilated (Brandl et al. 2001, 2004).

6 Cuticular Hydrocarbons and Genetic Differentiation Between Phenotypes

As already mentioned, termites show little morphological diversity, and thus cuticular hydrocarbons are welcomed characters for termite taxonomy and phylogeny. Several studies have demonstrated the potential of cuticular hydrocarbons in the delimitation of termite taxa (*Reticulitermes*: e.g., Haverty et al. 1999b; Takematsu and Yamaoka 1999; Page et al. 2002; *Coptotermes*: e.g., Haverty et al. 1990a; *Heterotermes*: Watson et al. 1989; *Zootermopsis*: e.g., Haverty and Thorne 1989; *Drepanotermes*: Brown et al. 1996; *Nasutitermes*: e.g., Haverty et al. 1990b; *Macrotermes*: Bagine et al. 1994; *Odontotermes*: Kaib et al. 1991). The use of cuticular hydrocarbons in chemotaxonomy assumes a rather fixed pattern of hydrocarbons within taxa and fixed differences between taxa. As shown above, cuticular hydrocarbon profiles could differ considerably between sympatric colonies within a supposed species (see also Haverty et al. 1999a; Bagine et al. 1994; Copren et al. 2005). Some authors have suggested that cuticular hydrocarbons are species-specific, and that different phenotypes reflect therefore sibling species (e.g., Haverty et al. 1996, 1999b; Haverty and Nelson 1997). Copren et al. (2005) found distinct hydrocarbon phenotypes within the *Reticulitermes hesperus* group occurring in California. The comparison of these phenotypes with a phylogeny of the termite group based on mitochondrial DNA revealed monophyly for most of the phenotypes and therefore phenotypes represent cryptic species. Similar results were obtained for *Reticulitermes* termites from the southeastern United States by

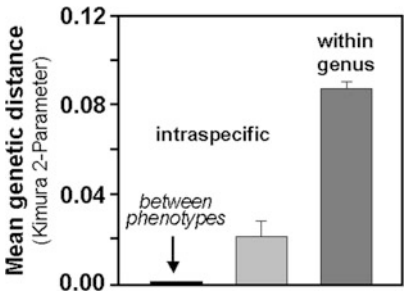


Fig. 12 Mean pairwise genetic distances (Kimura 2-parameter distances of a 658-bp fragment of the cytochrome *c* oxidase subunit I (COI) gene) between cuticular hydrocarbon phenotypes ($n = 19$), within species ($n = 8$), and between congeneric species ($n = 113$). For distance calculations between hydrocarbon phenotypes, sequences of *Macrotermes falciger*, *M. herus* and *M. subhyalinus* (West Africa) were considered. For distance calculations within and between species, our own and published sequences were used (for details, see Marten et al. 2009)

mapping hydrocarbon phenotypes to a phylogenetic tree based on mitochondrial DNA sequences (Jenkins et al. 2000).

These results from lower termites provided the first evidence that cuticular hydrocarbons are correlated to the phylogenetic pattern (e.g., *Zootermopsis*: Haverty et al. 1988; Haverty and Thorne 1989). Aggressive behavior between termites with different hydrocarbon composition can mediate reproductive isolation with the potential of sympatric speciation if alates use the same information for mate choice and exhibit agonistic responses to variation in cuticular hydrocarbons similar to those of workers. Therefore, we tested whether the sympatric phenotypes of *Macrotermes* correspond to cryptic species, as demonstrated for lower termites, using COI sequences (Marten et al. 2009). Despite strong differences in their compositions, different hydrocarbon phenotypes occurred within the same haplotype. Clearly, genetic differentiation between hydrocarbon phenotypes is very low and does not represent cryptic species in *Macrotermes* (Fig. 12). Such an intraspecific variability of hydrocarbon composition has also been observed in the giant cockroach *Macropanesthia rhinoceros* (Brown et al. 2000).

Our results on the genetic differentiation between hydrocarbon phenotypes contradict the observations in lower termites and suggest fundamental differences in the evolution and function of cuticular hydrocarbons between termites. Furthermore, the occurrence of discrete phenotypes within species questions the general value of cuticular hydrocarbons as characters in chemo-taxonomy of termites.

7 Conclusions

We investigated whether cuticular hydrocarbons have the potential to be involved in processes of behavioral isolation and therefore genetic differentiation. We found considerable variation in the composition of cuticular hydrocarbons among species

and among colonies of certain species of the higher termite genus *Macrotermes*. Variation within species was discrete with differences between phenotypes within species that exceeded differences between species. Furthermore, we provide some evidence that variations in cuticular hydrocarbon composition have a genetic component, although the variation of hydrocarbons within and between species may also have an environmental background. However, the environment varies in a continuous way, and environmental variation may not explain the existence of discrete phenotypes which occur even in sympatry. We found clear correlation between differences in cuticular hydrocarbon profiles and agonistic behavior within and between species. Individuals seem to adjust their behavioral responses according to the differences in hydrocarbon profiles. However, despite the general suitability of cuticular hydrocarbons in nestmate and species recognition, this system is not perfect. Separate colonies with similar hydrocarbon phenotypes often displayed little or no agonistic behavior. Thus, colonies use additional cues to maintain colony integrity. The reaction of termites during encounters depends not only on the hydrocarbon profile of the other termite but also on the context within the encounter occurs.

Although cuticular hydrocarbons fulfill most of the prerequisites for nestmate and species recognition, there was no evidence that cuticular hydrocarbons are in some ways connected or involved in speciation events of *Macrotermes* termites. Different hydrocarbon phenotypes even occurred within the same DNA haplotype. Swarming alates may not recognize conspecific partners by their cuticular hydrocarbon compositions. Instead, temporal separation of the nuptial flights may prevent mating between sympatric *Macrotermes* species (Wood 1981; Haverty et al. 2003). Furthermore, Peppuy et al. (2004) recently showed differences of sexual pheromones between two sympatric *Macrotermes* species, and the notion that diversity of pheromone is low in termites compared to ants may be premature. The specific function of the few known pheromones in termites could also be a matter of concentration and depends on the context (e.g., Bordereau et al. 1991; Laduguie et al. 1994). Nevertheless, our findings that phenotypes do not correspond to genetic differentiated lineages contrast with findings within lower termites and suggest fundamental differences in the evolution and function of cuticular hydrocarbons between lower and higher termites.

We propose a possible different function of cuticular hydrocarbon phenotypes in Macrotermitinae. The occurrence of different phenotypes within species even on a small spatial scale might possibly act as a defense strategy against inquilines (see Howard et al. 1982). Long-living termite colonies are a predictable resource for possible invaders, and termites might have evolved strategies to limit colony access for inquilines. As demonstrated above, the invaders have to mimic the colony-specific hydrocarbon pattern. A high variability of different phenotypes could make entering the colony more difficult for inquilines. This hypothesis is in agreement with our observation that cuticular hydrocarbon compositions often vary much more within a certain area than between areas.

Acknowledgments During our work on the genus *Macrotermes* several people helped conduct fieldwork, performed behavioral tests or helped to identify the hydrocarbons. We want to thank R. Bagine, S. Franke, W. Francke, and particularly A. Halwas, who did most of the gaschromatography. A native English speaker improved the language of our manuscript. We thank two anonymous reviewers and the handling editor for their comments. The work was supported by the German Science Foundation within the Priority Program 1127 (Radiations – The origin of biodiversity).

References

Adams ES (1991) Nest-mate recognition based on heritable odors in the termite *Microcerotermes arboreus*. Proc Natl Acad Sci USA 88:2031–2034

Akino T (2006) Cuticular hydrocarbons of *Formica truncorum* (Hymenoptera: Formicidae): description of new very long chained hydrocarbon components. Appl Entomol Zool 41: 667–677

Bagine RKN, Brandl R, Kaib M (1994) Species delimitation in *Macrotermes* (Isoptera: Macrotermitidae): Evidence from epicuticular hydrocarbons, morphology, and ecology. Ann Entomol Soc Am 87:498–506

Bagnères AG, Killian A, Clément J-L, Lange C (1991) Interspecific recognition among termites of the genus *Reticulitermes*: evidence for a role for the cuticular hydrocarbons. J Chem Ecol 17:2397–2420

Billen J, Morgan ED (1998) Pheromone communication in social insects: sources and secretions. In: Vander Meer RK, Breed MD, Espelie KE, Winston ML (eds) Pheromone communication in social insects: ants, wasps, bees, and termites. Westview, Boulder, Colorado, pp 3–33

Blomquist GJ, Tillman JA, Mpuru S, Seybold SJ (1998) The cuticle and cuticular hydrocarbons of insects: structure, function, and biochemistry. In: Vander Meer RK, Breed MD, Espelie KE, Winston ML (eds) Pheromone communication in social insects: ants, wasps, bees, and termites. Westview, Boulder, Colorado, pp 34–54

Bordereau C, Robert A, Bonnard O, Le Quere J-L (1991) 3Z, 6Z, 8E)-3, 6, 8-dodecatrien-1-ol: sex pheromone in a higher fungus-growing termite, Pseudacanthotermes spiniger (Isoptera, Macrotermitinae. J Chem Ecol 17:2177–2191

Bouillon A (1981) Mechanisms of species isolation in termites. In: Howse PE, Clément J-L (eds) Biosystematics of social insects. Academic, London, pp 297–308

Brandl R, Bagine RKN, Kaib M (1996) The distribution of *Schedorhinotermes lamanianus* (Isoptera: Rhinotermitidae) and its termitophile *Paraclystis* (Lepidoptera: Tineidae) in Kenya: its importance for understanding east African biogeography. Global Ecol Biogeogr 5:143–148

Brandl R, Hacker M, Bagine RKN, Kaib M (2001) Geographic variation of polygyny in the termite *Macrotermes michaelseni* (Sjostedt). Insect Soc 48:134–137

Brandl R, Hacker M, Bagine RKN, Kaib M (2004) Yearly variation in polygyny in the termite *Macrotermes michaelseni* (Sjostedt). Insect Soc 51:294–298

Brandl R, Hyodo F, von Korff-Schmising M, Maekawa K, Miura T, Takematsu Y, Matsumoto T, Abe T, Bagine RKN, Kaib M (2007) Divergence times in the termite genus *Macrotermes* (Isoptera: Termitidae). Mol Phylogenet Evol 45:239–250

Brown WV, Watson JAL, Lacey MJ (1996) A chemotaxonomic survey using cuticular hydrocarbons of some species of the Australian harvester termite genus *Drepanotermes* (Isoptera: Termitidae). Sociobiology 27:199–221

Brown WV, Rose HA, Lacey MJ (1997) The cuticular hydrocarbons of the soil burrowing cockroach *Geoscapheus dilatatus* (Saussure) (Blattodea: Blaberidae: Geoscapheinae) indicate species dimorphism. Comp Biochem Physiol B 118:549–562

Brown WV, Rose HA, Lacey MJ, Wright K (2000) The cuticular hydrocarbons of the giant soil-burrowing cockroach *Macropanesthia rhinoceros* Saussure (Blattodea: Blaberidae: Geoscapheinae): analysis with respect to age, sex and location. Comp Biochem Physiol B 127:261–277

Brune A (1998) Termite guts: the world's smallest bioreactors. Trends Biotechnol 16:16–21

Carlin N, Hölldobler B (1986) The kin recognition system of carpenter ants (*Camponotus*). I. Hierarchical cues in small colonies. Behav Ecol Sociobiol 19:123–134

Carlson DA, Service MW (1980) Identification of mosquitoes of Anopheles gambiae species complex A and B by analysis of cuticular components. Science 207:1089–1091

Chevan A, Sutherland M (1991) Hierarchical partitioning. Am Statist 45:90–96

Clément JL, Bagnères A-G (1998) Nestmate recognition in termites. In: Vander Meer RK, Breed MD, Espelie KE, Winston ML (eds) Pheromone communication in social insects: ants, wasps, bees, and termites. Westview Press, Boulder, Colorado, pp 126–155

Clément J-L, Bonavita-Cougourdan A, Lange C (1987) Nestmate recognition and cuticular hydrocarbons in *Camponotus vagus* Scop. In: Ede J, Rembold H (eds) Chemistry and biology of social insects. Papemy, Munich, Germany, pp 473–474

Copren KA, Nelson LJ, Vargo EL, Haverty MI (2005) Phylogenetic analyses of mtDNA sequences corroborate taxonomic designations based on cuticular hydrocarbons in subterranean termites. Mol Phylogenet Evol 35:689–700

Coyne JA (1996) Genetics of differences in pheromonal hydrocarbons between *Drosophila melanogaster* and *D. simulans*. Genetics 143:353–364

Dallerac R, Labeur C, Jallon J, Knipple D, Roelofs W (2000) A Δ9 desaturase gene with a different substrate specificity is responsible for the cuticular diene hydrocarbon polymorphism in *Drosophila melanogaster*. Proc Natl Acad Sci USA 97:9449–9454

Darlington JPEC (1994) Nutrition and evolution in fungus-growing termites. In: Hunt JH, Nalepa CA (eds) Nourishment and evolution in insect societies. Westview, Boulder, Colorado, USA

Donovan SE, Eggleton P, Dubbin WE, Batchelder M, Dibog L (2001) The effect of a soil-feeding termite, *Cubitermes fungifaber* (Isoptera: Termitidae) on soil properties: termites may be an important source of soil microhabitat heterogeneity in tropical forests. Pedobiologia 45:1–11

Dronnet S, Lohou C, Christides J-P, Bagnères A-G (2006) Cuticular hydrocarbon composition reflects genetic relationship among colonies of the introduced termite *Reticulitermes santonensis* Feytaud. J Chem Ecol 32:1027–1042

Dunn R, Messier S (1999) Evidence for the opposite of the dear enemy phenomenon in termites. J Insect Behav 12:461–464

Eggleton P (1999) Termite species description rates and the state of termite taxonomy. Insect Soc 46:1–5

Espelie KE, Berisford CW, Dahlsten DL (1996) Use of cuticular hydrocarbons in bark beetle parasitoid taxonomy: a study of *Roptrocerus xylophagorum* (Ratzeburg) (Hymenoptera: Torymidae) from the United States, Europe and Australia. Comp Biochem Physiol B 113:193–198

Estrada-Pena A, Castella J, Moreno JA (1994) Using cuticular hydrocarbon composition to elucidate phylogenies in tick populations (Acari, Ixodidae). Acta Trop 58:51–71

Ferveur JF, Jallon JM (1996) Genetic control of male cuticular hydrocarbons in *Drosophila melanogaster*. Genet Res 67:211–218

Florane CB, Bland JM, Husseneder C, Raina AK (2004) Diet-mediated inter-colonial aggression in the Formosan subterranean termite *Coptotermes formosanus*. J Chem Ecol 30:2559–2574

Gibbs AG, Pomonis JG (1995) Physical properties of insect cuticular hydrocarbons: the effects of chain length, methyl-branching and unsaturation. Comp Biochem Physiol B 112:243–249

Gibbs AG, Louie AK, Ayala JA (1998) Effects of temperature on cuticular lipids and water balance in a desert *Drosophila* – is thermal acclimation beneficial? J Exp Biol 210:71–80

Guo L, Quilici DR, Chase J, Blomquist GJ (1991) Gut tract microorganisms supply the precursors for methyl-branched hydrocarbon biosynthesis in the termite, *Zootermopsis nevadensis*. Insect Biochem 21:327–333

Hacker M, Kaib M, Bagine RKN, Epplen JT, Brandl R (2005) Unrelated queens coexist in colonies of the termite *Macrotermes michaelseni*. Mol Ecol 14:1527–1532

Hamilton WD (1964) The genetic evolution of social behaviour. J Theor Biol 71:1–52

Hardley NF (1978) Cuticular permeability of desert tenebrionid beetles: correlation with epicuticular hydrocarbons. Insect Biochem 8:17–22

Haverty MI, Thorne BL (1989) Agonistic behavior correlated with hydrocarbon phenotypes in dampwood termites, *Zootermopsis* (Isoptera: Termopsidae). J Insect Behav 2:523–543

Haverty MI, Nelson LJ (1997) Cuticular hydrocarbons of *Reticulitermes* (Isoptera: Rhinotermitidae) from California indicate undescribed species. Comp Biochem Physiol B 111:869–880

Haverty MI, Page M, Nelson LJ, Blomquist GJ (1988) Cuticular hydrocarbons of dampwood termites, *Zootermopsis*: Intra- and intercolony variation and potential as taxonomic characters. J Chem Ecol 14:1035–1058

Haverty MI, Nelson LJ, Page M (1990a) Cuticular hydrocarbons of four populations of *Coptotermes formosanus* Shiaki in the United States. J Chem Ecol 16:1635–1647

Haverty MI, Thorne BL, Page M (1990b) Surface hydrocarbon components of two species of *Nasutitermes* from Trinidad. J Chem Ecol 16:2441–2450

Haverty MI, Forschler BT, Nelson LJ (1996) An assessment of the taxonomy of *Reticulitermes* (Isoptera: Rhinotermitidae) from the southeastern United States based on cuticular hydrocarbons. Sociobiology 28:287–318

Haverty MI, Copren KA, Getty GM, Lewis VR (1999a) Agonistic behavior and cuticular hydrocarbon phenotypes of colonies of *Reticulitermes* (Isoptera: Rhinotermitidae) from Northern Carolina. Ann Entomol Soc Am 92:269–277

Haverty MI, Nelson LJ, Forschler BT (1999b) New cuticular hydrocarbon phenotypes of *Reticulitermes* (Isoptera: Rhinotermitidae) from the United States. Sociobiology 34:1–21

Haverty MI, Getty GM, Nelson LJ, Lewis VR (2003) Flight phenology of sympatric populations of *Reticulitermes* (Isoptera: Rhinotermitidae) in Northern California: disparate flight intervals indicate reproductive isolation among cuticular hydrocarbon phenotypes. Ann Entomol Soc Am 96:828–833

Holt JA, Lepage M (2000) Termites and soil properties. In: Abe T, Bignell DE, Higashi M (eds) Termites: evolution, sociality, symbioses, ecology. Kluwer, Dordrecht, Netherlands, pp 389–407

Howard RW (1993) Cuticular hydrocarbons and chemical communication. In: Stanley-Samuelson DW, Nelson DR (eds) Insects lipids: chemistry, biochemistry and biology. University of Nebraska Press, Lincoln, USA, pp 179–226

Howard RW, Blomquist GJ (1982) Chemical ecology and biochemistry of insect hydrocarbons. Annu Rev Entomol 27:149–172

Howard RW, Blomquist GJ (2005) Ecological, behavioral, and biochemical aspects of insect hydrocarbons. Annu Rev Entomol 50:371–393

Howard RW, McDaniel CA, Blomquist GJ (1980) Chemical mimicry as an integrating mechanism: cuticular hydrocarbons of a termitophile and its host. Science 210:431–433

Howard RW, McDaniel CA, Blomquist GJ (1982) Chemical mimicry as an integrating mechanism for three termitophiles associated with *Reticulitermes virginicus* (Banks). Psyche 89:157–168

Husseneder C, Brandl R, Epplen C, Epplen JT, Kaib M (1998) Variation between and within colonies in the termite: morphology, genomic DNA, and behaviour. Mol Ecol 7:983–990

Jenkins TM, Haverty MI, Basten CJ, Nelson LJ, Page M, Forschler BT (2000) Correlation of mitochondrial haplotypes with cuticular hydrocarbon phenotypes of sympatric *Reticulitermes* species from the southeastern United States. J Chem Ecol 26:1525–1542

Jmhasly P, Leuthold RH (1999) Interspecific colony recognition in the termites *Macrotermes subhyalinus* and *Macrotermes bellicosus* (Isoptera: Termitidae). Insect Soc 46:164–170

Kaib M (1985) Defense strategies of termites: a review exemplified by *Schedorhinotermes lamanianus*. Mitt Dtsch Ges Allg Angew Ent 4:302–306

Kaib M (2000) Chemical signals and communication in termites: a review. Mitt Dtsch Ges Allg Angew Ent 12:211–218

Kaib M, Brandl R (1992) Distribution, geographic variation and between-colony compatibility of *Schedorhinotermes lamanianus* in Kenya (Isoptera: Rhinotermitidae). In: Billen J (ed) Biology and evolution of social insects. Leuven University Press, Leuven, Belgium, pp 121–131

Kaib M, Brandl R, Bagine RKN (1991) Cuticular hydrocarbon profiles: a valuable tool in termite taxonomy. Naturwissenschaften 78:176–179

Kaib M, Heinze J, Ortius D (1993) Cuticular hydrocarbon profiles in the slave-making ant *Harpagoxenus sublaevis* and its hosts. Naturwissenschaften 80:281–285

Kaib M, Franke S, Francke W, Brandl R (2002) Cuticular hydrocarbons in a termite: phenotypes and a neighbour-stranger effect. Physiol Entomol 27:189–198

Kaib M, Jmhasly P, Wilfert L, Durka W, Franke S, Francke W, Leuthold RH, Brandl R (2004a) Cuticular hydrocarbons and aggression in the termite *Macrotermes subhyalinus*. J Chem Ecol 30:365–385

Kaib M, Kinuthia W, Bagine RKN, Brandl R (2004b) Chemical battles in the "castle of clay". Nature East Africa 34:12–15

Kirchner WH, Minkley N (2003) Nestmate discrimination in the harvester termite *Hodotermes mossambicus*. Insect Soc 50:222–225

Kistner DH (2001) Cladistic analysis and taxonomic revision of the termitophilous tribe Termitopaediini (Coleoptera: Staphylinidae) with remarks on their evolution and the behavior of some species. Sociobiology 38:1–278

Laduguie N, Robert A, Bonnard O, Vieau F, Le Quere J-L, Semon E, Bordereau C (1994) Isolation and identification of (3Z, 6Z, 8E)-3, 6, 8-dodecatrien-1-ol in Reticulitermes santonensis Feytaud (Isoptera, Rhinotermitidae): roles in worker trail-following and in alate sex-attraction behavior. J Insect Physiol 40:781–787

Lahav S, Soroker V, Hefetz A (1999) Direct behavioral evidence for hydrocarbons as ant recognition discriminator. Naturwissenschaften 86:246–249

Liang D, Silverman J (2000) "You are what you eat": Diet modifies cuticular hydrocarbons and nestmate recognition in the Argentine ant, *Linepithema humile*. Naturwissenschaften 87:412–416

Lockey KH (1988) Lipids of the insect cuticle: origin, composition and function. Comp Biochem Physiol B 89:595–645

Lockey KH (1991) Insect hydrocarbon classes: implications for chemotaxonomy. Insect Biochem 21:91–97

Lucas C, Pho DB, Jallon JM, Fresneau D (2005) Role of cuticular hydrocarbons in the chemical recognition between ant species in the *Pachycondyla villosa* species complex. J Insect Physiol 51:1148–1157

Marten A, Kaib M, Brandl R (2009) Cuticular hydrocarbon phenotypes do not indicate cryptic species in fungus-growing termites (Isoptera: Macrotermitinae). J Chem Ecol 35:572–579

Matsuura K (2001) Nestmate recognition mediated by intestinal bacteria in a termite, *Reticulitermes speratus*. Oikos 92:20–26

Obin MS (1986) Nestmate recognition cues in laboratory and field colonies of *Solenopsis invicta* Buren (Hymenoptera: Formicidae). Effect of environment and role of cuticular hydrocarbons. J Chem Ecol 12:1965–1975

Page RE, Metcalf RH, Metcalf RL, Erickson EH, Lampman RL (1991) Extractable hydrocarbons and kin recognition in honeybee (*Apis mellifera* L.). J Chem Ecol 17:745–756

Page M, Nelson LJ, Forschler BT, Haverty MI (2002) Cuticular hydrocarbons suggest three lineages in *Reticulitermes* (Isoptera: Rhinotermitidae) from North America. Comp Biochem Physiol B 131:305–324

Pan C, Mo J, Cheng M (2006) Influence of diet and soil on inter-colonial aggression of *Coptotermes formosanus* (Isoptera: Rhinotermitidae). Sociobiology 48:841–848

Pasteels JM, Bordereau C (1998) Releaser pheromones in termites. In: Vander Meer RK, Breed MD, Winston ML, Espelie KE (eds) Pheromone communication in social insects. Westview, Oxford, pp 193–215

Peppuy A, Robert A, Bordereau C (2004) Species-specific sex pheromones secreted from new sexual glands in two sympatric fungus-growing termites from northern Vietnam. *Macrotermes annandalei* and *M. barneyi*. Insect Soc 51:91–98

Pomeroy DE (1978) Abundance of large termite mounds in Uganda in relation to their environment. J Appl Ecol 15:51–63

Prestwich GD (1983) Chemical systematics of termite exocrine secretions. Annu Rev Ecol Syst 14:287–311

Raboudi F, Mezghani M, Makni H, Marrakchi M, Rouault JD, Makni M (2005) Aphid species identification using cuticular hydrocarbons and cytochrome *b* gene sequences. J Appl Entomol 129:75–80

Ruther J, Sieben S, Schricker B (2002) Nestmate recognition in social wasps: manipulation of hydrocarbon profiles induces aggression in the European hornet. Naturwissenschaften 89:111–114

Shelton TG, Grace JK (1996) Revision of agonistic behaviors in the Isoptera. Sociobiology 28:155–176

Shorey HH (1973) Behavioral responses to insect pheromones. Annu Rev Entomol 18:349–380

Singer TL (1998) Roles of hydrocarbons in the recognition systems of insects. Am Zool 38:394–405

Smith BH, Breed MD (1995) The chemical basis for nestmate recognition and mate discrimination in social insects. In: Cardé RT, Bell WJ (eds) Chemical ecology of insects 2. Chapman & Hall, New York, pp 287–317

Steiner FM, Schlick-Steiner BC, Nikiforov A, Kalb R, Mistrik R (2002) Cuticular hydrocarbons of *Tetramorium* ants from Central Europe: analysis of GC-MS data with self-organizing maps (SOM) and implications for systematics. J Chem Ecol 28:2569–2584

Su NY, Haverty MI (1991) Agonistic behavior among colonies of the Formosan subterranean termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae), from Florida and Hawaii: Lack of correlation with cuticular hydrocarbon composition. J Insect Behav 4:115–128

Su NY, Scheffrahn RH (2000) Termites as pests of buildings. In: Abe T, Bignell DE, Higashi M (eds) Termites: evolution, sociality, symbioses, ecology. Kluwer, Dordrecht, Netherlands, pp 437–453

Sugimoto A, Bignell DE, MacDonald JA (2000) Global impact of termites on the carbon cycle and atmospheric trace gases. In: Abe T, Bignell DE, Higashi M (eds) Termites: evolution, sociality, symbioses, ecology. Kluwer, Dordrecht, Netherlands, pp 409–435

Takahashi S, Gassa A (1995) Roles of cuticular hydrocarbons in intra- and interspecific recognition behaviour of two Rhinotermitidae species. J Chem Ecol 21:1837–1845

Takahashi A, Tsaur SC, Coyne JA, Wu CI (2001) The nucleotide changes governing cuticular hydrocarbon variation and their evolution in *Drosophila melanogaster*. Proc Natl Acad Sci USA 98:3920–3925

Takematsu Y, Yamaoka R (1999) Cuticular hydrocarbons of *Reticulitermes* (Isoptera: Rhinotermitidae) in Japan and neighboring countries as chemotaxonomic characters. Appl Entomol Zool 34:179–188

ter Braak CJF, Smilauer P (2002) Canoco for Windows version 4.5. Biometrics – plant research international, Wageningen, Netherlands

Thorne BL, Haverty MI (1991) A review of intracolony, intraspecific and interspecific agonism in termites. Sociobiology 19:115–145

Turelli M, Barton NH, Coyne JA (2001) Theory and speciation. Trends Ecol Evol 16:330–343

Vander Meer RK, Wojcik DP (1982) Chemical mimicry in the myrmecophilous beetle, *Myrmecophodius excavaticollis*. Science 218:806–808

Vander Meer RK, Morel L (1998) Nestmate recognition in ants. In: Vander Meer RK, Breed MD, Espelie KE, Winston ML (eds) Pheromone communication in social insects: ants, wasps, bees, and termites. Westview, Boulder, Colorado, pp 79–103

Watson JAL, Brown WV, Miller LR, Carter FL, Lacey MJ (1989) Taxonomy of *Heterotermes* (Isoptera: Rhinotermitidae) in south-eastern Australia: cuticular hydrocarbons of workers, and soldier and alate morphology. Syst Entomol 14:299–325

Wei J, Mo J, Pan C, Deng T, Cheng M, Chen C (2007) The intestinal microbes inducing the agonistic behavior of inter-colonial individuals in *Coptotermes formosanus* (Isoptera: Rhinotermitidae). Sociobiology 50:245–256

Wood TG (1981) Reproductive isolating mechanisms among species of *Microtermes* (Isoptera, Termitidae) in the Southern Guinea Savanna near Mokwa, Nigeria. In: Howse PE, Clément J-L (eds) Biosystematics of social insects. Academic, London, pp 309–325

Wood TG, Sands WA (1978) The role of termites in ecosystems. In: Brian MV (ed) Production ecology of ants and termites. Cambridge University Press, Cambridge, UK, pp 245–292

Ye GY, Li K, Zhu JY, Zhu GH, Hu C (2007) Cuticular hydrocarbon composition in pupal exuviae for taxonomic differentiation of six necrophagous flies. J Med Entomol 44:450–456

Electric Organ Discharge Divergence Promotes Ecological Speciation in Sympatrically Occurring African Weakly Electric Fish (*Campylomormyrus*)

Ralph Tiedemann, Philine G.D. Feulner, and Frank Kirschbaum

Abstract While speciation is often discussed mainly with regard to its geographic context, i.e., allopatric versus parapatric versus sympatric, the role of adaptation in speciation has been repeatedly emphasized in the recent past. Such an *ecological speciation*, in which populations diverge by disruptive selection, can occur in different geographic contexts. Here, we present a combined molecular genetic, electrophysiological, morphometric, and behavioral study on an adaptive radiation of African weakly electric fish (Genus *Campylomormyrus*). Multilocus genetic data, types of electric organ discharge (EOD), and morphometry identified several reproductively isolated clusters of specimens, i.e., different species, in sympatry. By this analysis, six previously described species could be confirmed, while at least one specimen appeared not to belong to any species described so far. *Campylomormyrus* is characterized by its elongated trunk-like snout used for preying on insect larvae. Morphometric analysis confirmed that divergence among species is mainly attributed to differences in snout morphology. Though direct evidence is still lacking, it appears reasonable to assume that these differences in the feeding apparatus translate into different diets and/or feeding habits. We could also demonstrate that mate choice occurs with regard to EOD and could hence have triggered

R. Tiedemann (✉)
Unit of Evolutionary Biology/Systematic Zoology, Institute of Biochemistry and Biology, University of Potsdam, Karl-Liebknecht-Strasse 24-25, Haus 26, 14476 Potsdam, Germany
e-mail: tiedeman@uni-potsdam.de

P.G.D. Feulner
Unit of Evolutionary Biology/Systematic Zoology, Institute of Biochemistry and Biology, University of Potsdam, Karl-Liebknecht-Strasse 24-25, Haus 26, 14476 Potsdam, Germany
Institute for Evolution and Biodiversity, Evolutionary Bioinformatics Group, Westfälische-Wilhelms-Universität Münster, Hüfferstrasse 1, 48149 Münster, Germany

F. Kirschbaum
Institute of Animal Sciences, Humboldt-University Berlin, Berlin, Germany
Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_15,

speciation by serving as a prezygotic isolation mechanism among species with divergent feeding apparatus. In addition, the extremely divergent EODs (100-fold difference in duration) among closely related species might also have a direct adaptive value, as these differences affect echolocation (the primary function of the EOD) and might hence alter the prey spectrum detected. Under such a scenario, the EOD as a single trait pleiotropically combines natural divergent selection (adaptation to divergent diet and/or feeding habits) and reproductive isolation (assortative mating with regard to EOD). EOD might hence constitute a "magic trait" which promoted ecological speciation in African weakly electric fish.

1 Introduction

The concept of ecological speciation invokes adaptive divergence, rather than geographic separation (i.e., allopatry) as a major driver of speciation (Schluter 2000). Under such a scenario, populations diverge from one another mainly because of disruptive selection. In comparison, under the classical allopatric speciation scenario, populations will diverge by random genetic drift; they may also experience adaptive divergence, but this is not necessary for an allopatric speciation to occur. While the idea of ecological speciation is not restricted to a particular geographic setting (Rundle and Nosil 2005), it becomes especially appealing under parapatric and sympatric situations. There are spectacular examples of sympatric speciation due to adaptive divergence in Cichlid fish, both in Africa (Schliewen et al. 1994) and Nicaragua (Barluenga et al. 2006). Most of these examples are, however, restricted to peculiar settings, e.g., small geologically young crater lakes with seemingly uniform environmental conditions. Not least because of the influential contributions of Ernst Mayr in the last century, ecological factors in speciation have been discussed, but considered to play a secondary role, relative to geographic factors (e.g., Mayr 1947, 1963; reviewed in Nosil 2008). Consequently, allopatric speciation is still today typically considered the default, the "null hypothesis" (Coyne and Orr 2004, p. 142), which remains valid unless explicitly rejected. In fact, concluding that potential cases of sympatric speciation should only be accepted, if "the biogeographic and evolutionary history [. . .] make the existence of an allopatric phase very unlikely" (Coyne and Orr 2004, p. 142) restricts – by definition – the possibility for sympatric speciation to special settings, like the crater lakes mentioned above.

Our argument here is that – if we adhere to such rigorous criteria – it is virtually impossible to make a case for sympatric ecological speciation in larger open habitats, like river systems. We investigate here a radiation in African weakly electric fish of the genus *Campylomormyrus*, for which – albeit they occur in the huge Congo River System – patterns of genetic, morphological, electrophysiological, and behavioral divergence are fully compatible with the concept of ecological speciation.

2 Taxonomy of *Campylomormyrus*

The mormyrid weakly electric fishes (Mormyridae) constitute one of the most diverse clades of freshwater fishes from Africa and the single largest known group of electric fishes, with at least 188 described species (Gosse 1984; Alves-Gomes and Hopkins 1997). Mormyrids comprise a well-supported monophyletic group which is endemic to Africa (Taverne 1972; Alves-Gomes and Hopkins 1997; Sullivan et al. 2000). Within the Mormyridae, the taxonomy of the genus *Campylomormyrus* has been particularly puzzling, as the number of accepted species varied between 3 and 16, based on morphological analyses by different authors (Table 1). Most of the 14 species considered valid in the most recent taxonomic revision (Poll et al. 1982) are endemic to a single river system, the Congo and its tributary streams (Table 1; Fig. 1). Only a single species (*Campylomormyrus phantasticus*) has been found not in the Congo Basin, but in the Sanaga river. A single further species (*Campylomormyrus tamandua*) is distributed across different river systems, including the Congo, Volta, Niger, and Tchad/Shari (Gosse 1984).

Prior to our work, only two species of *Campylomormyrus* (*C. numenius* and *C. tamandua*) have been included in molecular phylogenies (Sullivan et al. 2000; Lavoué et al. 2003). Our in-depth phylogenetic analysis within this genus was motivated by the observation that *Campylomormyrus* specimens of similar morphology (putatively assigned to *C. numenius*) can exhibit dramatically divergent adult electric organ discharges (EODs) (Schugardt and Kirschbaum 2002; Feulner

Table 1 Taxonomy of *Campylomormyrus* according to three different authors, their distribution, and type museum material used for morphometric comparison in our study (adapted from Feulner et al. 2007). *H, S, P* refer to inclusion of holotypus, syntypus, or paratypus in the analysis. Underlined taxa have been confirmed as separate species by our combined genetic/electrophysiological/morphometric analysis (Feulner et al. 2007)

Taverne 1972	Roberts and Stewart 1976	Poll et al. 1982	Distribution (Gosse 1984)	Type
C. alces	*C. mirus*	*C. alces*	Congo Basin	S
C. bredoi	*C. rhynchophorus*	*C. bredoi*	Lake Moero and Luapula River	H
C. cassaicus	*C. mirus*	*C. cassaicus*	Afflux Kasai River	P
C. christyi	*C. mirus*	*C. christyi*	Congo Basin	S
C. curvirostris	*C. rhynchophorus*	*C. curvirostris*	Congo Basin	H
C. elephas	*C. mirus*	*C. elephas*	Congo Basin	S
C. luapulaensis	*C. rhynchophorus*	*C. luapulaensis*	Upper Luapula	H
C. mirus	*C. mirus*	*C. mirus*	Congo Basin	H
C. numenius	*C. rhynchophorus*	*C. numenius*	Congo Basin	S
C. ibis	*C. rhynchophorus*	*C. numenius*	Congo Basin	S
		C. orycteropus	Lake Moero	H
C. phantasticus	*C. rhynchophorus*	*C. phantasticus*	Sanaga River	H
C. rhynchophorus	*C. rhynchophorus*	*C. rhynchophorus*	Congo Basin	S
C. compressirostris	*C. rhynchophorus*	*C. rhynchophorus*	Congo Basin	H
C. lualabaensis	*C. rhynchophorus*	*C. rhynchophorus*	Congo Basin	H
C. tamandua	*C. tamandua*	*C. tamandua*	Volta, Niger, Tchad/Shari and Congo Basin	H
C. tshokwe	*C. rhynchophorus*	*C. tshokwe*	Kasai River and afflux	H

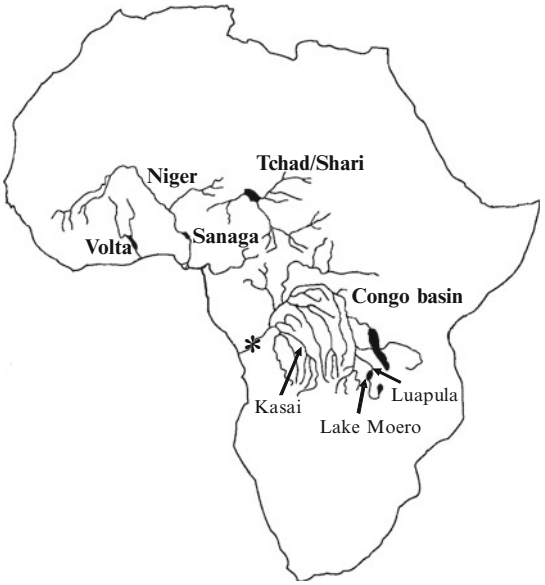


Fig. 1 African river systems in which *Campylomormyrus* occurs. Most species are endemic to the Congo Basin. *Asterisk* indicates the sampling location at Brazzaville/Kinshasa (from Feulner et al. 2007)

et al. 2006). With this discharge, the fish produce an electric field which is used for active electrolocation (von der Emde 1999). Each species (and sometimes each sex) produces its specific EOD, which is also used in social communication (Hopkins and Bass 1981; Kramer and Kuhn 1994; Werneyer and Kramer 2002) and pair formation (Bratton and Kramer 1989; Crawford 1992; Kramer and Kuhn 1994).

We specifically tested the hypothesis that *Campylomormyrus* specimens discharging differently as adults are reproductively isolated from one another. We analyzed altogether 109 *Campylomormyrus* specimens all occurring in a single region, i.e., Kinshasa/Brazzaville at the Lower Congo River, in the onset area of the lower rapids (* in Fig. 1; see Feulner et al. 2006, 2007, 2008 for details). For phylogenetic reconstruction, we sequenced two different molecular marker loci: the complete mitochondrial cytochrome b gene (1,142 base pairs, bp) and a part of the nuclear S7 ribosomal protein gene (895 bp; see Feulner et al. 2006 for experimental details). This analysis yielded a robust well-resolved phylogeny, in which four monophyletic clades (A, B, D, and F) separated out specimens in full accordance with their EOD (Fig. 2). In other words, if specimens were assigned to groups according to their EOD, these groups (except for C) were reciprocally monophyletic in our molecular phylogeny. As all our specimens occur in sym- or parapatry (stemming from a single site), this corroboration of EOD assignment by genetics already fulfils the criteria for species delineation (see, e.g., Milinkovitch et al. 2002). To further confirm reproductive isolation among these sympatrically occurring clades, we developed nuclear microsatellites specific for *Campylomormyrus* (Feulner et al. 2005) to evaluate our clade assignment by independent genetic data. Indeed, assignment of specimens to clusters by genotype information for 16 unlinked microsatellite (using STRUCTURE; Pritchard et al. 2000) confirmed

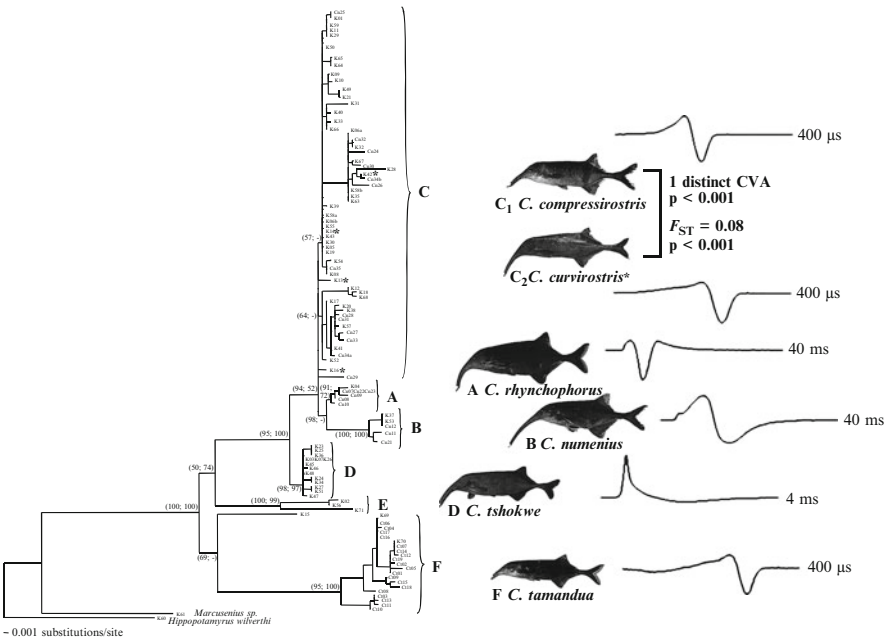


Fig. 2 Bayesian phylogeny based on 2,037 bp (mitochondrial cytochrome b and nuclear S7 genes). Photographs and adult EODs (notice differences in duration) are shown for those clades which could be assigned to described species. In clade C, the differentiation between *C. compressirostris* and *C. curvirostris* (the latter indicated by *asterisk*) is not resolved in the phylogenetic tree, but could be detected by significant differences in morphometric and microsatellite analyzes (from Feulner et al. 2008)

our clades A, B, D, and F (Feulner et al. 2007; Fig. 3). Also, clade C was separated out, but showed substantial heterogeneity among specimens.

We had now accumulated electrophysiological (EOD) and multi-locus genetic (mtDNA, S7, microsatellite) evidence that clusters A, B, D, and F were true species in sympatry. Still, it remained to be evaluated whether they comprise cryptic species behind the putative species *C. numenius* or whether they could be assigned to already described taxa. To settle this issue, we accessed type specimen information of all described *Campylomormyrus* species (Table 1). Ideally, we would have analyzed the same suite of molecular markers for these specimens. That, however, proved not to be feasible, as type specimens were preserved in formalin, a reagent highly destructive to DNA. We hence adopted a multivariate morphometric approach: Based on digital images of all (both ours and the type) specimens, we recorded *x*, *y* coordinates of 11 homologous landmarks (Fig. 4a) to capture information of body shape using TpsDig (Rohlf 2003). After correction for differences due to size, orientation, and position, these variables can be used in a canonical variates analysis (CVA) for stratification of specimens according to multivariate morphotypes (see Feulner et al. 2007 for methods). Interestingly, all clades A–F

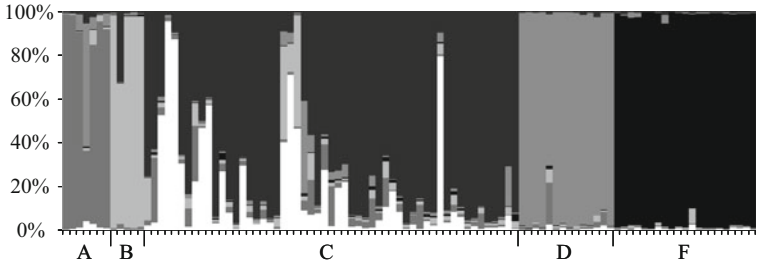


Fig. 3 STRUCTURE analysis of microsatellites clearly identifies clusters A, B, D, and F found in the phylogenetic analysis. Specimens within clade C appear heterogeneous. Data are shown for $k = 6$, the value with the highest likelihood (from Feulner et al. 2007)

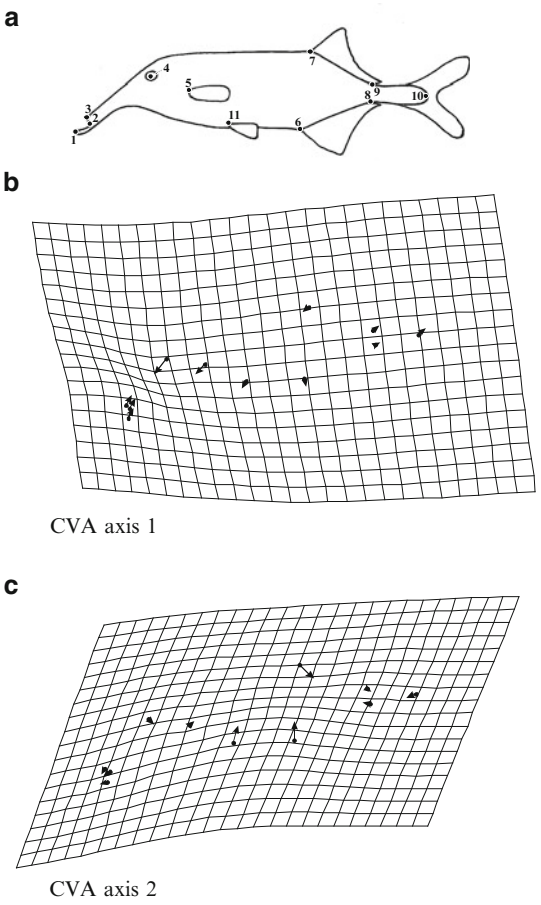


Fig. 4 Landmark configuration and displacement vectors that distinguish clades of *Campylomormyrus*. (**a**) The 11 landmarks chosen to analyze variability in body shape. (**b**, **c**) Deformation grid with relative displacement vectors visualizing the shape changes for each landmark captured by the first two CVA axes, due to which different groups can be discriminated (adapted from Feulner et al. 2007)

could be stratified according to the two principle axes of the CVA (Fig. 5a). Shape-based assignments following the method of Nolte and Sheets (2005) allowed us to assign our clades A, B, D, and F to type specimens of different species (Fig. 5a), while the status of clades C and E remained ambiguous. For clade E, the analysis revealed a tentative assignment to the *C. elephas* type specimen, with, however, too little certainty to be conclusive. In C, several specimens were deviant from the majority. To further investigate the taxonomic status of these specimens, we ran a principal component analysis (PCA) with C clade specimens only, which separated out two clades CI and CII. These clades were further stratified in a subsequent CVA (Fig. 5b). In addition, the two C clades were a posteriori compared with regard to their multi-locus microsatellite genotypes. Indeed, they were highly significantly diverged ($F_{ST} = 0.08$; $p < 0.001$; Fig. 5). Our morphometric assignment indicated that CI and CII comprise two separate species, i.e., *C. compressirostris* and *C. curvirostris* (Figs. 2 and 5b).

In summary, in our combined morphometric, genetic, and electrophysiological analysis we could identify six different previously described species of *Campylomormyrus*, all divergent genetically and morphometrically. Regarding the EOD, there was a standard type of very short pulses shared among not directly related species (i.e., *C. compressirostris*, *C. curvirostris*, and *C. tamandua;* Figs. 2 and 6). From this type, three species differ either by shape of the EOD (*C. tshokwe*) or by extreme elongation (about 100-fold) of single EOD pulses (*C. rhynchophorus*, *C. numenius*) (Fig. 6). Interestingly, the two species with the longest EOD (*C. rhynchophorus*, *C. numenius*) comprise sister taxa phylogenetically nested into the C cluster (which exhibits the standard EOD; Fig. 2). Note that our study assigned a genetically and morphometrically distinct group of specimens (clade CII) to the type specimen of *C. curvirostris*, thus tentatively supporting the existence of *C. curvirostris* as a separate species (as in Taverne 1972), contrary to Roberts and Stewart (1976) and Poll et al. (1982), who suggest lumping *C. curvirostris*-like specimens into *C. rhynchophorus*.

A single specimen (K15) separated out as diverged sister group of *C. tamandua* in the phylogenetic analysis (Fig. 2). This specimen also stood apart morphometrically (Fig. 5). As we were unable to assign it to any type specimen with sufficient confidence, we conclude that this specimen might belong to a so far undescribed species.

3 Indication for Ecological Speciation

Visual inspection reveals small, but consistent, differences in body shape among our *Campylomormyrus* specimens assigned to different clades by EOD and/or phylogenetic analysis (Fig. 2). These differences are especially apparent regarding the form and length of the trunk-like snout as well as the overall body height. In our CVA of 11 morphometric landmarks, these characters proved to account

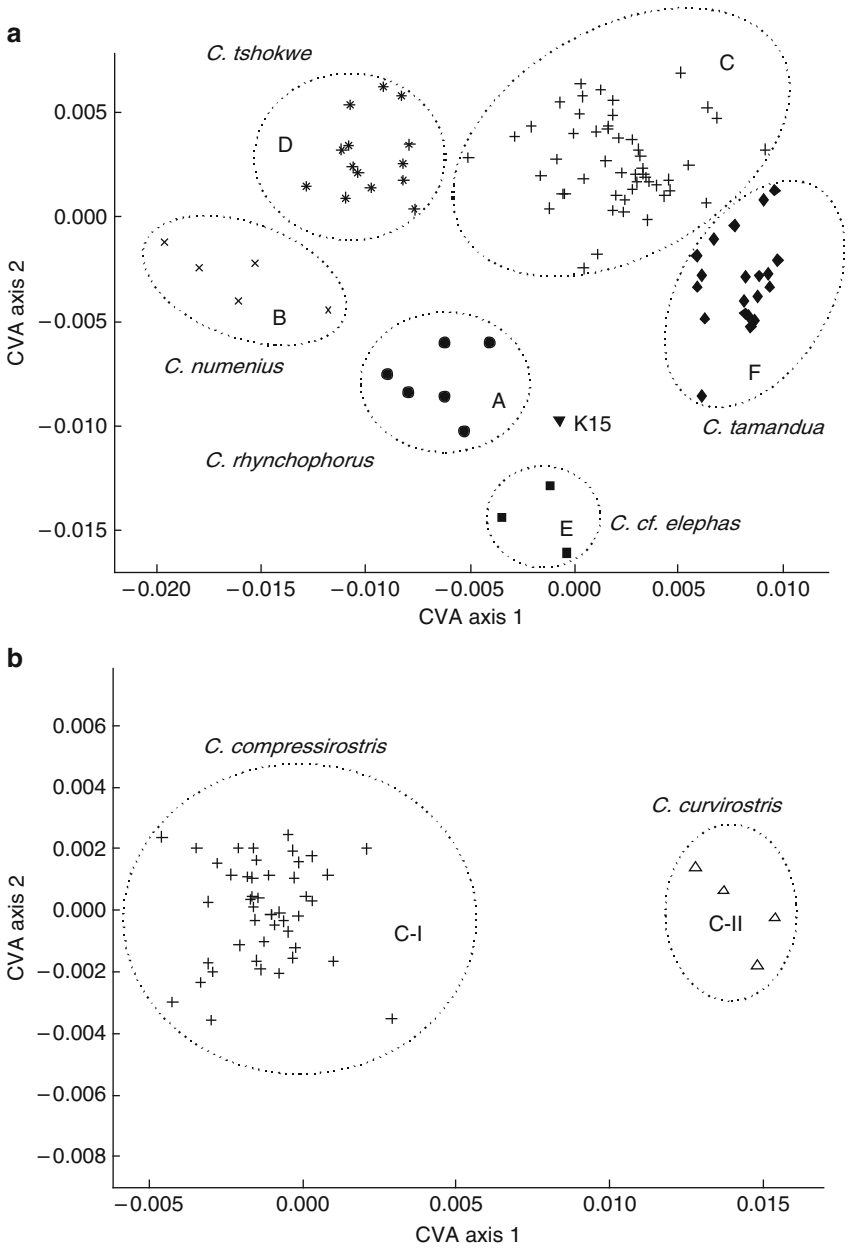


Fig. 5 (**a**) Results of the CVA conducted on morphological variables for all *Campylomormyrus* samples analyzed genetically. *Letters* (*A*–*F*) match coding of clades in the tree of Fig. 2. The main six different clades identified on genetic grounds are clearly separated due to the first two CVA axes. (**b**) CVA restricted to individuals of clade C in the tree of Fig. 2. Four individuals (K13–14–16–52) are clearly differentiated (from Feulner et al. 2007). Species names are given if clades could be morphometrically assigned to type specimens. Assignment of clade E to *C. elephas* was uncertain

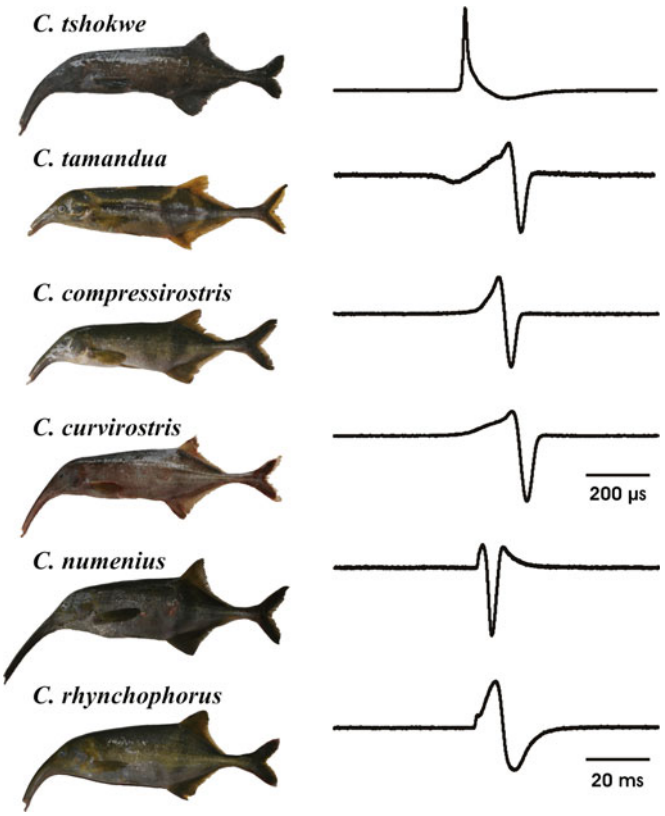


Fig. 6 Adult electric organ discharge (EOD) differentiation between *Campylomormyrus* species. Note that elongated EODs of *C. numenius* and *C. rhynchophorus* are shown on 100 times larger time scale, i.e., 20 ms instead of 200 µs (from Feulner et al. 2009b)

for most of the morphometric variation among our *Campylomormyrus* specimens: The first CVA axis (Lambda = 0.0007; v2 = 619.1097; $df = 108$; $P < 0.001$) described variation in trunk length, whereas the second axis (Lambda = 0.0076; v2 = 417.0081; $df = 85$; $P < 0.001$) was mainly related to body height (Feulner et al. 2007). These differences can be visualized in a deformation grid with relative displacement vectors representing the shape changes for each landmark captured by the first two CVA axes (Figs. 4b, c).

We argue here that, in particular, the divergence in trunk morphology may be adaptive: *Campylomormyrus* feed on insect larvae which burrow into or hide within interstitial spaces and holes in clay sediments of river channels (Marrero and Winemiller 1993). It is therefore reasonable to hypothesize that different trunk shapes are associated with different diets, as the accessibility of certain food items might depend on the morphology of the trophic apparatus. At present, we cannot verify this hypothesis by direct evidence, as neither feeding behavior nor stomach content have so far been analyzed in these nocturnal tropical fishes. Nevertheless,

all species identified on genetic grounds significantly differed in an important morphological trait, i.e., their trophic apparatus.

So far, information about the exact geographic distribution of the *Campylomormyrus* species analyzed here is scanty. What we do know is that all are described for the Congo basin. Admittedly, this currently available information is insufficient to put any speciation scenario into a convincing geographic perspective. Nonetheless, all these species do occur sympatrically at least in one part of their distribution range, i.e., at our sampling sites at Kinshasa/Brazzaville.

According to the competitive exclusion principle (Hardin 1960), it seems highly unlikely that all these co-occurring *Campylomormyrus* species should exploit the same environmental resources. On the contrary, as they exhibit significant differences in their trophic apparatus, it appears reasonable to assume that these differences translate into differences in feeding habits and/or diet. The radiation of *Campylomormyrus* can hence be considered adaptive (Schluter 2000; Feulner et al. 2007, 2008). Consequently, disruptive selection to adapt to the exploitation of different food resources is likely to have played a predominant role in this radiation. Such adaptive speciation can occur in different geographic contexts. It can involve an initial allopatric and a later sympatric (or parapatric) phase (Rundle and Nosil 2005); it can also be driven by habitat specialization (Rice 1987), a scenario sometimes called "microallopatric" (Smith 1965). One might argue that these considerations circumvent the question, "what, if anything, is sympatric speciation?", the title of a recent review by Fitzpatrick et al. (2008). In fact, "truly sympatric" versus "truly allopatric" speciation represent "opposite extremes of the geography of speciation" (Rundle and Nosil 2005). It might be over-simplistic to reduce our view of speciation to geography. Rather, it might be worth considering the respective effects of different evolutionary factors, in particular drift, gene flow, and selection (Fitzpatrick et al. 2008). In speciation scenarios where (disruptive) selection is assumed to play a major role, like in our *Campylomormyrus* example, the process can be termed *ecological speciation*, regardless of knowledge on the exact geographical context of the speciation (Rundle and Nosil 2005).

4 Electric Organ Discharge and Mate Choice

While we argue that the speciation process observed in our *Campylomormyrus* fish is *ecological* in the sense that it is presumably driven by disruptive selection as an adaptation to the exploitation of different food resources (Feulner et al. 2008), it remains to be discussed by which proximate mechanism reproductive isolation (as evidenced from the genetic studies; Feulner et al. 2007) is achieved. Given the sympatric/parapatric occurrence of the species studied here, an effective reproductive isolation mechanism should greatly facilitate speciation. Given the pronounced divergence in EOD among closely related species of *Campylomormyrus* (Figs. 2 and 6), we hypothesized that this character could function as a prezygotic isolation mechanism in these weakly electric fishes, if mating was assortative with regard to

EOD. If this was true, then we expected that the EOD should particularly diverge among closely related species.

We have no conclusive evidence regarding the character polarity of EOD characteristics, i.e., we do not definitely know which types are ancestral and which are derived. Nonetheless, applying the parsimony principle, one could indirectly assume that the short EOD pulses shown by many not directly related *Campylomormyrus* species (i.e., *C. compressirostris*, *C. curvirostris*, and *C. tamandua;* Figs. 2 and 6) might constitute the ancestral type, from which the shape-deviant and/or elongated EOD pulses produced by three species (*C. tshokwe*, *C. rhynchophorus*, and *C. numenius*) might have derived.

To test the function of the EOD for mate recognition, we exposed females of *C. compressirostris* in a choice experiment to conspecific and heterospecific males. The heterospecific males belonged either (1) to a distantly related species showing an EOD similar to that of *C. compressirostris* (i.e., *C. tamandua*) or (2) to a closely related species showing a divergent EOD (i.e., *C. rhynchophorus*; Figs. 2 and 6; see Feulner et al. 2009a for experimental details). To test if the EOD alone was sufficient to trigger female mate recognition, the males were removed and "substituted" by play-back of male-specific EODs (Feulner et al. 2009a).

In both these mate choice experiments, *C. compressirostris* females preferred conspecific males over males of the closely related, but divergently discharging *C. rhynchophorus* males. By contrast, *C. compressirostris* females neither discriminated between conspecific and the more distantly related, but similarly discharging *C. tamandua* males nor their playback signals (Fig. 7). Interestingly, male mate recognition (Arnegard et al. 2006) and species recognition (Markowski et al. 2008) in other mormyrid genera also showed such an asymmetric response. The evolution of a divergent longer EOD in *C. rhynchophorus* in conjunction with assortative mating might have promoted reproductive isolation and divergence between the closely related and sympatrically occurring *C. rhynchophorus* and *C. compressirostris*. Based on this evidence, we can conclude that *Campylomormyrus* has undergone a rapid ecological speciation with disruptive selection for diverse feeding apparatus, which was promoted by assortative mating based on strikingly different adult male electric signals (EODs), serving as an effective prezygotic isolation mechanism.

5 Electric Organ Discharge: A Magic Trait for Speciation?

So far, we have developed a scenario that (1) *Campylomormyrus* has diversified into several species by ecological speciation and (2) that this speciation was promoted by mate choice according to divergent EOD. As such, the two discussed character complexes relevant for speciation, i.e., (1) differentiation in the feeding apparatus and (2) divergence in the EOD appear correlated, rather than functionally (or genetically) linked. Theoretical models have shown that speciation from disruptive selection and assortative mating is possible even when variability of fitness and mate choice depends on different, independently inherited quantitative traits

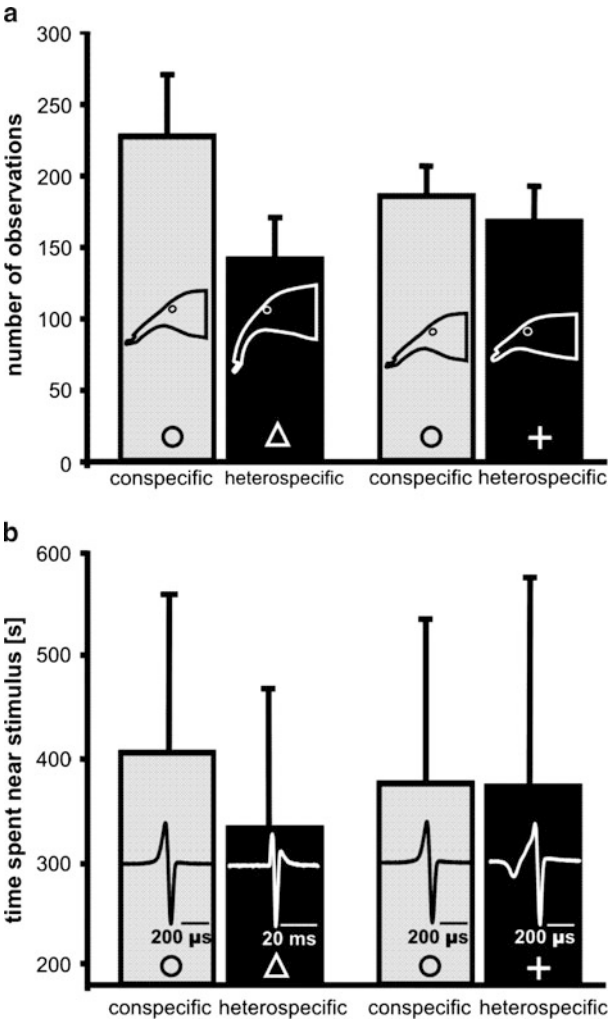


Fig. 7 (**a**) Fish–fish interactions ($n = 6$) and (**b**) fish-playback interactions ($n = 7$). Association behavior of *C. compressirostris* females with conspecific and heterospecific males (**a**) and EODs presented alone (**b**; mean ± s.e.). Different symbols refer to different species (*triangle*, *C. rhynchophorus*; *circle*, *C. compressirostris*; *plus*, *C. tamandua*) (from Feulner et al. 2009a)

(Kondrashov and Kondrashov 1999). However, the EOD might be a trait also allowing direct transmission of ecologically caused divergent selection to a form of reproductive isolation (Kirkpatrick and Ravigne 2002; Rundle and Nosil 2005). There is evidence that the EOD is involved in foraging for insect larvae in electric fish (von der Emde and Bleckmann 1998) and EOD duration might be adaptive for the detection of different preys (Meyer 1982; von der Emde and Ringer 1992). The dual function, (1) electrolocation used in foraging and (2) social communication,

including assortative preferences (as evidenced in this study), suggests that EOD is a trait shaped by disruptive natural selection, but pleiotropically also affecting pre-zygotic reproductive isolation. Such traits have been termed "magic" (Gavrilets 2004; Feulner et al. 2009b). Having the same trait (and hence the same genetic basis) for adaptive divergence and reproductive isolation greatly facilitate speciation, in particular with gene flow (i.e., under sympatric and parapatric conditions).

We conclude that our "magic trait" EOD promoted ecological speciation in our sympatrically occurring African weakly electric fish by (1) allowing for divergent adaptation to different food resources (due to changes in echolocation in conjunction with morphological differences in the feeding apparatus) and (2) additionally triggering pre-zygotic isolation as a clue for assortative mate choice.

Acknowledgments The studies reported in this article have been financed by the German Science Foundation (DFG, TI 349/1) and the University of Potsdam.

References

Alves-Gomes J, Hopkins CD (1997) Molecular insights into the phylogeny of Mormyriform fishes and the evolution of their electric organs. Brain Behav Evol 49:324–350

Arnegard ME, Jackson BS, Hopkins CD (2006) Time-domain signal divergence and discrimination without receptor modification in sympatric morphs of electric fishes. J Exp Biol 209:2182–2198

Barluenga M, Stolting KN, Salzburger W, Muschick M, Meyer A (2006) Sympatric speciation in Nicaraguan crater lake cichlid fish. Nature 439:719–723

Bratton BO, Kramer B (1989) Patterns of the electric organ discharge during courtship and spawning in the mormyrid fish, *Pollimyrus isidori*. Behav Ecol Sociobiol 24:349–368

Coyne JA, Orr HA (2004) Speciation. Sinauer, Sunderland

Crawford JD (1992) Individual and sex specificity in the electric organ discharges of breeding mormyrid fish (*Pollimyrus isidori*). J Exp Biol 164:79–102

Feulner PGD, Kirschbaum F, Tiedemann R (2005) 18 microsatellite loci for endemic African weakly electric fish (*Campylomormyrus*, Mormyridae) and their cross species applicability among related taxa. Mol Ecol Notes 5:446–448

Feulner PGD, Kirschbaum F, Schugardt C, Ketmaier V, Tiedemann R (2006) Electrophysiological and molecular genetic evidence for sympatrically occuring cryptic species in African weakly electric fishes (Teleostei: Mormyridae: *Campylomormyrus*). Mol Phyl Evol 39:198–208

Feulner PGD, Kirschbaum F, Mamonekene V, Ketmaier V, Tiedemann R (2007) Adaptive radiation in African weakly electric fish (Teleostei: Mormyridae: *Campylomormyrus*): a combined molecular and morphological approach. J Evol Biol 20:403–414

Feulner PGD, Kirschbaum F, Tiedemann R (2008) Adaptive radiation in the Congo River: an ecological speciation scenario for African weakly electric fish (Teleostei; Mormyridae; *Campylomormyrus*). J Physiol Paris 102:340–346

Feulner PGD, Plath M, Engelmann J, Kirschbaum F, Tiedemann R (2009a) Electrifying love: electric fish use species-specific discharge for mate recognition. Biol Lett 5:225–228

Feulner PGD, Plath M, Engelmann J, Kirschbaum F, Tiedemann R (2009b) Magic trait electric organ discharge (EOD) - dual function of electric signals promotes speciation in African weakly electric fish. Commun Integr Biol 2(4):1–3

Fitzpatrick BM, Fordyce JA, Gavrilets S (2008) What, if anything, is sympatric speciation? J Evol Biol 21:1452–1459

Gavrilets S (2004) Fitness landscapes and the origin of species. Princeton University Press, Princeton
Gosse JP (1984) Mormyriformes. In: Daget J, Gosse JP, van den Audenaerde DFE Thys (eds) Check-list of the freshwater fishes of Africa. ORSTOM/MRAC, Paris/Tervuren, pp 63–124
Hardin G (1960) The competitive exclusion principle. Science 131:1292–1297
Hopkins CD, Bass AH (1981) Temporal coding of species recognition signals in an electric fish. Science 212:85–87
Kirkpatrick M, Ravigne V (2002) Speciation by natural and sexual selection: models and experiments. Am Nat 159:S22–S35
Kondrashov AS, Kondrashov FA (1999) Interactions among quantitative traits in the course of sympatric speciation. Nature 400:351–354
Kramer B, Kuhn B (1994) Species recognition by the sequence of discharge intervals in weakly electric fishes of the genus *Campylomormyrus* (Mormyridae, Teleostei). Anim Behav 48:435–445
Lavoué S, Sullivan JP, Hopkins CD (2003) Phylogenetic utility of the first two introns of the S7 ribosomal protein gene in African electric fishes (Mormyroidea: Teleostei) and congruence with other molecular markers. Biol J Linn Soc 78:273–292
Markowski B, Baier B, Kramer B (2008) Differentiation in electrical pulse waveforms in a pair of sibling dwarf stonebashers *Pollimyrus castelnaui* and *P. marianne*: possible mechanisms and functions (Mormyridae, Teleostei). Behaviour 145:115–135
Marrero C, Winemiller KO (1993) Tube-snouted gymnotiform and mormyriform fishes – convergence of a specialized foraging mode in teleosts. Environ Biol Fish 38:299–309
Mayr E (1947) Ecological factors in speciation. Evolution 1:263–288
Mayr E (1963) Animal species and evolution. Harvard University Press, Cambridge
Meyer JH (1982) Behavioral-responses of weakly electric fish to complex impedances. J Comp Physiol 145:459–470
Milinkovitch MC, LeDuc R, Tiedemann R, Dizon A (2002) Applications of molecular data in cetacean taxonomy and population genetics with special emphasis on defining species boundaries in cetaceans. In: Evans PGH, Raga JA (eds) Marine mammals: biology and conservation. Kluwer/Plenum, New York, pp 325–359
Nolte AW, Sheets HD (2005) Shape based assignment tests suggest transgressive phenotypes in natural sculpin hybrids (Teleostei, Scorpaeniformes, Cottidae). Front Zool 2:11
Nosil P (2008) Ernst Mayr and the integration of geographic and ecological factors in speciation. Biol J Linn Soc 95:26–46
Poll M, Gosse JP, Orts (1982) Le genre *Campylomormyrus* Bleeker, 1874, étude systématique et description d'une espèce nouvelle (Pisces, Mormyridae). Bull Inst R Sci Nat Belg Biol 54:1–44
Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959
Rice WR (1987) Speciation via habitat specialization: the evolution of reproductive isolation as a correlated character. Evol Ecol 1:301–314
Roberts TR, Stewart DJ (1976) An ecological and systematic survey of fishes in the rapids of the lower Zaire or Congo river. Bull Mus Comp Zool 147:239–317
Rohlf JF (2003) TpsDig, digitize landmarks and outlines. Department of Ecology and Evolution, State University of New York at Stony Brook, Stony Brook
Rundle HD, Nosil P (2005) Ecological speciation. Ecol Lett 8:336–352
Schliewen UK, Tautz D, Paabo S (1994) Sympatric speciation suggested by monophyly of crater lake cichlids. Nature 368:629–632
Schluter D (2000) The ecology of adaptive radiation. Oxford University Press, Oxford
Schugardt C, Kirschbaum F (2002) Nilhechte des *Campylomormyrus numenius*-Formenkreises: Bemerkungen zur Ontogenese des Habitus, der elektrischen Entladung sowie zum Fortpflanzungsverhalten. In: Greven H, Riehl R (eds) Verhalten der Aquarienfische, vol 2. Birgit Schmettkamp, Bornheim, pp 137–146
Smith HM (1965) More evolutionary terms. Syst Zool 14:57–58

Sullivan JP, Lavoué S, Hopkins CD (2000) Molecular systematics of the African electric fishes (Mormyroidea: Teleostei) and a model for the evolution of their electric organs. J Exp Biol 203:665–683

Taverne L (1972) Ostéologie des genres *Mormyrus* Linné, *Mormyrops* Müller, *Hyperopisus* Gill, *Isichthys* Gill, *Myomyrus* Boulenger, *Stomatorhinus* Boulenger et *Gymnarchus* Cuvier considérations générales sur la systématique des poissons de l'ordre des Mormyriformes. MRAC, Tervuren

von der Emde G (1999) Active electrolocation of objects in weakly electric fish. J Exp Biol 202:1205–1215

von der Emde G, Bleckmann H (1998) Finding food: senses involved in foraging for insect larvae in the electric fish *Gnathonemus petersii*. J Exp Biol 201:969–980

von der Emde G, Ringer T (1992) Electrolocation of capacitive objects in 4 species of pulse-type weakly electric fish. 1. Discrimination performance. Ethology 91:326–338

Werneyer M, Kramer B (2002) Intraspecific agonistic interactions in freely swimming mormyrid fish, *Marcusenius macrolepidotus* (South African form). J Ethol 20:107–121

Ongoing Phenotypic and Genotypic Diversification in Adaptively Radiated Freshwater Crabs from Jamaica

Christoph D. Schubart, Tobias Weil, Jesper T. Stenderup, Keith A. Crandall, and Tobias Santl

Abstract The Caribbean island of Jamaica is home to a lineage of crabs (Crustacea: Decapoda: Brachyura: Sesarmidae) which have colonized the most unusual terrestrial habitats. Today, they can be found nesting in bromeliad leaf axils or empty snail shells, scurrying on the forest floor or among dry rock rubble, climbing on walls of deep cave systems, or digging burrows in the banks of mountain streams. In order to facilitate survival of their offspring in these remarkable and new habitats, complex behavioral adaptations have evolved, including feeding and protecting the offspring, and transport of empty snail shells into the nursery to buffer the pH and provide the necessary calcium carbonate [summarized in Diesel (Proc Roy Soc Lond B 264:1403–1406, 1997), Diesel and Schubart (Evolutionary ecology of social and sexual systems: crustaceans as model organisms. Oxford University Press, New York, pp 365–386, 2007)]. No other American sesarmid crab species shows this degree of terrestriality and elaborate brood care behavior. Instead, the most related species typically are inhabitants of marshes and mangroves and reproduce by releasing their larvae into the ocean, without any additional paternal obligation. Molecular phylogenetic analyses revealed that the Jamaican endemic sesarmids are monophyletic and thus the outcome of a single colonization event, which took place approximately 4.5 million years ago and was followed by a rapid radiation, speciation, and adaptation to ecological niches, which have not been used by crabs in any other parts of the world and required evolution of complex behavioral strategies to render survival in these habitats possible (Schubart et al. Nature 393:363–365, 1998a). This system can be considered a classic example of

C.D. Schubart (✉), T. Weil, and T. Santl
Biologie 1, Institut für Zoologie, Universität Regensburg, 93040 Regensburg, Germany
e-mail: christoph.schubart@biologie.uni-regensburg.de

J.T. Stenderup
Biologie 1, Institut für Zoologie, Universität Regensburg, 93040 Regensburg, Germany
Natural History Museum of Denmark, University of Copenhagen, 1350 København, Denmark

K.A. Crandall
Department of Biology, Brigham Young University, Provo, UT 84602-5255, USA

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_16,

an adaptive radiation of comparatively young age. It calls for research concerning the mechanisms and the pace of evolution to generate these highly specialized forms. In the present paper, we provide new data on evolutionary processes at the intraspecific level, which may help to understand the potential of diversification in these crabs. Furthermore, published and unpublished data available for these specialized crabs are summarized, giving evidence for the astounding diversity of evolutionary strategies and the dimension of biodiversity beyond the species level.

1 Introduction

The insight by Charles Darwin that animals and plants thriving on our planet are subject to continuous change as a result of natural selection and adaptation has been decisively influenced by endemic ground finches (Emberizidae: Geospizinae) from the Galapagos Islands. Thirteen of these birds were collected by Darwin in autumn 1835 during the voyage of the *HMS Beagle* and, shortly after the return to London, the ornithologist John Gould communicated to Darwin that the finches and mockingbirds from the different islands were distinct species and not just varieties (Sulloway 1982). The realization that each island had its own set of species with different adaptations prompted Darwin to write "Seeing this gradation and diversity of structure in one small, intimately related group of birds, one might really fancy that from an original paucity of birds in this archipelago, one species had been taken and modified for different ends" (Darwin 1845), thus considering a "transmutation of species" and later culminating in publication of his theory of natural selection (Darwin 1859). Nowadays, the Geospizinae are commonly referred to as "Darwin's finches" and are the most often cited example for so-called adaptive radiations.

Adaptive radiations thus belong to the earliest evidences for biological evolution and, next to Darwin's finches, honey creepers (Drepanidinae), fruit flies (Drosophilidae), and silverswords (Madiinae) from Hawaii, *Anolis* lizards (Iguanidae) from the Caribbean islands, as well as cichlid fishes from East African lakes are frequently reported and impressive examples of such evolutionary events (Schluter 2000). According to this author, an adaptive radiation is "the evolution of ecological and phenotypic diversity within a rapidly multiplying lineage". Possible factors causing adaptive radiations could be (1) the combination of diverse habitat and competition (divergent selection), (2) the evolution of novel advantageous features (innovation), and/or (3) the presence of ecological opportunity, like new habitats, combined with a lack of predation or competition (unoccupied niches). In most cases, such increase of diversity results in a number of species (or other taxa) of approximately the same age.

Schluter (2000) proposed four criteria to define and recognize adaptive radiations: (1) common ancestry, (2) rapid speciation, (3) phenotype-environment correlation, and (4) trait utility. The first two criteria are of phylogenetic nature, while the latter two criteria correlate present morphological and ecological characteristics

with natural selection. Schubart et al. (1998a) gave phylogenetic evidence for common ancestry and rapid speciation in a monophyletic group of endemic crabs of the family Sesarmidae from Jamaica, inhabiting diverse terrestrial and freshwater environments. This and previous studies (Hartnoll 1964; Schubart et al. 1997) provide examples of phenotype–environment correlation, suggesting that morphological differences between the species can be related to their habitats (e.g., flattened carapace in bromeliad crabs and elongated tactile legs in cave crabs). Trait utility, i.e., "evidence that morphological and physiological traits of species are indeed useful where they are employed" (Schluter 2000: p.11) becomes obvious when observing these animals in the field; for example, a bromeliad crab retreating into the narrowest part of a leaf axil, a mountain stream crab constructing burrows with its stout, strong and setose ambulatories, or a cave crab exploring its three dimensional environment in the dark by stretching its slender elongated legs in all directions (personal observations; Diesel and Schubart 2000: figs. 3 and 6). Also, differences in the evolution of abbreviated development (Hartnoll 1964; González-Gordillo et al. 2010) and of hyperosmotic regulation (Schubart and Diesel 1999) compared to their marine confamilial relatives can be considered as traits providing physiological and morphological advantages in the new environment. We thus consider all criteria fulfilled to classify the radiation of Jamaican land-dwelling crabs adaptive.

As a consequence of the prolonged time which is normally necessary for completion of speciation processes, most of our available evidence for adaptive radiations is post-hoc, i.e., the outcome of adaptive radiations. In the scientific literature, the term "adaptive radiation" is therefore mostly used when referring to past multiple speciation events with simultaneous ecological divergence. However, adaptive radiation is first manifested as variation among populations. Therefore, the study of ecological and genetic divergence between populations is a promising approach of gaining new insights into the fundamental processes that cause adaptive radiations (e.g., Huber et al. 2007). Post-hoc reconstructions and interpretations of adaptive radiations are often very difficult due to the relative rapidity of the involved speciation processes (e.g., Schubart et al. 1998a). Fast splits into several species may not allow diagnostic morphological or molecular characters to be established in the different evolutionary lineages. Morphological and genetic variation within the ancestral population is randomly distributed among the different lineages and, even with an absolute lack of gene flow, it will take a considerable evolutionary time before these lineages can be told apart. If this "lineage sorting" process (see Neigel and Avise 1986) is not completed, it may hinder reconstructions from rapid or recent speciation events.

The present study provides examples of ongoing morphological and genetic diversification within some of the Jamaican endemic freshwater crabs that evolved by adaptive radiation between 3 and 4.5 million years ago. This shows once more that in evolution nothing is ever completed but is in constant flux. Species formation and biological diversification is a continuous process from which we can only witness a very limited time window, attempting to understand the principles of the evolution of biodiversity.

2 Phenotypic Modification of Body Form in Response to Cave-Living

Jamaican caves are inhabited by different species of crabs, from which only one, *Sesarma verleyi* Rathbun 1914, is an obligatory troglobiont (i.e., restricted to caves), whereas others are troglophilic freshwater crabs that can be encountered in caves as well as outside them. One such example is *Sesarma windsor* Türkay and Diesel 1994. Originally described from the entire western-central region of Jamaica (Türkay and Diesel 1994), the occurrence of this species was later restricted to a single cave system in central Jamaica, its type locality Printed Circuit Cave (Trelawny), and the species was redescribed by Schubart et al. (1997), after recognizing morphological differences from other populations of freshwater crabs previously included within *S. windsor*. At that point, most of its morphological differences, as for example lighter coloration, longer appendages, smaller eye stalks, flatter and broader body form, were attributed to a cave-dwelling mode of life (Schubart et al. 1997), similar to, but not as pronounced as, in *S. verleyi*. Additional geographic sampling procured material which revealed that *S. windsor* occurs in four neighboring river systems (Hectors River, Quashies River, Mouth River, and Cave River) along the northeastern fringe of the Cockpit Country (Trelawny, Central Jamaica) (Schubart and Koller 2005) (Fig. 1). These rivers have in common that their beds are only in part exposed to the surface. All of them are characterized by water seepage into the calcareous rock and probable reappearance in the coastal plains (e.g., Rio Bueno) or along the submerged island shelf. The subterranean connections between these rivers still remain mostly unexplored. *S. windsor* was encountered in the surface streams of these rivers as well as in the entrance area of caves or deep within the accessible caves. Therefore, the question remained whether morphological characteristics as described by Schubart et al. (1997) are unique for the cave population or are a general characteristic of this species. Expressed differently: is the presumed troglophilic morphology of

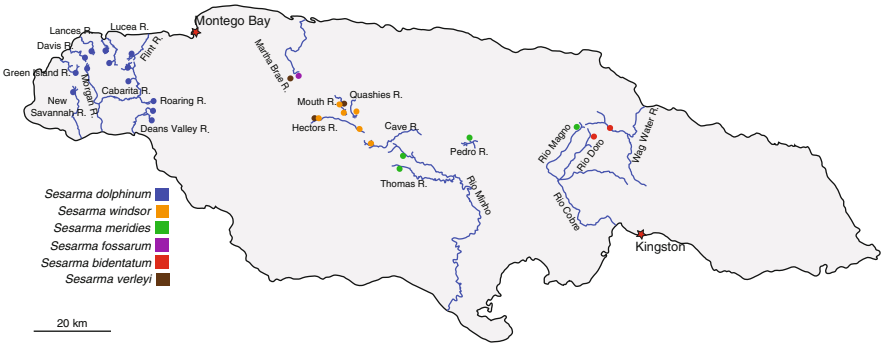


Fig. 1 Map of Jamaica showing selected rivers and localities where species for the current study have been collected

S. windsor consistent and also found in representatives thriving in surface waters, or are there short-term genetic or even phenotypic mechanisms allowing rapid evolution of a body form which is favorable in cave habitats?

To investigate this question, we compared morphometry and genetics of crabs from the Mouth River in central Jamaica, which has extended subterranean passages (Printed Circuit Cave, Harties Cave, Mouth River Maze) directly connected to nearby surface waters. Crabs from the subterranean and surface populations seem to differ in their overall morphological appearance. However, lack of gene flow with only a few meters distance between the populations appears unlikely and could only be explained by strong ecological exclusion. Genetic differentiation at such close geographic distances would be a striking example of parapatric separation, whereas genetic identity would represent an interesting case of phenotypic plasticity in the arthropod world.

2.1 Materials and Methods

Specimens of *Sesarma windsor* were collected over an 11-year period between 1993 and 2003 from a subterranean stream inside the Printed Circuit Cave (also known as Corner Cave or John Foden Cave) and outside the cave from the Mouth River. The Printed Circuit Cave is located to the southeast of the Mouth River sink near the village of Rock Spring and stretches across approximately 610 m length more or less parallel to the Mouth River. Like most of the other limestone caves in the Cockpit karst, the Printed Circuit Cave has many sinter terraces lining the river channel of the little cave stream. These sinter terraces are the habitat for the crabs living in the cave, while crabs outside the cave inhabit the beds and banks of the rivers. Collections were also carried out in the neighboring Quashies River, in the vicinity of the village Stettin (eastern Cockpit Country, Trelawny Parish). Freshwater crabs were killed on ice and preserved in 75–90% ethanol for subsequent measurements and genetic analyses.

2.1.1 Morphometric Data

Altogether, 58 adult crabs of both genders were available for the statistical analyses of morphometric data. Measurements of the studied material included the carapace width at the epibranchial tooth (CWT), the maximum width of the carapace (CW), the carapace length (CL), the body height (BH), the maximum width of the pleon (PW), the interorbital width (IOW), the length of the epibranchial tooth (ET), the length of the dactylus (DaL), the length (PrL), and the height of the propodus (PrH) from the larger chela, the merus length of the larger cheliped (MerL), the width (MW), and length (ML) of the meri of all walking legs (pereiopods 2–5) and the ventral length (ischium-dactylus) of the extended fourth pereiopod (4PpL). The data were tested for fit to a normal distribution by the Kolmogorov-Smirnov-Test

(Statistica, version 6.0). Because of the sexual dimorphism, measurements for chelae and pleon were tested separately for fit to a normal distribution for each gender. Statistical differences between ratios of these variables were tested with a *t* test. A discriminant analysis was carried out for an accurate differentiation between populations by choosing 14 sex-unrelated log-transformed morphometric variables. Hereby, the number of specimens was reduced to 47 as a consequence of missing variables in the overall dataset caused by absence of different body appendages in some specimens (e.g., single walking legs). Six specimens of *S. windsor* from the neighboring Quashies River were included as an outgroup and the discriminant analysis was carried out with Statistica. The variables PW, DL, PrL, PrH, and 4PpL were excluded from the discriminant analyses due to evident sex-dependent differences in size.

2.1.2 Genetic Data

Genomic DNA was isolated from muscle tissue of walking legs from 40 crabs (20 from the cave and 20 from outside) using the Puregene (Gentra Systems) extraction kit. Isolated DNA was precipitated with 100% ethanol, desalted with 70% ethanol, dried, and resuspended in TE-buffer. Selective amplification of a 1,059-basepair region from the mitochondrial cytochrome oxidase subunit I (Cox1) gene was carried out by polymerase-chain-reaction (PCR) with 35–40 cycles and the following temperature profile: 45 s denaturing at 94°C, 1 min annealing at 48–56°C, and 1 min extension at 72°C, preceded and concluded by 5 min initial denaturing and 10 min final extension. The following primers were used: COL6b (5′- ACA AAT CAT AAA GAT ATY GG-3′; Schubart and Huber 2006), COL8 (5′-GAY CAA ATA CCT TTA TTT GT-3′; Schubart 2009), CO1f (5′-CCT GCA GGA GGA GGA GAY CC-3′; Palumbi et al. 1991) as forward primers and COH6 (5′- TAD ACT TCD GGR TGD CCA AAR AAY CA-3′; Schubart and Huber 2006), COH8 (5′-TGA GGR AAA AAG GTT AAA TTT AC-3′; Schubart 2009), COH4 (5′-AAR RAT CCT RAD TTR CCA TAY CC-3′; Mathews et al. 2002), and CO1a (5′-AGT ATA AGC GTC TGG GTA GTC-3′; Palumbi et al. 1991) as reverse primers. PCR reactions were carried out in 25-µl volumes containing forward and reverse primers (1.25 µl of 20 µM), 1.25 mM dNTPs, 25 mM magnesium chloride, 10× buffer, 1U/µl Taq polymerase (MBI Fermentas), template DNA, and Millipore water. PCR products were purified with Millipore Montage™ PCR Centrifugal Filter Devices (Millipore) prior to cycle-sequencing. Sequence products were ethanol precipitated, resuspended in ddH_2O, and run on an ABI Prism™ 310 Genetic Analyzer (Applied Biosystems/Perkin Elmer) automated sequencer. Forward and reverse strands were sequenced for all samples. The sequences were analyzed with the program ABI Sequencing Analyses® 3.4 (Applied Biosystems) and sequence data aligned unambiguously (no indels) with the multi-sequence editing software XESEE (Cabot and Beckenbach 1989). Sequences of the different haplotypes have been deposited in the EMBL database (FN395290-FN395305).

Genetic heterogeneity within the populations was estimated as haplotype diversity ($h = 1 - \sum f\,\mathrm{i}^2$; f i being the frequency of the haplotype i; Avise, 2004). Pairwise ϕ_{ST} values among populations and analysis of molecular variance (AMOVA) were calculated with Arlequin version 3.1 (Excoffier et al. 2005). The phylogenetic relationships among haplotypes are graphically represented with a statistical parsimony network using the algorithm outlined in Templeton et al. (1992) and the software TCS (phylogenetic network estimation using statistical parsimony; Clement et al. 2000).

Three microsatellite loci currently available for the Jamaican Sesarmidae (Marcadé et al., unpublished; Heine 2006) were amplified for 25 animals from the caves versus 25 from outside the cave.

2.2 Results

2.2.1 Morphometrics

Highly significant morphometric differences between individuals of *Sesarma windsor* from the Printed Circuit Cave and the Mouth River population were revealed in all comparisons: ratios of single characters, sex specific characters, and in the overall discriminant analyses. The most obvious character to distinguish specimens of both populations is the length of the walking legs in both sexes, with specimens of the cave population having longer and more slender legs than those of the river population. The relative length of the fourth pereiopod in relation to the carapace width (4PpL/CW) is 1.75 ± 0.04 in the cave animals ($N_{PC} = 18$) and 1.65 ± 0.06 in the Mouth River ($N_{MR} = 24$). There are also highly significant differences in the length to width proportions of the meri of all walking legs, with cave animals always showing values above 2.5 and surface inhabitants below. These differences are so pronounced that standard deviations are non-overlapping, with the exception of the last pair of walking legs (Table 1). Furthermore, crabs of the river population have a significantly broader front in relation to the carapace width (IOW/CW $= 0.48 \pm 0.01$, $N_{MR} = 29$; IOW/CW $= 0.47 \pm 0.01$, $N_{PC} = 28$) and a significantly longer exorbital tooth in relation to the total carapace length (ET/CL $= 0.27 \pm 0.09$, $N_{MR} = 26$; ET/CL $= 0.18 \pm 0.01$, $N_{PC} = 28$).

Relations of the chelae and the pleon were analyzed separately for adult males and females to account for their sexual dimorphism. Male chelae show highly significant differences between both populations, being shorter and higher in surface animals, while female claws show no significant differences (Table 1). The trend for longer appendages in cave specimens is thus only followed in male cheliped fingers. One possible explanation for this phenomenon would be that the marked differences in male chelar height (higher in surface animals) can be attributed to sexual selection in the form of visual display during courtship, which takes place in the surface population, but is not possible in the cave.

Table 1 Comparison of morphometric relationships (mean ± standard deviation; n = sample size) of the Printed Circuit Cave and the Mouth River crab populations. Exclusively adult animals of both genders were measured. Statistical calculation based on unpaired two-tailed t test. Significant results are shown in bold

♂ and ♀	Printed Circuit Cave	Mouth River	p	t	df
IOW/CW	0.47 ± 0.01 (n = 28)	0.48 ± 0.01 (n = 29)	**0.002**	−2.41	55
4PpL/CW	1.75 ± 0.04 (n = 18)	1.65 ± 0.06 (n = 24)	<**0.0001**	6.49	40
2 ML/2 MW	2.57 ± 0.15 (n = 26)	2.28 ± 0.11 (n = 29)	<**0.0001**	8.18	53
3 ML/3 MW	2.63 ± 0.13 (n = 26)	2.35 ± 0.11 (n = 28)	<**0.0001**	8.93	52
4 ML/4 MW	2.66 ± 0.13 (n = 25)	2.36 ± 0.12 (n = 26)	<**0.0001**	8.8	49
5 ML/5 MW	2.51 ± 0.16 (n = 25)	2.29 ± 0.10 (n = 26)	<**0.0001**	5.91	49
ET/CL	0.18 ± 0.01 (n = 28)	0.27 ± 0.09 (n = 26)	<**0.0001**	−5.16	52
♂ PrL/PrH	1.81 ± 0.08 (n = 18)	1.75 ± 0.08 (n = 20)	**0.009**	2.65	37
♂ DaL/PrH	1.17 ± 0.05 (n = 18)	1.11 ± 0.05 (n = 20)	**0.0007**	3.67	37
♀ PrL/PrH	2.03 ± 0.11 (n = 10)	1.99 ± 0.03 (n = 9)	0.3	1.07	17
♀ DaL/PrH	1.24 ± 0.05 (n = 10)	1.22 ± 0.03 (n = 9)	0.25	1.18	17

In order to test for the overall differentiation between the two populations, a discriminant analysis was carried out using 14 sex-unrelated log-transformed variables and adding six specimens of *S. windsor* from the unconnected Quashies River as an outgroup. The dataset was subjected to canonical analyses as shown in Fig. 2. The discrimination between the groups was highly significant (Wilks´ Lambda: 0.0299, $F(28.74) = 12.650$, $p < 0.0001$). All populations could be correctly classified with a likelihood of 100%. The Mahalanobis distances are shorter between the Mouth River and the Printed Circuit Cave population than between both of these populations compared to the outgroup population from the Quashies River. The first canonical function (root1) accounted for 93.94% of the explained variance, the first two roots together for 100%.

2.2.2 Genetics

A 1,059-basepair fragment of the mitochondrial Cox1 gene was amplified and sequenced for a total of 20 specimens from each population. Among the 40 sequences, a total of 16 different haplotypes and 19 variable nucleotide positions could be identified. The statistical parsimony network constructed with TCS illustrates the genetic distances among the haplotypes (ht) of *S. windsor* (Fig. 3). The common haplotypes ht1 (3× PC, 4× MR), ht6 (4× PC, 3× MR), and ht7 (2× PC, 3× MR) occur in both populations with similar frequencies. Ht1 can be regarded as the ancestral haplotype, because six other haplotypes seem to have diverged from it (Crandall and Templeton 1993). From those six haplotypes, three (6, 7, 16) are again the starting point for more haplotypes. Ht4 shows the maximal distance to ht1. These two haplotypes are separated by eight substitutions. The largest distance between all recorded haplotypes is shown between ht4 and ht13, which are separated by 13 substitutions (Fig. 3, Table 2).

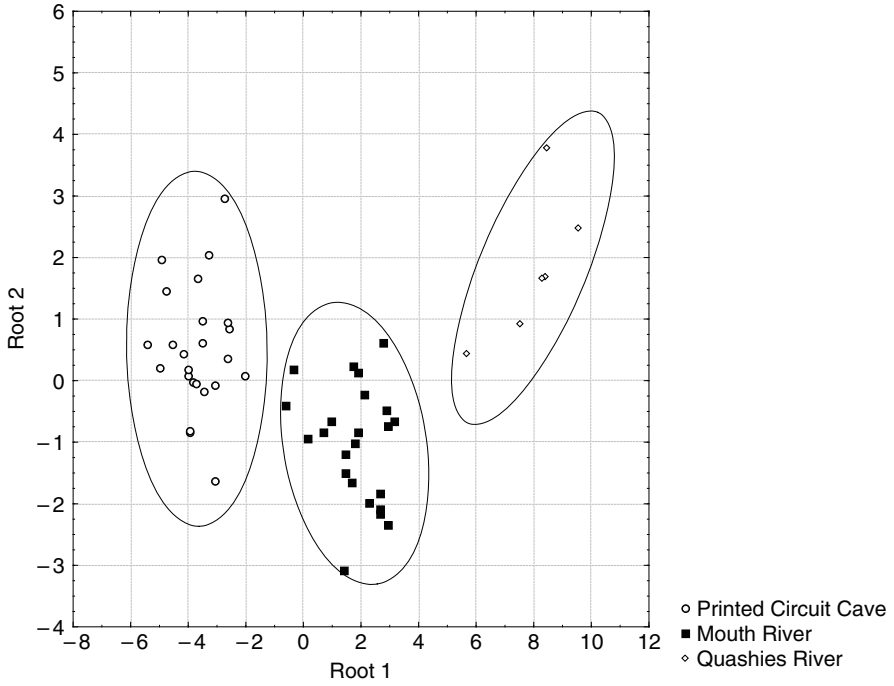


Fig. 2 Discriminant analysis showing morphometric differentiation between 53 specimens of *Sesarma windsor* (24 specimens, Printed Circuit Cave; 23, Mouth River; 6, Quashies River) according to 14 sex-unrelated log-transformed variables

Six haplotypes occur in both populations (1–4, 6–7) while five currently appear as private haplotypes for either the Printed Circuit Cave population (5, 8–11) or the Mouth River population (12–16). Among the haplotypes restricted to one of the two populations, the haplotypes ht12 and ht13 are the only ones to occur twice. The three most common haplotypes, ht1, ht6 and ht7 (accounting for 47.5% from the total sequences), are shared, and ht6 and ht7 are separated by single transitions from ht1. Transversions are found between shared haplotypes (2, 3, 4) as well as in haplotypes so far restricted to the cave (5, 8, 10). Four haplotypes result in an amino acid exchange according to the invertebrate codon usage (Table 2). Both populations are characterized by identical haplotype diversities of $h = 0.88$ and maximum sequence divergence of 13 mutational steps (1.23%) in the river population and 12 mutational steps (1.13%) in the cave population. The analysis of molecular divergence using AMOVA showed that there is no significant genetic difference between the cave and the river population ($p = 0.51$). The overall ϕ_{ST} of 0.09 indicates that gene flow between the populations is not restricted over longer time periods.

Likewise, three microsatellite markers failed to provide evidence for genetic differentiation between the cave population and the surface population. Originally designed for the bromeliad crab *Metopaulias depressus* Rathbun 1896, the microsatellite Met23 (Marcadé et al., unpublished) showed variability in the form of

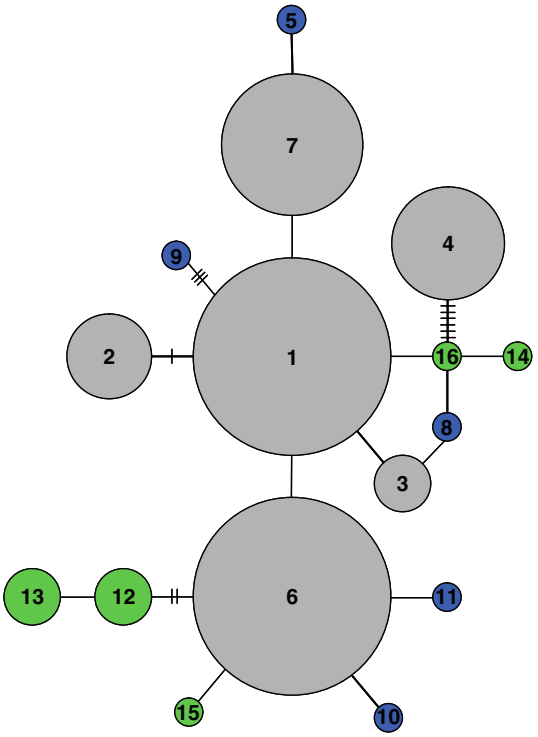


Fig. 3 Statistical parsimony network showing the genetic distances between haplotypes of the Mouth River population and the Printed Circuit Cave population of *Sesarma windsor*. The size of the circular area is equivalent to the frequency at which the haplotype occurs within the 40 examined specimens (Table 2). Each *connecting line* and each transverse *smaller line* illustrates a substitution. Transversions are shown with *bold lines*. Mouth River haplotypes are shown in *green*, Printed Circuit haplotypes in *blue* and haplotypes occurring in both populations in *gray*

25 alleles, but their distribution among 50 individuals suggests extensive gene flow between the cave and the surface population ($F_{ST} = 0.0065$, $p > 0.05$) and a strong heterozygote deficiency ($F_{IS} = 0.0065$, $p < 0.001$). Microsatellites MHA and MHE (Heine 2006) amplified regions with repeats, but they were of the same length in all tested individuals.

3 Genotypic Diversification Within Three Species of Freshwater Crabs

The adaptive radiation and consequent species diversity of terrestrial and freshwater crabs on Jamaica is not equalled by the crab faunas from the other Greater Antilles. Cuba, Hispaniola, and Puerto Rico; all have one or more species of endemic freshwater crabs belonging to the genus *Epilobocera* and the family Pseudothelphusidae. However, neither in terms of species numbers nor ecologically, are the freshwater crabs from these islands as diverse as the Jamaican fauna, despite the considerably larger size of some of these islands. Unlike the Sesarmidae, which are mostly coastal marine forms, the family Pseudothelphusidae is an old

Table 2 Variable positions within 16 haplotypes obtained out of 40 mitochondrial Cox1 sequences (1,059 bp) of the species *Sesarma windsor*. Comparison of nucleotide positions referring to haplotype 1. *Identical nucleotides compared to haplotype 1; *Ntot* frequency of the haplotype with regard to the total sample size ($n = 40$); N_{PC} frequency of the haplotype with regard to the Printed Circuit Cave sample size ($n = 20$); N_{MR} frequency of the haplotype with regard to the Mouth River sample size ($n = 20$); *Aa1* amino acid regarding to haplotype 1; *Aa2* amino acid after mutation

	Nucleotide position																					
Haplotypes	208	260	262	277	284	364	395	445	521	571	580	649	758	779	782	970	1017	1036	1054	Ntot	NPC	NMR
1	C	C	G	T	T	T	T	T	G	A	T	G	C	C	G	G	T	T	T	7	3 (0,15)	4 (0,20)
2	*	*	C	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	3	2 (0,10)	1 (0,05)
3	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	2	1 (0,05)	1 (0,05)
4	T	*	*	C	C	*	*	*	*	G	*	A	T	T	*	C	*	*	*	4	3 (0,15)	1 (0,05)
5	*	*	*	*	*	*	*	C	*	*	*	*	*	*	*	*	G	*	*	1	1 (0,05)	0
6	*	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	7	4 (0,20)	3 (0,15)
7	*	*	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	*	5	2 (0,10)	3 (0,15)
8	T	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	1	1 (0,05)	0
9	*	*	A	*	*	C	*	*	*	*	C	*	*	*	*	A	*	*	*	1	1 (0,05)	0
10	*	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	G	*	1	1 (0,05)	0
11	*	*	*	*	*	*	C	*	*	*	*	*	*	*	A	*	*	*	*	1	1 (0,05)	0
12	*	T	*	*	*	*	C	*	*	*	*	A	*	*	*	A	*	*	*	2	0	2 (0,10)
13	*	T	*	*	*	*	C	*	A	*	*	A	*	*	*	A	*	*	*	2	0	2 (0,10)
14	T	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	1	0	1 (0,05)
15	*	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	*	C	1	0	1 (0,05)
16	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	1	0	1 (0,05)
Codonposition	3	1	3	3	1	3	1	3	1	3	3	3	1	1	1	3	2	3	3			
Aa1									A						A		V	I				
Aa2									T						T		G	M				

lineage of exclusively freshwater crabs distributed throughout the neotropics (northern Brazil to southern Mexico). While representatives of the Pseudothelphusidae probably colonized the Lesser Antilles rather recently from the South American mainland via the Aves Ridge (similar to the description in Iturralde and MacPhee 1999), the Greater Antilles (with the exception of Jamaica) have a much older and more derived fauna of freshwater crabs, which morphologically most resemble the pseudothelphusids from Yucatán (Mexico). Colonization in this case may have taken place by means of vicariance (as described in Hedges 2001), when the Caribbean Plate was pushed eastward between the two American Plates in the Early Tertiary (see maps in Buskirk 1985). Currently, there are seven recognized endemic species of *Epilobocera* on Cuba (Capolongo and Pretzmann 2002; Ng et al. 2008), two on Hispaniola, and one on Puerto Rico (Chace and Hobbs 1969; Rodríguez and Williams 1995). The absence of *Epilobocera* from Jamaica can be explained by the fact that this island was almost completely submerged during part of the Mid Tertiary (see Buskirk 1985).

Most rivers and mountain streams of the different Greater Antillean islands are very similar in terms of soils, vegetation, and temperature. They appear to represent comparable ecological niches, which is also recognizable by the presence of the same species of freshwater shrimp (e.g., *Atya scabra*, *A. innocuous*, *Macrobrachium faustinum*, *M. carcinus*, *Xiphocaris elongata*) in rivers of Cuba, Hispaniola, Jamaica, and Puerto Rico (Chace and Hobbs 1969). These shrimps have a marine larval development which allows dispersal among the Caribbean islands (Reuschel and Schubart, submitted). In contrast, the freshwater crabs have a direct (Pseudothelphusidae) or an abbreviated larval development (Sesarmidae) (Hartnoll 1964; Chace and Hobbs 1969; Schubart et al. 2000; Anger and Schubart 2005; González-Gordillo et al. 2010). This allows for independent evolution and radiations on the different islands. The question remains, why on Jamaica such a high diversity of freshwater and terrestrial crabs evolved in a much shorter time period (4.5 my, according to Schubart et al. 1998a) compared to the crab fauna of the other Greater Antilles, which had a ten-fold longer time for diversification. This question will be addressed by comparative population genetic analyses. Do species of *Sesarma* and *Epilobocera* differ in their intraspecific diversification? Results for three Jamaican species will be presented here, while diversification of freshwater crabs from the other Greater Antilles is dealt with elsewhere (Rivera and Schubart, submitted; Schubart et al., submitted) and summarized later in this chapter.

3.1 Material and Methods

For three endemic freshwater species of *Sesarma* from Jamaica, specimens were collected from all rivers where the crabs are known to occur (Fig. 1). *Sesarma dolphinum* Reimer et al. 1998 is present in rivers from westernmost Jamaica (parishes of Hanover and Westmoreland). For this species, samples were obtained from 13 localities corresponding to nine independent river systems. The Cabarita

River is the largest river in the area with long tributaries in different elevated areas, but unsuitable habitat in the southern lowland where the tributaries merge. This species was selected to quantify genetic diversity and geographic heterogeneity in a relatively small area with many rivers that are known to be unconnected. This is not the case for *Sesarma windsor* Türkay and Diesel 1994 and *Sesarma meridies* Schubart and Koller 2005, which occur in rivers from the eastern Cockpit Country (parishes of Trelawny and Clarendon), with rivers draining to the north harboring *S. windsor*, while rivers draining to the south are inhabited by *S. meridies*. The latter species also occurs further to the east in the Rio Magno (St. Catherine Parish), a tributary of the Rio Cobre system, otherwise inhabited by *Sesarma bidentatum* Benedict 1892. *Sesarma meridies* is the most recently described species of Jamaican freshwater crab and currently also the youngest phylogenetic split (Schubart, unpublished). Its genetic and morphological distinction from *S. windsor* has been highlighted by Schubart and Koller (2005) with the help of DNA from two mitochondrial genes. However, it was not tested with nuclear markers whether these two closely related species may show signs of hybridization.

Between 1995 and 2005, more than 250 individuals from 20 different sampling sites were collected for this study. Whenever possible, a minimum of 10 individuals were collected per locality. Animals were killed on ice and transferred to 95% ethanol. DNA was extracted as described above (see 2.1). In this case, the complete nuclear ITS1-5.8SrRNA-ITS2 gene complex (ITS1-2) was amplified in order to compare the variable DNA of the non-coding internally transcribed spacers (ITS). The primers ITS-5 (here ITSL1) (5′-GGA AGT AAA AGT CGT AAC AAG G-3′, White et al., 1990); ITSL1b (5′-GTC GTA ACA AGG TTT CCG TAG-3′, new), ITSH1 (5′-TTC AGT CGC CCT TAC TAA GGG AAT CC-3′; new), and ITSH1b (5′-CCA GTT CAG TCG CCC TTA CT-3′, new) nested within 18SrRNA (ITSL1 and L1b) and 28SrRNA (ITSH1 and H1b) were used. In addition, internal primers ITSL2 (5′-AAG AAT ACC AGA TAC ATC GAC AA-3′; new) and ITSH4 (5′- TTG TCG ATG TAT CTG GTA TTC TT-3′; new), located within the 5.8S rRNA, were occasionally applied. The resulting amplified fragments had a length between 1,200 and 1,400 base pairs (bp), due to several microsatellite repeat motives varying in length (see Harris and Crandall 2000). ITS1-2 clones were also obtained from single individuals of the mountain stream crab from the western and central Cockpit Country, *Sesarma fossarum* Schubart et al. 1997 and from the cave crab *S. verleyi* to be used as outgroups. Sequences of the different clones have been deposited in the EMBL database (FN395306-FN395315, FN395609-FN396100).

For PCR, standard 25-µl reactions were used including 2.5 µl of 10× buffer, 2.5 µl of 1.25 mM dNTPs, 0.5 µl of both primers (20 µM), 2 µl of 25 mM $MgCl_2$, 1 µl of 0.5 U/µl TAQ and 15 µl of double distilled water. The reaction ran for 40 cycles with the program described in sect. 2.1.2, but 90 s elongation time and an annealing temperature of 50°C. Positive amplifications were prepared for cloning by treatment with an A-Addition kit from Invitrogen (Carlsbad CA) to add polyA ends. Cloning was performed using the TOPO-TA cloning kit from Invitrogen. Colonies which had successfully included the vector with the PCR insert were picked and transferred to 50 µl of ddH_2O. This solution was denatured for 10 min at

96°C, and 1 µl was used for a PCR with 35 cycles and 55°C as annealing temperature to control for cloning of the correct fragment. This PCR product was cleaned with PCR Cleanup Millipore plates (Millipore, Billerica, MA) and then cycle-sequenced in both directions using standard protocols. Sequences were read with an automated ABI 3730 sequencer.

Sequence chromatograms were edited and proofread for possible errors with Chromas Lite http://www.technelysium.com.au/chromas_lite.html. An alignment was created with the ClustalW plugin of BioEdit (Hall 1999). This alignment was manually checked and modified, because microsatellite regions were not always correctly treated by the automated alignment. For further analysis, all alignment files were converted to the nexus file format. An important part of the information of ITS sequences is based on the elevated number of indel events, which should be considered phylogenetic signal according to Simmons and Ochoterena (2000). The indels are often due to microsatellite repetitive elements of varying lengths and point mutations in the non-coding ITS1 and ITS2 region, whereas the 5.8 rRNA region remained practically unmodified. To render the indels useful for all tree search methods, the simple indel coding method (Simmons et al. 2001) was used, which was calculated with the program GapCoder (Young and Healy 2003). Two datasets were maintained separately in order to address two slightly different questions. The dataset of all ITS1-2 clones from *S. dolphinum* was used to measure nuclear genetic intraspecific diversification in a species occurring in many unconnected rivers, but in a relatively small area. In contrast, the dataset including all ITS1-2 clones of *S. windsor* and *S. meridies* was used to test for possible hybridization in two closely related and morphologically similar species, which occur in rivers separated by few kilometers and which partly seep into the underground, with unknown subterranean connections. Accordingly, these datasets were analyzed separately using a network method for the preprocessed data obtained with GapCoder. The great amount of variation within the ITS dataset did not allow use of the statistical parsimony algorithm of the TCS software. Instead, the software Splitstree version 4 (Huson and Bryant 2006) was used for construction of the networks. Pairwise F_{ST} values among populations were calculated with Arlequin version 3.1 (Excoffier et al. 2005). For an overall phylogenetic interpretation of the data, all the ITS1-2 clones were finally combined. In this case, a Bayesian radiation tree was constructed with Mr. Bayes (Huelsenbeck & Ronquist 2001) and confidence levels determined after running one million generations with four chains and a sample frequency of 100.

3.2 Results

3.2.1 *Sesarma dolphinum*

232 clones from 32 individuals of *Sesarma dolphinum* were sequenced including the entire ITS1-2 region. Up to 15 successfully sequenced clones were included in this analysis, with an ITS sequence length of between 1,191 and 1,244 bp (after excluding flanking rDNA regions). This places this species in an intermediate

position considering the length of ITS sequences found in decapod Crustacea (Harris and Crandall 2000). Two of the clones had large inserts with over 300 bp and were excluded from the analysis. After the conversion of the alignment data with GapCoder, we obtained an alignment of 1,408 sites (415 variable/173 parsimony-informative including flanking regions) and 223 haplotypes. Only ten alleles occurred more than once and no allele more than twice. The corresponding minimum spanning network is presented in Fig. 4. Most prominent is the separate non-overlapping position of clones from individuals of the Deans Valley River and the Roaring River, both being southeastern populations of *S. dolphinum*. Next closest, and connecting to the other populations, are clones from the Flint River and the upper Cabarita River from the northeastern range of *S. dolphinum*. At the other extreme of the network are the clones from the New Savannah River from the southwestern range of the species, which also cluster closely together. Close and partly overlapping with New Savannah River are the clones from the Green Island, Morgan, Davis, and Lances Rivers, and both Lucea Rivers. These are the northwestern populations of *S. dolphinum*. A marked intraspecific geographic structure can thus be recognized by this network. This is confirmed by the analysis of molecular variance, which resulted in significant differences between all river systems except for the neighboring Green Island and Morgan Rivers (Table 3)

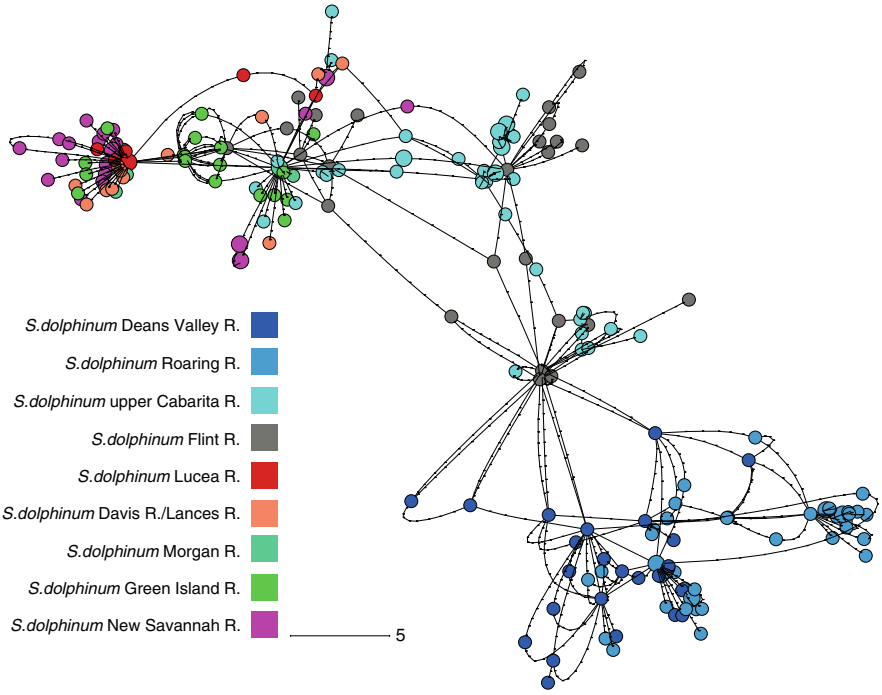


Fig. 4 Network constructed with Splitstree representing complete ITS1-5.8S-ITS2 alleles from different populations of *Sesarma dolphinum*

Table 3 Pairwise F_{ST} values among populations and analysis of molecular variance (AMOVA) based on nuclear sequences of the ITS1-5.8S-ITS2 complex as calculated with Arlequin version 3.1 (Excoffier et al. 2005). Upper matrix: *Sesarma dolphinum*; lower matrix: *S. meridies* and *S. windsor*. *n* clones, *k* individuals

	Flint R. ($n = 24$)	Lucea R. ($n = 4$)	Davis R. ($n = 14$)	Morgan R. ($n = 5$)	Green Island R. ($n = 29$)	New Savannah R. ($n = 17$)	Deans Valley R. ($n = 29$)	Roaring R. ($n = 44$)	upper Cabarita R. ($n = 48$)
Flint R. ($k = 3$)		0.37514	0.42624	0.36819	0.31784	0.21282	0.4424	0.32427	0.04277
Lucea R. ($k = 2$)	$p < 0.0001$		0.07404	0.11646	0.01120	0.20766	0.62805	0.52456	0.363
Davis R. ($k = 2$)	$p < 0.0001$	$p < 0.05$		0.11129	0.0792	0.18119	0.63125	0.50048	0.40927
Morgan R. ($k = 2$)	$p < 0.0001$	$p < 0.05$	$p < 0.01$		0.05366	0.11531	0.58909	0.46086	0.35564
Green Island R. ($k = 4$)	$p < 0.0001$	$p < 0.05$	$p < 0.01$	$p = 0.05581$		0.13191	0.55874	0.46254	0.3258
New Savannah R. ($k = 4$)	$p < 0.0001$	$p < 0.0001$	$p < 0.01$	$p < 0.01$	$p < 0.0001$		0.42403	0.33825	0.23344
Deans Valley R. ($k = 5$)	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$		0.17929	0.40655
Roaring R. ($k = 6$)	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.01$		0.30156
Upper Cabarita R. ($k = 5$)	$p < 0.05$	$p < 0.0001$	$p < 0.0001$	$p < 0.01$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	

	S.meridies Rio Minho ($n = 28$)	*S.meridies* Pedro R. ($n = 19$)	*S.meridies* Magno R. ($n = 11$)	*S.windsor* Cave R. ($n = 42$)	*S.windsor* Hectors R. ($n = 43$)	*S.windsor Quahies R.* ($n = 52$)	*S.windsor* Mouth R. ($n = 51$)
S.meridies Rio Minho ($k = 6$)		0.19341	0.53127	0.80596	0.8315	0.77479	0.70757
S.meridies Pedro R. ($k = 3$)	$p < 0.0001$		0.48769	0.80741	0.83586	0.75119	0.70184
S.meridies Magno R. ($k = 3$)	$p < 0.0001$	$p < 0.0001$		0.80419	0.83458	0.74416	0.69193
S.windsor Cave R. ($k = 8$)	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$		0.15558	0.31952	0.27955
S.windsor Hectors R. ($k = 5$)	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$		0.26282	0.2535
S.windsor Quahies R. ($k = 5$)	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$		0.15865
S.windsor Mouth R. ($k = 4$)	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	

The pairwise F_{ST} reflect the degree of differentiation between the different populations and are in agreement with the network representation.

3.2.2 *Sesarma windsor* and *Sesarma meridies*

The ITS1-2 complex was also used to depict the genetic population structure of *Sesarma windsor* and *Sesarma meridies* from central Jamaica. Overall, 260 clones from 31 individuals were obtained. The length of the ITS gene in these species varied between 1,196 and 1,226 bp (after exclusion of flanking regions of rDNA). After gap-coding, the length of the aligned data was 1,427 sites (314 variable/142 parsimony-informative, including flanking regions) and revealed 23 haplotypes. Most haplotypes occurred only once and the most common haplotype was found 11 times. The minimum spanning network shown in Fig. 5 and the distance tree in Fig. 6 show that the two species are well separated into two well-delimited clouds which are connected by a simple long line of missing genotypes. This confirms species status for both species and refutes the possibility of hybridization, due to close ranges and possible subterranean contact. In both species, no division into the different river systems is readily recognizable, but certain parts of the network are dominated by specific river systems or sets of rivers. This is also true for the clones from the Rio Magno, a locality which is geographically most distant from all the other sampling points. AMOVA, in contrast, confirms significant genetic differentiation of all analyzed river systems (Table 3), confirming the high degree of intraspecific differentiation among Jamaican river crabs.

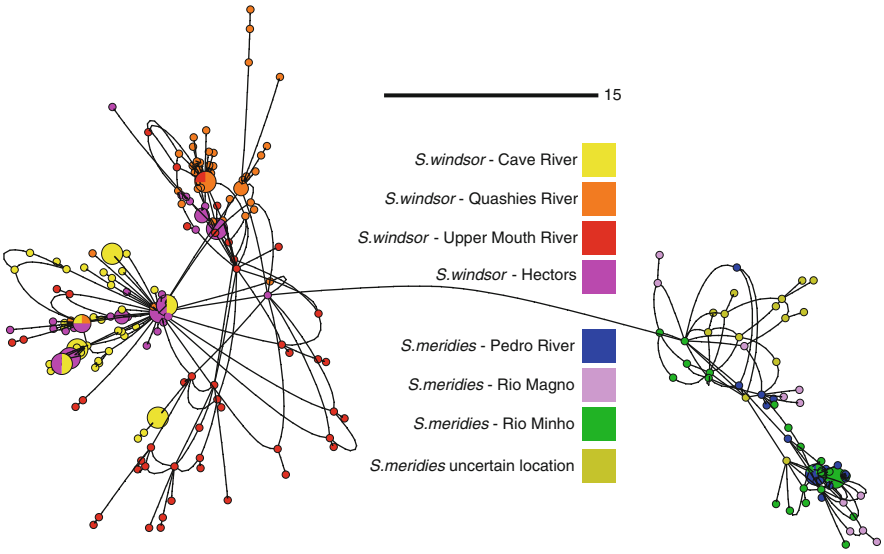


Fig. 5 Network constructed with Splitstree representing complete ITS1-5.8S-ITS2 alleles from different populations of *Sesarma windsor* and *S. meridies*

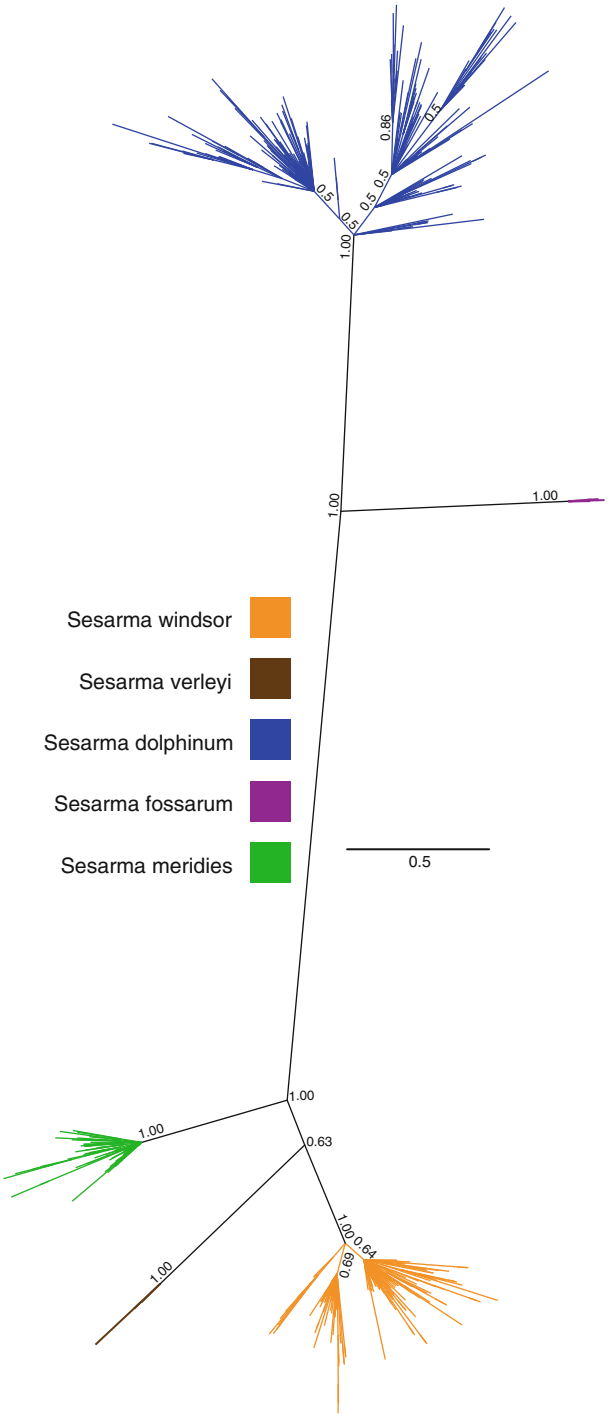


Fig. 6 Bayesian radiation tree constructed with MrBayes (one million generations) depicting phylogenetic relationships among ITS1–5.8S-ITS2 alleles of selected Jamaican endemic Sesarmidae

3.3 Discussion

Most phylogeographic and many population genetic studies of animals are carried out with mitochondrial (mt) sequences, especially of the cytochrome oxidase subunit 1 or the mt control region. This is primarily due to its overall higher mutation rate compared to the nuclear DNA. MtDNA is relatively stable and occurs in numerous copies per cell. This, and the fact that many universal primers are available for a number of genes, makes mtDNA relatively easy to amplify for a wide range of organisms. Due to its uni-parental inheritance (normally from the maternal side) and its consequent single-copied occurrence in most mitochondria, mtDNA is furthermore relatively easy to interpret. However, the discovery of selective sweeps, mitochondrial introgressions and nuclear copies of mtDNA (numts) have sounded a note of caution when interpreting phylogeographic or population genetic results exclusively based on mtDNA. The need for complimentary data from the nuclear genome is thus more urgent than ever. A number of molecular techniques are available that use information from the nuclear genome to distinguish between individuals or populations from within a species, like microsatellites, AFLPs, etc. Establishing microsatellite libraries is costly and sometimes tricky, the corresponding primers only being useful for a narrow range of species. Furthermore, the information from microsatellites as well as AFLPs is indirect, based on length differences or specific patterns of DNA fragmentation. Using the full sequence information in its original composition is still only possible by sequencing DNA. However, very few nuclear DNA regions are variable enough, but still universally amplifiable, to be used as intraspecific genetic markers. Internally transcribed spacers (ITS) represent an important exception. They are non-coding and therefore allow mutations without alteration of their function as buffers between the ribosomal genes 18S rRNA, 5.8S rRNA, and 28 rRNA. These neighboring genes, on the other hand, provide highly conserved flanking regions in which suitable primers can be easily designed. The fact that some primers, which are commonly used for amplifying animal ITS, were originally designed for fungi is good evidence for the universality of the flanking regions. Two ITS genes are always found in the complex ITS1–5.8rRNA-ITS2, but the length of ITS1 and ITS2 varies tremendously among major taxa and to a lesser extent among species, individuals, or even copies within individuals. The main reason why ITS is not more frequently applied for population studies is probably precisely the fact that several copies of different lengths can be present within individuals, which results in overlapping signals when directly sequencing PCR products. Harris and Crandall (2000) found several short repetitive elements in the genome of the crayfish genera *Orconectes* and *Procambarus*, which were considered microsatellites according to their structure. This was also the case in both ITS genes of the Jamaican crabs studied here, as well as freshwater crabs of the family Pseudothelphusidae (see Schubart et al., submitted) and the family Parathelphusidae (see Kolbinger, Koller and Schubart, unpublished). To obtain clean sequences of the different alleles from one individual, PCR products need to be cloned prior to sequencing. When this was

done in different insect (Culicidae by Wesson et al. 1992, *Cicindela dorsalis* by Vogler and DeSalle 1994, Coccinelidae by Schulenburg et al. 2001) or crustacean (cambarid crayfish by Harris and Crandall 2000, sesarmid and pseudothelphusid freshwater crabs in current study and Schubart et al., submitted) taxa, it turned out that more than two (from each parent) alleles were always present. It is known that the eukaryotic ribosomal DNA (rDNA) array typically consists of several hundred tandemly repeated copies of the transcription unit, encoding 18S-ITS1-5.8S-ITS2-28SrRNA. However, these copies are believed to be genetically homogenized by concerted evolution (e.g., Hillis et al. 1991). Williams et al. (1988) considered the possibility that homogenization via concerted evolution may take longer evolutionary times than commonly believed. Vogler and DeSalle (1994) found large intra-individual variation of ITS1-sequences in the tiger beetle *Cicindela dorsalis*, obscuring the phylogeographic signal within the Gulf of Mexico and along the Atlantic coast, and concluded that there is a lack of an efficient homogenization system. Also, the data by Harris and Crandall (2000) and the present study argue against at least rapidly acting concerted evolution in decapod crustaceans. While our ITS phylogeny allowed us to distinguish two closely related species, *Sesarma windsor* and *S. meridies*, its use to reveal phylogeographic structure is limited due to the fact that intra-individual variation was in part large enough to include sequences from the entire spectrum of variability within the species. We therefore agree with previous authors that the use of ITS for phylogeography and population genetics is diminished if multiple copies per individual are present and rDNA is not consistently homogenized by concerted evolution.

4 Additional Findings and Outlook

A relatively young adaptive radiation of land-dwelling crabs on a small tropical island offers many opportunities to study mechanisms and consequences of such a radiation. Not all the results can be presented within the frame of this book chapter; however, a few of them will be summarized here in order to show the progress after 1998, when Schubart et al. (1998a) provided final evidence that the Jamaican crab diversity is indeed the consequence of adaptive radiation.

Between 1994 and 2005, five new species of Jamaican freshwater crabs have been described based on morphology and genetics. Previously, they were all thought to belong to the then one described species of freshwater crab from Jamaican mountain streams, *Sesarma bidentatum*. The six species of freshwater crabs now recognized are allopatric and have non-overlapping distributional ranges that are arranged from west to east of the island as follows: *S. dolphinum* Reimer et al. 1998; *S. fossarum* Schubart et al. 1997; *S. windsor* Türkay and Diesel 1994; *S. meridies* Schubart and Koller 2005; *S. bidentatum* Benedict 1891; *S. ayatum* Schubart et al. 1998b. At first sight, these six species are of similar morphologies, but the specialist's eye will rapidly recognize important differences, like the aforementioned body shape of *Sesarma windsor* populations from caves, different

degrees of pubescence on the walking legs, different tuberculation patterns on the claws, and also differently pronounced sexual dimorphism in some of the species, indicating different behaviors during courtship and/or male–male interactions. For example, the realization that crab females from the Rio Magno have clearly smaller chelae than males was the reason that they could immediately be identified as *S. meridies*, despite being found in a tributary of the Rio Cobre, which is otherwise inhabited by *S. bidentatum,* due to the fact that the latter species, and *S. ayatum*, have no or very faint sexual dimorphism. This could later be confirmed by other morphological and molecular results and currently constitutes the only example for two species being found in tributaries from the same larger river system (see Fig. 1), but without evidence for mixing as further confirmed in this study with the results from nuclear DNA of the Rio Magno population of *S. meridies*. Interestingly, *S. meridies* is still found in a western tributary and *S. bidentatum* in the eastern one, thus following the overall geographic trend of the species and allowing us to draw a distribution limit line between them. The lower Rio Cobre is a lowland river without much structure consisting of fast flowing and muddy waters during the rainy season. It is thus not suitable for mountain crabs, preventing a mixture of adults from both species in this part of the connected water system. However, it must be assumed that early ontogenetic stages of the crabs, which have no appendages as holdfasts (zoea larvae) or are too small and weak to withstand currents (juveniles), are regularly washed down into the main river and some of them later find their way back into the more suitable parts of the river system. Currently, it remains unclear how regularly this happens and whethter the clean separation of these two species is maintained by directed migration (smell of familiar tributaries) or by exclusive competition.

A similar case, but at the intraspecific level, could be demonstrated with the data presented here on population differentiation within *Sesarma dolphinum*. The Cabarita River system harbors crab populations in several of its tributaries of which four were sampled for this study (Fig. 1, Table 3). Two of these populations from the upper Cabarita River were genetically identical and closely related to the Flint River, which drains to the north but has its headwaters in immediate vicinity of the ones of the Cabarita River draining south from there. Another population from the Cabarita drainage is the one from the upper Morgan River, a tributary gathering its waters in the northwest area of the distribution of *S. dolphinum*. Interestingly, this population could not be statistically separated from the nearby Green Island River draining to the northeast (Table 3), but was clearly distinct from the other three populations from the Cabarita system. Finally, the population from the Roaring River in the southeast of the distributional range of *S. dolphinum*, and joining the Cabarita River in the southern plains, can also be genetically separated from the other populations of the Cabarita River and is instead more closely related to the nearby population from Deans Valley River with independent drainage into the ocean.

All these findings provide clear evidence that these crabs are more likely to migrate across the headwaters than in the large beds of the lowland rivers, where the corresponding tributaries merge. At the same time, it gives evidence for the clear geographic structure which is already recognizable at the intraspecific level and

may explain the high diversification potential of the Jamaican crabs as one factor leading to the present species richness. Parallel to these studies, we are and have been carrying out phylogeographic and population genetic studies on diversification of the freshwater crabs from the other Greater Antilles. Thereby, it becomes evident that their intraspecific diversification potential is indeed reduced in comparison to the Jamaican crabs, despite being the much older lineage of freshwater crabs and probably having had much more time on the respective islands to evolve regional endemisms. Cook et al. (2008) studied phylogeography of the freshwater crab *Epilobocera sinuatifrons* (A. Milne-Edwards, 1866) from Puerto Rico with the mitochondrial Cox1 gene and found very limited differentiation for a freshwater-dwelling crab. This could be confirmed by current studies of Santl (2009) and Schubart et al. (submitted) using the more variable NDH1 mitochondrial DNA and ITS1-2 sequences in a similar pattern as shown in the current study. In this species, there is a gradient from western to eastern Puerto Rico, and populations from the extremes are significantly different from each other, but there is a clear isolation by recognizable distance pattern, when including intermediate populations. Rivera and Schubart (submitted) studied population genetics of the freshwater crab *Epilobocera haytensis* Rathbun, 1893 from Hispaniola with similar conclusions as for *E. sinuatifrons*. On one hand, there is significant genetic structure in 20 different rivers of the island and a clear west–east differentiation related to the Cordillera Central. However, this differentiation is lower than expected for such a large island and also shows isolation by distance patterns. Only a population of the Río Ocoa appears to have been isolated for a longer time, without deserving distinct species status. The number of species currently recognized for Cuba (Capolongo and Pretzmann 2002; Ng et al. 2008) is probably overestimated. *E. armata* is not a valid species and the genetic differences between some of the other species are not large enough to justify species status (Schubart et al., in progress). These findings suggest that crabs of the genus *Epilobocera* must maintain relatively constant gene flow between rivers, which is counteracting the regional differentiation, if freshwater crabs live in isolated river systems over extended periods (see, for example, Shih et al 2006). This gene flow is probably achieved by regular overland migrations, and this has indeed been observed repeatedly and recently documented by us with trapping experiments (Rivera and Schubart, unpublished).

The intra- and interspecific diversification of Jamaican freshwater crabs described here is the same as that found in many other marine, limnic, and terrestrial organisms, and follows the typical patterns known from "standard" allopatric differentiation and speciation: i.e., lack of gene flow followed by local specialization and genetic differentiation in the separated populations due to genetic drift, resulting in long-term reproductive isolation without necessary ecological specialization. The description of the five new species of freshwater crabs is thus the first instance in which the adaptive radiation of Jamaican crabs is documented as being accompanied or driven by "simple" allopatric speciation. It also demonstrates how difficult it is to separate the non-adaptive from adaptive radiations. A closer examination of some of the river species will show early adaptations to a new environment, as, for example, in the cave-living populations of *Sesarma*

windsor (see above). Also, in the textbook examples of adaptive radiation, as in Darwin's finches and other organisms known to have evolved through adaptive radiations, allopatric separation in combination with the subsequent adaptations often plays a fundamental role as can be seen from the fact that many of the examples are from organisms living on nearby islands, where gene flow is highly reduced and organisms can in part specialize due to reduced homogenizing gene flow. Sympatric speciation can take place during adaptive radiations, but it is definitely not a condition for defining adaptive radiations.

The exact processes of speciation may be better understood if all the speciation events are clearly documented in a phylogenetic tree. However, as mentioned above, adaptive radiations are normally very fast processes, and different diversification processes (geographic and ecological) may take place within a single species at the same time leading to non-dichotomous net-like reticulate phylogenetic relationships. The phylogeny of the Jamaican crabs published in Schubart et al. (1998a) suggests such a fast radiation with a long branch and high bootstrap support leading to the monophyletic clade of all Jamaican endemic species, but short shared branches and low confidence values within the clade of Jamaican endemics. Currently, we are in the process of improving this phylogeny by adding sequences of more genes to this phylogenetic reconstruction. The most recent preliminary phylogeny based on more than 4,300 bp (12S, 16S, and 28S rRNA, 1,200 bp Cox1, and ITS1–5.8S-ITS2) strengthens the insight from earlier trees that neither the similar looking crabs from mountain streams nor the more specialized terrestrial forms are monophyletic, but that their diversification follows a strong geographic pattern (Schubart, in progress). That means that the allopatric differentiation in fresh waters played an important role from early on, but was accompanied by the evolution of ecologically more specialized forms that evolved regionally from the corresponding river species ancestor. For example, the rock rubble crab from the easternmost John Crow Mountains, *Sesarma cookei* Hartnoll 1971, is most closely related to a clade formed by the river crabs *S. bidentatum* (today mostly Blue Mountains) and *S. ayatum* (today exclusively John Crow Mountains) and not to the similar looking rock rubble crabs from central (*S. jarvisi* Rathbun 1914) and western (*Sesarma* sp.n.) Jamaica. Morphological convergence is thus another issue that needs to be taken into account when studying radiations and may be a factor in maintaining similar morphologies in the mountain stream species, despite their early divergence in the overall phylogenetic tree.

New endemic crab species have been regularly added to the list of the Jamaican fauna during the last years, and the question arises whether we have seen the end of it. Compared to the diversity of "real" terrestrial organisms like snails (Goodfriend 1986; Rosenberg and Muratov 2006), amphibians (Hedges 1989), and reptiles (Hedges and Burnell 1990), the Jamaican crabs are not an exceptionally species-rich group. However, for a recent marine invader, they probably are. So far, all new Jamaican species have been described using morphological methods, sometimes aided by molecular results (e.g., Schubart & Koller 1998b; Schubart and Koller 2005). However, in the future, it will become more and more common practice to recognize species solely based on their genetic differentiation. This way, Bond and

Sierwald (2002) revealed cryptic species among Jamaican millipedes. For the Jamaican mountain stream crabs, most rivers have been sampled, and intraspecific diversity is being described for the six currently recognized species. Reimer et al. (1998), when describing *S. dolphinum* as new, noticed that animals from Galloway (Deans Valley River) had a slightly different morphology and considered the possibility of placing them into a separate subspecies. Our molecular results presented here, and additional ones based on mtDNA by Santl (2009), confirm the distinctness of the animals from Deans Valley River. Compared to the differences that we are finding between already described species (e.g., *S. meridies* and *S. windsor*), we do recognize a lower level of differentiation. However, the populations from the Deans Valley-Roaring River represent an evolutionary significant unit which is geographically, morphologically, and genetically separable from other units within *S. dolphinum*. Therefore a subspecies status may indeed be justifiable. Also, among the more specialized Jamaican species, population genetics are still revealing the possible existence of undescribed species. This is the case in the bromeliad crab *Metopaulias depressus* as indicated by Heine (2006) and Rivera (2007) and currently being investigated with nuclear markers. Even more pronounced species-level distinction is discernible in the snail shell crab *Sesarma jarvisi*, for which a new species is currently being described based on mitochondrial and nuclear markers as well as on morphology (Schubart, in preparation). This will add to the spectacular ecological and biological diversity of Jamaican crabs, which have descended from a single lineage of marine ancestor approximately 4.5 million years ago and will not stop in their divergence and long-term species formation, if human environmental destruction, as currently seen in Jamaican forests, does not destroy part of the diversity before it can even be described and appreciated.

Acknowledgements The first author is especially grateful to his former advisor Rudi Diesel who introduced him to the world of the Jamaican crabs and contributed decisively to this manuscript with many early discussions and collecting trips. We would also like to thank all the other people who helped collect in the field over the years, among them Jens Reimer, Gernot Bäurle, Martina Schuh, Gary Rosenberg, Rene Brodie, Klaus Anger, Silke Reuschel, and Luise Heine, as well as the Discovery Bay Marine Laboratory and the University of the West Indies for continuing support. Special thanks are also due to the team of the Crandall Laboratory, who supported two of us, while cloning a maximum of ITS alleles in relatively short time intervals and associated support from the US National Science Foundation (EF-0531762). This study was financially supported by research project Schu 1460/3 to CDS within the DFG Priority Program 1127 "Adaptive Radiation-Origin of Biological Diversity".

References

Anger K, Schubart CD (2005) Experimental evidence of food-independent larval development in endemic Jamaican freshwater-breeding crabs. Physiol Biochem Zool 78:246–258

Avise JC (2004) Molecular markers, natural history, and evolution. Sinauer Associates, Sunderland, Massachusetts

Benedict JE (2004) Decapod Crustacea of Kingston Harbour. Johns Hopkins University Circular 11:77

Bond JE, Sierwald P (2002) Cryptic speciation in the *Anadenobolus excisus* millipede species complex on the island of Jamaica. Evolution 56:1123–1135

Buskirk R (1985) Zoogeographic patterns and tectonic history of Jamaica and the northern Caribbean. J Biogeo 12:445–461

Cabot EL, Beckenbach AT (1989) Simultaneous editing of multiple nucleic acid and protein sequences with ESEE. Comp Appl Biosci 5:233–234

Capolongo D, Pretzmann G (2002) Süßwasserkrabben von Cuba. Agemus Nachrichten 67b:1–4

Chace Jr FA, Hobbs Jr HH (1969) The freshwater and terrestrial decapod crustaceans of the West Indies with special reference to Dominica. In: Bredin-Archbold-Smithsonian Biological Survey of Dominica. Bull US Natl Mus 292:1–258

Clement M, Posada D, Crandall K (2000) TCS: a computer program to estimate gene genealogies. Mol Ecol 9:1657–1660

Cook BD, Pringle CM, Hughes JM (2008) Phylogeography of an island endemic, the Puerto Rican freshwater crab (*Epilobocera sinuatifrons*). J Hered 99:157–164

Crandall KA, Templeton AR (1993) Empirical tests of some predictions from coalescent theory with applications to intraspecific phylogeny reconstruction. Genetics 134:959–969

Darwin C (1845) Journal of researches into the natural history and geology of the countries visited during the voyage of H.M.S. Beagle round the world, under the Command of Capt. Fitz Roy, R.N, 2nd edn. John Murray, London

Darwin C (1859) On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life, 1st edn. John Murray, London

Diesel R (1997) Maternal control of calcium concentration in the larval nursery of the bromeliad crab, *Metopaulias depressus* (Grapsidae). Proc R Soc Lond B 264:1403–1406

Diesel R, Schubart CD (2000) Die außergewöhnliche Evolutionsgeschichte jamaikanischer Felsenkrabben. Biologie in unserer Zeit 30:136–147

Diesel R, Schubart CD (2007) The social breeding system of the Jamaican bromeliad crab *Metopaulias depressus*. In: Duffy JE, Thiel M (eds) Evolutionary ecology of social and sexual systems: Crustaceans as model organisms. Oxford University Press, New York, pp 365–386

Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.1: An integrated software package for population genetics data analysis. Evol Bioinf 1:47–50

González-Gordillo JI, Anger K, Schubart CD (2010) Morphology of the larval and first juvenile stages of two Jamaican endemic crab species with abbreviated development, *Sesarma windsor* and *Metopaulias depressus* (Decapoda: Brachyura: Sesarmidae). J Crust Biol 30:101–121

Goodfriend GA (1986) Radiation of the land snail genus *Sagda* (Pulmonata: Sagdidae): comparative morphology, biogeography and ecology of the species of north-central Jamaica. Zool J Linn Soc 87:367–398

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Harris DJ, Crandall KA (2000) Intragenomic variation within ITS1 and ITS2 of freshwater crayfishes (Decapoda: Cambaridae): implications for phylogenetic and microsatellite studies. Mol Biol Evol 17:284–291

Hartnoll RG (1964) The freshwater grapsid crabs of Jamaica. Proc Linn Soc Lond 175:145–169

Hartnoll RG (1971) *Sesarma cookei* n. sp., a grapsid crab from Jamaica (Decapoda, Brachyura). Crustaceana 20:257–262

Hedges SB (1989) An island radiation: allozyme evolution in Jamaican frogs of the genus *Eleutherodactylus* (Leptodactylidae). Carib J Sci 25:123–147

Hedges SB (2001) Caribbean biogeography: an overview. In: Woods CA, Sergile FE (eds) Biogeography of the West Indies: patterns and perspectives. CRC Press, Boca Raton, Florida

Hedges SB, Burnell KL (1990) The Jamaican radiation of *Anolis* (Sauria: Iguanidae): An analysis of relationships and biogeography using sequential electrophoresis. Carib J Sci 26:31–44

Heine L (2006) Genetische Untersuchung der Sozialstruktur und zur Phylogeographie bei der Bromelienkrabbe Metopaulias depressus (Decapoda: Brachyura). Diploma thesis, Universität Regensburg

Hillis DM, Moritz C, Porter A, Baker RJ (1991) Evidence for biased gene conversion in concerted evolution of ribosomal DNA. Science 251:308–310

Huber SK, de Leon LF, Hendry AP, Bermingham E, Podos J (2007) Reproductive isolation of sympatric morphs in a population of Darwin's finches. Proc R Soc Lond B 274:1709–1714

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754–755

Huson DH, Bryant D (2006) Application of phylogenetic networks in evolutionary studies. Mol Biol Evol 23:254–267

Iturralde MA, MacPhee RDE (1999) Paleogeography of the Caribbean region: implications for Cenozoic biogeography. Bull Am Mus Nat Hist 238:1–95

Marcade I, Klinkicht M, Diesel R (unpublished) Larvae dumping in the bromeliad crab, *Metopaulias depressus* (Decapoda: Grapsidae): Mothers care for unrelated young

Mathews LM, Schubart CD, Neigel JE, Felder DL (2002) Genetic, ecological, and behavioural divergence between two sibling snapping shrimp species (Crustacea: Decapoda: *Alpheus*). Mol Ecol 11:1427–1437

Neigel JE, Avise JC (1986) Phylogenetic relationships of mitochondrial DNA under various models of speciation. In: Nevo E, Karlim S (eds) Evolutionary processes and theory. Academic, New York, pp 515–534

Ng PKL, Guinot D, Davie PJF (2008) Systema Brachyurorum: Part I. An annotated checklist of extant brachyuran crabs of the world Raffles Bull Zool Suppl 17:1–286

Palumbi SR, Martin A, Romano S, McMillan WO, Stice L, Grabowski G (1991) The simple fool's guide to PCR. A collection of PCR protocols, version 2. University of Hawaii, Honolulu

Rathbun MJ (1896) Description of a new genus and four new species of crabs from the West Indies. Proc US Natl Mus 19:141–144

Rathbun MJ (1914) New species of crabs of the families Grapsidae and Ocypodidae. Proc US Natl Mus 47:69–85

Reimer J, Schubart CD, Diesel R (1998) Description of a new freshwater crab of the genus *Sesarma* Say, 1817 (Brachyura: Grapsidae: Sesarminae) from western Jamaica. Crustaceana 71:186–196

Reuschel S, Schubart CD (submitted) Genetic variability in the freshwater shrimp *Xiphocaris elongata* (Crustacea: Caridea) does not reflect morphological or geographical patterns

Rivera NT (2007) Evolution of intraspecific diversity: a comparison of genetic and geographic structure in Epilobocera haytensis and Metopaulias depressus (Crustacea: Decapoda: Brachyura). Diploma thesis, Universität Regensburg

Rivera NT, Schubart CD (submitted) Phylogeography of the freshwater crab *Epilobocera haytensis* (Brachyura: Pseudothelphusidae) from Hispaniola reveals limited gene flow among different river systems

Rodríguez G, Williams AB (1995) *Epilobocera wetherbeei*, a new species of freshwater crab (Decapoda: Brachyura: Pseudothelphusidae) from Hispaniola. Proc Biol Soc Wash 108:76–83

Rosenberg G, Muratov IV (2006) Status report on the terrestrial Mollusca of Jamaica. Proc Acad Nat Sci Philadelphia 155:117–161

Santl T (2009) Comparative diversification potential of an old and a young lineage of freshwater crabs on two Caribbean islands explained at the population level. PhD Dissertation, Universität Regensburg

Schluter D (2000) The ecology of adaptive radiations. Oxford University Press, Oxford

Schubart CD (2009) Mitochondrial DNA and decapod phylogenies; the importance of pseudogenes and primer optimization. In: Martin JW, Crandall KA, Felder DL (eds) Crustacean Issues 18: Decapod Crustacean Phylogenetics. Taylor & Francis/CRC Press, Boca Raton, Florida, pp 47–65

Schubart CD, Cuesta JA, Diesel R, Felder DL (2000) Molecular phylogeny, taxonomy, and evolution of nonmarine lineages within the American grapsoid crabs (Crustacea: Brachyura). Mol Phylogenet Evol 15:179–190

Schubart CD, Diesel R (1999) Osmoregulation and the transition from marine to freshwater and terrestrial life: a comparative study of Jamaican crabs of the genus *Sesarma*. Arch Hydrobiol 145:331–347

Schubart CD, Diesel R, Hedges SB (1998a) Rapid evolution to terrestrial life in Jamaican crabs. Nature 393:363–365

Schubart CD, Huber MGJ (2006) Genetic comparisons of German populations of the stone crayfish, *Austropotamobius torrentium* (Crustacea: Astacidae). Bull Franç Pêche Piscic 380–381:1019–1028

Schubart CD, Koller P (2005) Genetic diversity of freshwater crabs (Brachyura: Sesarmidae) from central Jamaica with description of a new species. J Nat Hist 39:469–481

Schubart CD, Reimer J, Diesel R (1998b) Morphological and molecular evidence for a new endemic freshwater crab, *Sesarma ayatum* sp. n., (Grapsidae, Sesarminae) from eastern Jamaica. Zool Scr 27:373–380

Schubart CD, Reimer J, Diesel R, Türkay M (1997) Taxonomy and ecology of two endemic freshwater crabs from western Jamaica with the description of a new *Sesarma* species (Brachyura: Grapsidae: Sesarminae). J Nat Hist 31:403–419

Schubart CD, Rivera NT, Crandall KA, Santl T (submitted) Comparing phylogeographic structure of freshwater crabs from two Caribbean islands: Puerto Rico versus Jamaica. In: Held C, Koenemann S, Schubart CD (eds) Crustacean Issues 19: Phylogeography and population genetics in Crustacea. Taylor & Francis/CRC Press, Boca Raton, Florida

Schulenburg JHGvd, Hancock JM, Pagnamenta A, Sloggett JJ, Majerus MEN, Hurst GDD (2001) Extreme length and length variation in the first ribosomal internal transcribed spacer of ladybird beetles (Coleoptera: Coccinellidae). Mol Biol Evol 18:648–660

Shih H-T, Hung HC, Schubart CD, Chen CA, Chang H-W (2006) Intraspecific diversity of the endemic freshwater crab *Candidiopotamon rathbunae* (Crustacea: Decapoda, Brachyura, Potamidae) reflects five million years of geological history of Taiwan. J Biogeo 33:980–989

Simmons MP, Ochoterena H (2000) Gaps are characters in sequenced-based phylogenetic analyses. Syst Biol 49:369–381

Simmons MP, Ochoterena H, Carr TG (2001) Incorporation, relative homoplasy, and effect of gap characters in sequence-based phylogenetic analysis. Syst Biol 50:454–462

Sulloway FJ (1982) Darwin and his finches: the evolution of a legend. J Hist Biol 15:1–53

Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. Genetics 132:619–633

Türkay M, Diesel R (1994) Description of a new species of *Sesarma* from Jamaica with notes on its occurrence and biology (Crustacea: Decapoda: Brachyura). Senckenberg Biol 74:157–161

Vogler AP, DeSalle R (1994) Evolution and phylogenetic information content of the ITS-1 region in the tiger beetle *Cicindela dorsalis*. Mol Biol Evol 11:393–405

Wesson DM, Porter CH, Collins FH (1992) Sequence and secondary structure comparisons of ITS rDNA in mosquitoes (Diptera: Culicidae). Mol Phylogenet Evol 1:253–269

White TJ, Bruns T, Lee SW, Taylor G (1990) In: White TJ, Innis M, Gelfand DH, Sninsky JJ, Innis MA (Eds) PCR protocols: A guide to methods and applications. Academic, San Diego, 1990, pp 315–324

Williams SC, DeBry RW, Feder JL (1988) A commentary on the use of ribosomal DNA in systematic studies. Syst Zool 37:60–62

Young ND, Healy J (2003) GapCoder automates the use of indel characters in phylogenetic analysis. BMC Bioinformatics 4:1–6

The Herring Gull Complex (*Larus argentatus - fuscus - cachinnans*) as a Model Group for Recent Holarctic Vertebrate Radiations

Dorit Liebers-Helbig, Viviane Sternkopf, Andreas J. Helbig†, and Peter de Knijff

Abstract Under what circumstances speciation in sexually reproducing animals can occur without geographical disjunction is still controversial. According to the ring species model, a reproductive barrier may arise through "isolation-by-distance" when peripheral populations of a species meet after expanding around some uninhabitable barrier. The classical example for this kind of speciation is the herring gull (*Larus argentatus*) complex with a circumpolar distribution in the northern hemisphere. An analysis of mitochondrial DNA variation among 21 gull taxa indicated that members of this complex differentiated largely in allopatry following multiple vicariance and long-distance colonization events, not primarily through "isolation-by-distance".

In a recent approach, we applied nuclear intron sequences and AFLP markers to be compared with the mitochondrial phylogeography. These markers served to reconstruct the overall phylogeny of the genus *Larus* and to test for the apparent biphyletic origin of two species (*argentatus, hyperboreus*) as well as the unexpected position of *L. marinus* within this complex. All three taxa are members of the herring gull radiation but experienced, to a different degree, extensive mitochondrial introgression through hybridization. The discrepancies between the mitochondrial gene tree and the taxon phylogeny based on nuclear markers are illustrated.

1 Introduction

Ernst Mayr (1942), based on earlier ideas of Stegmann (1934) and Geyr (1938), proposed that reproductive isolation may evolve in a single species through

D. Liebers-Helbig (✉) and V. Sternkopf
Deutsches Meeresmuseum, Katharinenberg 14-20, 18439 Stralsund, Germany
e-mail: dorit.liebers@meeresmuseum.de

P. de Knijff
Department of Human Genetics, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands
e-mail: knijff@lumc.nl

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_17,

"isolation-by-distance" when peripheral populations meet after expanding around a large, uninhabitable area. This mode of speciation through "circular overlap" (Mayr 1942) was later termed the "ring species" model (Cain 1954). Geographic overlap between taxa that are elsewhere connected through interbreeding populations is an essential element of this model, because it is ongoing gene flow that distinguishes ring species from cases of allopatric speciation that happen to be arranged in a roughly circular fashion (Irwin et al. 2001a).

The classical example of the ring species model was originally based upon the herring gull complex (Mayr 1942). This group comprises more than 20 taxa of large gulls (Haffer 1982) which together occupy a circumpolar breeding range in the northern hemisphere. Phenotypically, taxa differ most obviously in body size and in

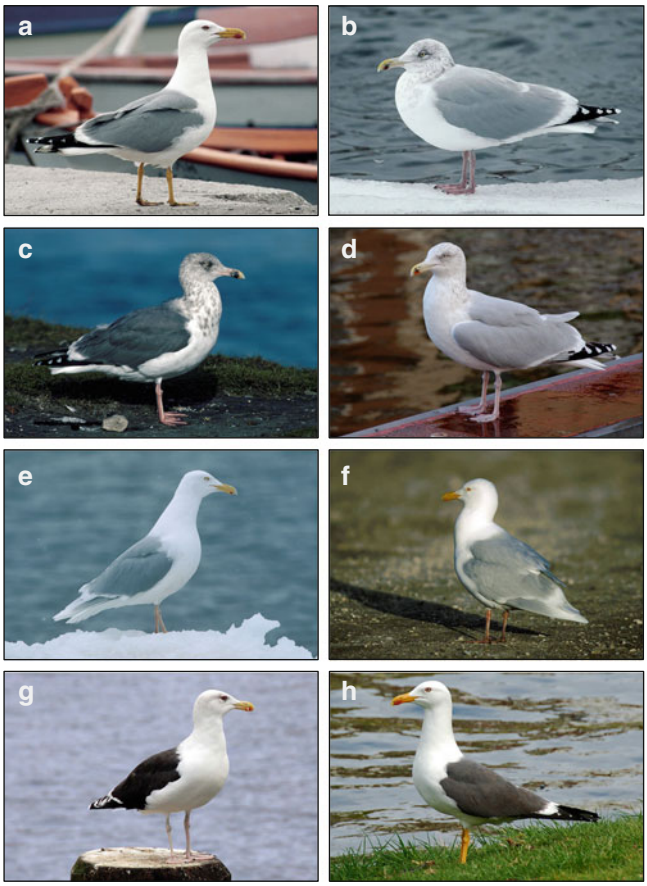


Fig. 1 Examples of phenotypic variation within the herring gull complex. (**a**) *L. michahellis*, Lesbos, Greece, (**b**) *L. smithsonianus*, Newfoundland, Canada, (**c**) *L. a. argentatus*, Rotterdam, Netherlands, (**d**) *L. a. argenteus*, Leiden, Netherlands, (**e**) *L. hyperboreus,* Svalbard, (**f**) *L. hyperboreus*, Churchill, Canada, (**g**) *L. marinus,* Greifswald, Germany, (**h**) *L. fuscus,* Leiden, Netherlands. Photos: P. de K. (**a**–**c**, **e**, **f**), V.S. (**d**, **g**, **h**)

the darkness of their dorsal plumage (see Fig. 1), which varies from pale gray to black (Malling Olsen and Larsson 2003). According to Mayr's (1942) model, the group originated in the Aralo-Caspian region, from where gulls spread in three directions (Fig. 2): (1) west via the Mediterranean into the Atlantic giving rise to Mediterranean (*michahellis*) and Atlantic (*atlantis*) herring gulls; (2) east toward Inner Asia giving rise to the Mongolian gull (*mongolicus*) and (3) north to the Arctic Ocean. Along the north Eurasian coasts, the ancestral population expanded both ways: (1) west across Scandinavia towards Britain and Iceland differentiating into dark-mantled lesser black-backed gulls (*fuscus)*, and (2) east all the way to the North Pacific giving rise to progressively paler-mantled forms, *taimyrensis* (Taimyr), *birulai* and *vegae* (eastern Siberia), and into North America (*smithsonianus*). Following the last Glacial Maximum, North American herring gulls are supposed to have crossed the North Atlantic and invaded Europe, where

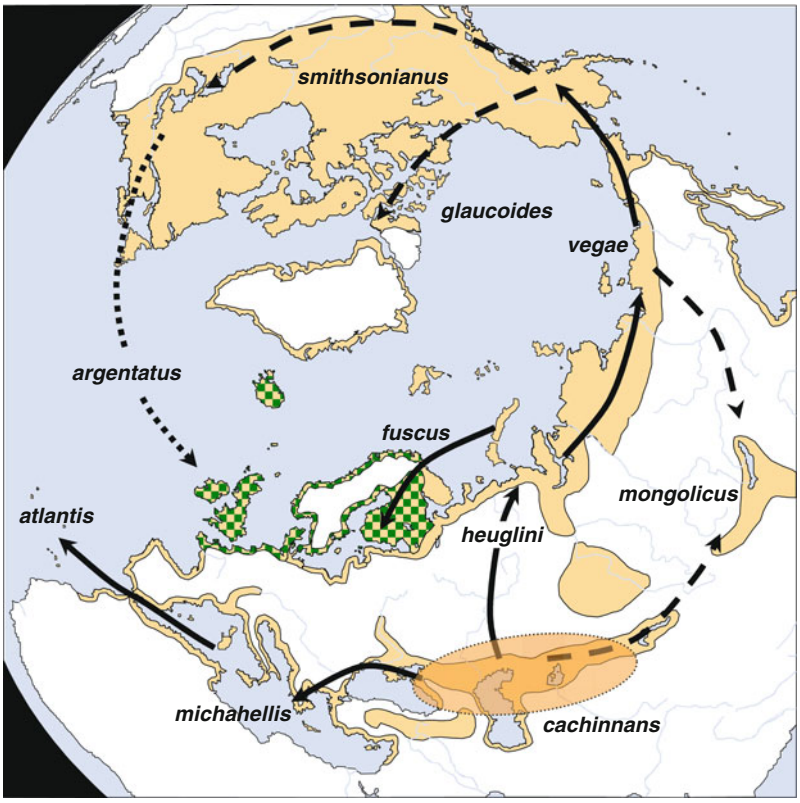


Fig. 2 Mayr's (1942) ring species model about the differentiation and colonization history of the herring gull complex. Mayr assumed a single Aralo-Caspian refugium (*pale orange oval*) and a most recent invasion of herring gulls from North America to Europe, where they now overlap with lesser black-backed gulls (*checker-board pattern*). *Arrows* indicate inferred colonization routes with temporal progression from ancient to most recent events indicated by *solid*, *broken* and *stippled arrows*, respectively

they now overlap with lesser black-backed gulls (Mayr 1942; Geyr 1938). Mayr envisioned all taxa of the circumpolar chain to be connected by gene flow, while herring and lesser black-backed gulls in Europe, the hypothetical endpoints of the ring, have reached full reproductive isolation and now coexist as distinct species.

Previous attempts to test the ring species model in these gulls were inconclusive due to the low amount of variation recovered from allozymes (Snell 1991) and short, conservative segments of mitochondrial DNA (Crochet et al. 2002). Our work has shown for the "yellow-legged" *L. cachinnans* subgroup (Liebers et al. 2001) and for the dark-mantled *L. fuscus* subgroup (Liebers and Helbig 2002) that partial reproductive barriers have arisen in situ between neighboring taxa both within the northern (Arctic Ocean – NE Atlantic) and the southern (Mediterranean – Aralo-Caspian) chain of taxa that Mayr (1942) hypothesized to be interconnected by gene flow.

There are a few well-documented cases of ring species (see, e.g., Irwin et al. 2001b for another avian example), most of which indicate that repeated allopatric fragmentation of large, roughly circular ranges is likely to lead to the evolution of multiple reproductive barriers *before* circular overlap and isolation-by-distance do. In particular, the well-known *Ensatina* complex of salamanders in western North America (Wake 1997), as well as our results obtained so far for the herring gull complex, best fit such a scenario. This means that fragmentation of circular ranges, most likely due to repeated episodes of glacial advance and retreat, may be an important mechanism in the early stages of a radiation in diverse groups of vertebrates.

We have already completed a comprehensive study of mitochondrial DNA (mtDNA) variation of the herring gull complex (Liebers et al. 2004). The aim of the present project, funded by the German Research Foundation for 4 years, was to use nuclear genetic markers (AFLP genotyping, intron sequences) to test a number of hypotheses derived from the previous study. The AFLP technique has been shown to be also effective in a number of different groups of birds, e.g., in gulls (de Knijff et al. 2001), *Phylloscopus* warblers (Bensch et al. 2002), house finch (Wang et al. 2003), *Hippolais* warblers (Secondi et al. 2006), New World crossbills (Parchman et al. 2006), and golden- and blue-winged warblers (Vallender et al. 2007). The same holds for the resolution of autosomal (intron) sequences, not only for closely related avian species groups such as *Phylloscopus* warblers (Bensch et al. 2006) and *Aquila* eagles (Helbig et al. 2005), but also for resolving higher order Avian phylogenies (Hackett et al. 2008).

2 State of the Art

We investigated the phylogeography of 20 northern hemisphere gull taxa (Fig. 3) and one southern large gull (*L. dominicanus*) using 1.5 kb of mitochondrial DNA sequence. This included the entire cytochrome b gene and a hypervariable part of the mitochondrial control region (HVR-1), which had previously been shown to

be highly informative about recent evolutionary differentiation of various taxa (Liebers and Helbig 1999, 2002; Liebers et al. 2001).

The haplotype network (Fig. 4) shows extensive genetic divergence within this complex of gulls. Analysis of molecular variance (Excoffier et al. 1992) indicated strong segregation of haplotypes along taxonomic, i.e., phenotypically defined, boundaries. The significant taxonomic and geographic structure enables us to interpret the evolutionary history of these gulls based on mitochondrial genetic variation. The western gull (*Larus occidentalis*) of the North American west coast (range not shown in Fig. 3) was found to have highly divergent haplotypes relative to all other taxa in this study and is regarded as the outgroup. Nested Clade Analysis (NCA; Templeton 1998) indicated that the ancestral ingroup population was divided by an allopatric fragmentation event leading to the evolution of two major clades: clade I centered in the North Atlantic and clade II with a circumpolar distribution. Assuming a cytochrome b divergence rate of 1.6% per million years (Fleischer et al. 1998), the initial vicariance event occurred some 308,000 (95% CI: 102,000–602,000) years ago. *L. argentatus* and *L. cachinnans* are the two taxa currently containing the most highly divergent, and the earliest-branching, haplotype lineages (Fig. 4). This indicates that they are direct descendants of the two ancestral populations. If current breeding ranges are any indication, ancestors of clade I probably lived in the north-eastern Atlantic (current range of *argentatus*), those ancestral to clade II lived in the Aralo-Caspian region (current range of *cachinnans*; Fig. 5).

As indicated by NCA, the Aralo-Caspian population (ancestors of clade II) spread by contiguous range expansion toward the north Eurasian coast, then west up to Britain and Iceland (*fuscus* range) and east throughout northern Siberia (*vegae, schistisagus*) and North America (*smithsonianus, glaucescens, glaucoides;* Fig. 5). In accordance with Mayr's theory, sharing of haplotypes between adjacent taxa in this circumpolar range indicates ongoing gene flow. However, we find no support for the key element of the ring species hypothesis, i.e., a transatlantic invasion of North American herring gulls *(smithsonianus)* into Europe. No haplotypes typical of, or derived from, Nearctic *smithsonianus* were found anywhere in the European *argentatus* population, not even in Iceland. The endpoints of the circumpolar ring of interbreeding taxa, therefore, do not overlap. Furthermore, yellow-legged gulls of the Atlantic Islands (*atlantis*), Mediterranean Sea (*michahellis*) and Asia Minor (*armenicus*) are derived from North Atlantic (clade I), not Aralo-Caspian ancestors (contra Mayr 1942).

Two more aspects of the mitochondrial phylogeny are surprising. First, three distinct species previously thought to derive from phylogenetically older ancestors are nested within the herring gull complex: great black-backed gull (*L. marinus*) and glaucous gull (*L. hyperboreus*), which overlap extensively in breeding range between themselves and with other members of the complex, and the southern hemisphere kelp gull (*L. dominicanus*). The latter evolved via long-distance colonization from the same ancestral population as the lesser black-backed gull (Fig. 4), suggesting that the ancestors of kelp gulls were highly migratory as nominate lesser black-backed gulls still are today.

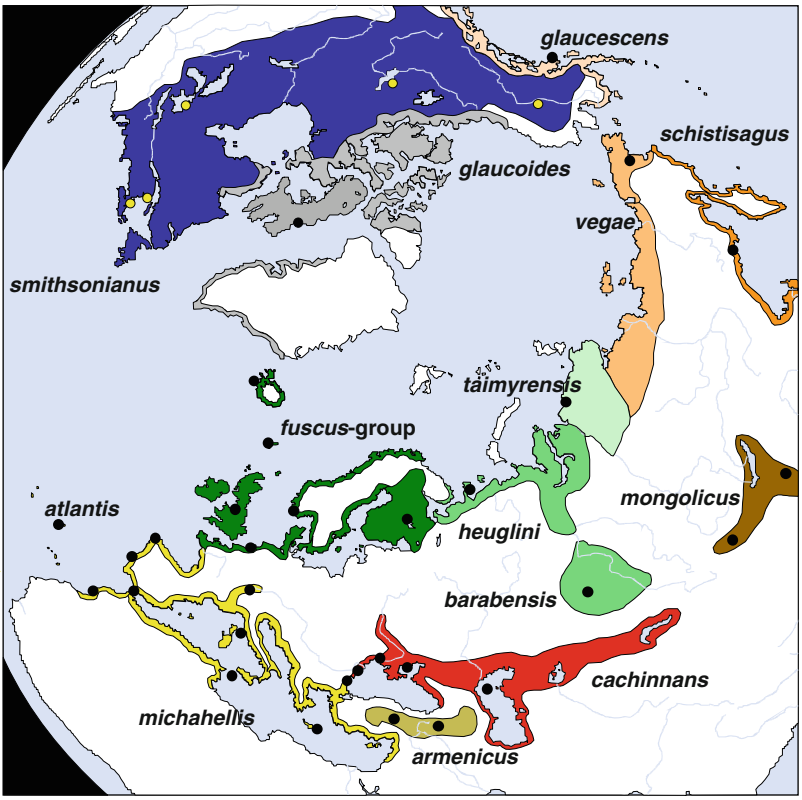


Fig. 3 Breeding ranges and sampling locations (*dots*) of the gull taxa investigated. For reasons of clarity, extensively overlapping ranges are shown on different maps, e.g., for *L. argentatus* in Fig. 8 and for *L. marinus*, and *L. hyperboreus* in Fig. 9. Ranges of *L. occidentalis* and *L. dominicanus* are not shown

Second, two taxa are biphyletic in the mtDNA network: populations of European *argentatus* (pale blue in Fig. 4) and of *hyperboreus* (dark gray) each contain haplotypes of both major clades. This can be explained by retention of ancestral polymorphisms and/or by mitochondrial gene flow that occurred after the initial split into two separate refugia.

In the case of *argentatus*, there is evidence that both processes were involved. Nuclear AFLP markers (de Knijff et al. 2001) showed *argentatus* to be most closely related to clade I taxa (*atlantis*/*michahellis*). Within clade I, *argentatus* haplotypes within the *marinus*-cluster probably reflect ancient polymorphism. The geographically widespread occurrence of clade II haplotypes in extant *argentatus* populations appears to be the footprint of a past gene flow episode. Birds derived from the Aralo-Caspian refugium, possibly members of a pre-*heuglini* population, must have hybridized, perhaps briefly, with the ancestral *argentatus* population from the North Atlantic. The introgressed clade II mitochondrial lineage persisted and diversified to this day in the *argentatus* population.

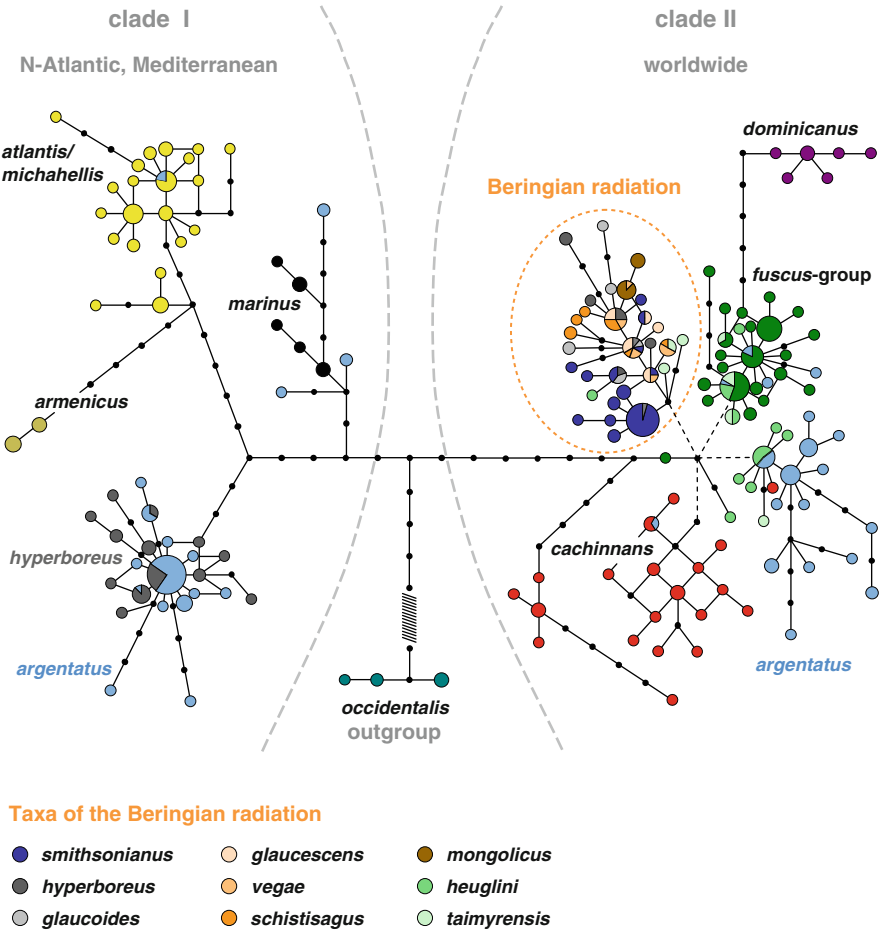


Fig. 4 Median-joining network (Bandelt et al. 1999) of 160 concatenated mtDNA haplotypes (cytochrome b, HVR-1) identified in this study. *Larus occidentalis* was designated as the outgroup. *Colors* represent taxa as shown in Fig. 3, Fig. 8 (*L. argentatus*), and Fig. 9 (*L. marinus* and *L. hyperboreus*)

The apparent biphyly of glaucous gulls (*hyperboreus*) is more problematic. In Palearctic birds, we found only haplotypes closely related to or shared with European *argentatus* (clade I), while Nearctic *hyperboreus* only contained a variety of haplotypes shared with North American and Pacific taxa (clade II). Hybridization between glaucous and herring gulls has been observed in Iceland (Ingolfsson 1970; Vigfúsdottir et al. 2008) and north-western Canada (Spear 1987) and may have led to mitochondrial introgression.

We found no close relationship between mitochondrial genetic distance and reproductive isolation. The most divergent taxon in terms mitochondrial DNA, *L. occidentalis*, hybridizes extensively with one of the ingroup taxa, *L. glaucescens*,

Fig. 5 Alternative model of the colonization history of the herring gull complex based on the mitochondrial DNA-sequences. Two ancient refugia are inferred (*pale green* and *pale orange oval*). Current ranges of taxa derived from Atlantic refugium are shown in *green*, those derived from Aralo-Caspian refugium are in *orange*, *checker-board pattern* shows areas of overlap. No invasion of herring gulls from North America to Europe occurred. *L. marinus* developed reproductive isolation in allopatry (probably in north-eastern North America) before making secondary contact with North American *smithsonianus* and Eurasian *argentatus*/*fuscus*. Two separate colonization events from the Atlantic into the Mediterranean led to the differentiation of *armenicus* and, much later, *michahellis*. *Arrows* as in Fig. 2

along the west coast of North America (Bell 1997). On the other hand, our data do not support the traditional view of great black-backed gull (*L. marinus*) being an outgroup relative to the herring gull complex. Although *L. marinus* is clearly reproductively isolated from all species it co-occurs with, in the mitochondrial network it is nested among taxa several of which hybridize: *argentatus* x *hyperboreus* (Ingolfsson 1970; Spear 1987), *michahellis* x *graellsii* (van Swelm 1998), *cachinnans* x *argentatus* (Panov and Monzikov 1999), and, earlier in the twentieth century, *argentatus* x *fuscus* (Tinbergen 1953).

3 Recent Progress

Mitochondrial DNA alone, due its maternal and clonal mode of inheritance, potentially being subject to selective sweeps and stochastic effects of lineage sorting, is not sufficient to reconstruct robustly the phylogeography and sequence of divergence events leading to a recent radiation such as that of the herring gull complex (e.g., Sattler and Braun 2000; Ballard and Whitlock 2004). We therefore studied and still work on autosomal, i.e., biparentally inherited nuclear markers, in order to

- Compare the nuclear genomic with the mitochondrial phylogeny.
- Compare levels of nuclear versus mitochondrial gene flow.
- Identify classes of markers that segregate at the earliest stages of the speciation process and may thus be generally applicable to the study of recent radiations in birds and perhaps other vertebrates.

3.1 Phylogenetic Framework for the Herring Gull Complex

Question: What species not included in our study so far might be part of the herring gull complex? The herring gull complex has traditionally been seen as a northern circumpolar group, but we have already shown that one southern hemisphere species (*L. dominicanus*) is in fact part of it. This may well be true for a number of other species (Fig. 6) that so far were not regarded as part of the complex either because of their smaller body size (*L. californicus, L. canus*) or due to their southern hemisphere distribution (*L. pacificus*).

To answer this question and, equally importantly, in order to check for congruence between phylogenetic signal between mitochondrial and nuclear genomes, we broadened the taxon sampling both for mitochondrial sequences (cytochrome b gene, HVR-1) and nuclear intron sequences (LDH intron 3, VLD intron 9, GAP intron 11, BRM intron 15) to provide a detailed phylogenetic framework for the herring gull complex (Sternkopf et al., in preparation).

Results: It turned out that *L. californicus*, although clearly smaller than all other members of the herring gull group identified so far, is indeed a member of this group (Fig. 7), while *L. canus* and *L. delawarensis* together form the sister group of the complex. More specifically, *L. californicus* is a member of clade II of the overall mitochondrial network (see Fig. 4) and is derived within the Beringian radiation which diversified primarily in the North Pacific–North American region (de Knijff et al. 2005; Sternkopf et al., in preparation).

The large Australian species *L. pacificus*, although superficially similar to *L. occidentalis* and *L. marinus* of the Holarctic and *L. dominicanus* of the southern hemisphere, is not a member of the complex. It forms a clade with *L. crassirostris* and *L. belcheri*, two other Pacific species with which it shares a black subterminal tail band in adult plumage. This character does not occur in adult plumages in the herring gull complex.

Fig. 6 Known members and new candidates for the herring gull radiation. (**a**) The kelp gull (*L. dominicanus*) is a southern hemisphere representative of the herring gull complex, nested within clade II. (**b**) The Pacific gull (*L. pacificus*) resembles the kelp gull at first glance. It inhabits the coasts of Australia and Tasmania. (**c**) Common gull (*L. canus*) and (**d**) California gull (*L. californicus*) are smaller gulls that breed throughout the Palearctic and in interior North America, respectively. Photos: (**a**) E. Stich, King George Island, Antarctica. (**b**) N. Murray, Phillip Island, Australia. (**c**) P. de K., Rotterdam, Netherlands. (**d**) P. de K., Palo Alto, California

Overall, the phylogeny of the Laridae has been much better resolved than in previous attempts (Crochet et al. 2000; Pons et al. 2005). Monophyly of the genus *Larus* was fully supported (blue triangle in Fig. 7), but only to the exclusion of the little gull (previously called *L. minutus* but renamed as *Hydrocoleus minutus*; cf. Helbig 2005). This species is a member of the basal clade of non-*Larus* gulls (*Rissa, Xema, Pagophila, Creagrus*). Our results strongly support a sister taxon relationship between little gull and Ross's gull (*Rhodostethia rosea)*. As a taxonomic consequence, we propose to exclude little gull from the genus *Larus* and instead group it together with the Ross's gull into the genus *Hydrocoleus* (Sternkopf et al., in preparation).

Within the mitochondrial control region, a larger insert (31 base pairs) was shared by the "masked gulls" (*L. genei–ridibundus* group), yielding additional support (beyond single nucleotide variation) for the monophyly of this group. The biphyly of large gulls is also apparent in the combined mitochondrial and nuclear genomic sequences, further supporting the two-refugia model of their phylogenetic history (Fig. 5).

3.2 *Population History of "polyphyletic" Taxa in the Mitochondrial Gene Tree*

Question: Several taxa were represented by a biphyletic pattern in the mitochondrial–genetic network, most notably *L. argentatus* and *L. hyperboreus* (see Fig. 4), but as

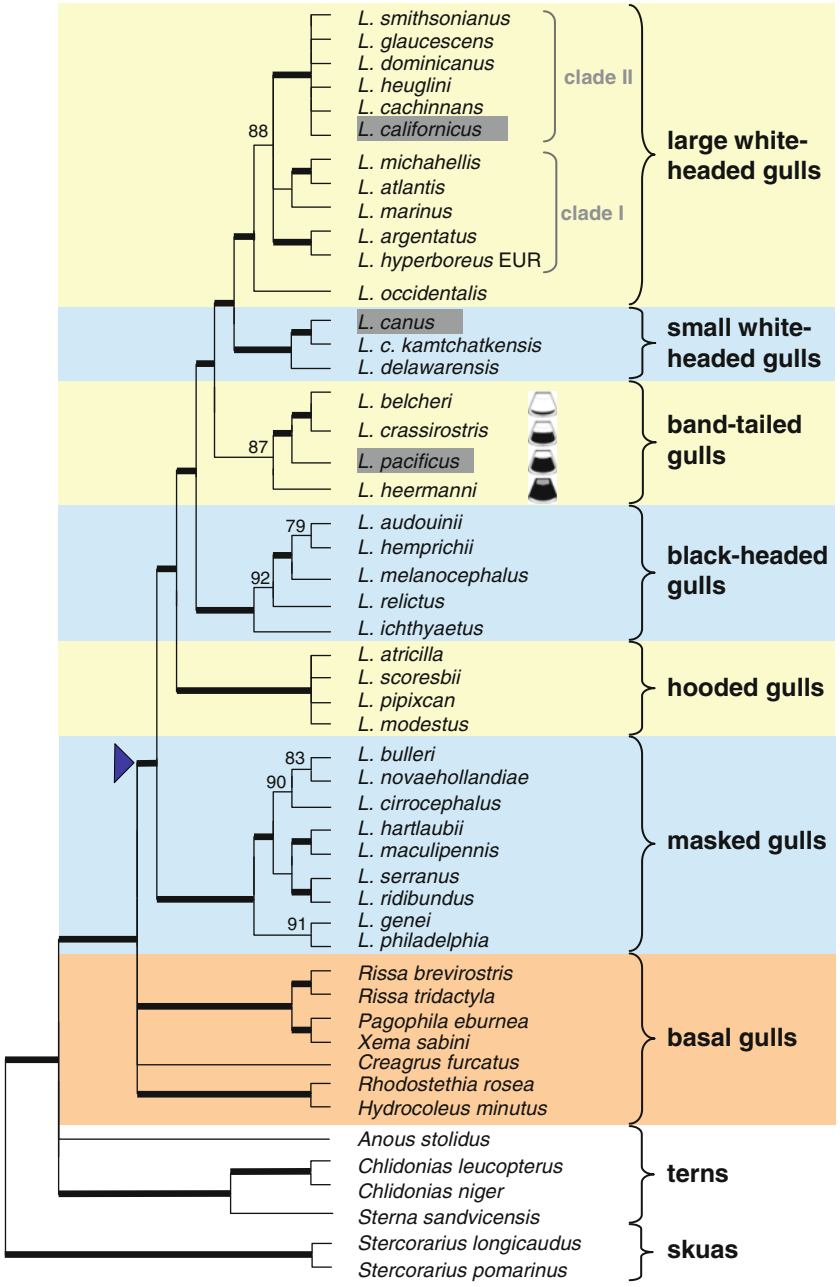


Fig. 7 Bayesian consensus tree of gulls of the family Laridae based on concatenated mitochondrial (cytochrome b, HVR-1) and nuclear intron (LDH 3, VLD 9, GAP 11, BRM 15) sequences. Numbers on branches are support of Bayesian posterior probabilities (GTR+γ model, 20,000 generations), *thick* branches indicate support values higher than 95. *L. californicus, L. canus,* and *L. pacificus*, three species suspected as possible members of the herring gull complex, are *shaded*. Monophyly of the genus *Larus* is shown by a *blue triangle*

we discovered in the meantime, this is now also true of *L. marinus* (Sternkopf et al., in preparation). The question arises whether individuals belonging to different clades in the mitochondrial gene tree but co-occurring in the same geographic area form a single or separate reproductive communities?

In the mitochondrial network, *L. argentatus* (pale blue in Fig. 4) shows a biphyletic origin and strong geographic substructure within its Palearctic range. Clade I haplotypes (green portion in Fig. 8) predominate in the northern populations, clade II haplotypes in the south (orange portion in Fig. 8; Sternkopf et al., in preparation).

Glaucous gull *L. hyperboreus*, which is also biphyletic in the network (dark gray in Fig. 4), shows an even more pronounced segregation of mitochondrial haplotypes (Fig. 9). All North American birds exhibit clade II haplotypes (orange portion in Fig. 9), while European populations, except two (wintering, not breeding) birds

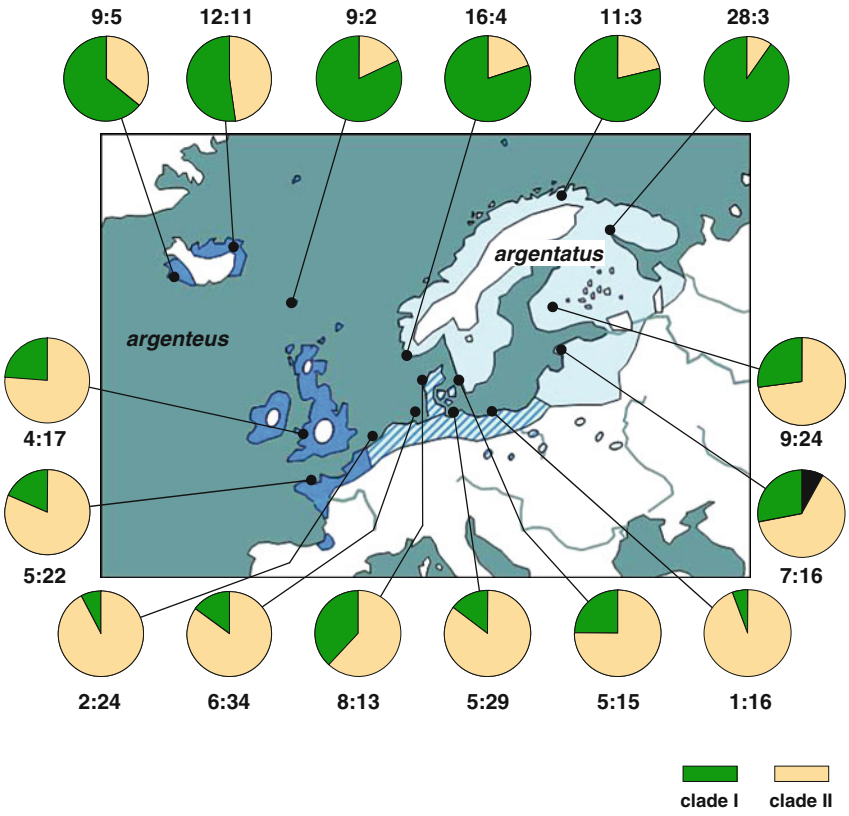


Fig. 8 Breeding ranges, sampling locations (*dots*) and haplotype composition (*pie charts*) of herring gulls throughout Europe ($n = 377$). Areas of intermixing between the two subspecies *argentatus* and *argenteus* are *shaded*. *Colors* in *pie charts* correspond to clade I (*green*) and clade II (*orange*) haplotypes in the mitochondrial network (see Fig. 4). The *black* portion in the Estonian population represents two individuals carrying a southerly distributed *cachinnans*-type sequence

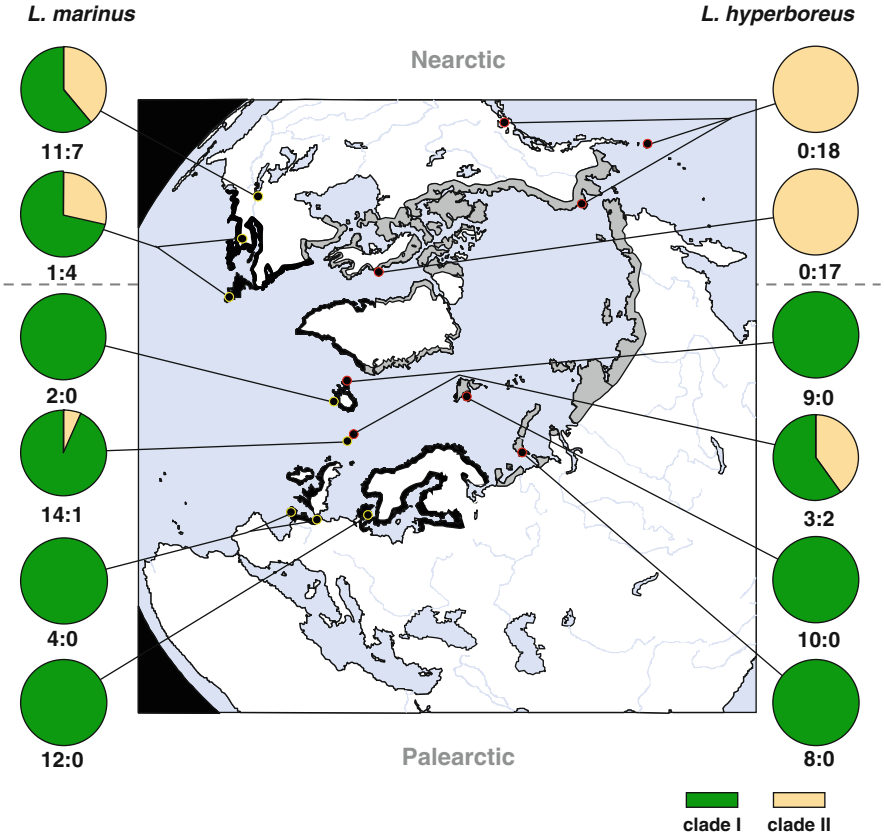


Fig. 9 Breeding ranges, sampling locations (*dots*) and haplotype composition (*pie charts*) of *L. marinus* ($n = 56$) and *L. hyperboreus* ($n = 67$). *Colors* in *pie charts* correspond to clade I (*green*) and clade II (*orange*) haplotypes in the mitochondrial network (see Fig. 4)

from the Faeroe Islands, carry clade I haplotypes (green portion in Fig. 9). Either the Nearctic or the Palearctic population acquired its mitochondrial haplotypes through hybridization – in North America with *smithsonianus*, or in Europe with *argentatus*.

Of the four European great black-backed gull *L. marinus* populations, all but one individual from the Faeroe Islands display clade I haplotypes (green portion in Fig. 9). Among North American *L. marinus*, clade I still predominates but clade II haplotypes account for a substantial amount of sequence variation.

We decided to use the amplified fragment length polymorphisms (AFLP) technique (Vos et al. 1995) because it allows the screening of a large number of autosomal loci and has proven to be very informative (Bensch and Akesson 2005).

Overall results: An extensive AFLP screening among seven large gull taxa yielded 17 primer combinations that could be scored with sufficient reliability. The resulting AFLP matrix consisted of the 1/0 scores of 230 loci among 369

individuals (Sternkopf et al., in preparation). We performed a principal component analysis (PCA), assuming within-group correlation. The plot of the first three principal components values, explaining 72.7% of the total data variation, is depicted in Fig. 10. This clearly demonstrates that five taxa, including *argentatus* and *marinus*, are genetically well defined and form distinct groups. The two remaining taxa, *smithsonianus* and *hyperboreus*, display partially overlapping clusters strongly suggesting a close autosomal genetic affinity.

3.2.1 Evolution of Herring Gulls in Europe

By means of AFLP loci, it was not possible to reproduce the strong biphyletic distribution among European herring gulls *L. argentatus* as was observed using mitochondrial sequences (Fig. 4). We were unable to detect significant differences in AFLP frequencies between herring gulls grouped according to mtDNA-defined clade I versus clade II (results not shown). Also, when we compared AFLP frequency patterns between northern and southern herring gull populations (see Fig. 8), no significant differences were found (results not shown). Even if we compare the two most extreme colonies with almost pure clade I mtDNA haplotypes from the White Sea and the almost pure clade II mtDNA haplotypes from the Netherlands, no significant AFLP distribution differences could be observed.

On the contrary, the PCA analysis (Fig. 10) and several Structure analysis runs (Pritchard et al. 2000; Falush et al. 2007) illustrate, that clade I and clade II herring gulls were not separable (results not shown), but formed a uniform cluster separate of the other six species (Fig. 10). Population differentiation and nuclear gene flow relationships also showed that *L. argentatus* from both mitochondrial clades were not significantly differentiated from each other, while they are from the other species (Sternkopf et al., in preparation).

These results confirm our prediction that clade I and clade II herring gulls form a single reproductive community. Their biphyly in the mitochondrial gene tree must, therefore, be the footprint of past gene flow between birds derived from different ancestral refugia.

This supports our original hypothesis (Liebers et al. 2004) that birds derived from the Aralo-Caspian refugium, possibly members of a pre-*heuglini* population, must have hybridized with the ancestral *argentatus* population from the North Atlantic. The introgressed clade II mitochondrial lineage persisted and has diversified to this day in the *argentatus* population.

3.2.2 Phylogeographic History of Circumpolar Breeding Glaucous Gulls

Among the circumpolar breeding glaucous gull L. *hyperboreus*, we see an even more distinct geographic distribution of mitochondrial haplotypes. All *hyperboreus* from Nearctic breeding colonies display exclusively clade II haplotypes, whereas Palearctic birds display clade I sequences (Fig. 9). The only two Palearctic

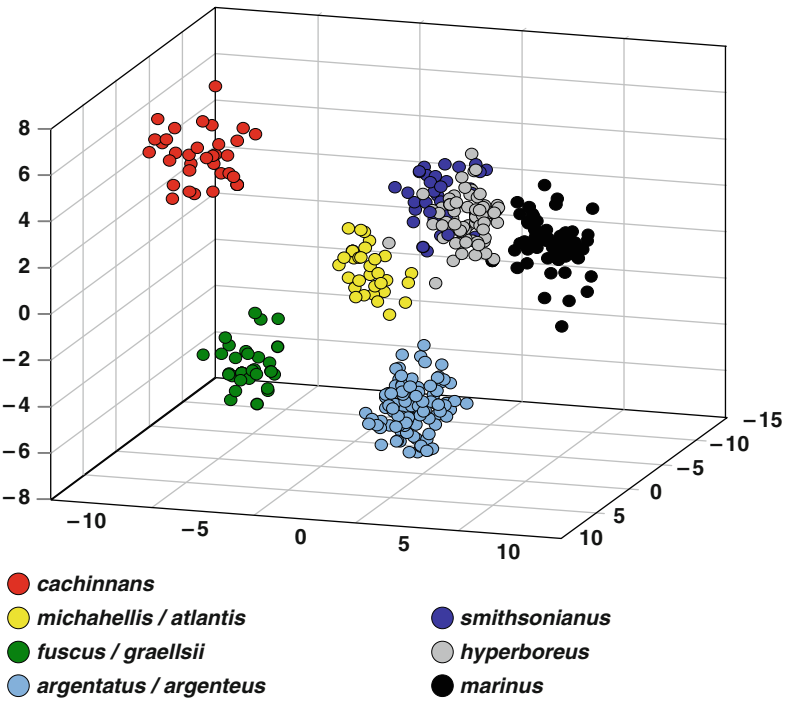


Fig. 10 Principal component analysis of total 1/0 AFLP data matrix ($n = 369$ individuals). The *axis* represents the first three principal component values

hyperboreus with clade II sequences were individuals wintering on the Faeroe Islands. The nuclear AFLP-markers do not co-segregate with the mitochondrial ones and show no differentiation of Nearctic versus Palearctic populations. On the contrary, Nearctic and Palearctic birds group together and overlap significantly with North American herring gulls (*L. smithsonianus*) indicating that both taxa are each other's closest relatives based on autosomal marker (Fig. 10).

Most probably, glaucous gull is an original member of the Beringian radiation (Fig. 4) that originated and diversified along the North Pacific/north-west Arctic coasts of North America and north-east of Russia. Only very recently, e.g., after the last deglaciation event of the Nearctic, could *hyperboreus* have spread into northern Europe where it came into contact with *argentatus* birds of clade I (see Fig. 8, green portion). Palearctic populations of *hyperboreus* must have acquired their mitochondrial haplotypes through hybridization with *argentatus* in Europe. So far, our Palearctic sampling reaches as far east as Taimyr. To fully resolve the circumpolar expansion, we should also include samples from north-eastern Siberia. Obviously, only with those samples can we reconstruct the complete population history and introgression process of *hyperboreus*.

Already at this stage of research, glaucous gulls constitute a textbook example of "mitochondrial take-over", e.g., complete replacement of the original mitochondrial haplotypes (clade II) through hybridization with birds of the other clade.

3.2.3 Colonization Pattern of Greater Black-Backed Gulls

Nuclear markers confirmed the phylogenetic position of *marinus* within the herring gull complex (Fig. 7) contrary to the traditional view of great black-backed gull being a distinct outgroup, although *L. marinus* is fully reproductively isolated from all species with which it co-occurs (Liebers et al. 2004).

Based on mitochondrial sequences, *marinus* shows a biphyletic structure. Palearctic populations exclusively carry clade I, with a single exception on the Faeroe Islands, while Nearctic populations display a substantial proportion of clade II haplotypes (Fig. 9). Based on AFLP loci, it is not possible to reliably distinguish between North American and European *marinus* (Sternkopf et al., in preparation).

We suggest that *marinus* developed originally in northern Europe as a member of clade I. Only very recently, it colonized the coasts of eastern North America. The combined mitochondrial and AFLP results strongly suggest a brief period of hybridization in the Nearctic with members of the Beringian radiation, after which *marinus* rapidly became reproductively isolated again. This process involved only a few individuals and lasted only for a limited number of generations. Clade II mitochondrial haplotypes, that invaded the population via introgression, are now captured in the genepool of *marinus* and mask the true phylogenetic history of this species.

3.2.4 Summary

Mallet (2005) suggested that about 10% of animal species, mostly the younger ones, hybridize and hence continue to exchange genetic material through introgression. Many studies of closely related species reveal introgression, mostly of maternally inherited mitochondrial DNA. For example, in mammals, it was first discovered in house mice (Ferris et al. 1983; Prager et al. 1993), followed by a growing list of species, e.g., voles (Tegelström 1987), deer (Cathey et al. 1998), hares (Thulin et al. 1997; Melo-Ferreira et al. 2005), and chipmunks (Good et al. 2008). In birds, introgression through hybridization has been proven in *Hippolais* warblers (Secondi et al. 2006), *Vermivora* warblers (Vallender et al. 2007), and Caribbean banabaquit (Bellemain et al. 2008).

The consequences of such cryptic hybridization broadly echo and emphasize that extreme caution is needed when interpreting single gene genealogies, especially those based on mitochondrial DNA alone (Brito and Edwards 2008). The biphyletic representation of herring gulls, glaucous gulls and great black-backed gulls in the mitochondrial haplotype network provides a striking illustration of how discrepancies can arise between a gene tree (in this case, based on mitochondrial

DNA) and a taxon phylogeny (based on autosomal markers). The fact that some species, apparently due to past gene flow episodes, contain highly divergent mitochondrial haplotypes suggests that lineage sorting could have quite different and unpredictable outcomes (Gadagkar et al. 2005; Rokas and Carroll 2005).

In closely related species, such as the herring gull radiation, only a multilocus analysis of mitochondrial and nuclear markers can probably reveal the complex history of population subdivision and gene flow. Our results highlight the importance of using information from independent genetic markers when evaluating the evolutionary history of an adaptive radiation.

4 Future Perspectives

Despite the use of extensive mitochondrial DNA sequence variation, autosomal intron sequence variation, and a large number of independently segregating autosomal AFLP loci, we were unable to completely reconstruct the ancient demographic events resulting in the present day biphyletic position of three members of the large gull radiation: herring gull *L. argentatus*, glaucous gull *L. hyperboreus*, and great black-backed gull *L. marinus*. For a much better insight into the – probably relatively recent – events giving rise to the present day genetic variation observed in the herring gull radiation, much more autosomal DNA information is required. Here, we see two clearly distinct approaches. The first is the identification of a high number of genome-wide distributed autosomal co-dominant single nucleotide polymorphisms (SNPs) that could be used to reconstruct the genetic affinities among closely related groups of individuals. Such an approach can be applied successfully as has been clearly demonstrated by a number of studies using many thousands of SNPs among distantly and closely related human populations (Jakobsson et al. 2008; Lao et al. 2008; Li et al. 2008; Novembre et al. 2008). Another approach involves the use of relatively short but highly polymorphic non-recombining stretches of autosomal introns and/or exons that could serve as multiple mini-haplotype blocks. Such autosomal haplotypes, when sufficiently polymorphic, are ideal not only to retrace subtle gene flow processes among populations but will also facilitate the relative timing of such processes. For both approaches, there are now a growing number of biotechnological solutions, including extremely high-throughput sequencing (Vera et al. 2008), and the identification of conserved genome fragments by simply comparing the growing number of (nearly) completely sequenced whole genomes (Backström et al. 2008).

In addition, divergence levels at autosomal versus Z-chromosomal loci could be compared to test whether reinforcement played a significant role in the speciation process of large gulls. We hypothesize that differentiation has progressed further on the Z-chromosome than on the autosomes because Z-chromosomal loci are more likely to be related to, or linked to, loci causing reproductive incompatibilities (Servedio and Sætre 2003; Sætre et al. 2003; Borge et al. 2005; Mank et al. 2007; Qvarnström and Bailey 2009). Finally, contrasting genetic signatures from avian

W- and Z-chromosomes can be informative for inferring and reconstructing sex-specific gene flow patterns in large gulls.

To this end, we have already identified ~35,000 short autosomal genome fragments among four different members of the herring gull complex that could serve as the basis of further research using the approaches described above.

Acknowledgements We would like to thank all colleagues who provided samples or helped during field work, the Institute of Ecology (University of Jena) for use of laboratory facilities and M. Ritz for his help with generating the AFLP profiles. M. Braun and S. Bensch made helpful comments on previous versions of this manuscript. This research was supported by two grants from the German Research Foundation to D.L.H. (LI 1049/1-1 and 1-2).

References

Backström N, Karaiskou N, Leder EH, Gustafsson L, Primmer CR, Qvarnström A, Ellegren H (2008) A gene-based genetic linkage map of the collared flycatcher (*Ficedula albicollis*) reveals extensive synteny and gene-order conservation during 100 million years of avian evolution. Genetics 179:1479–1495

Ballard JW, Whitlock MC (2004) The incomplete natural history of mitochondria. Mol Ecol 13:729–744

Bandelt H-J, Forster P, Röhl A (1999) Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 16:37–48

Bell DA (1997) Hybridization and reproductive performance in gulls of the *Larus glaucescens-occidentalis* complex. Condor 99:585–594

Bellemain E, Bermingham E, Ricklefs R (2008) The dynamic evolutionary history of the banana-quit (*Coereba flaveola*) in the Caribbean revealed by multiple analysis. BMC Evol Biol 8:240. doi:10.1186/1471-2148-8-240

Bensch S, Akesson M (2005) Ten years of AFLP in ecology and evolution: why so few animals? Mol Ecol 14:2899–2914

Bensch S, Helbig AJ, Salomon M, Seibold I (2002) Amplified fragment length polymorphism analysis identifies hybrids between two subspecies of warblers. Mol Ecol 11:473–481

Bensch S, Irwin DE, Irwin JH, Kvist L, Akesson S (2006) Conflicting patterns of mitochondrial and nuclear DNA diversity in *Phylloscopus* warblers. Mol Ecol 15:161–171

Borge T, Webster MT, Andersson G, Sætre GP (2005) Contrasting patterns of polymorphism and divergence on the Z chromosome and autosomes in two *Ficedula* flycatcher species. Genetics 171:1861–1873

Brito PH, Edwards SV (2008) Multilocus phylogeography and phylogenetics using sequence-based markers. Genetica. doi:10.1007/s10709-008-9293-3

Cain AJ (1954) Animal species and their evolution. Hutchinson House, London

Cathey JC, Bickham JW, Patton JC (1998) Introgressive hybridization and nonconcordant evolutionary history of maternal and paternal lineages in North American deer. Evolution 52:1224–1229

Crochet P-A, Bonhomme F, Lebreton J-D (2000) Molecular phylogeny and plumage evolution in gulls (Larini). J Evol Biol 13:47–57

Crochet P-A, Lebreton J-D, Bonhomme F (2002) Systematics of large white-headed gulls: patterns of mitochondrial DNA variation in western European taxa. The Auk 119:603–620

de Knijff P, Denkers F, Swelm ND, van Kuiper M (2001) Genetic affinities within the *Larus argentatus* assemblage revealed by AFLP genotyping. J Mol Evol 52:85–93

de Knijff P, Helbig AJ, Liebers D (2005) The Beringian connection: speciation in the herring gull assemblage of North America. Birding 37:402–411
Excoffier L, Smouse P, Quattro J (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479–491
Falush D, Stephens M, Pritchard JK (2007) Inference of population structure using multilocus genotype data: dominant markers and null alleles. Mol Ecol Notes 7:574–578
Ferris SD, Sage RD, Huang CM, Nielsen JT, Ritte U, Wlson AC (1983) Flow of mitochondrial DNA across a species boundary. Proc Natl Acad Sci USA 80:2290–2294
Fleischer RC, McIntosh CE, Tarr CL (1998) Evolution on a volcanic conveyor belt: using phylogenetic reconstructions and K-Ar-based ages of the Hawaiian Islands to estimate molecular evolutionary rates. Mol Ecol 7:533–545
Gadagkar SR, Rosenberg MS, Kumar S (2005) Inferring species phylogenies from multiple genes: concatenated sequence tree versus consensus gene tree. J Exp Zool (Mol Dev Evol) 304B:64–74
Geyr von Schweppenburg H (1938) Zur Systematik der *fuscus-argentatus*-Möwen. J Orn 86:345–365
Good JM, Hird S, Reid N, Demboski JR, Steppan SJ, Martin-Nims TR, Sullivan J (2008) Ancient hybridization and mitochondrial capture between two species of chipmunk. Mol Ecol 17:1313–1327
Hackett SJ, Kimball RT, Reddy S et al (2008) A phylogenomic study of birds reveals their evolutionary history. Science 320:1763–1768
Haffer J (1982) Systematik und Taxonomie der *Larus argentatus* - Artengruppe. In: von Blotzheim UN Glutz, Bauer KM (eds) Handbuch der Vögel Mitteleuropas, vol 8. Aula, Wiesbaden, pp 502–514
Helbig AJ (2005) Möwen und Seeschwalben. In: Bauer H-G, Bezzel E, Fiedler W (eds) Kompendium der Vögel Mitteleuropas, vol 1, Nonpasseriformes. Aula, Wiesbaden, pp 573–574
Helbig AJ, Kocum A, Seibold I, Brown MJ (2005) A multi-gene phylogeny of aquiline eagles (Aves: Accipitriformes) reveals extensive paraphyly at the genus level. Mol Phyl Evol 35:147–164
Ingolfsson A (1970) Hybridization of glaucous gulls *Larus hyperboreus* and herring gull *L. argentatus* in Iceland. Ibis 112:340–362
Irwin DE, Irwin JH, Price TD (2001a) Ring species as bridges between microevolution and speciation. Genetica 112–113:223–243
Irwin DE, Bensch S, Price TD (2001b) Speciation in a ring. Nature 409:333–337
Jakobsson M, Scholz SW, Scheet P et al (2008) Genotype, haplotype and copy-number variation in worldwide human populations. Nature 451:998–1003
Lao O, Lu TT, Nothnagel M, Junge O et al (2008) Correlation between genetic and geographic structure in Europe. Curr Biol 18:1241–1248
Li JZ, Absher DM, Tang H, Southwick AM et al (2008) Worldwide human relationships inferred from genome-wide patterns of variation. Science 319:1100–1104
Liebers D, Helbig AJ (1999) Phänotypische Charakterisierung und systematische Stellung der Armenienmöwe *Larus armenicus*. Limicola 13:281–321
Liebers D, Helbig AJ (2002) Phylogeography and colonization history of lesser black-backed gulls (*Larus fuscus*) as revealed by mtDNA sequences. J Evol Biol 15:1021–1033
Liebers D, Helbig AJ, de Knijff P (2001) Genetic differentiation and phylogeography of gulls in the *Larus fuscus - cachinnans* group (Aves: Charadriiformes): inferences from mitochondrial control region sequences. Mol Ecol 10:2447–2462
Liebers D, de Knijff P, Helbig AJ (2004) The herring gull complex is not a ring species. Proc R Soc Lond B 271:893–901
Mallet J (2005) Hybridization as an invasion of the genome. Trends Ecol Evol 20:229–237
Malling Olsen K, Larsson H (2003) Gulls of Europe, Asia and North America. Helm identification guides. Black, London

Mank JE, Axelsson E, Ellegren H (2007) Fast-X on the Z: Rapid evolution of sex-linked genes in birds. Gen Res 17:618–624

Mayr E (1942) Systematics and the origin of species. Dover, New York

Melo-Ferreira J, Boursot P, Suchentrunk F, Ferrand N, Alves PC (2005) Invasion from the cold past: extensive introgression of mountain hare (*Lepus timidus*) mitochondrial DNA into three other hare species in northern Iberia. Mol Ecol 14:2459–2464

Novembre J, Johnson T, Bryc K et al (2008) Genes mirror geography within Europe. Nature 456:98–101

Panov EN, Monzikov DG (1999) Intergradation between the herring gull *Larus argentatus* and the southern herring gull *Larus cachinnans* in European Russia. Russ J Zool 78:334–348

Parchman TL, Benkman CW, Britch SC (2006) Pattern of genetic variation in the adaptive radiation of New World crossbills (Aves: Loxia). Mol Ecol 15:1873–1887

Pons J-M, Hassanin A, Crochet P-A (2005) Phylogenetic relationships within Laridae (Charadriiformes: Aves) inferred from mitochondrial markers. Mol Phyl Evol 37:686–699

Prager EM, Sage RD, Gyllensten U et al (1993) Mitochondrial DNA sequences diversity and the colonization of Scandinavia by house mice from East Holstein. Biol J Linn Soc 50:85–122

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

Qvarnström A, Bailey RI (2009) Speciation through evolution of sex-linked genes. Heredity 102:4–15

Rokas A, Carroll SB (2005) More genes or more taxa? The relative contribution of gene number and taxon number to phylogenetic accuracy. Mol Biol Evol 22:1337–1344

Sattler GD, Braun MJ (2000) Morphometric variation as an indicator of genetic interactions between Black-capped and Carolina chickadees at a contact zone in the Appalachian Mountains. Auk 117:427–444

Sætre GP, Borge T, Lindroos K, Haavie J, Sheldon BC, Primmer C, Syvanen AC (2003) Sex chromosome evolution and speciation in *Ficedula* flycatchers. Proc R Soc Lond B 270: 53–59

Secondi J, Faivre B, Bensch S (2006) Spreading introgression in the wake of a moving contact zone. Mol Ecol 15:2463–2475

Servedio MR, Sætre G-P (2003) Speciation as a positive feedback loop between postzygotic and prezygotic barriers to gene flow. Proc R Soc Lond B 270:1473–1479

Snell RR (1991) Interspecific allozyme differentiation among north Atlantic white-headed larid gulls. Auk 108:319–328

Spear LB (1987) Hybridization of glaucous and herring gulls at the Mackenzie Delta, Canada. Auk 104:123–125

Stegmann B (1934) Ueber die Formen der großen Möwen ("subgenus *Larus*") und ihre gegenseitigen Beziehungen. J Orn 82:340–380

Sternkopf V, Liebers-Helbig D, Ritz M, Helbig AJ, de Knijff P (in preparation) Introgressive hybridization and non-concordant evolutionary history of mitochondrial and nuclear DNA in the herring gull complex.

Sternkopf V, Liebers-Helbig D, Helbig AJ, de Knijff P (in preparation) Phylogeny of the genus *Larus* revised.

Tegelström H (1987) Transfer of mitochondrial DNA from the northern red-backed vole (*Clethrionymys rutilus*) to the bank vole (*Clethrionymys glareolus*). J Mol Evol 24:218–227

Templeton AR (1998) Nested clade analysis of phylogeographic data: testing hypotheses about gene flow and population history. Mol Ecol 7:381–397

Thulin CG, Jaarola M, Tegelström H (1997) The occurrence of mountain hare mitochondrial DNA in wild brown hares. Mol Ecol 6:463–467

Tinbergen N (1953) The herring gull's world. Collins, London

Vallender R, Robertson RJ, Friesen VL, Lovette IJ (2007) Complex hybridization dynamics between golden-winged and blue-winged warblers (*Vermivora chrysoptera* and *Vermivora*

pinus) revealed by AFLP, microsatellite, intron an mtDNA markers. Mol Ecol 16: 2017–2029

van Swelm ND (1998) Status of yellow-legged gull *Larus michahellis* as a breeding bird in the Netherlands. Sula 12:199–202

Vera JC, Wheat CW, Fescemyer HW, Frilander MJ, Crawford DL, Hanski I, Marden JH (2008) Rapid transcriptome characterization for a nonmodel organism using 454 pyrosequencing. Mol Ecol 17:1636–1647

Vigfúsdottir F, Pálsson S, Ingolfsson A (2008) Hybridization of glaucous gull (*Larus hyperboreus*) and herring gull (*Larus argentatus*) in Iceland: mitochondrial and microsatellite data. Philos Trans R Soc Lond B 363:2851–2860

Vos P, Hogers R, Bleeker M et al (1995) AFLP: a new technique for DNA fingerprinting. Nucl Acids Res 23:4407–4414

Wake DB (1997) Incipient species formation in salamanders of the Ensatina complex. Proc Natl Acad Sci USA 94:7761–7767

Wang Z, Baker AJ, Hill GE, Edwards SV (2003) Reconciling actual and inferred population histories in the House Finch (*Capodacus mexicanus*) by AFLP analysis. Evolution 57: 2852–2864

Genetic Divergence and Evolution of Reproductive Isolation in Eastern Mediterranean Water Frogs

Jörg Plötner, Thomas Uzzell, Peter Beerli, Çiğdem Akın, C. Can Bilgin, Cornelia Haefeli, Torsten Ohst, Frank Köhler, Robert Schreiber, Gaston-Denis Guex, Spartak N. Litvinchuk, Rob Westaway, Heinz-Ulrich Reyer, Nicolas Pruvost, and Hansjürg Hotz

Abstract Water frogs [genus *Pelophylax* (*Rana*)] that occur around the eastern Mediterranean Sea provide an opportunity to study early stages of speciation. The geography of the eastern Mediterranean region has changed dramatically since the Middle Miocene as a result of motions of adjoining lithospheric plates and regional-scale vertical crustal motions (uplift and subsidence). For several hundred thousand years between 6 and 5 million years ago (Mya), the Mediterranean basin was isolated from the Atlantic Ocean, and became desiccated (the Messinian Salinity Crisis; MSC). Geological data suggest that the endemic water frog lineage on Cyprus was isolated by the flooding of the Mediterranean basin by salt water at the end of the MSC, circa 5.5–5.3 Mya. This suggests a rate of uncorrected genetic

J. Plötner (✉), T. Ohst, and R. Schreiber
Museum für Naturkunde, Leibniz-Institut für Evolutions- und Biodiversitätsforschung an der Humboldt-Universität zu Berlin, Invalidenstraße 43, 10115 Berlin, Germany
e-mail: joerg.plotner@mfn-berlin.de

T. Uzzell
Laboratory for Molecular Systematics and Ecology, Academy of Natural Sciences, 1900 B. F. Parkway, Philadelphia, PA 19103, USA

P. Beerli
Department of Scientific Computing, Florida State University, Tallahassee, FL 32306-4120, USA

Ç. Akın and C.C. Bilgin
Department of Biology, Biodiversity and Conservation Laboratory, Middle East Technical University, 06531 Ankara, Turkey

C. Haefeli, G.-D. Guex, H.-U. Reyer and N. Pruvost
Institut für Evolutionsbiologie und Umweltwissenschaften, Universität Zürich-Irchel, Winterthurerstrasse 190, 8057 Zurich, Switzerland

F. Köhler
Museum für Naturkunde, Leibniz-Institut für Evolutions- und Biodiversitätsforschung an der Humboldt-Universität zu Berlin, Invalidenstraße 43, 10115 Berlin, Germany
Department of Research, Australian Museum, 6 College Street, Sydney 2010, NSW, Australia

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_18,

divergence of approximately 1.1% per million years (My). Divergence time estimates based on this rate are in good agreement with the chronology of events in the history of crustal deformation and landscape development in the eastern Mediterranean region.

Despite a high similarity in morphology, eastern Mediterranean water frogs show considerable genetic divergence, indicating the existence of several evolutionary species at varied levels of differentiation. Based on two mitochondrial (mt) genes (ND2 and ND3), several lineages have been identified: *Pelophylax bedriagae*, *P. cretensis*, *P. epeiroticus*, *P. ridibundus* (Europe), six Anatolian lineages, all provisionally subsumed under the name *P.* cf. *bedriagae*, and a distinct lineage restricted to Cyprus. Genetic data from transition zones in eastern Greece/western Anatolia, south-western Anatolia, and south-eastern Anatolia, in concert with the results of female choice experiments, indicate that antihybridization mechanisms are only weakly developed in eastern Mediterranean water frogs. Genetic incompatibility, as expressed by average hatching rate of heterospecific crosses, increases with genetic divergence measured by uncorrected distance estimated from mtDNA sequences. Hatching rates of heterospecific crosses show an extremely high variability, however, and viable F1 hybrids originated from almost all crosses. We conclude that speciation in eastern Mediterranean water frogs follows the allopatric model and has been closely associated with the geodynamic evolution of the Mediterranean since the Middle Miocene (i.e., since ~11 Mya).

Keywords Water frogs · Pelophylax (Rana) · Eastern mediterranean · Genetic diversity · Geology · Divergence time · Genetic incompatibilities · Antihybridization mechanisms · Speciation

S.N. Litvinchuk
Institute of Cytology, Russian Academy of Sciences, Tikhoretsky prospekt 4, 194064 St. Petersburg, Russia

IRES, Newcastle University, Newcastle-upon-Tyne NE1 7RU, UK
H. Hotz
Museum für Naturkunde, Leibniz-Institut für Evolutions- und Biodiversitätsforschung an der Humboldt-Universität zu Berlin, Invalidenstraße 43, 10115 Berlin, Germany
Institut für Evolutionsbiologie und Umweltwissenschaften, Universität Zürich-Irchel, Winterthurerstrasse 190, 8057 Zurich, Switzerland

IRES, Newcastle University, Newcastle-upon-Tyne NE1 7RU, UK
H. Hotz
Museum für Naturkunde, Leibniz-Institut für Evolutions- und Biodiversitätsforschung an der Humboldt-Universität zu Berlin, Invalidenstraße 43, 10115 Berlin, Germany
Institut für Evolutionsbiologie und Umweltwissenschaften, Universität Zürich-Irchel, Winterthurerstrasse 190, 8057 Zurich, Switzerland

1 Introduction

Species are real entities in nature, originating in an interminable process called speciation, but their history is difficult to reconstruct because of many unknown parameters and events. Speciation is an adaptive process driven either by natural or by sexual selection; it involves complex genetic, ecological, and behavioral mechanisms. Several modes of speciation have been proposed: (1) allopatric speciation including bottleneck-induced speciation (reviewed by Carson and Templeton 1984), (2) sympatric speciation (reviewed by Bolnick and Fitzpatrick 2007), and (3) speciation via hybridization (e.g., Bullini 1994, Seehausen 2004; Baak and Rieseberg, 2007). In almost all cases, however, conventional evolutionary forces such as mutation, selection, and drift lead to distinct lineages of ancestor-descendant populations with specific gene pools and distinct evolutionary tendencies.

Speciation usually begins with the accumulation of genetic differences between isolated populations. If such differentiated populations come into secondary contact, the differences between them may favor the evolution of antihybridization and anticompetition mechanisms (Remington 1968), which reduce wasteful matings between the newly sympatric populations and decrease the competition between them. Neither speciation itself nor the establishment of these sequelae to speciation necessarily depends on the degree of genetic divergence of two lineages; single genes or gene complexes, rather than genomes as a whole, may be the units of speciation (e.g., Wu 2001).

Water frogs [genus *Pelophylax* (*Rana*)] distributed around the eastern Mediterranean Sea are an ideal group of vertebrates for studying speciation processes, especially in their early stages, because they represent a genetically and phylogenetically complex biological radiation comprising different levels of molecular and organismal differentiation. Furthermore, the geomorphological complexity of the region allows for analysis of the effects of geological processes and environmental change on genetic diversification and speciation. In particular, the formation of mountain ranges such as the Northern Anatolian Mountains (the Pontide Mountains, parallel to the Black Sea coast) and the Taurus Mountains (from south-west Anatolia, along the Mediterranean coast to the northern part of south-east Anatolia), the uplift of the Anatolian plateau between these mountain ranges, and the development of other more localized mountain ranges in uplifting blocks bounded by active fault zones may have had a considerable impact on the current distribution and genetic diversity of water frogs and other organismal groups in this area. Cyclic climate change, including the repeated glaciations in the last ~3 My at high northern latitudes in Europe and western Asia (e.g., Ehlers and Gibbard 2007), probably reduced the size of populations and isolated them in southern refugial areas (Hewitt 2000, 2004). In addition, contact zones between distinct genetic lineages can be used to test for the effects of allopatric divergence upon secondary contacts.

Eastern Mediterranean water frogs occur on many islands, separated from the mainland by effective salt-water barriers. Because frog skin is readily permeable to the ions in salt water, these barriers prevent exchange of individuals between island

and mainland populations. Water frogs have been introduced into many European countries (e.g., Schmeller et al. 2007; Holsbeek et al. 2008; Ohst 2008) but, provided that no individuals were introduced onto the islands by humans or arrived via rafting (e.g., Vences et al. 2003; Heinicke et al. 2007), and provided that the original populations on the adjacent mainlands have not been replaced by other lineages, the age of salt-water barriers isolating pairs of water frog populations should correspond to the minimum time that such pairs of populations have been genetically isolated (Beerli et al. 1996).

During a 6-year study, we have estimated, for several population pairs, the amount of genetic divergence using different sets of molecular markers, and have analyzed the role of genetic incompatibility and antihybridization mechanisms for speciation in eastern Mediterranean water frogs. The frogs studied represent at least four known and several cryptic undescribed species: one species of the *Pelophylax ridibundus* group and *P. epeiroticus* in the Peloponnese, *P. cretensis* (Crete), *P. bedriagae* (The Levant), a form restricted to Cyprus, and six more-or-less differentiated *ridibundus*-like forms distributed in Anatolia and on some Mediterranean islands. These latter six forms and the Cypriot form have provisionally been subsumed under the name *P.* cf. *bedriagae* (e.g., Plötner et al. 2001, Haefeli et al., unpublished) because their systematic status is not yet clear (Akın et al., in press).

2 Materials and Methods

2.1 Taxon Sampling

Frogs were collected at different localities in Anatolia and Greece including the islands Chios, Crete, Cyprus, Evvoia, Ikaria, Karpathos, Kerkyra (Corfu), Kythira, Lesvos, Rhodos, Samos, and Zakynthos (Table 1). Detailed locality information was published by Beerli et al. (1996), Haefeli (2005), Akın (2007), Plötner et al. (2001, 2009), and Akın et al. (2010).

2.2 Estimation of Genetic Divergence

Genetic divergence was estimated using three independent datasets: allozymes (products of up to 33 independent loci; Beerli et al. 1996, and unpublished data), nucleotide and amino acid sequences of two mitochondrial genes (NADH dehydrogenase subunits 2 and 3 or ND2 and ND3; e.g., Plötner et al. 2008, Akın et al., in press), and nucleotide sequences of serum albumin intron 1 (SAI1) and a non-LTR chicken repeat 1-like retrotransposon (*Rana*CR1) that was detected in SAI1 of water frog species (Plötner et al. 2009).

Table 1 Genetic distances between population pairs estimated on the basis of nucleotide (nu) and amino acid (aa) sequences of two mitochondrial genes (ND2, ND3), and by protein electrophoretic data (D^*_{Nei}, Beerli et al. 1996). Minimum times of geological isolation are compared with putative split times estimated from mean p distances using a rate of uncorrected genetic divergence of 1.1% per My. In parentheses: number of individuals/number of haplotypes. *cre: P. cretensis* (from Demati, Kastelli, Lavris, and Skinias), cf. *bed: P.* cf. *bedriagae*, *epe: P. epeiroticus*, *per: P. perezi*, *sah: P. saharicus*, *kur: P. kurtmuelleri* (individuals from the Peloponnese originated from the following populations: Kalanistra, Kaminia, Kavasilas, Lake Stimfalias, Nea Manolada, and Skala)

Population pair		Estimated split time (ND2 + ND3) [Mya]	Minimum time of geological isolation [Mya]	Population pair	p Distance (nu) [%]			p Distance (aa) [%]			D^*_{Nei}
					Min	Mean±SD	Max	Min	Mean±SD	Max	
Spain (1/1)	Morocco (9/9)	14.8	5.3	*per – sah*	16.18	16.33±0.114	16.47	9.17	9.28±0.156	9.39	0.550
Crete (9/7)	Akçapinar (4/3)	9.7–13.4	5.3 or 10.0	*cre* – cf. *bed*	10.53	10.71±0.135	10.89	4.38	4.60±0.215	4.81	0.584
	Peloponnese (10/6)			*cre – kur*	11.18	11.44±0.178	11.69	5.25	5.47±0.220	5.69	0.698
	Lechena (2/1)			*cre – epe*	14.60	14.74±0.114	14.89	7.88	7.99±0.156	8.10	0.530
Cyprus (3/2)	Ceyhan, Cevlik (4 /4)	5.6–6.0	5.3	cf. *bed* – cf. *bed*	6.39	6.46±0.070	6.53	3.49	3.60±0.156	3.71	–
	Silifke (2 /1)			cf. *bed* – cf. *bed*	6.17	6.17±0.000	6.17	3.49	3.60±0.156	3.71	–
	W-Syria (3/3)			cf. *bed – bed*	6.61	6.85±0.357	7.26	2.63	3.06±0.345	3.50	–
North Cyprus (2/1)	Silifke (2/1)	1.0–3.6	5.3	cf. *bed* – cf. *bed*	1.09	1.09±0.000	1.09	1.53	1.53±0.000	1.53	–
	Ceyhan, Cevlik (4 /4)			cf. *bed* – cf. *bed*	3.92	4.02±0.112	4.14	3.06	3.06±0.000	3.06	–
Karpathos (3/1)	Akçapinar (4/3)	0.43–1.3	1.8–3.0	cf. *bed* – cf. *bed*	1.38	1.42±0.050	1.45	0.87	0.98±0.156	1.09	0.26
	Fethiye, Kas, Antalia (4/4)			cf. *bed* – cf. *bed*	0.36	0.47±0.094	0.58	0.44	0.55±0.156	0.66	–
Rhodos (2/1)	Akçapinar (4/3)			cf. *bed* – cf. *bed*	1.38	1.42±0.050	1.45	0.87	0.98±0.156	1.09	0.18
	Fethiye, Kas, Antalia (4/4)			cf. *bed* – cf. *bed*	0.36	0.47±0.094	0.58	0.44	0.55±0.156	0.66	–

(*continued*)

Table 1 (continued)

Population pair		Estimated split time (ND2 + ND3) [Mya]	Minimum time of geological isolation [Mya]	Population pair	p Distance (nu) [%]			p Distance (aa) [%]			D^*_{Nei}
					Min	Mean±SD	Max	Min	Mean±SD	Max	
Andros (3/1)	Aliartos (3/3)	0.24–1.53	0.2–0.45	cf. *bed* – cf. *bed*	0.29	0.36±0.075	0.44	–	–	–	0.01
	Evvoia (3/2)			cf. *bed* – cf. *bed*	0.22	0.33±0.156	0.44	0.0	0.11±0.156	0.22	0.03
Ikaria (3/1)	Özbasi (3/1)			cf. *bed* – cf. *bed*	1.60	1.60±0.000	1.60	0.88	0.88±0.000	0.88	–
	Samos (2/2)			cf. *bed* – cf. *bed*	1.68	1.68±0.000	1.68	1.10	1.21±0.156	1.32	0.06
Kythira (3/1)	Peloponnese (10/6)			*kur* – *kur*	0.15	0.26±0.090	0.36	0.22	0.33±0.156	0.44	0.02
Chios (3/1)	Foça (3/3)	0.16–0.90	0.012–0.025	cf. *bed* – cf. *bed*	0.29	0.65±0.317	0.87	0.0	0.22±0.220	0.44	–
Evvoia (3/2)	Aliartos (3/3)			*kur* – *kur*	0.15	0.45±0.178	0.65	0.0	0.11±0.156	0.22	0.04
Kerkyra (2/1)	Igoumenitsa, Ioannina, Aitolikon (6/4)			*epe* – *epe*	0.36	0.99±1.028	2.18	0.44	0.88±0.615	1.31	–
Lesvos (4/3)	Edremit (2/2)			cf. *bed* – cf. *bed*	0.80	0.94±0.115	1.09	0.22	0.33±0.156	0.44	–
Samos (2/2)	Özbasi (3/1)			cf. *bed* – cf. *bed*	0.51	0.51±0.000	0.51	0.22	0.33±0.156	0.44	–
Zakynthos (5/3)	Peloponnese (10/6)			*kur* – *kur*	0.0	0.18±0.135	0.36	0.0	0.22±0.220	0.44	–

DNA sequences were aligned using CLUSTAL V (Higgins et al. 1992). For sequence statistics, the programs MEGA4 (Tamura et al. 2007) and DnaSP 5.0 (Librado and Rozas 2009) were used. Regression analyses were performed using Statgraphics Plus 4.1 (Statistical Graphics, StatPoint, Herndon, Virginia). Monophyletic groups were identified by Bayesian inference using the program MrBayes (Ronquist and Huelsenbeck 2003; Huelsenbeck and Ronquist 2005), with a GTR + I + G model of sequence evolution. For the phylogenetic tree, we augmented the sequences of Mediterranean water frogs (including *P. bedriagae*, the endemic Cypriot lineage, and all the Anatolian lineages of the *P. ridibundus* group) with sequences of *P. epeiroticus* and *P. cretensis* as outgroups.

2.3 Estimation of Rates and Times of Divergence Using a Non-constant Molecular Clock

BEAST (Drummond and Rambaut 2007), which takes all samples into account and allows for variable mutation rates among species lineages, was used with the mtDNA data to compare four geological scenarios and to re-estimate the divergence rate used for pairwise estimates (Akın et al., in press). In the Bayesian framework of BEAST, the times of internal nodes of the phylogeny can be associated with prior distributions that reflect prior knowledge about the time of speciation events associated with that node. We explored the power of our data to distinguish between four alternative scenarios concerning the putative isolation time of Crete with wide (half of the mean) and narrow (0.3) standard deviations:

1. Crete9w; isolation time $= 9.0 \pm 4.5$ Mya,
2. Crete9n; isolation time $= 9.0 \pm 0.3$ Mya,
3. Crete5w; isolation time $= 5.3 \pm 2.65$ Mya,
4. Crete5n; isolation time $= 5.3 \pm 0.3$ Mya.

The isolation time of Cyprus was set to 5.3 ± 0.3 Mya in all scenarios. BEAST was run for each scenario using a relaxed clock model that allows for differences between evolutionary rates among lineages; the rates were drawn from a lognormal prior distribution assuming that rates are not correlated. Each scenario was run five times with a burn-in of 10^6 iterations and 10^7 sampled iterations; subsequently, the five replicates were combined in Tracer (http://tree.bio.ed.ac.uk/software/tracer/) and analyzed; marginal likelihood estimates were then used to calculate Bayes factors (Kass and Raftery 1995), which were used to compare the different geological scenarios. Mutation rate was estimated using the TN93 model with site rate variation.

2.4 Crossing Experiments

To assess the amounts of genetic incompatibility between taxa, artificial inter- and intraspecific crosses were performed using standard methods (e.g., Berger et al.

1982, 1994). After reaching the free-swimming stage (stage 25; Gosner 1960) tadpoles were counted and hatching rates were calculated.

2.5 Bioacoustic Investigations

Recordings of advertisement calls were made in an artificial pond using a directional microphone (SONY ECM 707) and analyzed with Avisoft SASLab Pro version 4.2. Eleven call parameters were measured on the audio spectrograms and oscillograms (Haefeli 2005; Haefeli et al., unpublished). Five call parameters (call duration, pulse groups per call, duration of pulse groups, pulses per pulse group, interval between pulse groups) were directly compared with data published by H. Schneider and collaborators (reviewed by Plötner 2005). Temperature-dependent call parameters were predicted for a temperature of 20 °C, using the regression models published by Joermann et al. (1988), Schneider (1997), and Schneider and Sinsch (1992).

2.6 Female Choice Experiments

Female choice experiments were performed with 29 females from Crete (*P. cretensis*) and 26 lake frog females (*P. kurtmuelleri*) from the Peloponnese, following the experimental design developed by Roeseli and Reyer (2000). Two inwardly-facing speakers were placed 97 cm from the center of the arena on radii with an angle of 120° between them; the speakers were 160 cm apart. The basin was divided into 24 equal-sized sectors. Females were individually exposed to alternating vocalizations, with one loudspeaker playing the call of *P. cretensis*, the other the call of *P. kurtmuelleri* from the Peloponnese. The position of the females in the arena was registered every 7 s for 12 min. The overall interest of females was measured by the relative time a female spent in either loudspeaker sector, compared to the value expected under a random distribution. Haefeli (2005) provided additional details.

3 Results and Discussion

3.1 Genetic Diversity of Eastern Mediterranean Water Frogs

The extensive genetic diversity detected in eastern Mediterranean water frogs indicates the existence of several distinct lineages that may represent several evolutionary species (sensu Wiley 1978, 1981). Besides the main mitochondrial haplogroups (MHGs) specific for *P. epeiroticus* and *P. cretensis*, six additional

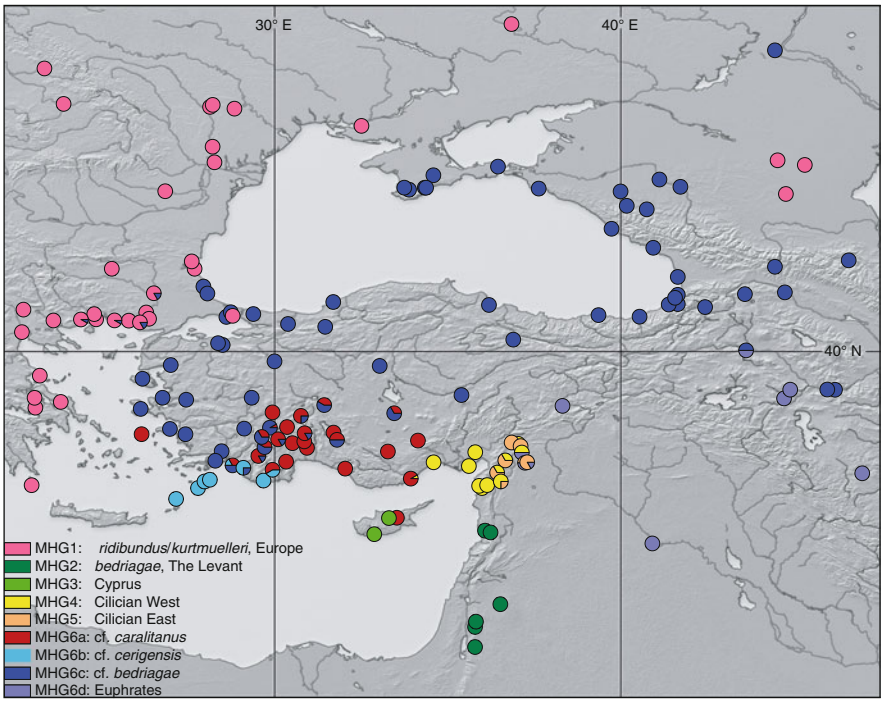
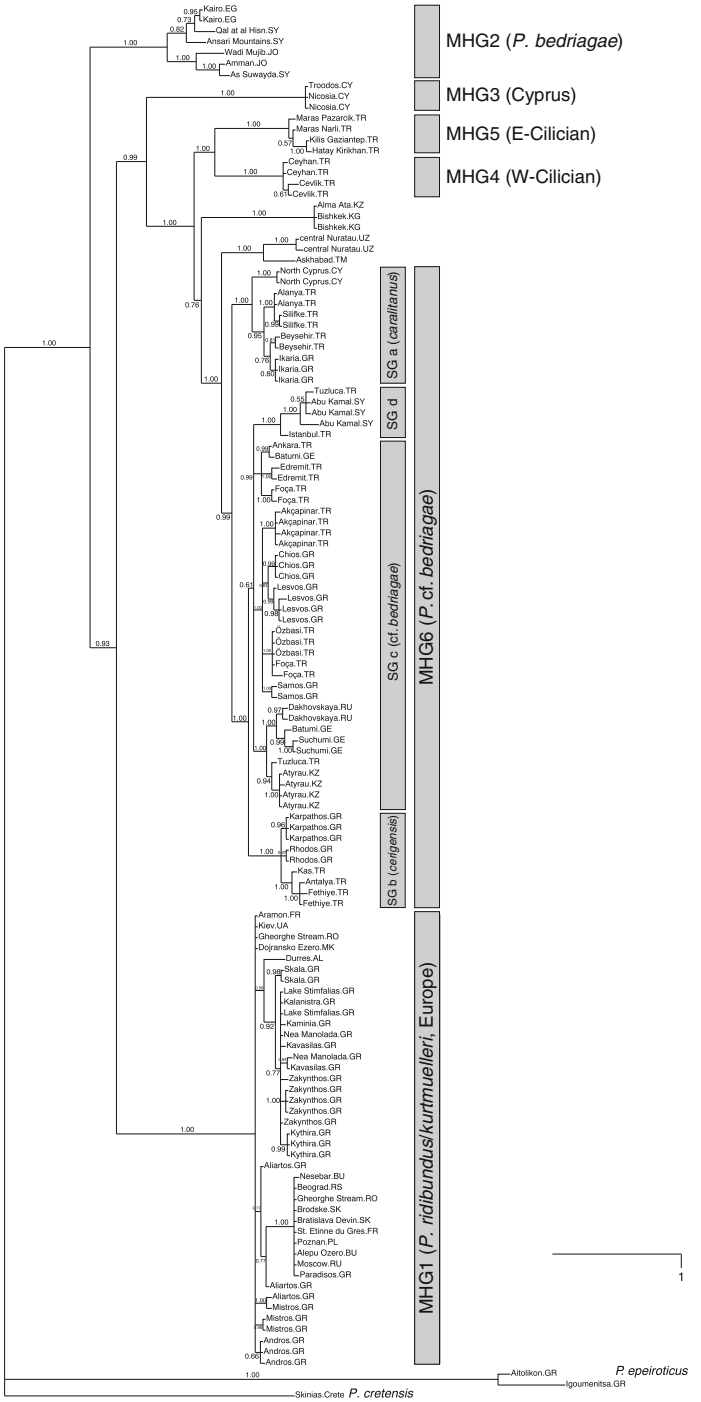


Fig. 1 Distribution of the main ND2/ND3 haplogroups (MHG) and subgroups of water frogs (genus *Pelophylax*) in the eastern Mediterranean region MHGs of *P. cretensis* and *P. epeiroticus* are not plotted.

MHGs can be distinguished among eastern Mediterranean water frogs (Akın et al., in press). These MHGs are defined by pairwise uncorrected *p* distances >3.5%; they are supported by specific geographic distribution (Fig. 1) and clade probabilities of 1.0 (Fig. 2).

MHG1 is composed of European *P. ridibundus*, including *P. kurtmuelleri* from the Balkan Peninsula. MHG2 comprises haplotypes of *P. bedriagae*, a species distributed in the Levant (Plötner 2005); haplotypes of this group are found in western Syria, Jordan, and the Nile delta. MHG3 comprises haplotypes from Cyprus. MHG4 haplotypes occur primarily in the Cilician plain of south-east Turkey, but also east of the Amanos Mountains, whereas MHG5 haplotypes only occur east of the Amanos Mountains. MHG4 and MHG5 represent sister clades with a sequence divergence of approximately 3.7%. Haplotypes of MHG6 are distributed from north-eastern Greece (Akın et al., in press; Hotz et al., in preparation) to central Russia; they are also found in Iran and Syria (Plötner et al. 2001, unpublished data). In some areas, MHG6 occurs syntopically with haplotypes of other groups, for example, with MHG4 in the western Cilician plain, with MHG5 in the eastern Cilician plain (Akın et al., in press), and with MHG1 in north-eastern Greece (Hotz et al., in preparation).


MHG2 (P. bedriagae)
MHG3 (Cyprus)
MHG5 (E-Cilician)
MHG4 (W-Cilician)
SG a (caralitanus)
SG d
SG c (cf. bedriagae)
SG b (cerigensis)
MHG6 (P. cf. bedriagae)
MHG1 (P. ridibundus/kurtmuelleri, Europe)
P. epeiroticus
P. cretensis
Kairo.EG
Qal at al Hisn.SY
Ansari Mountains.SY
Wadi Mujib.JO
Amman.JO
As Suwayda.SY
Troodos.CY
Nicosia.CY
Maras Pazarcik.TR
Maras Narli.TR
Kilis Gaziantep.TR
Hatay Kirikhan.TR
Ceyhan.TR
Cevlik.TR
Alma Ata.KZ
Bishkek.KG
central Nuratau.UZ
Askhabad.TM
North Cyprus.CY
Alanya.TR
Siirkke.TR
Beysehir.TR
Ikaria.GR
Tuzluca.TR
Abu Kamal.SY
Istanbul.TR
Ankara.TR
Batumi.GE
Edremit.TR
Foça.TR
Akçapinar.TR
Chios.GR
Lesvos.GR
Ozbasi.TR
Samos.GR
Dakhovskaya.RU
Suchumi.GE
Atyrau.KZ
Karpathos.GR
Rhodos.GR
Kas.TR
Antalya.TR
Fethiye.TR
Aramon.FR
Kiev.UA
Gheorghe Stream.RO
Dojransko Ezero.MK
Durres.AL
Skala.GR
Lake Stimfalias.GR
Kalanistra.GR
Kaminia.GR
Nea Manolada.GR
Kavasilas.GR
Zakynthos.GR
Kythira.GR
Aliartos.GR
Nesebar.BU
Beograd.RS
Brodske.SK
Bratislava Devin.SK
St. Etinne du Gres.FR
Poznan.PL
Alepu Ozero.BU
Moscow.RU
Paradisos.GR
Mistros.GR
Andros.GR
Altolikon.GR
Igoumenitsa.GR
Skinias.Crete
1

MHG6 can be divided into four subgroups (6a–d). Subgroup 6a is specific for a form that, because of particular morphological, karyological, bioacoustic, and enzymological characteristics (e.g., Jdeidi 2000; Alpagut Keskin and Falakalı Mutaf 2006), was assumed to represent a separate species, *P. caralitanus* (Jdeidi 2000; Jdeidi et al. 2001; Plötner 2005). Haplotypes of this group are distributed in south-western and south-central Anatolia, but are also found on the island of Ikaria and in northern Cyprus. They occur syntopically at the boundaries of their range with individuals carrying other haplotypes: with subgroup 6b to the south-west, with subgroup 6c to the north and west, and with MHG4 to the southeast (Akın et al., in press).

Subgroup 6b is characteristic of water frogs from Karpathos and Rhodos. Based on protein electrophoretic data, frogs inhabiting these islands were described as a separate species (*P. cerigensis*) by Beerli et al. (1994). Haplotypes of this subgroup are not restricted to Karpathos and Rhodos, however, but are also found in the coastal region of south-western Anatolia (Ohst 2001; Akın et al., in press).

Subgroup 6c has a wide distribution from eastern Greece in the west, beyond Anatolia to the Caspian Sea in the east, including Atyrau, the type locality of *P. ridibundus* on the northern shore of the Caspian Sea (Akın et al., in press; Plötner and Litvinchuk, unpublished data). The sequence divergence between haplotypes from Atyrau and haplotypes of Central European and Balkan lake frogs (MHG1) is about 6.5%, an indication that lake frogs from Atyrau and Central Europe are not conspecific. Haplotypes of subgroup 6c were also detected on several Mediterranean islands near the Anatolian coast, for example Chios, Lesvos, and Samos.

Haplotypes of subgroup 6d are found in the Tigris and Euphrates catchments of north-eastern Syria, eastern Anatolia and western Iran (Akın et al., in press; Plötner, unpublished data).

3.2 Genetic Divergence Between Population Pairs

Genetic divergence between selected population and species pairs (Table 1), calculated on the basis of protein electrophoretic data (Beerli et al. 1996), sequences of the mitochondrial genes ND2 and ND3 (Plötner et al. 2001; Akın et al., in press;

Fig. 2 (continued) Phylogenetic relationships of eastern Mediterranean water frogs estimated with Bayesian inference. The tree is based on 1,378 base pairs from the protein coding mitochondrial genes ND2 and ND3. The values at the branches are posterior probabilities for the clade to the right of that branch. The scale bar represents one expected mutation per site. *Pelophylax epeiroticus* and *P. cretensis* were used as outgroups. *MHG* Main haplogroup, *SG* subgroup. Country abbreviations (ISO-Code): *AL* Albania; *BU* Bulgaria; *CY* Cyprus; *EG* Egypt; *FR* France; *GE* Georgia; *GR* Greece; *JO* Jordan; *KG* Kyrgyzstan; *KZ* Kazakhstan; *MK* Macedonia; *PL* Poland; *RO* Romania; *RS* Republic Serbia; *RU* Russia; *SK* Slovakia; *TM* Turkmenistan; *TR* Turkey; *UA* Ukraine; *UZ* Uzbekistan

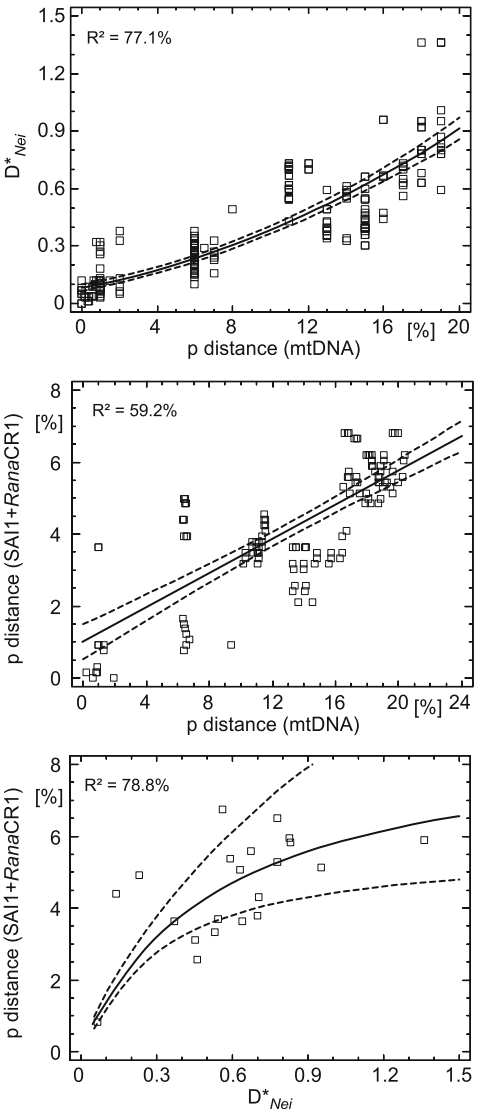


Fig. 3 Relationships between genetic distances based on mitochondrial (mt) DNA (ND2+ND3), nuclear (n) DNA of serum albumin intron 1 (SAI1) and *Rana*CR1, and protein electrophoretic data. Uncorrected p distances were calculated for both mtDNA and nuDNA. For protein electrophoretic data, modified Nei distances (D^*_{Nei}) were estimated as proposed by Hillis (1984). *Above* Uncorrected p distance (mtDNA) vs D^*_{Nei}. $D^*_{Nei} = (0.28 + 3.35*p_{mtDNA})^2$, $r = 0.88$, F-ratio = 709.8, $p < 0.01$. Middle: p_{mtDNA} vs p_{nDNA}. $p_{nDNA} = 0.01 + 0.24*p_{mtDNA}$, $r = 0.77$, F-ratio = 191.0, $p < 0.01$. *Below* D^*_{Nei} vs p_{nDNA}. $p_{nDNA} = 1/(11.2 + 6.06/D^*_{Nei})$, $r = 0.89$, F-ratio = 70.6, $p < 0.01$. *Solid line* regression line. *Broken lines* 95% confidence limits

Plötner unpublished data), and sequences of the nuclear markers SAI1 and *Rana*CR1 (Plötner et al. 2009) are significantly correlated with each other (Fig. 3).

We envisage that environmental changes associated with the Messinian Salinity Crisis (MSC) had a significant effect on the development of the genetically distinct frog populations in the study region. Until ~6 Mya the Mediterranean Sea was connected to the Atlantic, the connection being via shallow seaways across southern Spain and northern Morocco, not via the Strait of Gibraltar, which did not yet

exist (e.g., Krijgsman et al. 1999a). Uplift of Spain and Morocco gradually closed these seaways, so that around 6 Mya (5.96 Mya according to Krijgsman et al. 1999b) the Mediterranean basin became isolated from the Atlantic Ocean. Because evaporation was no longer balanced by inflow from the Atlantic, the Mediterranean basin became desiccated at this time, marking the start of the MSC. Isolation was initially intermittent (during global sea-level minima that were contemporaneous with maxima of high-latitude glaciation, restricted at this time to the Antarctic) but became complete by ~5.6 Mya (at 5.59 Mya according to Krijgsman et al. 1999b); with complete isolation, what is now the Mediterranean sea floor (in places ~3 km or more below modern sea-level) was covered by vast playas (salt pans) in which salts such as halite (NaCl) and gypsum ($CaSO_4$) were precipitated (Fig. 4). This was an extremely hostile environment, with estimated surface temperatures as high as ~80 °C; frogs could not have survived there. These conditions probably lasted for less than 100,000 years, however, and were followed (at 5.50 Mya according to Krijgsman et al., 1999b) by partial flooding of the Mediterranean basin (known as the "Lago-Mare" stage of the MSC), which created a low-salinity lacustrine realm that lasted circa 200,000 years. It has been proposed that the influx of freshwater to create the Lago-Mare paleo-lake was a consequence of temporary capture, by the Mediterranean basin with its reduced base-level, of the river Danube (e.g., Kvasov 1983). Alternative explanations, relating to increased rainfall in surrounding regions as a result of global climate change, have also been put forward (e.g., van der Laan et al. 2006; Hilgen et al. 2007). The Lago-Mare phase ended when,

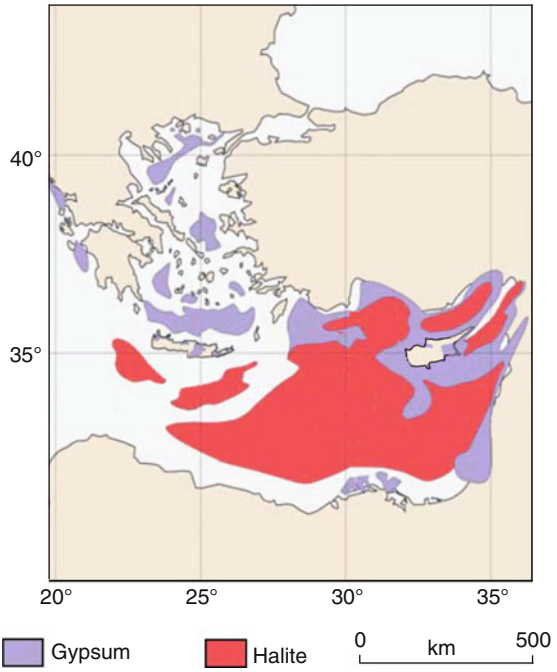


Fig. 4 Map of the eastern Mediterranean basin showing the distribution of evaporite deposits from the Messinian salinity crisis, modified from part of Fig. 1 of Hilgen et al. (2007). Modern coastlines are shown for location purposes and do not correspond to contemporaneous coastlines; the map does not attempt to restore changes in the relative positions and shapes of parts of the region caused by post-Messinian plate motions and related crustal deformation

apparently as a result of headward erosion of rivers in the westernmost Mediterranean (e.g., Loget et al. 2005), the Atlantic Ocean breached into the Mediterranean basin through what is now the Strait of Gibraltar and rapidly restored its water level to that of the global ocean. We adopt as the timing of this flooding event 5.33 Mya, from Krijgsman et al. (1999b), Hilgen et al. (2007), and others.

The environmental conditions during the Messinian in and around Cyprus were described in detail by Robertson (1998). During the evaporitic phase of the MSC, this island was largely surrounded by playas (Fig. 4); during the subsequent Lago-Mare phase, waters of the paleo-lake covered these areas. Sediments of the Lago-Mare facies are found both offshore of Cyprus in boreholes and onshore in low-lying parts of the island; the latter deposits reflect subsequent uplift of the island, the water surface during the Lago-Mare phase having been far below modern sea-level. The Kyrenia (Beşparmak) mountain range of northern Cyprus is structurally connected to the Misis mountain range east of Adana in southern Turkey by the Misis-Kyrenia Fault Zone (MKFZ), which forms part of the boundary between the Turkish and African plates (Fig. 5). This fault zone became active around the beginning of the MSC (e.g., Robertson et al. 2004; Westaway et al. 2008) and forms a significant linear ridge, both onshore in Cyprus and offshore to the northeast. As depicted schematically in Fig. 4, it appears to have protruded above the level of evaporite deposition during the MSC. We infer that it also remained above water level during the subsequent Lago-Mare phase, enabling frogs to migrate from Anatolia into Cyprus; this frog population became isolated when the normal sea-level was restored.

One may contrast this situation with that of Crete. As indicated in Fig. 4. Crete was surrounded by Messinian evaporite depocenters, parts of the island being low enough at the time (before subsequent uplift took place) to be covered by evaporite. Cosentino et al. (2007) have shown that much of Crete was subsequently inundated during the Lago-Mare phase. There is no evidence, however, of any adjoining

Fig. 5 Map of the eastern Mediterranean region, showing the boundaries (*red lines*) of the African (*AF*), Arabian (*AR*), Eurasian (*EU*), and Turkish (*TR*) plates. *MKFZ*: Misis-Kyrenia Fault Zone

geological structure, analogous to the MKFZ for Cyprus, that could permit migration of frogs to or from Crete at this time; Crete was evidently an island, surrounded by tens or hundreds of kilometres of open salt water in all directions. The local frog population (*P. cretensis*) probably became isolated before the MSC and survived its evaporitic phase, presumably in what are now the highest parts of the island, which were well above the contemporaneous playa level.

We conclude that the geological data strongly support a Messinian origin for Cypriot frogs and a potentially older isolation for frogs on Crete. The lower age bound for the isolation of Cyprus is the time of the end-MSC flooding at about 5.33 Mya; its upper bound is 5.50 Mya, the start of the Lago-Mare phase of the MSC. The Cyprus frogs (MHG3) differ in homologous positions of their ND2 +ND3 genes from Anatolian frogs (MHG6) by approximately 6%. The rate of development of uncorrected genetic divergence can thus be estimated as between 1.09% per My (6.0%/5.50 My) and 1.13% per My (6.0%/5.33 My) or approximately 1.1% per My.

3.3 Estimation of Confidence Limits for Divergence Times of Basic Lineages Using a Nonconstant Molecular Clock

The BEAST analysis (2.3) revealed only small statistical differences between the four geological scenarios; most credibility intervals of the time of divergence for particular clades overlap (Akın et al., in press). Crete appears to have become isolated before Cyprus but the posterior distributions of the divergence times overlap considerably. The scenario with strongly peaked prior distributions for the isolation of Crete and Cyprus achieved the highest marginal log likelihood (CRETE9n: $-10{,}511.035 \pm 0.164$), but the other scenarios produced very similar values: CRETE9w has $-10{,}511.474 \pm 0.177$, CRETE5w has $-10{,}511.623 \pm 0.183$, and CRETE5n has $-10{,}514.081 \pm 0.182$. The largest difference among scenarios is 3.046 log likelihood units; using Kass and Raftery's (1995) guidelines, this difference suggests that the scenarios CRETE5n and CRETE9n are different. The other scenarios, however, are only 0.588 and 0.439 log likelihoods units apart from CRETE9n, suggesting that they cannot be rejected. Selecting among the four scenarios, using Kass and Raftery's (1995) approach, results in probabilities of 0.445, 0.287, 0.247, and 0.021 for CRETE9n, CRETE9w, CRETE5w, and CRETE5n, respectively. Thus, although the CRETE9n scenario (involving isolation of Crete circa 9 Mya and Cyprus circa 5.3 Mya) is the most probable scenario considered, other possible scenarios (e.g., isolation of Crete earlier than Cyprus, but not circa 9 Mya) cannot be ruled out. The same is true for the separation of the western Mediterranean species pair *P. saharicus* (North Africa) and *P. perezi* (Iberian Peninsula) which is suggested to have occurred before 5.3 Mya (Table 1; Akın et al., in press). The present data are sufficient to reveal the order of the divergence events, but insufficient for exact correlation of geological events with species divergences.

BEAST also calculates a posterior estimate of the average expected mutation rate. The average mutation rate estimate for the best model was 0.00943 mutations per site per My with a coefficient of variation of 1.112 indicating a standard deviation of 0.0105. A standard deviation of the magnitude of the mean suggests considerable mutation rate heterogeneity among lineages in the phylogeny. A comparison using Bayes factors of the strict clock model versus the relaxed clock model with independent evolutionary rates and no divergence time constraints shows considerable support for the non-clock model (Akın et al., in press).

3.4 Evolution of Anatolian Water Frog Populations

Our sequence data are insufficient to choose among the four alternative scenarios for isolation of Crete from mainland areas, but they provide relatively strong support for the divergence sequence of the mitochondrial lineages themselves. The application of a constant divergence rate, however, offers plausible hypotheses on historical events that influenced the evolution of genetic diversity in Mediterranean water frogs, especially in Anatolian populations. If the divergence rate of 1.1% per My is applied to all Anatolian frogs, several lineages appear to have diverged between approximately 4 and 1 Mya (5.3–1.6 Mya, according to the BEAST analysis used by Akın et al., in press). The Cilician clades MHG4 and 5 may have split from the rest of the Anatolian populations (MHG6) first, with a divergence of about 4.0% from MHG6 populations and 3.7% from each other; these values correspond to divergence times of approximately 3.6 and 3.4 Mya, respectively (5.3–3.5 and 4.4–1.6 Mya, according to the BEAST analysis). Populations with haplotypes of MHG6 were probably isolated from the rest of the Anatolian populations by uplift of the central Taurus Mountains during the Late Miocene and Pliocene (Jaffey and Robertson 2005), although the timing of this uplift is not well constrained. The Cilician clades were probably separated from each other by the uplift of the Amanos Mountains, which began at ∼3.7 or ∼3.6 Mya as a result of a change in the pattern of plate motions in the region (Seyrek et al. 2008a; Westaway et al. 2008).

The observed rates of divergence imply a time window of 2.0–1.4 My (1.6–1.1 My, according to a more sophisticaed TrN + G model of sequence evolution used by Akın et al., in press) for diversification of the subgroups (a–d) of MHG6, so the radiation of these Anatolian lineages appears to have taken place around the Pliocene–Pleistocene boundary. This was a time of significant global cooling (e.g., Head and Gibbard 2005; Ehlers and Gibbard 2007), the boundary being currently defined at the first influx of cold-climate marine mollusc and ostracod taxa into the Mediterranean Sea, as recorded in sediments in southern Italy (e.g., Aguirre and Pasini 1985). In Britain, a dramatic increase in fluvially-transported sediment occurred at this time (e.g., Rose et al. 2002), thought to reflect a reduction in vegetation cover and, as a result, increased transport of sediments into rivers (e.g., Bridgland and Westaway 2007a, b). Resulting from more rapid erosion

consistent with the climate change around this time, increased rates of uplift occurred in some regions, for instance north of the Black Sea (e.g., Bridgland and Westaway 2007b), on the northern Arabian Platform in south-eastern Turkey and adjacent parts of Syria (Demir et al. 2007, 2008; Westaway et al. 2009), and in the eastern Taurus Mountains (Seyrek et al. 2008b). These processes may have reduced distributional ranges and enhanced the isolation of different lineages (Akın et al., in press).

The subgroups of MHG6 have largely contiguous geographical ranges of varying sizes, but the first three forms (cf. *caralitanus*, cf. *cerigensis*, and cf. *bedriagae*) are all found in south-western Anatolia. South-western Anatolia has often been proposed as a glacial refugium (e.g., Kosswig 1955; Schmidtler 1998) because of the presence of relict or endemic taxa, including the tree *Liquidambar* (Öztürk et al. 2008), the fishes *Aphanius* (Hrbek et al. 2002) and *Pseudophoxinus* (Hrbek et al. 2004), the salamander *Lyciasalamandra* (Veith and Steinfartz 2004), the mountain frog *Rana tavasensis* (Veith et al. 2003), and the snake *Vipera* (*ursinii*) *anatolica* (Joger et al. 1992). The presence, sometimes syntopically, of these closely related Anatolian water frog subgroups in this region probably indicates that two or more subgroups of MHG6 found refuge here during Pleistocene glaciations.

3.5 Genetic Incompatibilities

Genetic incompatibilities and conflicts of single genes, in conjunction with ecological factors, may result in lowered viability or fertility of hybrids or of the offspring of hybrids (e.g., Uzzell and Ashmole 1970; Guex et al. 2001, 2002; Orr 2005). The amount of genetic incompatibility can often be estimated by crossing experiments. The results of such experiments, however, have to be interpreted with caution because artificial crosses may differ from hybridization in nature in respect to fitness parameters such as hatching success, vitality, and fertility of hybrids. Among the factors that may have influenced the results of our crossing experiments, the long time of transport (up to 4 weeks) of parental individuals, artificial hormone treatment of females, and environmental conditions in the laboratory (e.g., food composition or water chemistry) could have had significant effects on the results. Although all crosses were performed with the same method, the physiological responses to artificial treatment were probably different among individuals and species. From the free-swimming stage (stage 25; Gosner 1960) to the onset of metamorphosis, water quality, density of individuals, and food supply are important parameters that influence the performance of larvae. In captivity, juvenile water frogs often suffer from bacterial and fungal infections resulting in high mortality, especially during and immediately after metamorphosis. We therefore mainly considered the hatching rate (stage 25) as a parameter of genetic incompatibility (Table 2), although it is known from former crossing experiments that viability of F_1 hybrids in water frogs is a weak estimator for genetic incompatibilities (e.g.,

Table 2 Hatching rates (minimum, geometric mean, maximum) of intra- and interspecific water frog crosses and uncorrected *p* distances (p) estimated for the forms crossed on the basis of two mitochondrial genes (ND2 and ND3). *cre: P. cretensis*, cf. *bed: P.* cf. *bedriagae* (Anatolia), *epe: P. epeiroticus*, *les: P. lessonae*, *kur: P. kurtmuelleri* (Balkan), *shq: P. shqipericus*, *n:* number of crosses

Female		Male		*n*	Hatching Rate [%]			p [%]
Species	Origin	Species	Origin		Min	Mean	Max	
Interspecific crosses								
cre	Ski	cf. *bed*	Akç	12	13.2	48.8	98.8	10.68
cf. *bed*	Akç	*cre*	Ski	4	22.5	46.1	88.9	
cre	Ski	*kur*	Ska	12	6.9	42.7	99.4	11.36
kur	Kav, Lec, Ska	*cre*	Ski	5	58.1	75.5	94.1	11.40
cre	Ski	*epe*	Kil, Lec	3	21.3	42.6	67.8	14.72
epe	Kil, Lec	*cre*	Ski	7	15.9	45.9	97.5	
cf. *bed*	Sil	*cre*	Ski	1	–	89.8	–	10.93
cf. *bed*	Sil	cf. *bed*	Cyp (N)	1	–	98.5	–	1.09
cf. *bed*	Cyp (N)	cf. *bed*	Sil	1	–	34.8	–	
les	Rog	*epe*	Igo, Lec	3	18.0	39.8	91.4	16.84
les	Rog	*kur*	Kav	1	–	66.9	–	15.13
les	Poz	*shq*	Vir	3[a]	31.7	59.2	94.6	8.52
shq	Vir	*les*	Poz	3[a]	89.4	91.4	91.7	
les	Poz	cf. *les*	Pia	6[a]	25.5	62.7	96.2	4.93
cf. *les*	Pia	*les*	Poz	3[a]	56.0	92.1	99.2	
Intraspecific crosses								
cre	Ski	*cre*	Ski	1	–	99.3	–	0
epe	Ker	*epe*	Igo	2	16.4	47.9	73.9	0.40
kur	Ska	*kur*	Ska	1	–	95.0	–	0
kur	Kav	*kur*	Zak	1	–	96.0	–	0.18

Localities: *Akç*: Akçapinar (Turkey); *Cyp (N):* North Cyprus (Turkey); *Igo:* Igoumenitsa (Greece); *Kav:* Kavasilas (Greece); *Ker:* Kerkyra (Greece); *Kil:* Kilini (Greece); *Lec:* Lechena (Greece); *Pia:* Piana di Catania (Italy, Sicily); *Poz:* Poznań (Poland); *Rog:* Rogaczewo (Poland); *Sil:* Silifke (Turkey); *Ska:* Skala (Greece); *Ski:* Skinias (Crete); *Vir:* Virpazar (Montenegro); *Zak:* Zakynthos (Greece)

[a]Data from Berger et al. (1994)

Günther 1973; Kawamura and Nishioka 1979, 1986; Berger et al. 1982, 1994); fertility of F_1 hybrids and the viability of progeny from backcrosses between F_1 hybrids and their parental species are more appropriate parameters to reveal genetic incompatibilities in water frogs.

Hatching rates of both intra- and interspecific crosses of different combinations showed extremely high variability; the values obtained for the combination *P. cretensis* female x *P. ridibundus* male, for example, ranged from 6.9 to 99.4%.

The significant negative but relatively weak correlation between the average hatching rate and the uncorrected pairwise genetic distance estimated from mtDNA (Fig. 6) probably reflects gradual genetic divergence of populations in allopatry. The R^2 statistic indicates that the model as fitted explains only about 25% of the variability in hatching rate; prediction of hatching rates for certain distance (p) values seems to be error prone. This is also seen if the proposed linear regression model is used to estimate the theoretical *p* value at which the hatching rate is zero

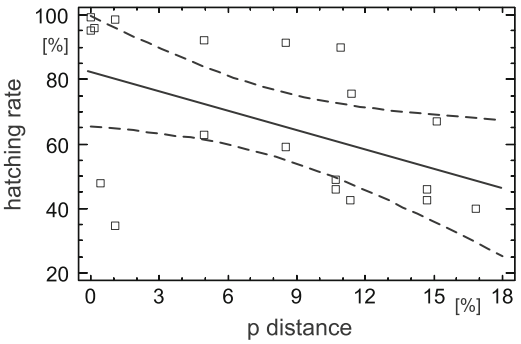


Fig. 6 Relationship between hatching rate (Table 2) and uncorrected p distance estimated from the mt genes ND2 and ND3 ($r = -0.50$, F-ratio $= 5.61$, $p < 0.05$). *Solid line* regression line. *Broken lines* 95% confidence limits

(ca. 41%). Such high divergence values are not observed, even between species that are completely isolated reproductively. For example, the ND2 genes of Central European *P. ridibundus* and the Palearctic brown frog species *Rana temporaria* show a sequence divergence of approximately 24%. Similar values (~21%) were obtained between the mtDNA sequences (ND2+ND3) of western Palearctic water frogs and the eastern Palearctic water frog species *P. nigromaculatus* and *P. plancyi* (Plötner and Ohst, unpublished data). As shown by several crossing experiments, the genomes of eastern Palearctic water frogs are almost incompatible with those of western Palearctic water frog species; there is usually complete isolation by F_1 hybrid inviability and sterility in crosses between the two groups (Kawamura et al. 1972; Kawamura and Nishioka 1979). These data clearly indicate that around a divergence level of 20%, ND genes of ranid frogs are saturated by multiple substitutions. Even at lower levels of divergence, mtDNA sequence divergence seems to be a rather inappropriate parameter to predict the degree of genetic incompatibility in western Palearctic water frogs.

Relatively high hatching rates seen in certain heterotypic crosses between genetically distinct species (e.g., *P. ridibundus* x *P. cretensis*) indicate that individual genomes can be more or less compatible. Natural interspecies F_1 hybrids, when observed, may originate from single exceptional crosses. This assumption is supported by the results of interspecies crosses, for example between *P. nigromaculatus* from Japan and *P. lessonae* from Luxembourg (Kawamura et al. 1972; Kawamura and Nishioka 1979). Normally, almost all embryos resulting from such crosses die before hatching. A few hybrids, however, were able to complete metamorphosis and some even reached maturity. Similar results were obtained by Berger et al. (1994), who performed interspecies water frog crosses, $F_1 \times F_1$ crosses, and backcrosses between F_1 hybrids and their parental species. For example, hatching success of crosses between *P. shqipericus* and *P. epeiroticus* varied between circa 74 and 97%. Two backcrosses, one to each parental species (using an F_1 hybrid individual from a *P. shqipericus* × *P. epeiroticus* cross that had a hatching rate of 74.4%), resulted in hatching rates of 42.6 and 97.3%.

If the high reproduction rate of female water frogs is taken into consideration (one female can lay several hundred to several thousand eggs per breeding season; e.g., Berger and Uzzell 1980), only a few successful interspecific crosses could be

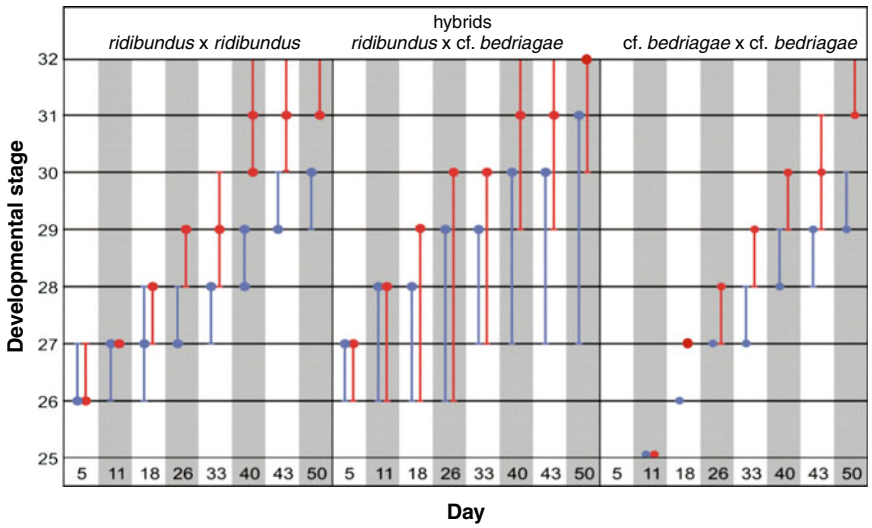


Fig. 7 Developmental stages (Gosner 1960) of F_1 larvae from homo- and heterospecific experimental crosses at different times after fertilization. *Red line* larvae kept in the laboratory. *Blue line* larvae kept outdoors. Points = mode. From Ohst (2008), modified

sufficient to maintain the occurrence of F_1 hybrids in areas of sympatry. The role of individual genome compatibility for hybrid fitness was also demonstrated by an experiment in which the development of F_1 individuals resulting from homo- and heterospecific crosses was analyzed under different environmental conditions (Ohst 2008). Compared to the homotypic progeny obtained from crosses of Central European *P. ridibundus* (MHG1) and of Anatolian lake frogs (MHG6c), F_1 larvae from a heterospecific cross between a Central European *P. ridibundus* female and an Anatolian *P.* cf. *bedriagae* male exhibited a greater heterogeneity in rate of larval development (Fig. 7). Whether hybrid inviability and sterility in western Palearctic water frogs is caused by single genes or is an additive effect of different loci remains to be investigated.

3.6 Antihybridization Mechanisms

Antihybridization mechanisms (also known as prezygotic or premating isolating mechanisms) serve to prevent the combination of two genetically incompatible genomes; they evolve because hybrids with maladapted diploid genomes have a lower fitness than their parental species. Genetic incompatibility between two or more lineages is often caused by the accumulation of genetic differences in allopatry. In contrast, antihybridization mechanisms, which minimize wastage of gametes as a result of heterospecific matings (e.g., Coyne and Orr 1998), are favored by

selection only when interbreeding occurs; they are, therefore, a sequela to speciation that is necessary to establishing sympatry of genetically incompatible lineages (Remington 1968).

In anuran amphibians, antihybridization mechanisms often include various elements of courtship and mating behavior, for example mating or advertisement calls of males that enable females to discriminate between conspecific and heterospecific males. Antihybridization mechanisms are only adaptive under sympatry. Nevertheless, they, or at least elements of them, may have already evolved in allopatry as indicated by the correlation between differences in advertisement calls and p distances (Fig. 8; Haefeli et al., unpublished).

While for many frog species, the mating call is known to be a major determinant of pair formation and thus syngamy (reviewed by Schneider and Sinsch 2007), its role as an integral element of antihybridization mechanisms in water frogs is not fully understood. For example, preliminary mate choice experiments with females from Crete and the Peloponnese, which represent genetically distinct species (*P. cretensis* and *P. kurtmuelleri*) with specific mating calls (Fig. 9), did not reveal a female preference for mating calls of conspecific males (Haefeli 2005; Haefeli et al., unpublished; Fig. 10). Despite the presence of female choice for mating, males can cause competition and sexual coercion because of highly male-biased operational sex ratios at crowded breeding sites (Bergen et al. 1997).

Although the behavior of females in such choice experiments is possibly biased by the artificial environments and/or the physiological conditions of the animals, field observations have also shown that quite distinct mating calls do not always prevent heterospecific matings and hybridization in areas of sympatry, for instance between *P. kurtmuelleri* and *P. epeiroticus* (Hotz and Uzzell 1982) or between

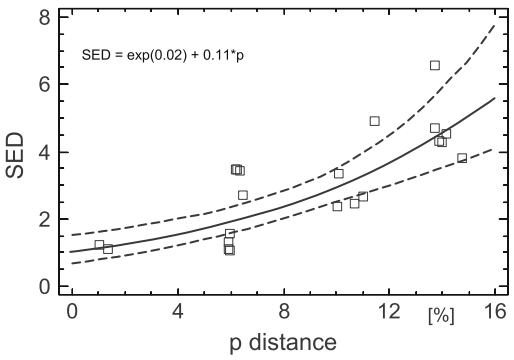


Fig. 8 Correlation between the Standard Euclidean Distance (SED) calculated on the basis of five mating call parameters of different water frog species (data from Joermann et al. 1988; Akef and Schneider 1989; Schneider and Sinsch 1992; Schneider and Haxhiu 1994; Schneider 1997, 1999; Haefeli 2005) and the uncorrected p distance obtained from the mt genes ND2 and ND3 of the same species ($r = 0.79$, F ratio $= 31.72$, $p < 0.01$). The R^2 statistic indicates that the model as fitted explains 62.5% of the variability in SED after transforming to a logarithmic scale to linearize the model. *Solid line* regression line. *Broken lines* 95% confidence limits

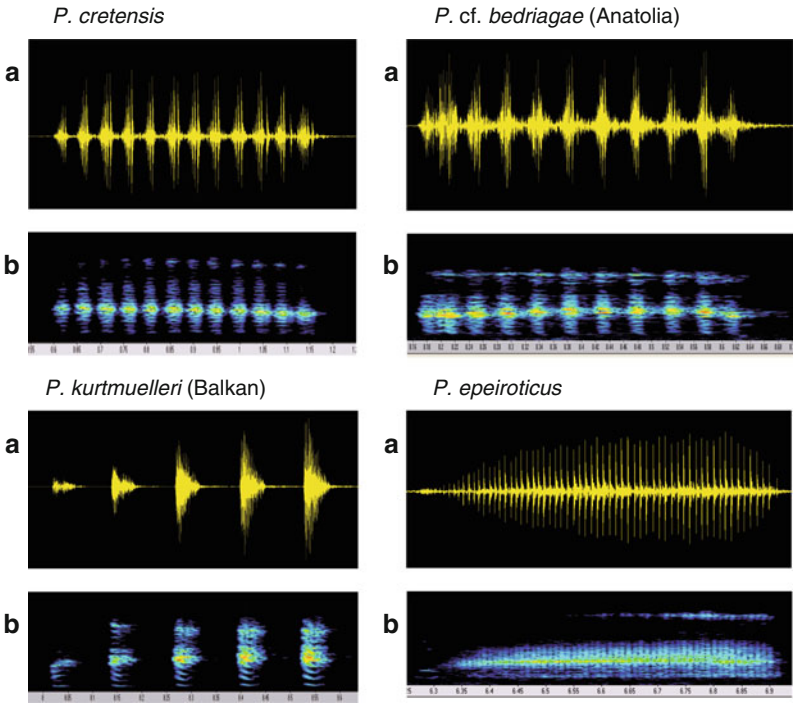


Fig. 9 Wave forms (**a**) and audio spectrograms (**b**) of mating calls recorded from *P. cretensis*, *P.* cf. *bedriagae* (Turkey), *P. kurtmuelleri* from the Peloponnese, and *P. epeiroticus* recorded at water temperatures of 26 °C (*cretensis*), 27 °C (cf. *bedriagae*), 28 °C (*kurtmuelleri*), and 26.5 °C (*epeiroticus*). From Haefeli (2005)

P. ridibundus and *P. lessonae* (Günther et al. 1991). Similarly, genetic investigations of two distinct groups with different mating calls in a transition zone, extending across north-eastern Greece and European Turkey, indicate nearly random mating among frogs in an area of secondary contact near the river Nestos (Beerli 1994; Hotz et al., in preparation). Similar observations have also been made in transition zones in Anatolia (Akın et al. 2010), such as in south-western Anatolia (MHG4 and 6) and east of the Amanos Mountains (MHG4, 5, and 6), where mtDNA haplotypes from distinct lineages are found syntopically in many populations. These observations are also supported by other findings in south-western Anatolia; individuals with the *caralitanus*-specific orange-colored ventral maculation have haplotypes of either subgroup 6b or 6c; and individuals with a black-spotted or white-colored venter may have *caralitanus*-specific haplotypes of subgroup 6a (Akın et al. 2010).

On the other hand, the low frequency of natural hybrids in western Greece, where *P. epeiroticus* and *P. kurtmuelleri* share the same habitats (e.g., Hotz and Uzzell 1982; Schneider et al. 1984; Berger et al. 1994), or in the Danube delta where *P. ridibundus* and *P. lessonae* occur sympatrically (Günther et al. 1991),

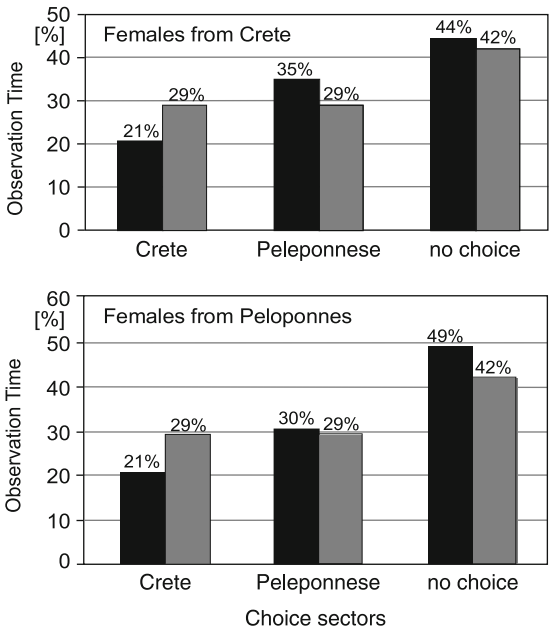


Fig. 10 Percentage of observation time that females of *P. cretensis* ($n = 26$) and of Balkanic *P. kurtmuelleri* ($n = 29$) spent in loudspeaker areas defined as conspecific and heterospecific and in no choice (indifferent) areas. *Black bars* observed values, *gray bars* expected values under random distribution. From Haefeli (2005)

speaks in favor of effective antihybridization mechanisms probably linked with the mating system, including mating calls. This assumption is consistent with empirical observations in western Greece where *P. epeiroticus* and *P. kurtmuelleri* occur syntopically but form separate choruses in the same aquatic habitat and at the same time. The choruses contained only single individuals of the foreign species and calling of *P. epeiroticus* choruses did not stimulate the calling activity of *P. kurtmuelleri* choruses (Kordges 1988). Similar observations were made by Günther (1982) in Lake Skutari (Montenegro) where *P. shqipericus* lives synptopically with *P. kurtmuelleri*.

3.7 *Implications for Water Frog Systematics*

Our genetic data suggest several new hypotheses on water frog systematics as a basis for future investigations. Most surprisingly, although all four individuals from the type locality of *P. ridibundus* (Atyrau, Kazakhstan) possessed mtDNA typical of the Anatolian clade (Fig. 2), they were all heterozygous for the nuclear markers SAI1 and *Rana*CR1, with one allele characteristic of Anatolian water frogs, the other of Central European lake frogs (Plötner et al. 2009). We therefore hypothesize that the northern Caspian Sea region represents a transition zone where different lineages have come into secondary contact and randomly interbreed.

As our molecular data clearly demonstrate, Anatolian water frogs and *P. bedriagae* from the Levant are not conspecific as proposed by Schneider and

co-workers (e.g., Schneider and Sinsch 1999). A sequence divergence in the mitochondrial ND2 and ND3 genes of approximately 6% (Ohst 2001; Akın et al., in press) and significant differences in the non-coding markers SAI1 and *Rana*CR1 (Plötner et al. 2009) clearly indicate that *P. bedriagae* and Anatolian water frogs represent distinct evolutionary species. Similarly, the Cilician MHG4 and 5 together represent a distinct clade separate from both *P. bedriagae* and the Anatolian (MHG6) lineages (Fig. 2). It is not yet clear, however, whether the observed degree of differentiation between MHG4, 5 and 6 (3.7–4.0%) warrants species status for the Cilician populations, jointly or separately.

On the other hand, the small genetic divergence values observed among the four subgroups (a–d) of the huge Anatolian MHG6 (1.5–2.2%) suggest that the subgroups are not distinct species. For instance, although populations belonging to subgroup b of MHG6 were described as a separate species under the name of *P. cerigensis* because of the presence of unique protein-coding alleles in Karpathos and Rhodos populations (Beerli et al. 1994), the samples used in this analysis were not fully representative of populations of that subgroup; specimens from the neighboring Anatolian populations from the coastal parts of the Antalya and Muğla provinces (Turkey), with exactly or very similar mitochondrial haplotypes, were not included in this study. The overall divergence between *cerigensis* and neighboring Anatolian subgroups does not exceed 1.3% (Table 1). The validity of *P. cerigensis* as a distinct species is not supported by our findings.

Similarly, although several authors (Jdeidi 2000; Jdeidi et al. 2001; Plötner 2005) have suggested raising *caralitanus* (MHG6a) to species status based on particular morphological, karyological, bioacoustic, and enzymological characteristics (e.g., Jdeidi 2000; Alpagut Keskin and Falakalı Mutaf 2006), its observed genetic distances to related forms and the extensive apparent hybridization with neighboring water frog populations (Akın et al. 2010) indicate that this suggestion was probably premature. Overall, despite, for example, Frost's (2008) treatment of the above-mentioned taxa as distinct species, the evolutionary relationships within Anatolian lineages require a taxonomic revision.

Two different lineages are represented on Cyprus, one closely related to Anatolian (MHG6) frogs of subgroup a, and the other (MHG3) unique to the island and of older origin. The genetic distinctness (ca. 6–7% pairwise distance; Table 1) and well-supported monophyly (Fig. 2) of the latter lineage confirms a separate species status for that group (Plötner et al. 2001; Plötner 2005). This hypothesis is also supported by sequence data obtained from SAI1 and *Rana*CR1 (Plötner and Akın, unpublished results). The same is true for Balkan lake frogs (Plötner et al. 2009), which were originally separated from Central European *P. ridibundus* on the basis of bioacoustic traits and named *Rana balcanica* by Schneider et al. (1993). Because morphological, protein electrophoretic, and mtDNA data have revealed only small differences between Balkan and Central European lake frogs, the systematic status of Balkan frogs has been questioned (e.g., Beerli et al. 1996; Plötner 1998, 2005; Plötner and Ohst 2001). Differences between the genomes of Balkan and Central European lake frogs are also expressed by crossing experiments with *P. lessonae*: while interspecies crosses between Central European *P. ridibundus*

and *P. lessonae* lead to hybridogenetic hybrids, crosses between Balkan lake frogs and *P. lessonae* result in non-hybridogenetic hybrids that are almost all sterile (Hotz et al. 1985; Berger et al. 1994). The results of crosses between Central European and Balkan lake frogs (Berger 1999, and unpublished data) also support the hypothesis that the two forms represent distinct species: backcross individuals (F_1 hybrids × parental species) are sterile and the F_2 progeny are not viable. We therefore propose to recognize the Balkan frogs as a separate species for which the name *Pelophylax kurtmuelleri* Gayda, 1940 is available (Dubois and Ohler 1994).

3.8 Conclusions and Prospects

Genetic divergence among eastern Mediterranean water frogs, including differences in advertisement calls, appears to be the result of gradual genetic divergence in allopatry, closely associated with the geodynamic evolution of the Mediterranean since the Middle Miocene (i.e., since circa 11 Mya). Dispersal between populations has been prevented by the development of localized mountain ranges, waterless plateaux, and salt-water barriers that have originated as a result of plate motions, regional uplift and subsidence, and environmental changes such as the MSC. In addition, cyclic climatic events during the last 3 My further restricted the migration pattern and resulted in subdivision among populations, especially in the eastern Mediterranean region. We hypothesize three main factors to be important in speciation of eastern Mediterranean water frogs: (1) accumulation of genetic differences during periods of allopatry in glacial refugia and via subdivision of populations by the development of localized mountain barriers or regional uplift; (2) natural selection and adaptation imposed by different environments; and (3) stochastic effects associated with colonization and an extreme reduction in population size. The latter factor is thought to play an important role for several island populations (e.g., Rhodos and Karpathos) while the first and second factors appear to promote the evolution of most mainland populations. The extreme morphological stasis of the eastern Mediterranean water frogs suggests low adaptive phenotypic evolution. Despite this high similarity in morphology, they show considerable genetic divergence (1.5–6.9%), indicating the existence of several cryptic species with varied degrees of differentiation.

Hybridization, observed, for example, in eastern Greece and western Anatolia (Hotz et al., in preparation) and in the western Peloponnese (Hotz and Uzzell 1982), can be interpreted as results of secondary contacts between already divergent lineages. Under sympatry, one can expect a more rapid divergence of traits associated with antihybridization mechanisms, for example, mating signals. Because differences in advertisement calls have typically not diverged more rapidly than genetic traits (Fig. 8), natural selection, and drift in allopatry are apparently the main forces for the evolution of antihybridization mechanisms in water frogs, rather than sexual selection. Upon secondary contact, however, isolating mechanisms might be reinforced (Dobzhansky 1940), as seen in the sympatric species

P. kurtmuelleri/*P. epeiroticus* which have quite different mating calls (Fig. 9): *P. epeiroticus* mating calls are characterized by many very short pulse groups with few pulses per group (cf. Schneider et al. 1984) whereas mating calls of Balkan *P. kurtmuelleri* are characterized by a few relatively long pulse groups with many pulses per group (Schneider et al. 1984, 1993; Schneider and Sinsch 1992). We do not know, however, whether antihybridization mechanisms, genetic incompatibilities, or both cause the low frequency of hybrids between these species. Future work will also elucidate the role of ecological and demographic factors in causing speciation in Mediterranean water frogs.

Acknowledgments We thank Rainer Günther (Berlin) for constructive comments on the manuscript. Grateful thanks are due to Metin Bilgin (Urbana-Champaign) for DNA sequencing and the development of a new DNA extraction protocol. We thank Christina Scheib (Eberswalde), Sylvia Glaser (Berlin), Kai-Joachim Schultze (Berlin), and Ronny Knop (Berlin) for technical assistance. For providing water frog samples, we thank Wolfgang Böhme (Bonn), Petr Kotlik (Libechov), Peter Mikuliček (Prague), and Mathias Stöck (Lausanne). Tissue samples from Greece were made available by the Greek Ministry of Rural Development and Food, kindly mediated by Doris Tippmann (Embassy of the FRG, Athens). This research was supported by the Deutsche Forschungsgemeinschaft (grants PL 213/3-1, 3-2, 3-3) and the Swiss National Fund (grants 31-37579.93, 31-59144.99, 31-103903/1 and 31-64004.00). Çiğdem Akın and Can Bilgin were supported by METU Research Fund (BAP-08-11-DPT-2002K120510). Peter Beerli was partly supported by the joint NSF/NIGMS Mathematical Biology programme under NIH grant R01 GM 078985 and by NSF grant DEB 0822626. Spartak N. Litvinchuk was partly supported by a DAAD grant under A/03/06782.

References

Aguirre E, Pasini G (1985) The pliocene-pleistocene boundary. Episodes 8:116–120

Akef MSA, Schneider H (1989) The eastern form of *Rana ridibunda* (Anura: Ranidae) inhabits the Nile delta. Zool Anz 223:129–138

Akın Ç (2007) Detection of species boundaries in the *Rana ridibunda* complex of southwestern Turkey using mitochondrial ND3 marker. M. Sc. Thesis, Middle East Technical University, Ankara, Turkey

Akın Ç, Bilgin M, Bilgin CC (2010) Discordance between ventral colour and mtDNA haplotype in the water frog *Rana* (*ridibunda*) *caralitana*, 1988 Arıkan. Amphibia-Reptilia 31:9–20

Akın Ç, Bilgin CC, Beerli P, Westaway R, Ohst T, Litvinchuk SN, Uzzell T, Bilgin M, Hotz H, Guex G-D, Plötner J (in press) Phylogeographic patterns of genetic diversity in eastern Mediterranean water frogs have been determined by geological processes and climate change in the Late Cenozoic. J Biogeography

Alpagut Keskin N, Falakalı Mutaf B (2006) Rod-shaped bivalents indicate assemblage among Anatolian water frog populations. Amphibia-Reptilia 27:47–53

Baak EJ, Rieseberg LH (2007) A genomic view of introgression and hybrid speciation. Cur Opin Gen Dev 17:513–518

Beerli P (1994) Genetic isolation and calibration of an average protein clock in the western Palearctic water frogs of the Aegean region. PhD thesis, University of Zurich, Switzerland

Beerli P, Hotz H, Uzzell T (1996) Geological dated sea barriers calibrate a protein clock for Aegean water frogs. Evolution 50:1676–1687

Beerli P, Hotz H, Tunner HG, Heppich S, Uzzell T (1994) Two new water frog species from the Aegean Islands Crete and Karpathos (Amphibia, Salientia, Ranidae). Notulae Naturae 470:1–9

Bergen K, Semlitsch RD, Reyer H-U (1997) Hybrid female mating are directly related to the availability of *Rana lessonae* and *Rana esculenta* males in experimental populations. Copeia 1997:275–283

Berger L (1999) Relationships of western Palearctic water frog taxa based on crossing experiments. In: III. International symposium on genetics, systematics and ecology of western Palearctic water frogs, Berlin, Abstract (unpublished)

Berger L, Uzzell T (1980) The eggs of European water frogs (*Rana esculenta* complex) and their hybrids. Folia Biol (Kraków) 28:3–25

Berger L, Uzzell T, Hotz H (1982) Crossing experiments between some western Palearctic species of water frogs (Salientia: Ranidae). Vertebr Hung 21:33–45

Berger L, Uzzell T, Hotz H (1994) Postzygotic reproductive isolation between Mendelian species of European water frogs. Zool Pol 39:209–242

Bolnick DI, Fitzpatrick BM (2007) Sympatric speciation: models and empirical evidence. Annu Rev Ecol Evol Syst 38:459–487

Bridgland DR, Westaway R (2007a) Climatically controlled river terrace staircases: a worldwide Quaternary phenomenon. Geomorphology 98:285–315

Bridgland DR, Westaway R (2007b) Preservation patterns of late Cenozoic fluvial deposits and their implications: results from IGCP 449. Quatern Int 189:5–38

Bullini L (1994) Origin and evolution of animal hybrid species. Trends Ecol Evol 9:422–425

Carson HL, Templeton AR (1984) Genetic revolutions in relation to speciation phenomena: the founding of new populations. Annu Rev Ecol Syst 15:97–131

Cosentino D, Gliozzi E, Pipponzi G (2007) The late Messinian Lago-Mare episode in the Mediterranean Basin: preliminary report on the occurrence of Paratethyan ostracod fauna from central Crete (Greece). Geobios 40:339–349

Coyne JA, Orr HA (1998) The evolutionary genetics of speciation. Philos Trans R Soc Lond B 353:287–305

Demir T, Seyrek A, Westaway R, Bridgland D, Beck A (2008) Late Cenozoic surface uplift revealed by incision by the River Euphrates at Birecik, southeast Turkey. Quatern Int 186:132–163

Demir T, Westaway R, Bridgland D, Pringle M, Yurtmen S, Beck A, Rowbotham G (2007) Ar-Ar dating of Late Cenozoic basaltic volcanism in northern Syria: implications for the history of incision by the river Euphrates and uplift of the northern Arabian platform. Tectonics 26, 10.1029/2006TC001959

Dobzhansky T (1940) Speciation as a stage in evolutionary divergence. Am Nat 74:312–321

Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 7:214. doi:10.1186/1471-2148-7-214

Dubois A, Ohler A (1994) Frogs of the subgenus *Pelophylax* (Amphibia, Anura, Genus *Rana*): a catalogue of available scientific names, with comments on name-bearing types, complete synonymies, proposed common names, and maps showing all type localities. Zool Pol 39:139–204

Ehlers J, Gibbard PL (2007) The extent and chronology of Cenozoic global glaciation. Quatern Int 164–165:6–20

Frost DR (2008) Amphibian species of the world: an online reference. Version 5.2 (15 July 2007). Electronic database available via http://research.amnh.org/herpetology/amphibia/index.html. American Museum of Natural History, New York, USA

Gosner KL (1960) A simplified table for staging anuran embryos with notes on identification. Herpetologica 16:183–196

Günther R (1973) Über die verwandtschaftlichen Beziehungen zwischen den europäischen Grünfröschen und den Bastardcharakter von *Rana esculenta* L. (Anura). Zool Anz 190:250–285

Günther R (1982) Ergebnisse experimenteller Kreuzungen zwischen Wasserfröschen (Anura, Ranidae) aus verschiedenen Ländern Europas und Mittelasiens. Vertebr Hung 21:157–167
Günther R, Plötner J, Tetzlaff I (1991) Zu einigen Merkmalen der Wasserfrösche (*Rana* synkl. *esculenta*) des Donau-Deltas. Salamandra 27:246–265
Guex GD, Hotz H, Semlitsch R (2002) Deleterious alleles and differential viability in progeny of natural hemiclonal frogs. Evolution 56:1036–1044
Guex GD, Hotz H, Uzzell T, Semlitsch R, Beerli P, Pascolini R (2001) Developmental disturbances in *Rana esculenta* tadpoles and metamorphs. Mitt Mus Nat kd Berl, Zool Reihe, 77:79–86
Haefeli C (2005) Variation in advertisement calls among geographically isolated water frogs. Diploma Thesis, University Zurich
Haefeli C, Hotz H, Guex G-D, Uzzell T, Beerli P, Plötner J, Reyer H-U (unpublished manuscript) Advertisement calls and genetic divergence among water frog species in the eastern Mediterranean
Head MJ, Gibbard PL (2005) Early-Middle Pleistocene transitions: the land and ocean evidence. Geol Soc Lond Spec Publ 247:1–18
Hewitt GM (2000) The genetic legacy of the quaternary ice ages. Nature 405:907–913
Hewitt GM (2004) Genetic consequences of climatic changes in the Quaternary. Philos Trans R Soc Lond B 359:183–195
Higgins DG, Bleasby AJ, Fuchs R (1992) CLUSTAL V: improved software for multiple sequence alignment. CABIOS 8:189–191
Hilgen FJ, Kuiper K, Krijgsman W, Snel E, van der Laan E (2007) Astronomical tuning as the basis for high resolution chronostratigraphy: the intricate history of the Messinian salinity crisis. Stratigraphy 4:231–238
Hillis DM (1984) Misuse and modification of Nei's genetic distance. Syst Zool 33:238–240
Holsbeek G, Mergeay J, Hotz H, Plötner J, Volckaert M, de Meester L (2008) A cryptic invasion within an invasion and widespread introgression in the European water frog complex: consequences of uncontrolled commercial trade and weak international legislation. Mol Ecol 17:5023–5035
Hotz H, Uzzell T (1982) Biochemically detected sympatry of two water frog species: two different cases in the Adriatic Balkans (Amphibia: Ranidae). Proc Acad Nat Sci Phila 134:50–79
Hotz H, Mancino G, Bucci-Innocenti S, Ragghiati M, Berger L, Uzzell T (1985) *Rana ridibunda* varies geographically in inducing clonal gametogenesis in interspecies hybrids. J Exp Zool 236:199–210
Hrbek T, Küçük F, Frickey T, Stölting KN, Wildekamp RH, Meyer A (2002) Molecular phylogeny and historical biogeography of the *Aphanius* (Pisces, Cyprinodontiformes) species complex of central Anatolia, Turkey. Mol Phyl Evol 25:125–137
Hrbek T, Stölting KN, Bardakçı F, Küçük F, Wildekamp RH, Meyer A (2004) Plate tectonics and biogeographical patterns of the *Pseudophoxinus* (Pisces: Cypriniformes) species complex of central Anatolia, Turkey. Mol Phyl Evol 32:297–308
Huelsenbeck JP, Ronquist F (2005) Bayesian analysis of molecular evolution using MrBayes. In: Nielsen R (ed) Statistical methods in molecular evolution. Springer, New York, pp 183–232
Jaffey N, Robertson A (2005) Non-marine sedimentation associated with oligocene-recent exhumation and uplift of the central taurus mountains, S Turkey. Sediment Geol 173:53–89
Jdeidi T (2000) Enzyme polymorphism, morphometric and bioacoustic studies in water frog complex in Turkey. PhD thesis, Middle East Technical University, Ankara, Turkey
Jdeidi T, Bilgin CC, Kence M (2001) New localities extend the range of *Rana bedriagae caralitana* Arıkan, 1988 (Anura: Ranidae) further west and suggest specific status. Turk J Zool 25:153–158
Joger U, Hermann H-W, Nilson G (1992) Molecular phylogeny and systematics of viperine snakes. II. A revision of the *Vipera ursinii* complex. In: Korsos Z, Kiss J (eds) Proc sixth ordinary general meeting SEH. Hungarian Natural History Museum, Budapest, pp 239–244

Joermann G, Baran I, Schneider H (1988) The mating call of *Rana ridibunda* (Amphibia: Anura) in western Turkey: bioacoustic analysis and taxonomic consequences. Zool Anz 220:225–232

Kass RE, Raftery AE (1995) Bayes factors. J Am Stat Ass 90:773–795

Kawamura T, Nishioka M (1979) Isolating mechanisms among the water frog species distributed in the Palearctic region. Mitt Zool Mus Berlin 55:171–185

Kawamura T, Nishioka M (1986) Hybridization experiments among *Rana lessonae*, *Rana ridibunda* and *Rana esculenta*, with special reference to hybridogenesis. Sci Rep Lab Amphibian Biol Hiroshima Univ 8:117–271

Kawamura T, Nishioka M, Kuramoto M (1972) Interspecific hybrids between Japanese and European pond frogs. Sci Rep Lab Amphibian Biol, Hiroshima Univ 1:277–301

Kordges T (1988) Beobachtungen am Epirusfrosch (*Rana epeirotica*), dem neuen griechischen Wasserfrosch. In: Günther R, Klewen R (eds) Beiträge zur Biologie und Bibliographie (1960–1987) der europäischen Wasserfrösche. Jb Feldherp, Beiheft 1:135–143

Kosswig C (1955) Zoogeography of the near East. Syst Zool 4:49–73

Krijgsman W, Hilgen FJ, Raffi I, Sierro FJ, Wilson DS (1999a) Chronology, causes and progression of the Messinian salinity crisis. Nature 400:652–655

Krijgsman W, Langereis CG, Zachariasse WJ, Boccaletti M, Moratti G, Gelati R, Iaccarino S, Papani G, Villa G (1999b) Late neogene evolution of the Taza-Guercif Basin (Rifian Corridor, Morocco) and implications for the Messinian Salinity Crisis. Mar Geol 153:147–160

Kvasov DD (1983) Causes of the marked regression of the Black and Caspian seas about five million years ago. Oceanology 23:331–335

Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics. doi:10.1093/bioinformatics/btp187

Loget N, van den Driessche J, Davy P (2005) How did the Messinian Salinity Crisis end ? Terra Nova 17:414–419

Öztürk M, Çelik A, Güvensen A, Hamzaoğlu A (2008) Ecology of tertiary relict endemic *Liquidambar orientalis* Mill. forests. For Ecol Manag 256:510–518

Orr HA (2005) The genetic basis of reproductive isolation: insights from *Drosophila*. Proc Natl Acad Sci USA 102(Suppl 1):6522–6526

Ohst T (2001) Untersuchungen zur stammesgeschichtlichen Entwicklung des westpaläarktischen Wasserfroschkomplexes (Anura, Ranidae) auf der Grundlage von DNA-Sequenzen des mitochondrialen ND2- und ND3-Gens. Diplomarbeit, Humboldt-Universität, Berlin

Ohst T (2008) Genetische Einflüsse allochthoner Wasserfrösche auf endemische Wasserfroschpopulationen (*R.* kl. *esculenta* Komplex). Diss, Humboldt-Universität, Berlin

Plötner J (1998) Genetic diversity in mitochondrial 12S rDNA of western Palearctic water frogs (Anura, Ranidae) and implications for their systematics. J Zool Syst Evol Res 36:191–201

Plötner J (2005) Die westpaläarktischen Wasserfrösche. Von Märtyrern der Wissenschaft zur biologischen Sensation. Z f Feldherpetologie, Beiheft 9, Laurenti, Bielefeld

Plötner J, Ohst T (2001) New hypothesis on the systematics of the western Palearctic water frog complex (Anura: Ranidae). Mitt Mus Nat kd Berl, Zool Reihe 77:5–21

Plötner J, Ohst T, Böhme W, Schreiber R (2001) Divergence in mitochondrial DNA of Near Eastern water frogs with special reference to the systematic status of Cypriote and Anatolian populations (Anura, Ranidae). Amphibia-Reptilia 22:397–412

Plötner J, Uzzell T, Beerli P, Spolsky C, Ohst T, Litvinchuk SN, Guex G-D, Reyer H-U, Hotz H (2008) Widespread unidirectional transfer of mitochondrial DNA: a case in western Palearctic water frogs. J Evol Biol 21:668–681

Plötner J, Köhler F, Uzzell T, Beerli P, Schreiber R, Guex G-D, Hotz H (2009) Evolution of serum albumin intron-1 is shaped by a 5′ truncated non-long terminal repeat retrotransposon in western Palearctic water frogs (Neobatrachia). Mol Phyl Evol 53:784–791

Remington CL (1968) Suture-zones of hybrid interaction between recently joined biotas. Evol Biol 2:321–428

Robertson AHF (1998) Late Miocene paleoenvironments and tectonic setting of the southern margin of Cyprus and the Eratosthenes Seamount. Proc Ocean Drill Program Sci Results 160:453–463

Robertson A, Ünlügenç ÜC, İnan N, Taşlı K (2004) The Misis-Andırın complex: a mid-tertiary melange related to late-stage subduction of the Southern Neotethys in S Turkey. J Asian Earth Sci 22:413–453

Roeseli M, Reyer H-U (2000) Male vocalization and female choice in the hybridogenetic *Rana lessonae/Rana esculenta* complex. Anim Behav 60:745–755

Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574

Rose J, Candy I, Moorlock BSP, Wilkins H, Lee JA, Hamblin RJO, Lee JR, Riding JB, Morigi AN (2002) Early and early Middle Pleistocene river, coastal and neotectonic processes, southeast Norfolk, England. Proc Geol Assoc 113:47–67

Schmeller DS, Pagano A, Plénet S, Veith M (2007) Introducing water frogs – Is there a risk for indigenous species in France? C R Biol 330:684–690

Schmidtler JF (1998) Verbreitungsstrukturen der Herpetofauna im Taurus-Gebirge, Türkei (Amphibia; Reptilia). Faunistische Abh Staatl Mus Tierkunde Dresden 21(Suppl):133–148

Schneider H (1997) Calls and reproductive behaviour of the water frogs of Damascus, Syria (Amphibia: Anura: *Rana bedriagae* Camerano, 1882). Zool Middle East 15:51–66

Schneider H (1999) Calls of the Levantine frog, *Rana bedriagae*, at Birket Ata, Israel (Amphibia: Anura). Zool Middle East 19:101–116

Schneider H, Haxhiu I (1994) Mating-call analysis and taxonomy of the water frogs in Albania (Anura: Ranidae). Zool Jb Syst 121:248–262

Schneider H, Sinsch U (1992) Mating call variation in lake frogs referred to as *Rana ridibunda* Pallas, 1771. Taxonomic implications. Z zool Syst Evol-forsch 30:297–315

Schneider H, Sinsch U (1999) Taxonomic reassessment of Middle Eastern water frogs: bioacoustic variation among populations considered as *Rana ridibunda, R. bedriagae* or *R. levantina*. J Zool Syst Evol Res 37:57–65

Schneider H, Sinsch U (2007) Contribution of bioacoustics to the taxonomy of the Anura. In: Heathwole H (ed) Amphibian biology, vol 7. Surrey Beatty, Chipping Norton, pp 2893–2932

Schneider H, Sofianidou TS, Kyriakopoulou-Sklavounou P (1984) Bioacoustic and morphometric studies in water frogs (genus *Rana*) of lake Ioannina in Greece, and description of a new species (Anura, Amphibia). Z zool Syst Evol-forsch 22:349–366

Schneider H, Sinsch U, Sofianidou TS (1993) The water frogs of Greece. Bioacoustic evidence for a new species. Z zool Syst Evol-forsch 31:47–63

Seehausen O (2004) Hybridization and adaptive radiation. Trends Ecol Evol 19:198–207

Seyrek A, Demir T, Pringle M, Yurtmen S, Westaway R, Bridgland D, Beck A, Rowbotham G (2008a) Late Cenozoic uplift of the Amanos Mountains and incision of the middle Ceyhan river gorge, southern Turkey; Ar-Ar dating of the Düziçi basalt. Geomorphology 97:321–355

Seyrek A, Westaway R, Pringle M, Yurtmen S, Demir T, Rowbotham G (2008b) Timing of the Quaternary Elazığvolcanism, eastern Turkey, and its significance for constraining landscape evolution and surface uplift. Turkish J Earth Sci 17:497–541

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Uzzell T, Ashmole NP (1970) Suture-zones: an alternative view. Syst Zool 19:197–199

van der Laan E, Snel E, de Kaenel E, Hilgen FJ, Krijgsman W (2006) No major deglaciation across the Miocene-Pliocene boundary: integrated stratigraphy and astronomical tuning of the Loutja section (Bou Regreg area, NW Morocco). Paleoceanography 21:PA3011. doi:10.1029/2005PA001193

Vences M, Vieites DR, Glaw F, Brinkmann H, Kosuch J, Veith M, Meyer A (2003) Multiple overseas dispersals in amphibians. Proc R Soc Lond B 270:2435–2442

Veith M, Steinfartz S (2004) When non-monophyly results in taxonomic consequences - the case of *Mertensiella* within the Salamandridae (Amphibia: Urodela). Salamandra 40:67–80

Veith M, Schmidtler F, Kosuch J, Baran Ü, Seitz A (2003) Paleoclimatic changes explain Anatolian mountain frog evolution: a test for alternating vicariance and dispersal events. Mol Ecol 12:185–189

Westaway R, Demir T, Seyrek A (2008) Geometry of the Turkey-Arabia and Africa-Arabia plate boundaries in the latest Miocene to Mid-Pliocene: the role of the Malatya-Ovacık Fault Zone in eastern Turkey. eEarth 3:27–35

Westaway R, Guillou H, Seyrek A, Demir T, Bridgland D, Scaillet S, Beck A (2009) Late Cenozoic surface uplift, basaltic volcanism, and incision by the river Tigris around Diyarbakır, SE Turkey. Int J Earth Sci 98:601–625

Wiley EO (1978) The evolutionary species concept reconsidered. Syst Zool 27:17–26

Wiley EO (1981) Phylogenetics: the theory and practice of phylogenetic systematics. Wiley, New York

Wu C-I (2001) The genetic view of the process of speciation. J Evol Biol 14:851–865

Inferring Multiple Corsican *Limax* (Pulmonata: Limacidae) Radiations: A Combined Approach Using Morphology and Molecules

Barbara Nitz, Gerhard Falkner, and Gerhard Haszprunar

Abstract Slugs of the genus *Limax* (Gastropoda: Stylommatophora) show a highly complicated genital system and reproductive behaviour probably triggering radiation and speciation. Pre-studies have revealed two so far largely undescribed species groups of *Limax* in Corsica. In order to clear up the phylogeny and evolutionary history of these radiations, we used a combination of molecular techniques and morphological characters. The two independent species groups of Corsican *Limax* species are monophyletic, and consist of six to ten species each, most of them new to science. The first species group, the endemic *Wolterstorffi*-group, can be differentiated by COI-Sequences, whereas COI-sequences fail to discriminate species of the *Corsicus*-group, which also has representatives in the Apennine Peninsula. This pattern suggests a much younger radiation of the *Corsicus*-group. Two hitherto unrecognized species on the adjacent islands of Elba and Capraia are described in an appendix.

Keywords *Limax* · *Corsicus*-group · *Wolterstorffi*-group · Corsica · Elba · Capraia · Apennine Peninsula · Endemism · Radiations · COI-Sequences · Molecular systematics · DNA barcoding · New species

B. Nitz (✉)
Zoologische Staatssammlung München, Münchhausenstr. 21, 81247 München, Germany
Institute of Epidemiology, Helmholtz Center München, German Research Center for Environmental Health, München/Neuherberg, Germany
e-mail: Barbara.Nitz@zsm.mwn.de

G. Falkner
Staatliches Museum für Naturkunde Stuttgart, Rosenstein 1, 70191 Stuttgart, Germany
Muséum National d'Histoire Naturelle Paris, Département Systématique et Evolution. UMS "Taxonomie et collections", 55 rue de Buffon, 75005 Paris, France
e-mail: Falkner@malaco.de

G. Haszprunar
Zoologische Staatssammlung München, Münchhausenstr. 21, 81247 München, Germany
e-mail: haszi@zsm.mwn.de

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_19,

1 Introduction

The genus *Limax* (Gastropoda: Pulmonata: Stylommatophora) is distributed mainly in Europe, with emphasis on southern Europe and Alpine regions (Falkner et al. 2001; Manganelli et al. 1995). These nocturnal slugs are quite large animals (6–30 cm) and feed mainly on fungi, terrestrial algae, lichens and dead plant material, but are also partly carnivorous. Up to now, species have been defined by external morphology and mainly by their complex genital anatomy.

The unique and highly complicated copulation behaviour has already been described in detail, e.g. by Gerhardt (1933, 1934, 1937). Copulation is highly sensitive: sometimes a 20% difference in penis length hinders a successful copulation (G.F., personal observation). Different species vary in the length and sculpture of their penes and also in copulation behaviour.

Estimates about species numbers vary, ranging from about 15 species (Schileyko 2003) up to 40 species (Wiktor 2001) for the whole distribution range. However, in contrast to these quite low numbers, Manganelli et al. (1995) list 18 species just for Italy. Most *Limax* species, especially the ones with a Mediterranean distribution, have small and fragmented ranges and are thus endangered by habitat destruction (burning of woods, urban development).

Current knowledge of the *Limax* fauna of Corsica is quite poor. Moquin-Tandot (1855) described *Limax corsicus* based on external characters with a type locality in Bastelica, Corsica. The name *Limax corsicus* was used by Lessona and Pollonera (1882) not only for specimens from Corsica, but they also applied the name to various *Limax* specimens from Northern Italy. However, Simroth (1910) considered *L. corsicus* to be a synonym of the common, widespread species *L. maximus* Linnaeus, 1758. Today, *L. corsicus* is regarded as a species distributed not only in Corsica but also on various Italian Islands like Sardinia and Capraia (Giusti and Mazzini 1971) and in the region of Tuscany (Giusti and Mazzini 1971). The name is generally applied for *Limax* specimens with red sole fields and brownish to creamy body colouration.

In addition to *L. corsicus*, two other *Limax* species are listed for Corsica (Holyoak 1983; Réal and Réal-Testud 1988): *L. maximus* and *L. cinereoniger* Wolf, 1803; both species have a large distribution range all over Europe, and the synanthropic *L. maximus* even occurs overseas.

Based on thorough field studies, breeding experiments, copulation observations and morphological investigations, Falkner (2001) and Falkner et al. (2002) assumed a total diversity of about nine *Limax* species probably endemic for Corsica and most of them new to science. The species form two groups, with four and five species respectively, probably representing two independent island radiations. However, morphological discrimination of *Limax* species is still difficult due to high colour variability and the fact that only fully mature specimens can be considered for genital comparisons. This leads to doubtful species identifications in collections and bio-inventories. A fast and unequivocal method of recognition of new or undetected species in the genus is required to facilitate new insights into species composition and protection of these slugs.

The standard barcode gene, cytochrome *c* oxidase subunit I (COI), is used not only for species re-identification but also proposed for the discovery of new species (Hebert et al. 2003a). This works quite well in the majority of animal groups; more than 95% of species possess unique COI barcode sequences and species level identification is possible in most cases (Hajibabaei et al. 2007; see also Waugh 2007 for a summary). Exceptions are found for example in the Cnidaria (Hebert et al. 2003b) and in insects (Whitworth et al. 2007; Elias et al. 2007).

For species discovery and (re-)identification via DNA barcoding, two general approaches are used. Firstly, tree-based methods should reveal the identity of unknown samples by their position in an established phylogeny (Hebert et al. 2003a, b). The second approach is to use a threshold value of sequence divergence to separate intraspecific from interspecific variation. This threshold value can be inferred in two ways. It may be based on a fixed threshold value, e.g. 3% sequence difference (Hebert et al. 2003 a, b), alternatively a threshold of ten times the average of intraspecific divergence has been proposed (Hebert et al. 2004). However, recent studies have shown high error rates in species delineation based on DNA barcoding alone, strongly suggesting a use of DNA sequences only in combination with solid taxonomic foundations and in an integrative taxonomy approach (Meyer and Paulay 2005; Meier et al. 2006).

In our study combining molecules and morphology, COI sequences should help to clear up the status of the Corsican *Limax* species/populations. To test the validity of new species in Corsica and to assign unidentified specimens to known species, we sequenced specimens of already described species (*L. corsicus* from its type locality on Corsica, *L. senensis* Pollonera, 1890 and *L. ciminensis* Pollonera, 1890 from their respective type localities on the Italian mainland, *L. cinereoniger* from its type locality in Germany and *L. maximus*) and specimens of the unknown and potentially new Corsican species. For comparison, we included several *Limax* species/populations from the Apennine Peninsula and from some Tyrrhenian islands, and also one of the most basal *Limax* species, *L. wohlberedti* Simroth, 1900 (B.N., personal observation).

2 Materials and Methods

2.1 Collection and Treatment of Specimens

Most Corsican *Limax* specimens were collected by the authors (G.F. and B.N.). In some cases, it was possible to document and photograph the copulation behaviour in the natural habitat and in captivity. Complementary European *Limax* specimens were collected for comparison and genetic differentiation or borrowed from other collections (see list of material in the Supplement). For the institutions from which we obtained material, the following standardised abbreviations (in brackets) are used: Istituto di Zoologia dell'Università di Siena (IZSI); Muséum National

d'Histoire Naturelle, Paris (MNHN); Museum of Natural History, Wrocław University (MNHW); Natur-Museum Luzern (NMLU); Naturhistorisches Museum Wien (NMW); Nationaal Natuurhistorisch Museum Leiden (RMNH); Staatliches Museum für Naturkunde Stuttgart (SMNS); Zoologisches Museum Hamburg (ZMH); Zoologische Staatssammlung München (ZSM).

To infer the phylogenetic position of the Corsican *Limax* species within the genus *Limax*, representatives of other limacid genera [*Lehmannia marginata* (O. F. Müller, 1774), *Limacus flavus* (Linnaeus, 1758)] and as outgroup, the vitrinid *Vitrina pellucida* (O. F. Müller 1774), were included in the genetic part of the study.

Most of the collected animals were photographed alive. Tissue samples for DNA extraction were taken alive from the left side of the mantle. This procedure is only minimally invasive so that the living slugs survived without problems. In preserved specimens, tissue was taken from either the body wall or from the left side of the mantle. For preservation, the animals were relaxed and killed in water or in a mixture of water and 2–3 drops of a solution of the synthetic tenside SUPRALAN-UF (three parts SUPRALAN-UF – a fatty alcohol polyglycol ether; Bauer Handels-GmbH, Adetswil, Switzerland – to two parts water). Preparations of slugs with everted penes were obtained with a bit of luck by drowning animals which were ready to copulate. The eversion of the penis is furthered by a quick reduction of oxygen in the drowning water obtained by drowning several animals together and slight regular movement of the jar combined with very gentle warming. The method of Colosi (1919) to use a veratric solution has not yet been tested. This method should produce slugs with everted penes and possibly also provides a procedure to study the morphology of the penial combs and the surface structures of the penes. With both methods, a complete eversion of the uttermost tip of the penis is not reached. This seems to have functional morphological reasons which also play a role in the conditioning of sperm and has to be further investigated by thin sections. All animals were fixed and preserved in ethanol. Morphological studies followed standard procedures.

Material is deposited in the ZSM, in the SMNS (Coll. Falkner) and in the MNHN. DNA elutions are stored in the DNA Bank of the ZSM (see http://www.zsm.mwn.de/dnabank/).

2.2 DNA Sequence Analysis

DNA was isolated from a small piece of tissue sampled from the mantle or body wall of the slugs using a QIAGEN extraction kit (Qiagen Blood and Tissue Kit). About 650 nucleotides of the mitochondrial cytochrome *c* oxidase subunit I gene (COI) were amplified by polymerase chain reactions (PCR) for all taxa using the primer set: mtCOI-1F-54 (5′-TTTCAACAAAYCATAARGATATTGG-3′) and mtCOI-1R-53 (5′-AAYACCAATAGAAATTATAGCATAAA-3′). The primers were based on the COI universal primers (Folmer et al. 1994) and the primers used by

Hyman et al. (2007) and were assessed using the computer program *Alignment* 1.2 (Engels 1993). The PCR conditions were: 92°C for 4 min, then 40 cycles of 92°C for 1 min, 50°C for 1 min, 72°C for 1 min and final elongation 72°C for 5 min.

PCR products were purified with one of three techniques, depending on the quality and intensity of the PCR results: a Qiagen DNA purification kit (Ultra Clean Band Excision Purification kit) or with ExoSapIt [PCR product was incubated at 37°C for 30 min and then at 85°C for 15 min with 5 units of Exonuclease I (ExoI; Amersham) and 0.5 unit Shrimp Alkaline Phosphatase (SAP; Amersham) to cleave nucleotides one at a time from the ends of excess primers and to inactivate single nucleotides (Werle et al. 1994)]. The purified PCR products were amplified with the same primers as above with a BigDye v3.1 Terminator Cycle Sequencing Kit, cleaned up with SephadexG-50 Superfine columns (GE Healthcare) and sequenced using an Applied Biosystems 3730 capillary automated sequencer according to the standard protocol. Sequences were assembled and proofread using Sequencher™ (Gene Codes), manually aligned in the program Se-Al v. 2.0a11 (Rambaut 1996) and deposited in GenBank (for accession numbers see list of material in the Supplement). The alignment was trimmed to 615 nucleotides, starting with position 40 of the reference taxon *Biomphalaria glabrata* (Say, 1818) (GenBank number NC 005439) and finishing at position 655.

Prior to phylogenetic analysis, the data were partitioned into first, second and third codon sites. Model selection was made using comparisons of hierarchical Likelihood Ratio Tests and Akaike Information Criterion scores in *MrModeltest* 2.3 (Nylander 2004). The general time-reversible (GTR) model with eight discrete gamma (Γ) categories and a proportion of invariant (I) sites (GTR+Γ8+I) was used. Markov Chain Monte Carlo (MCMC) sampling was carried out in *MrBayes* 3.1.2 (Ronquist and Huelsenbeck 2003) for 1,000,000 generations (four simultaneous chains, sample frequency 50, burn-in 100,000 generations). Majority-rule consensus trees were calculated from the sampled sets of trees.

The phylogenetic trees were rooted on *Vitrina pellucida*, because Vitrinidae appear to be the most basal family in the superfamily Limacoidea (Hausdorf 1998).

Inter- and intra-specific genetic distances were calculated with MEGA version 4.0 (Tamura et al. 2007) using the Kimura 2-parameter model (K2P), the most effective model when distances are low (Nei and Kumar 2000).

3 Results

3.1 Morphological and Copulation Studies

Based on preliminary results, two species groups can be defined: the *Wolterstorffi*-group (Fig. 1a–e) and the *Corsicus*-group sensu lato (Fig. 2a–e).

Representatives of the *Wolterstorffi*-group (named after *L. wolterstorffi* Simroth, 1900) are generally small animals (less than 10 cm, mostly about 8 cm), mostly dark

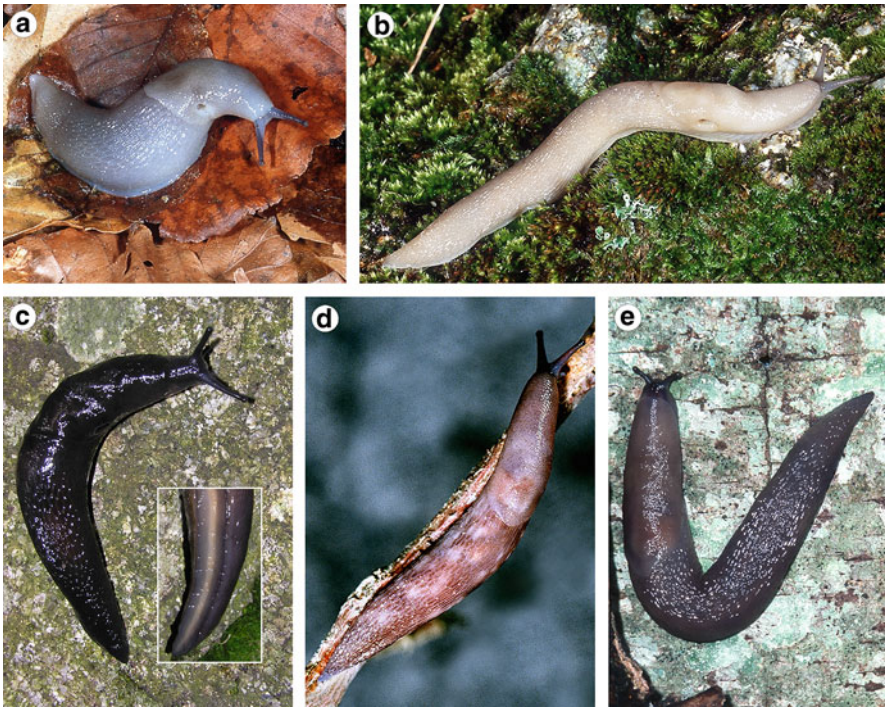


Fig. 1 Habitus photos of different colour morphs of the *Wolterstorffi*-group. All detailed pictures are approximately 2/3 natural size. (**a**, **b**) *Limax vizzavonensis* n. nom. (**a**) Specimen from Vizzavona near Cascade des Anglais (no. 12 on map, Fig. 10); (**b**) specimen from Ruine de Sorba (no. 10 on map), *creamy whitish*, but no albino; (**c**) specimen (F1) from Monte Rotondo near Petra Piana (no. 9 on map), *deep black* morph, in*sert* showing the *dark* lateral sole fields (photograph, courtesy C. M. Brandstetter); (**d**) specimen from Vallée de la Restonica at Tuani morph with irregular bright spots; (**e**) specimen from Porto, ravin du Riù (no. 3 on map), *brownish-grey* morph with metallic lustre (photograph, courtesy M. Falkner)

to uniformly black lacking distinct patterning and never show red pigmentation on body or sole. Hatchlings and early juveniles so far investigated exhibit a diffuse body colour entirely lacking lateral bandings ("Stammbinden" sensu Simroth; Fig. 3a–d). This lack has been verified by observations of eight populations (Monte Cinto, Citadelle of Corte, Porto, Vallée de la Restonica, Monte Rotondo, Bergeries de Baccialojo, Vizzavona, Plateau de Coscione), and defines for Corsica a discriminating character of the *Wolterstorffi*-group. In the whole genus *Limax*, this character has only been observed outside Corsica for *L. ianninii* Giusti, 1973, and *L. brandstetteri* Falkner, 2008, two unicoloured basal species within the *Limax maximus* group. Additionally, breeding experiments within the *Corsicus*-group with animals from Corsica (11 populations) and continental Italy (9 populations) showed the constant presence of "Stammbinden" at least in the early developmental stages (Fig. 3e–g); this character is shared with the majority of the *Limax* species. The morphological examination of the *Wolterstorffi*-group shows a huge variety in

Fig. 2 Habitus photos of different colour morphs in the *Corsicus*-group sensu lato. All detail pictures are approximately 2/3 natural size. (**a**) *Limax corsicus*, topotype from Bastelica (no. 28 on map, Fig. 11); (**b**) specimen from Vizzavona (no. 26 on map), morph with interrupted banding and diffuse bright spots on the mantle; (**c**,**d**) Vallée de la Restonica at Tuani, (no. 25 on map): (**c**) juveniles of two sympatric colour morphs which show a different phenology, although they are genetically not distinguishable: the largest specimen of the dark cohort is photographed together with the most retarded specimen of the reddish cohort; (**d**) adult specimen with *red sole*; (**e**) specimen from the Castagniccia near Croce, morph with creamy whitish sole; this rufinless morph is restricted to the central Castagnicca and is dominant at the Monte San Petrone

penis length in preserved specimens. For example, the observed penis length of L. sp. (Porto) (Fig. 4a) is approximately twice the body length. In contrast to this long and thin penis, the penis of *L. wolterstorffi* is less than the body length and very thick (Fig. 4b). Based on the findings of the morphological analyses, eight to ten species can be distinguished, although data on the reproductive behaviour are still entirely missing.

In the C*orsicus*-group s. l., specimens of Corsica and of the adjacent Apennine Peninsula are present. Although the species in this species group have specific copulation features with a huge range in penis length (Figs. 5 and 6), they share a distinct mode of sperm transfer through the extended penes, whereas in other species groups (e.g. *cinereoniger*- or *maximus*-group), the penis is everted with the sperm mass already in the tip. Up to now, morphological criteria have failed to

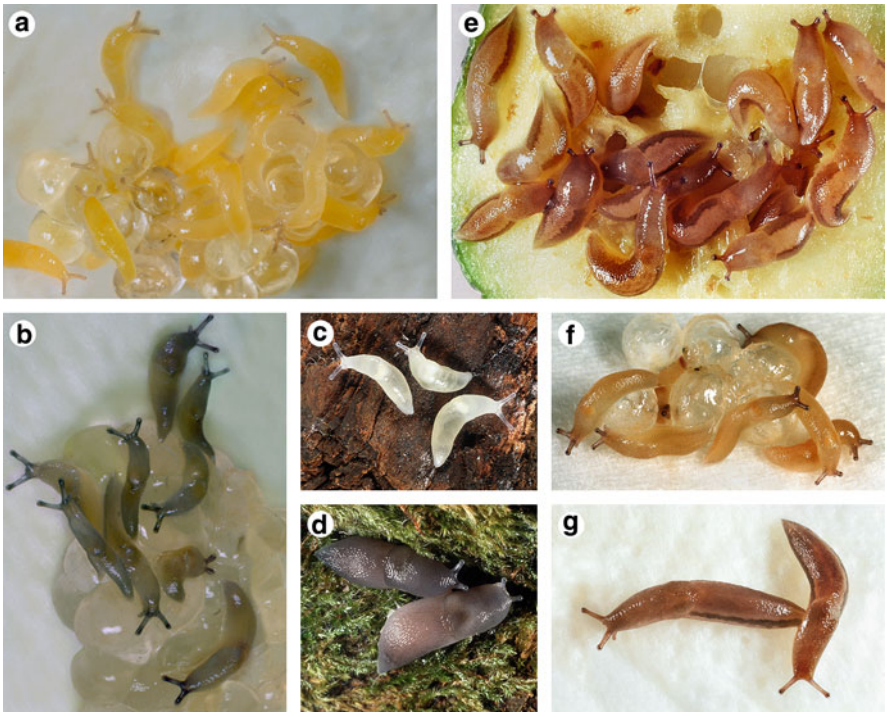


Fig. 3 Chromatic development of juveniles. All detailed pictures are approximately two times natural size. (**a**–**d**) Offspring from representatives of the Wolterstorffi-group: no traces of "Stammbinden." (**a**) F1 of a specimen from the Plateau de Coscione, photographed 1 h after the end of hatching, no pigment is developed by the embryos in the eggs; (**b**) F1 of a specimen from the Monte Rotondo, photographed shortly after hatching, the pigmentation of the body starts already in the eggs; (**c**) F1 of a specimen from the Citadelle of Corte, photographed 1 day after hatching; (**d**) the same animals as in (**c**) 4 weeks later. (**e**–**g**) Offspring from representatives of the Corsicus-group (Cap Corse/Tuscany group as an example): "Stammbinden" are always present. (**e**) F1 of a specimen from Furiani, 4 days after hatching, feeding on cucumber; (**f**) F1 of a specimen from Pietrabugno, Casevecchie, 5 days after hatching, the lateral body bands are present but very weakly developed; (**g**) F1 of a specimen from Furiani, 3 weeks after hatching

further divide this group, but molecular data (see below) distinguish an Endemic *Corsicus*-group with strictly Corsican representatives (Figs. 5b, c, d, 6b, c, and 7a) and the Cap Corse/Tuscany-group (Figs. 5e, 6e, and 7b) with representatives on Corsica and the Apennine Peninsula. For the Endemic *Corsicus*-group itself, the comparison of penis morphology and copulation modes clearly shows severe morphological differences that legitimate the assumption of at least five different species in Corsica. Species *L.* sp. (Bonifatu) and *L.* sp. (Tuani) for example, both positioned in this group, represent species with very distinct genital differences (Figs. 5b, c, 6b, c, and 8b).

Morphological characters in the Cap Corse/Tuscany-group reveal the existence of at least two species for Corsica. The specimens of the locality of Furiani *L.* sp.

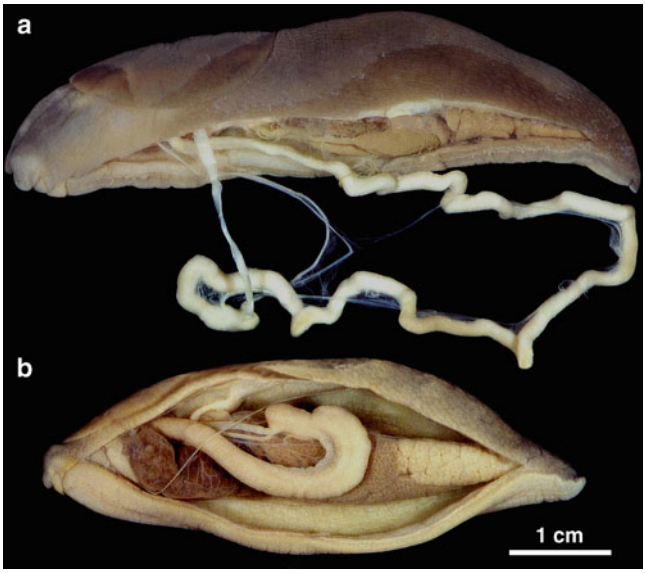


Fig. 4 Dissection photographs of the two extreme forms in the *Wolterstorffi*-group. (**a**) *Limax* sp. (Porto); the animals of this new species have an extreme long and thin penis. (**b**) *Limax wolterstorffi* (topotype): penis short and massy

(Cap Corse A), for example, show a unique copulation mode (Figs. 5e and 6e) and a very special penis morphology (Fig. 7b).

3.2 Sequence Analysis

The results of the phylogenetic analysis (Fig. 9) show monophyly for both the family Limacidae (including *Limax*, *Limacus* and *Lehmannia*) and the genus *Limax* (posterior probability, PP 100%). The basal part of the *Limax* clade is well resolved, with *L. wohlberedti* and *L. cinereoniger* diverging basally (PP 100%, 100%). All species representing European non-Corsican lineages (without the Italian relatives of the Corsicans) are clearly distinct from their nearest neighbours [*L. brandstetteri*, *L. maximus, L. cinereoniger, L. ianninii, L. wohlberedti*, *L.* sp. (Mte. Altissimo), *L.* sp. (Mte. Baldo), *Limacus flavus*] and form monophyletic groups that are in most cases supported by posterior probabilities of 100%. The phylogenetic reconstruction strongly supports the preliminary assumption of two independent Corsican/Tyrrhenian species groups: a mixed group from some Italian islands and the mainland and Corsica (arrow) and an endemic Corsican species group (bar G). This latter group (*Wolterstorffi*-group) is well resolved and monophyletic (PP 100%). The already described species (*L. wolterstorffi* and *L. vizzavonensis* n. nom.) and also the unnamed species [like *L.* sp. (Coscione), *L.* sp. (Porto) and *L.* sp. (Restonica)] show well -supported monophyletic separation.

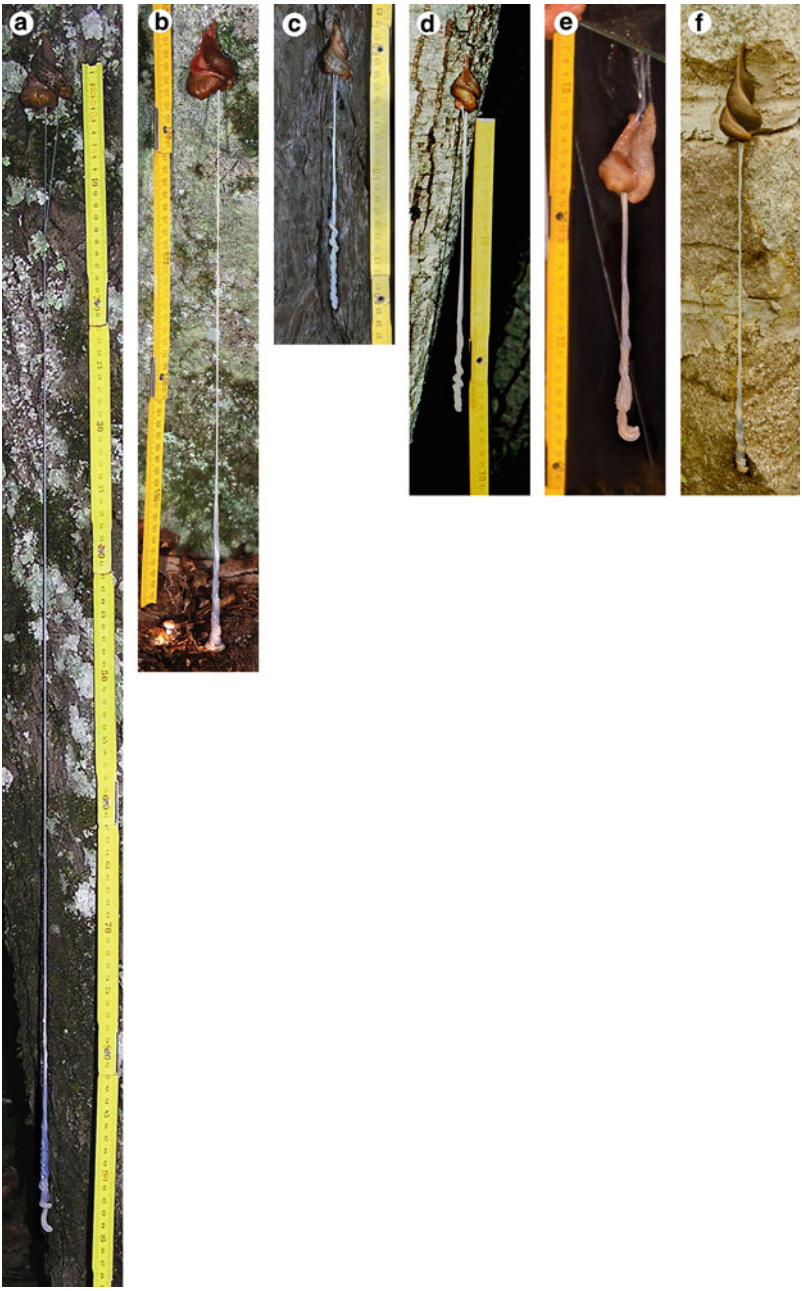


Fig. 5 Maximum extension of the entwined penes during copulation in the *Corsicus*-group sensu lato. All to scale. (**a**) Montagnola Senese, Tuscany, 92.5 cm (photograph, courtesy C. M. Brandstetter); (**b**) Bonifatu (no. 23 on map, Fig. 11), 50 cm; (**c**) Tuani, Restonica Valley (no. 25 on map), 19 cm; (**d**) Marmuccio, Castagniccia, 27 cm; (**e**) Furiani-Marinella (no. 21 on map), 22 cm; (**f**) Capraia (no. 31 on map), estimated length 28.0 cm (photograph, courtesy F. Giusti)

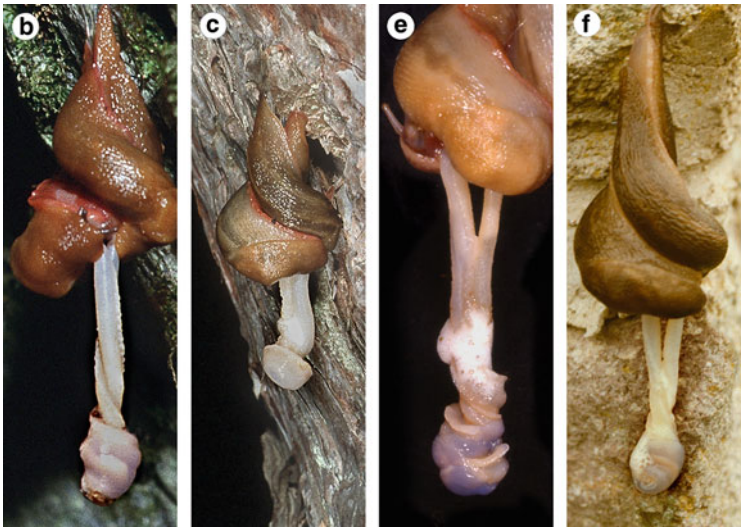


Fig. 6 Morphology of the penes in the *Corsicus*-group s. l. during or shortly after sperm exchange. *Lettering* coincides with couples in Fig. 2. All detailed pictures are approximately 2/3 natural size. Estimated penis lengths: (**b**) 5 cm; (**c**) 2 cm; (**e**) 6.5 cm; (**f**) 4 cm (**f**: photograph, courtesy F. Giusti)

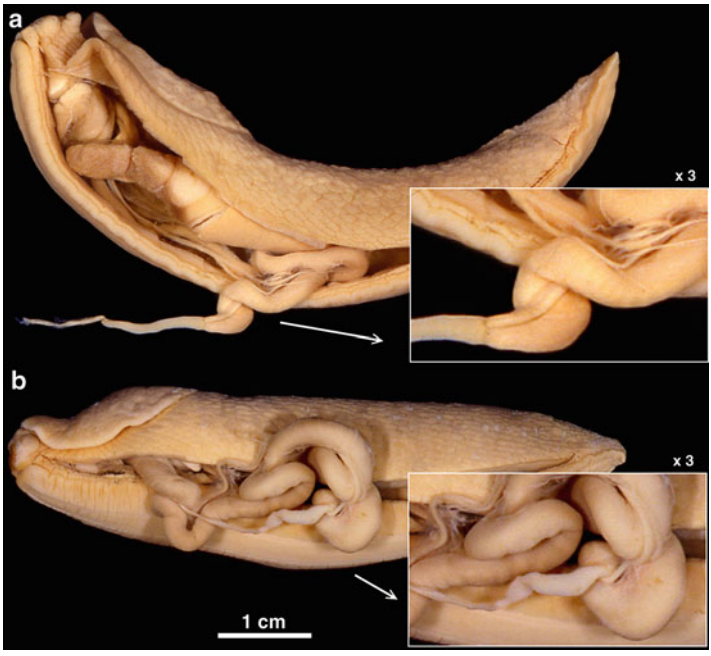


Fig. 7 Examples of anatomical specialisation in the *Corsicus*-group. (**a**) Specimen from Sperono (no. 30 on map), leg. E.Th.J. Ripken 1996; terminal insertion of retractor and vas deferens, morphology of the penis tip corresponding to *L. corsicus* s. str.; (**b**) specimen from Furiani-Marinella (no. 21 on map, Fig. 11), F1; distinct coecum and lateral insertion of retractor and vas deferens

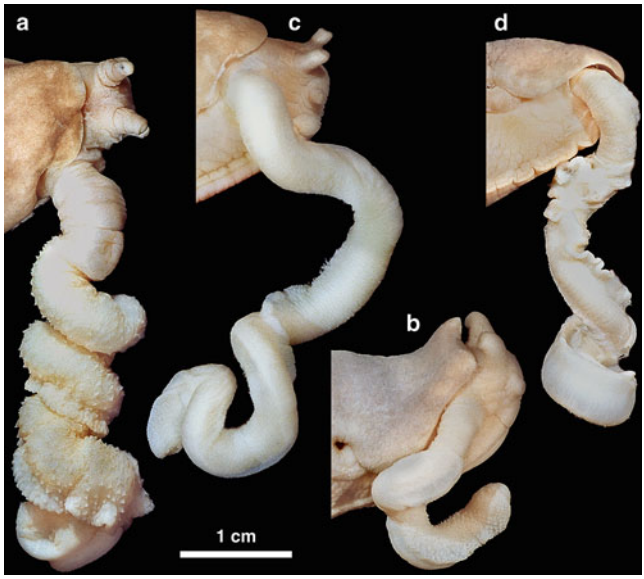


Fig. 8 Morphology of everted penes of clearly distinguishable forms of the *Corsicus*-group from different sampling places. (**a**) Truggia (no. 27 on map, Fig. 11); (**b**) Bonifatu (no. 23 on map), not fully everted; (**c,d**) Two sympatric forms from Grigione (no. 19 on map)

In contrast, species differences within the other species group (arrow) are less supported. The analysis reveals a large group of mixed species and populations from Corsica, the Apennine Peninsula, and several other Tyrrhenian islands (Sardinia, Capraia, Elba): the *Corsicus*-group sensu lato. Within this grouping, we have distinct monophyletic clades for the two species from the islands of Capraia and Elba (*L. giustii* n. sp. and *L ilvensis* n. sp., see Appendix). Further on, several non-Corsican groups are formed by specimens from the Apennine Peninsula [*ciminensis*-group: bar F, "sp. 2" of Italian Checklist (Manganelli et al. 1995): bar E, *senensis*-group: bar D, group of "Fossil Islands" ("Isole fossili" sensu Lanza 1984): bar B].

The Corsican specimens split into two clades: the endemic *Corsicus*-group (bar C) and the Cap Corse/Tuscany-group (bar A). This latter, monophyletic clade (PP 100%) forms an unresolved group including specimens from Tuscany (Apennine Peninsula) and specimens from Cap Corse (the most northern part of Corsica).

The Endemic *Corsicus*-group (PP 91%) comprises specimens only from Corsica, namely the whole area of Hercynian Corsica and the southern part of Alpine Corsica (see also Figs. 10 and 11).

The sequence divergence within the three species groups (*Wolterstorffi*-group, Endemic *Corsicus*-group, Cap Corse/Tuscany-group) is 3.9, 0.1 and 0.1% respectively (Table 1). The sequence divergence between the groups is 10.8% for the *Wolterstorffi*-group and the Endemic *Corsicus*-group. Between the *Wolterstorffi*-group and the Cap Corse/Tuscany-group, there is a sequence divergence of 10.8%

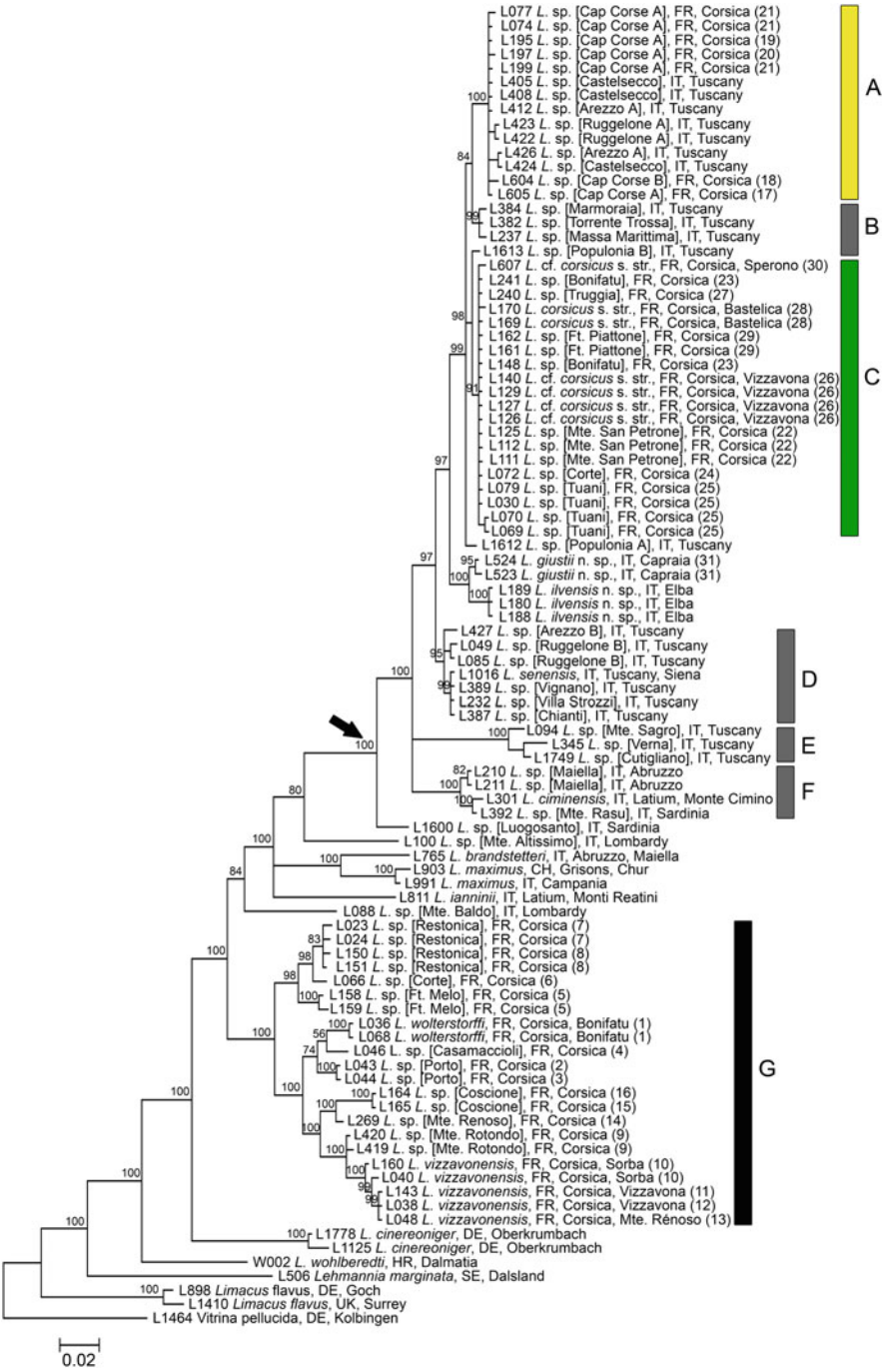


Fig. 9 Majority-rule consensus tree from the Bayesian inference analysis of the COI data. Posterior probabilities are marked above the branches. *Arrow Corsicus*-group sensu lato. *Bar A*

as well. For the Endemic *Corsicus*-group and the Corse/Tuscany-group, the sequence divergence between them is 1.4%.

Interspecific divergence within the *Wolterstorffi*-group ranges from 1.1 to 6.8% (Table 2), with an average value of 3.9%.

3.3 Distribution

All known distribution sites of the *Wolterstorffi*-group (Fig. 10) are located in mountainous habitats in the Hercynian Corsica (the geologically ancient, crystalline part of Corsica; Fig. 10 insert). Both the Endemic *Corsicus*-group and the Corsican species of the Cap Corse/Tuscany-group have their ecological preference in the montane forest zone. The Endemic *Corsicus*-group is found in the middle and southern part of Corsica, whereas the Corsican specimens of the Cap Corse/Tuscany-group are restricted to the Cap Corse region, the most northern part of the island (Fig. 11). The distribution range of the Italian specimens of the Cap Corse/Tuscany-group also comprises habitats in Tuscany.

4 Discussion

4.1 Biogeographical Scenarios

Today's distribution pattern of the *Limax* species in the Tyrrhenian area has certainly been influenced by geological history. The polyphyly of Sardinian and Corsican groups implies that there were several independent colonisation events on the islands of Sardinia and Corsica as well as on the smaller islands closer to mainland Italy. Although a direct scaling of the splitting events in the tree is currently not possible, the geohistory of both islands suggest only a few colonisation events:

Corsica was colonised by *Limax* at least three times. The first radiation of *Limax*, the *Wolterstorffi*-group, is (according to current knowledge) endemic to the Hercynian Corsica, suggesting a very ancient colonisation from a European mainland stock. Accordingly, this group probably has its origin on the French mainland and has been split from mainland taxa by the rotation of the Corsica–Sardinia microplate (Alvarez 1972; Durand-Delga 1974). The time frame for this event is during the Eocene or Oligocene at the latest (~30–21 Mya). A test of this hypothesis would be

Fig. 9 (continued) Cap Corse/Tuscany-group. *Bar B* group of fossil islands. *Bar C* Endemic *Corsicus*-group. *Bar D senensis*-group. *Bar E* group of "sp. 2" of Italian Checklist (Manganelli et al. 1995). *Bar F ciminensis*-group. *Bar G Wolterstorffi*-group

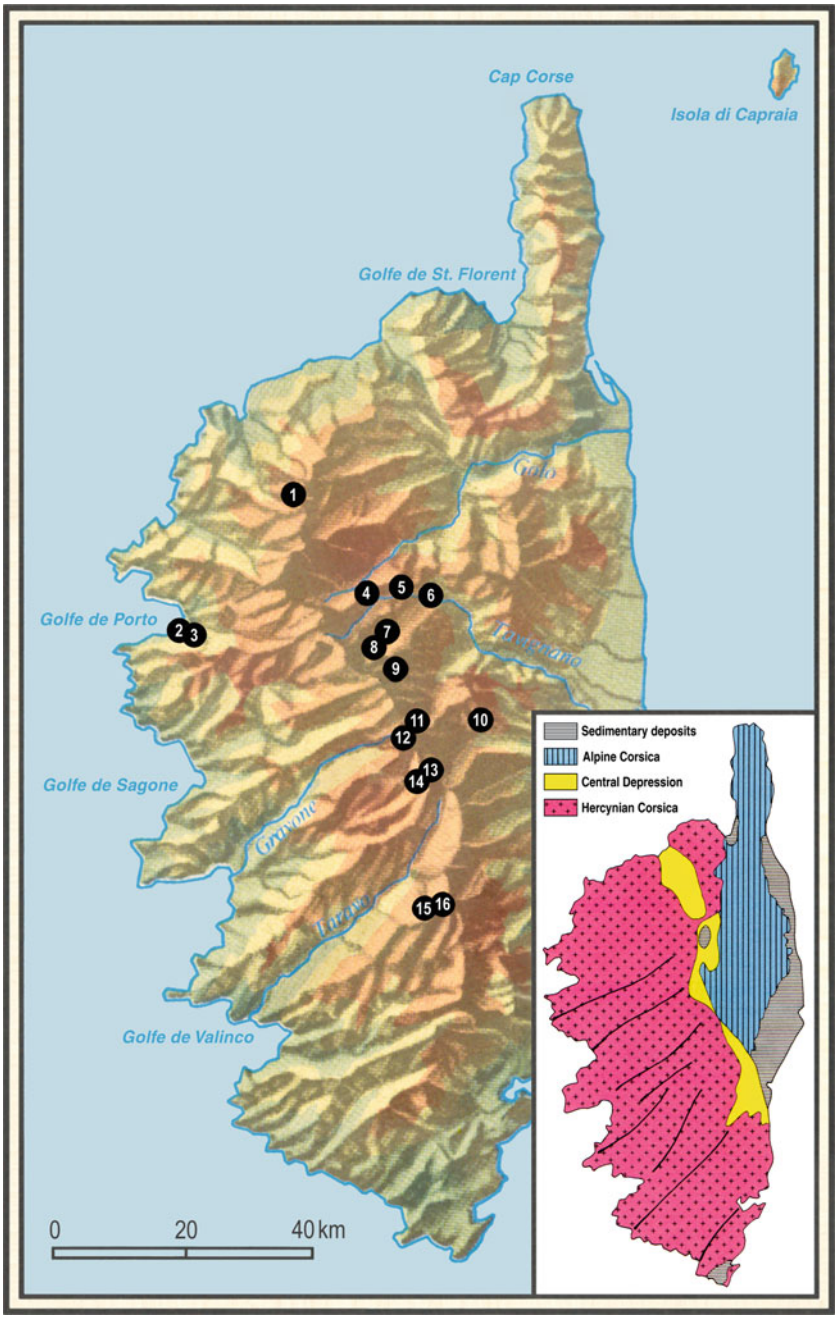


Fig. 10 Localities of the studied populations of the *Limax wolterstorffi*-group. Map base MNHN (modified). *Insert* Simplified geology after A. Gautier (1983). (*1*) Cirque de Bonifatu, 610 m (loc. typ. *wolterstorffi*); (*2*) Porto, D 81 direction Piana; (*3*) Porto, ravin du Riù; (*4*) Casamaccioli, 990 m; (*5*) Forêt de Melo, 1,300 m; (*6*) Corte, Citadelle, 450 m; (*7*) Vallée de la Restonica, 900–1,000 m;

the finding of sister taxa of the *Wolterstorffi*-group in southern France – a matter for future studies.

An additional, second lineage of *Limax* derived from the above-mentioned ancient European stock and followed the already (Miocene) formed and exposed chains of the Western and Ligurian Alps further along the Apennine chain (cf. Rook et al. 2006: Fig. 2) and radiated in middle Italy (Latium, Campania). This lineage – in our tree being represented by subsequent deviation of *Limax maximus* s. lat., *L. ianninii*, and *L.* sp. (Mte. Altissimo) (corresponding to "*Limax* sp. 3" of the Italian Checklist by Manganelli et al. 1995) – gave rise to all later colonisations of Corsica as well as the colonisation of Sardinia and the small islands close to Italy (see below).

Further land bridges enabling *Limax* to enter Corsica probably occurred only during the Pleistocene; this colonisation scenario is based on the following considerations: First, despite dense sampling, there is no Corsican taxon belonging to the above-mentioned Latium-Sardinia radiation. Accordingly, Corsica was probably not connected to either Sardinia or Latium during the late Miocene. Second, a major marine transgression during the Pliocene made any terrestrial faunistic exchange unlikely. Third, only the marine regressions following the onset of ice ages in the Pleistocene offered land connections again. Fourth, the low genetic differences of members of the Endemic *Corsicus*-group imply a recent radiation. And, fifth, the two main Corsican radiations (the *Wolterstorffi*-group and the Endemic *Corsicus*-group) are genetically clearly distinct suggesting a considerable long-term separation of these species groups.

Therefore, this second Corsican colonisation took probably place in the (Early or) Middle Pleistocene (780–130 ka). Interestingly, this now endemic group of the *Corsicus*-group seems to have initially reached only the Hercynian (i.e. older) area of Corsica, suggesting that there was still no connection to the younger Alpine Corsica (northeast Corsica). The Alpine Corsica was either separated from Hercynian Corsica and the northern Ligurian-Ocean landbridge by a small marine channel, or was still not tectonically lifted up high enough to reach the sea surface (Cavazza et al. 2001; Brunet et al. 2000; Danišík et al. 2007).

On the Italian mainland, the last remnants of this second colonisation wave are the Limaces of the "Fossil Islands" – this western Tuscan area was drowned during the Pliocene except for a number of mountain peaks above sea level (Brunet et al. 2000; Cipollari et al. 1999; Brogi 2008).

The massive regression of sea-level during the (Middle or) Late Pleistocene (probably Würm glaciation, 115,000–10,000 BP) possibly enabled the youngest, third colonisation of the Corsicus-group s. l. (the Cap Corse/Tuscany group), which

Fig. 10 (continued) (*8*) Vallée de la Restonica, 1,080 m; (*9*) Monte Rotondo, near Petra Piana, 1,850 m; (*10*) Ruine de Sorba, 1,254 m; (*11*) Vizzavona, right bank of the Vecchio, 850 m; (*12*) Vizzavona, 1,120–1,190 m (loc. typ. *minimus*); (*13*) Monte Renoso, Bastani, 2,090 m; (*14*) Monte Renoso, Vitalacia, 1,800 m; (15*)* Forêt de Coscione, 1,340 m; (*16*) Plateau de Coscione, 1,360 m

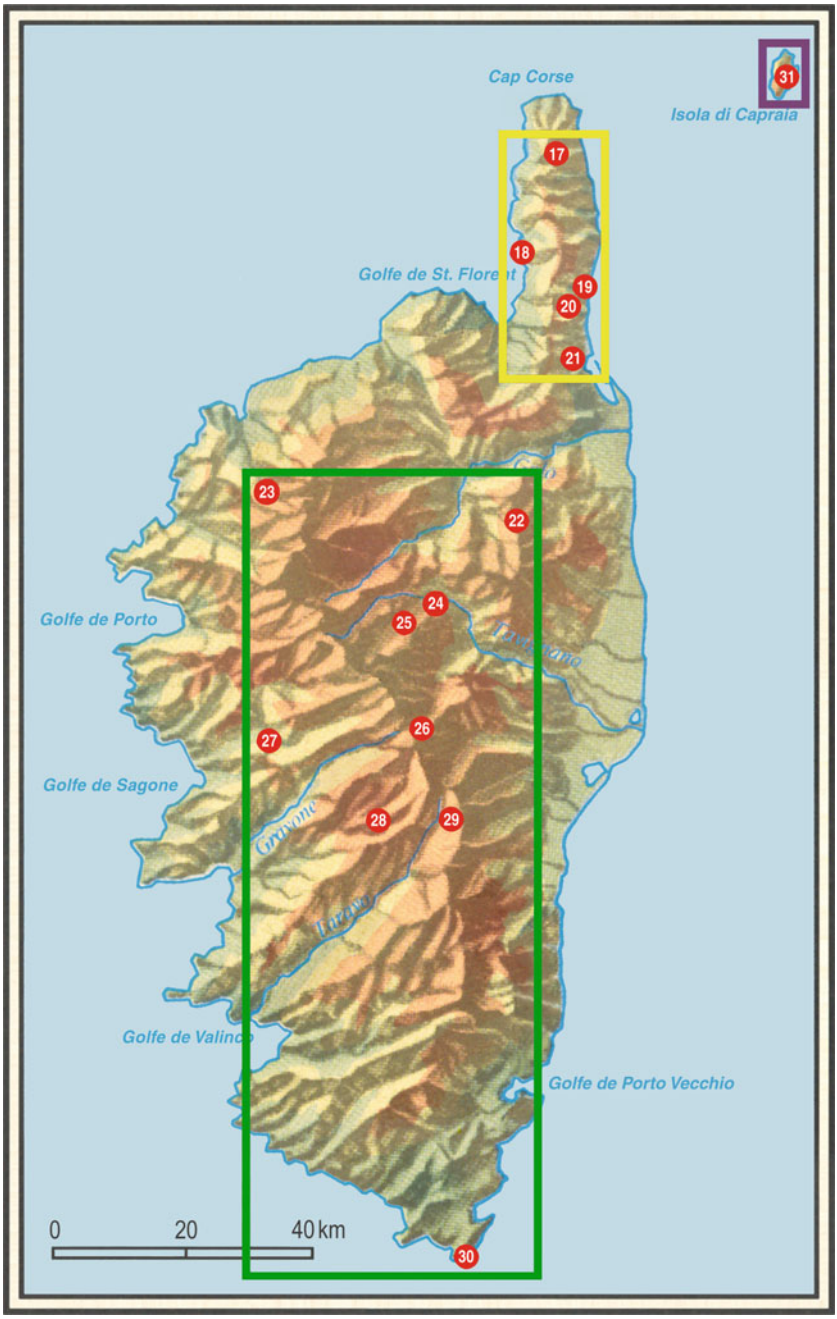


Fig. 11 Localities of the studied populations of the *Limax corsicus*- group. Map base MNHN (modified). *Green frame* Endemic *Corsicus*-group; *yellow frame* Cap Corse/Tuscany-group; *lilac frame* Capraia isolate. (*17*) Vallée de la Méria; (*18*) Nonza; (*19*) Vallée du Grigione; (*20*) Pietrabugno; (*21*) Furiani-Marinella; (*22*) Monte San Petrone, 1,060 m; (*23*) Bonifatu, 550 m;

Table 1 Percentage nucleotide sequence divergence (K2P distances) at COI within and between the Corsican/Tuscan species groups. (*n* number of specimens in each group)

Species group	*n*	Within species groups	Between groups		Cap Corse/ Tuscany-group
			Wolterstorffi-group	Endemic *Corsicus*-group	
Wolterstorffi-group	22	3.9			
Endemic *Corsicus*-group	20	0.1	10.8		
Cap Corse/ Tuscany-group	14	0.1	10.8	1.4	

presumably entered Corsica in the northeastern Alpine part of the island, the closest part of Corsica to the Italian mainland.

Because of the basal phylogenetic position of Sardinian *Limax* compared with Corsican taxa, we currently prefer the following hypothesis for the origin of the Sardinian species. Sardinia was probably colonised by two lineages of *Limax* in the late Upper Miocene (∼ around 5 Mya), during the extensive period of lowest sea-level following a large-scale evaporation of the Mediterranean Sea ("Messinian salinity crisis"). Freshwater drainage systems in the shallow exposed areas and brackish conditions in deeper basins ("Lago-Mare" environment) resulted in land bridges. The origin of the colonisation of Sardinia with *Limax* was on the Italian mainland, presumably northern Latium, which was connected via the exposed northern parts of the Tyrrhenian oceanic crust (Jolivet et al. 2008; Govers et al. 2009).

The smaller islands of Capraia and Elba as well as the above-mentioned "Fossil Islands" in Tuscany remained isolated (or partly still drowned) during the Pliocene, but probably already became connected repeatedly with mainland Italy during the Early Pleistocene cooling periods (1.8–0.78 Mya) which resulted in moderate marine regressions. Both the lineage of the first *Corsicus* radiation now endemic to Hercynian Corsica and the southern part of Alpine Corsica ("endemic *Corsicus*-group") as well as the later Cap Corse/Tuscan radiation probably derived from the "Fossil Islands" area. The geographical isolation of Capraia and Elba led to the formation of two distinct sister-species: *L. giustii* n. sp. and *L. ilvensis* n. sp. (see Appendix). Another group of palaeoendemics, pulmonate snails of the genus *Tacheocampylea* L. Pfeiffer, 1877, shows a similar distribution pattern (Giusti 2007) with endemic species in Corsica, Capraia and Sardinia.

The outlined interpretation of the various colonisation events by *Limax* spp. in the west Mediterranean area are in full agreement with paleontological evidence for faunal exchange of Mammalia between paleobiogeographic provinces in Italy (Rook et al. 2006) as well as phylogenetic studies carried out with Amphibia (Zhang et al. 2008; Meijden et al. 2009; Stöck et al. 2008), and Reptilia (Mayer

Fig. 11 (continued) (*24*) Corte, west of Citadelle, 430 m; (*25*) Tuani, Vallée de la Restonica, 624 m; (*26*) Vizzavona, 860 m; (*27*) Truggia, Vallée du Liamone; (*28*) Bastelica, 770 m (loc. typ. *corsicus*); (*29*) Forêt de Piattone, 1,035 m; (*30*) Sperono, west of Bonifacio; (*31*) Capraia

Table 2 Percentage nucleotide sequence divergence (K2P distances) at COI between the species of the *Wolterstorffi*-group

Species	*L.* sp. (Restonica)	*L. wolterstorffi*	*L. vizzavonensis*	*L.* sp. (Porto)	*L.* sp. (Casamaccioli)	*L.* sp (Corte)	*L.* sp (Ft. Melo)	*L.* sp (Coscione)	*L.* sp (Mte. Renoso)	*L.* sp (Mte. Rotondo)
L. sp. (Restonica)										
L. wolterstorffi	4									
L. vizzavonensis	5.3	4.9								
L. sp. (Porto)	4.8	2.1	4.5							
L. sp. (Casamaccioli)	4.5	1.9	4.4	2.1						
L. sp (Corte)	1.2	4.1	5.5	3.7	4.3					
L. sp (Ft.Melo)	2	4.3	5.7	4.7	4.9	2.1				
L. sp (Coscione)	6.8	4.6	4.9	4.8	4.6	6.4	6.4			
L. sp (Mte. Renoso)	4.6	2.9	3.5	2.9	2.7	4.3	4.7	2.1		
L. sp (Mte. Rotondo)	4.7	3.7	1.1	3.3	3.5	4.8	5	4.2	2.3	

and Pavlicev 2007) though these studies vary in their interpretation of events and their timing.

4.2 Species Boundaries

The assumptions of species groups and species on Corsica were inferred with mutual benefit from morphology and from sequence analyses of a fragment of the mitochondrial gene cytochrome *c* oxidase subunit I. The latter also enables tree reconstruction and provided the basis of our hypotheses of multiple colonisation of Corsica. In addition, our study provides insights into the benefits and limits of standard COI-barcoding:

In the case of the *Wolterstorffi*-group (and the majority of other *Limax* species groups; B.N., personal observation), standard species barcoding (i.e. re-identification and detection of further species by partial COI-sequencing) could be established. In all tested cases, the COI-based tree resolves the same species that were detected by morphological characters. The sequence divergences within this group are in most cases (33 of 45 pairings; cf. Table 2) higher than 3% between species, although all species can be connected by values below 3%.

However, the younger Endemic *Corsicus*-group and the Cap Corse/Tuscany-group containing specimens from Corsica and the Apennine Peninsula clearly show the limits of DNA standard barcoding concerning re-identification with COI. Despite the lack of resolution in the molecular tree, morphological and copulation characters suggest that the Endemic *Corsicus*-group comprises at least five species, including the genuine *L. corsicus* s. str. from the type locality.

Both latter mentioned *corsicus*-groups with quite recent radiation share very similar COI sequences (0.1% sequence divergence in these groups); an uncritical barcoding approach would underestimate the real number of species determined by genital anatomy and reproductive behaviour.

The current case is a significant example that, even within a single genus, species boundaries can substantially differ at the molecular level.

4.3 Evolutionary Considerations

The low genetic diversity in contrast to distinct genital anatomy and copulation features suggests an accelerated speciation rate of the two younger radiations compared to the *Wolterstorffi*-group. This acceleration might be triggered by extrinsic and intrinsic agents. First, increased rate of fragmentation of habitats of the deeper part of Corsica (contrary to the Hercynian part) by sea level changes. Alternatively, there might have been genetic exchange between populations or species in statu nascendi from the Apennine Peninsula and the island of Corsica (maybe also very recently by human influence). And second, rapid establishment of species boundaries by strong sexual selection being also reflected by an extremely

complicated copulation mode with sperm transfer through the extended penes. The unique and complex copulation behaviour and the associated morphological characters like penis length and shape are diagnostic criteria for each species. The discriminating nature of the copulatory organs is also obvious in the sympatric occurrence of different *Limax* species on Corsica.

5 Conclusions

The combined approach of morphological characters and COI-sequencing revealed multiple colonisation and three independent radiations of Corsica by *Limax*. In addition, our study provides a case showing benefits and pitfalls of COI barcoding within a single genus: except for the young radiations in Corsica and in Tuscany, standard barcoding provides sufficient resolution to identify the other *Limax* species and has lead to the molecular confirmation of two hitherto unrecognised insular endemics which are described in the Appendix. Additionally, the results establish a framework to facilitate the selection of specimens for future phylogenetic analyses with more genes. In summary, the present study shows the necessity for a combined morphological–molecular approach or an integrative taxonomy.

Acknowledgments This work was supported by the DFG (German Science Foundation) through the grant HA 2598/7 to G.H. within the Priority Programme "Radiations" (SPP 1127).

We have to thank a huge number of people (named in the material list) for kindly collecting or providing additional material for us. We are grateful to all collection managers and staff of various institutions for providing us with material and assistance. Dr. Isabel Hyman (Sydney, Australia) commented on the manuscript and gave us valuable input and hints for molecular work. We are extremely grateful to Dr. Markus Moser (Bayerische Staatssammlung für Paläontologie und Geologie München) for very helpful contributions on the geohistory of Corsica and the adjacent areas. Margrit Falkner gave us continuous support and a generous donation, which finally allowed the addition of crucial sequences. These three sequences have been elaborated by the commercial enterprise kmbs München.

We are very grateful to Prof. Dr. Folco Giusti (Univ. Siena) for kindly providing material and valuable comments and advices on the taxonomy of Italian *Limax*.

We acknowledge Janine Serafini (Société des Sciences historiques et naturelles de la Corse), Bernard Recorbet (DIREN Corsica), the late Bernard Roché (DIREN Corsica), Achille Pioli (Corte, ONF), Lucien Borelli (Furiani Marinella), Jean-Claude Gabrielli (ONF), and Emilio Vichera (Golfe de Sperono) for very helpful and cordial support on site.

References

Alvarez W (1972) Rotation of the Corsica-Sardinia microplate. Nature Phys Sci 235:103–105

Brogi A (2008) Kinematics and geometry of Miocene low-angle detachments and exhumation of the metamorphic units in the hinterland of the Northern Apennines (Italy). J Struct Geol 30:2–20

Brunet C, Monié P, Jolivet L, Cadet J-P (2000) Migration of compression and extension in the Tyrrhenian Sea, insights from 40Ar/39Ar ages on micas along a transect from Corsica to Tuscany. Tectonophysics 321:127–155

Cavazza W, Zattin M, Ventura B, Zuffa GG (2001) Apatite fission-track analysis of Neogene exhumation in northern Corsica (France). Terra Nova 13:51–57

Cipollari P, Cosentino D, Gliozzi E (1999) Extension- and compression-related basins in central Italy during the Messinian Lago-Mare event. Tectonophysics 315:163–185

Colosi G (1919) L'azione della veratrina sui Gasteropodi terrestri e la specificità di Limax maximus e Limax cinereo-niger. Arch Zool Exp Gen 58:45–48

Danišík M, Kuhlemann J, Dunkl I, Székely B, Frisch W (2007) Burial and exhumation of Corsica (France) in the light of fission track data. Tectonics 26, TC1001, pp 1–24

Durand-Delga M (1974) La Corse. In: Debelmas J (ed) Géologie de la France, vol 2. Doin, Paris, pp 465–478

Elias M, Hill RI, Willmott KR, Dasmahapatra KK, Brower AV, Mallet J, Jiggins CD (2007) Limited performance of DNA barcoding in a diverse community of tropical butterflies. Proc Biol Soc Lond B 274:2881–2889

Engels WR (1993) Contributing software to the internet: the amplify program. Trends Biochem Sci 18:448–450

Falkner G (2001) The genus Limax in Corsica: an unexpected diversity and its threats (Gastropoda, Limacidae). In: Salvini-Plawen L, Voltzow J, Sattmann H, Steiner G (eds) Abstracts of the world congress of malacology in Vienna 2001. Unitas Malacologica, Vienna, p 99

Falkner G, Bank RA, von Proschwitz T (2001) Check-list of the non-marine molluscan species-group taxa of the states of Northern, Atlantic, and Central Europe (CLECOM I). Heldia 4:1–76

Falkner G, Ripken TEJ, Falkner M (2002) Mollusques continentaux de France. Liste de Référence annotée et Bibliographie. Collection Patrimoines Naturels 52, Paris

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3:294–299

Gautier A (1983) Roches et paysages de la Corse. Découverte de la Nature, Collection des Guides des Parcs Naturels Régional de France, Publications du Parc Naturel Régional de la Corse et BRGM, Ajaccio et Orléans

Gerhardt U (1933) Zur Kopulation der Limaciden. I. Mitteilung. Z Morphol Ökol Tiere 27:401–450

Gerhardt U (1934) Zur Kopulation der Limaciden. II. Mitteilung. Z Morphol Ökol Tiere 28:229–258

Gerhardt U (1937) Weitere Untersuchungen zur Sexualbiologie der Limaciden. Z Morphol Ökol Tiere 32:518–541

Giusti F (2007) La Chiocciola di Capraia, ovvero Tacheocampylaea tacheoides (Pollonera, 1909). http://test.isoladicapraia.it/

Giusti F, Mazzini M (1971) Notulae Malacologicae XIV. I Molluschi delle Alpi Apuane. Elenco delle specie viventi con descrizione di una nuova specie: Vitrinobrachium baccettii n. sp. Lav Soc Ital Biogeogr (NS) 1:202–335

Govers R, Meijer P, Krijgsman P (2009) Regional isostatic response to Messinian salinity crisis events. Tectonophysics 463:109–129

Hausdorf B (1998) Phylogeny of the Limacoidea sensu lato (Gastropoda: Stylommatophora). J Molluscan Stud 64:35–66

Hajibabaei M, Singer GA, Hebert PD, Hickey DA (2007) DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. Trends Genet 23:167–172

Hebert PD, Cywinska A, Ball SL, deWaard JR (2003a) Biological identifications through DNA barcodes. Proc R Soc Lond B 270:313–321

Hebert PD, Ratnasingham S, deWaard JR (2003b) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc R Soc Lond B 270:S96–S99

Hebert PD, Stoeckle MY, Zemlak TS, Francis CM (2004) Identification of birds through DNA barcodes. PLoS Biol 2:e312

Holyoak DT (1983) Distribution of land and freshwater Mollusca in Corsica. J Conch 31:235–251

Hyman IT, Ho SYW, Jermiin LS (2007) Molecular phylogeny of Australian Helicarionidae, Euconulidae and related groups (Gastropoda: Pulmonata: Stylommatophora) based on mitochondrial DNA. Mol Phyl Evol 45:792–812

Jolivet L, Augier R, Faccenna C, Negro F, Rimmele G, Agard P, Robin C, Rossetti F, Crespo-Blanc A (2008) Subduction, convergence and the mode of backarc extension in the Mediterranean region. Bull Soc Geol Fr 179:525–550

Lanza B (1984) Sul significato biogeografico delle isole fossili, con particolare riferimento all'Arcipelago pliocenico della Toscana. Atti Soc ital Sci nat 125:145–158

Lessona M, Pollonera C (1882) Monografia dei limacidi italiani. Mem Accad Sci Torino 35:49–128

Manganelli G, Bodon M, Favilli L, Giusti F (1995) Gastropoda pulmonata. In: Minelli A, Ruffo S, La Posta S (eds) Checklist delle specie della fauna italiana, vol 16. Calderini, Bologna, pp 1–60

Mayer W, Pavlicev M (2007) The phylogeny of the family Lacertidae (Reptilia) based on nuclear DNA sequences: convergent adaptations to arid habitats within the subfamily Eremiainae. Mol Phylogenet Evol 44:1155–1163

Meier R, Shiyang K, Vaidya G, Ng PKL (2006) DNA barcoding and taxonomy in Diptera: a tale of high intraspecific variability and low identification success. Syst Biol 55:715–728

Meijden A van der, Chiari Y, Mucedda M, Carranza S, Corti C, Veith M (2009) Phylogenetic relationships of Sardinian cave salamanders, genus Hydromantes, based on mitochondrial and nuclear DNA sequence data. Mol Phylogenet Evol 51:399–404

Meyer CP, Paulay G (2005) DNA barcoding: error rates based on comprehensive sampling. PLoS Biol 3:2229–2238

Moquin-Tandot A (1855–1856) Histoire Naturelle des Mollusques terrestres et fluviatiles de France, vol 2. J.-B. Baillière, Paris

Nei M, Kumar S (2000) Molecular evolution and phylogenetics. Oxford University Press, Oxford

Nylander JAA (2004) MrModeltest v2. Program distributed by the author. Evolutionary biology centre, Uppsala University, Uppsala

Pollonera C (1896) Appunti di Malacologia IX Sui Limacidi della Corsica. Boll Mus Zool Anat comp r Univ Torino 11(264):1–5

Rambaut A (1996) Se-Al: sequence alignment editor. Available at http://evolve.zoo.ox.ac.uk

Réal G, Réal-Testud A-M (1988) La malacofaune terrestre de l'île de Corse: Historique et inventaire actualisé. Haliotis 18:43–54

Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574

Rook L, Gallai G, Torre D (2006) Lands and endemic mammals in the late Miocene of Italy: constrains for paleogeographic outlines of Tyrrhenian area. Palaeogeogr Palaeocl Palaeoecol 238:263–269

Schileyko AA (2003) Treatise on recent terrestrial pulmonate molluscs, 11. Trigonochlamydidae, Papillodermidae, Vitrinidae, Limacidae, Bielziidae, Agriolimacidae, Boettgerillidae, Camaenidae. Ruthenica Suppl 2:1467–1626

Simroth H (1900) Ueber einige Nacktschnecken von Montenegro und Corsica. Nachr-Bl Dtsch Malak Ges 32:77–85, 97–107

Simroth H (1910) Nacktschneckenstudien in den Südalpen. Abh Senckenb Naturf Ges 32:275–348

Stöck M, Dubey S, Klütsch C, Litvinchuk SN, Scheidt U, Perrin N (2008) Mitochondrial and nuclear phylogeny of circum-Mediterranean tree frogs from the Hyla arborea group. Mol Phylogenet Evol 49:1019–1024

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Waugh J (2007) DNA barcoding in animal species: progress, potential and pitfalls. Bioessays 29:188–197

Werle E, Schneider C, Renner M, Völker M, Fiehn W (1994) Convenient single-step, one tube purification of PCR products for direct sequencing. Nucleic Acids Res 22:4354–4355
Whitworth TL, Dawson RD, Magalon H, Baudry E (2007) DNA barcoding cannot reliably identify species of the blowfly genus Protocalliphora (Diptera: Calliphoridae). Proc Biol Sci 274:1731–1739
Wiktor A (2001) The slugs of Greece (Arionidae, Milacidae, Limacidae, Agriolimacidae - Gastropoda Stylommatophora) Fauna Graeciae 8. Natural History Museum of Crete, Hellenic Zoological Society, Irakleio
Zhang P, Papenfuss TJ, Wake MH, Qu L, Wake DB (2008) Phylogeny and biogeography of the family Salamandridae (Amphibia: Caudata) inferred from complete mitochondrial genomes. Mol Phylogenet Evol 49:586–597

Supplement: List of Material

The corresponding map points of locality in Figs. 9 and 10 are shown in parentheses.

L023, L024: *Limax* sp. (Restonica); FR, Corsica, Restonica Valley (7); leg. B. & H. Nitz, 2004; ZSM Mol 20071660, ZSM Mol 20071661, GenbankNo. GQ145497, GQ145498.

L030, L079: *Limax* sp. (Tuani); FR, Corsica, Restonica Valley (25); leg. B. & H. Nitz, 2004; ZSM Mol 20071662, ZSM Mol 20071663; GenbankNo. GQ145499, GQ145515.

L036: *Limax wolterstorffi* (Simroth, 1900); FR, Corsica, Bonifatu (1); leg. M. & G. Falkner, 2000; MNHN; GenbankNo. GQ145500.

L038: *Limax vizzavonensis*; FR, Corsica, Vizzavona, Cascade des Anglais (12); leg. M. & G. Falkner, 2000; MNHN; GenbankNo. GQ145501.

L040: *Limax vizzavonensis*; FR, Corsica, Sorba (10); leg. M. & G. Falkner, 2000; MNHN; GenbankNo. GQ145502.

L043, L044: *Limax* sp. (Porto); FR, Corsica, Porto (2; 3); leg. M. & G. Falkner, 2000; MNHN; GenbankNo. GQ145503, GQ145504.

L046: *Limax* sp. (Casamaccioli); FR, Corsica, Casamaccioli (4); leg. M. & G. Falkner, 2000; MNHN; GenbankNo. GQ145505.

L048: *Limax vizzavonensis*; FR, Corsica, Bastani/Monte Renoso (13); leg. B. & J. Recorbet, 2003; MNHN; GenbankNo. GQ145506.

L049: *Limax* sp. (Ruggelone B); IT, Tuscany, Com. Talla, località Ruggelone; leg. W. Weidinger, 2003; SMNS ZI 0071837; GenbankNo. GQ145507.

L066: *Limax* sp. (Corte); FR, Corsica, Corte, Citadelle (6); leg. M. & G. Falkner, F1; Coll. Falkner SMNS ZI 0071838; GenbankNo. GQ145508.

L068: *Limax wolterstorffi*; FR, Corsica, Bonifatu (1); leg. M. & G. Falkner, 2002; MNHN; GenbankNo. GQ145509.

L069, L070: *Limax* sp. (Tuani); FR, Corsica, Restonica Valley (25); leg. M. & G. Falkner, 2000; MNHN; GenbankNo. GQ145510, GQ145511.

L072: *Limax* sp. (Corte); FR, Corsica, Corte, Citadelle (24); F2; Coll Falkner; SMNS; ZI 0071839 GenbankNo. GQ145512.

L074: *Limax* sp. (Cap Corse A); FR, Corsica, Furiani (21); F1; Coll. Falkner; SMNS ZI 0071840; GenbankNo. GQ145513.
L077: *Limax* sp. (Cap Corse A); FR, Corsica, Furiani (21); F3; Coll. Falkner; SMNS ZI 0071841; GenbankNo. GQ145514.
L085: *Limax* sp. (Ruggelone B); IT, Tuscany, Com. Talla, località Ruggelone; F1; Coll. Falkner; SMNS ZI 0071842; GenbankNo. GQ145516.
L088: *Limax* sp. (Mte. Baldo); IT, Lombardy, Monte Baldo; leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071664; GenbankNo. GQ145517.
L094: *Limax* sp. (Mte. Sagro); IT, Tuscany, Alpi Apuane, Monte Sagro; leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071665; GenbankNo. GQ145518.
L100: *Limax* sp. (Mte. Altissimo); IT, Lombardy, Monte Altissimo; leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071666; GenbankNo. GQ145519.
L111, L112, L125: *Limax* sp. (Mte. San Petrone); FR, Corsica, Castagniccia, Monte San Petrone (22); leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071667 - ZSM Mol 20071669; GenbankNo. GQ145520, GQ145521, GQ145522.
L126, L127, L129, L140: *Limax* cf. *corsicus* s. str.; FR, Corsica, Vizzavona (26); leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071670 - ZSM Mol 20071673; GenbankNo. GQ145523, GQ145524, GQ145525, GQ145526.
L143: *Limax vizzavonensis*; FR, Corsica, Vizzavona (11); leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071674; GenbankNo. GQ145527.
L148: *Limax* sp. (Bonifatu); FR, Corsica, Bonifatu (23); leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071675; GenbankNo. GQ145528.
L150, L151: *Limax* sp. (Restonica); FR, Corsica, Restonica Valley (8); leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071676, ZSM Mol 20071677; GenbankNo. GQ145529, GQ145530.
L158, L159: *Limax* sp. (Ft. Melo); FR, Corsica, Fôret de Melo (5); leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071678, ZSM Mol 20071679; GenbankNo. GQ145531, GQ145532.
L160: *Limax vizzavonensis*; FR, Corsica, Sorba (10); leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071680; GenbankNo. GQ145533.
L161, L162: *Limax* sp. (Ft. Piattone); FR, Corsica, Fôret de Piattone (29); leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071681, ZSM Mol 20071682; GenbankNo. GQ145534, GQ145535.
L164: *Limax* sp. (Coscione); FR, Corsica, Plateau de Coscione (16); leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071683; GenbankNo. GQ145536.
L165: *Limax* sp. (Coscione); FR, Corsica, Fôret de Coscione (15); leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071684; GenbankNo. GQ145537.
L169, L170: *Limax corsicus* s. str.; FR, Corsica, Bastelica (28); leg. G. Falkner, B. Nitz & B. Recorbet, 2004; ZSM Mol 20071685, ZSM Mol 20071686; GenbankNo. GQ145538, GQ145539.
L180, L188, L189: *Limax ilvensis* n. sp.; IT, Elba; leg. E. Schwabe & J. Bohn, 2004; ZSM Mol 20071687 - ZSM Mol 20071689; GenbankNo. GQ145540, GQ145541, GQ145542.

L195: *Limax* sp. (Cap Corse A); FR, Corsica, Grigione near Bastia (19); leg. M. & G. Falkner, 2000; MNHN; GenbankNo. GQ145543.
L197: *Limax* sp. (Cap Corse A); FR, Corsica, Pietrabugno near Bastia (20); leg. M. & G. Falkner, 2000; MNHN; GenbankNo. GQ145544.
L199: *Limax* sp. (Cap Corse A); FR, Corsica, Furiani (21); F1; Coll. Falkner; SMNS ZI 00718; GenbankNo. GQ145545.
L210, L211: *Limax* sp. (Maiella); IT, Abruzzo, Maiella; leg. C.M. Brandstetter, 2004; SMNS ZI 0071844, ZI 0071861; GenbankNo. GQ145546, GQ145547.
L232: *Limax* sp. (Villa Strozzi); IT, Tuscany, Villa Strozzi near San Gimignano; leg. M. & G. Falkner, 1992; SMNS ZI 0071845; GenbankNo. GQ145548.
L237: *Limax* sp. (Massa Marittima); IT, Tuscany, Massa Marittima; leg. M. & G. Falkner, 1999; SMNS ZI 0071846; GenbankNo. GQ145549.
L240: *Limax* sp. (Truggia); FR, Corsica, Truggia, Liamone-Valley (27); leg. M. & G. Falkner, 2000; MNHN; GenbankNo. GQ145550.
L241: *Limax* sp. (Bonifatu); FR, Corsica, Bonifatu (23); leg. M. & G. Falkner, 2000; MNHN; GenbankNo. GQ145551.
L269: *Limax* sp. (Mte. Renoso); FR, Corsica, Monte Renoso (14); leg. B. Recorbet, 2000; MNHN; GenbankNo. GQ145552.
L301: *Limax ciminensis*; IT, Latium, Monte Cimino; leg. G. Falkner & C.M. Brandstetter, 2005; SMNS ZI 0071847; GenbankNo. GQ145553.
L345: *Limax* sp. (Verna); IT, Tuscany, Chiusi della Verna; leg. W. Weidinger, 2005; SMNS ZI 0071848; GenbankNo. GQ145554.
L382: *Limax* sp. (Torrente Trossa); IT, Tuscany, Fontebagni/Torrente Trossa; leg. G. Falkner & C.M. Brandstetter, 2005; SMNS ZI 0071849; GenbankNo. GQ145555.
L384: *Limax* sp. (Marmoraia); IT, Tuscany, Montagnola Senese, Marmoraia; leg. G. Falkner & C. M. Brandstetter, 2005; SMNS ZI 0071850; GenbankNo. GQ145556.
L387: *Limax* sp. (Chianti); IT, Tuscany, Castellina in Chianti; leg. G. Falkner & C.M. Brandstetter, 2005; SMNS ZI 0071851; GenbankNo. GQ145557.
L389: *Limax* sp. (Vignano); IT, Tuscany, Vignano near Siena; leg. G. Falkner & C.M. Brandstetter, 2005; SMNS ZI 0071852; GenbankNo. GQ145558.
L392: *Limax* sp. (Mte. Rasu); IT, Sardinia, Monte Rasu; leg. B. & H. Nitz, 2005; ZSM Mol 20071690; GenbankNo. GQ145559.
L405, L408, L424: *Limax* sp. (Castelsecco); IT, Tuscany, Castelsecco near Arezzo; leg. G. Falkner & C.M. Brandstetter, 2005; SMNS ZI 0071853, ZI 0071863, ZI 0071864; GenbankNo. GQ145560, GQ145561, GQ145567.
L412: *Limax* sp. (Arezzo A); IT, Tuscany, Arezzo, Podere Redi; leg. G. Falkner & C.M. Brandstetter, 2005; SMNS ZI 0071854; GenbankNo. GQ145562.
L419, L420: *Limax* sp. (Mte. Rotondo); FR, Corsica, Monte Rotondo (9); leg. B. Recorbet, 2005; MNHN; GenbankNo. GQ145563, GQ145564.
L422, L423: *Limax* sp. (Ruggelone A); IT, Tuscany, Com. Talla, località Ruggelone; leg. W. Weidinger, 2005; SMNS ZI 0071855, ZI 0071862; GenbankNo. GQ145565, GQ145566.

L426: *Limax* sp. (Arezzo A); IT, Tuscany, Arezzo, Villa Fiorita; leg. G. Falkner & C.M. Brandstetter, 2005; ZSM Mol 20071691; GenbankNo. GQ145568.
L427: *Limax* sp. (Arezzo B); IT, Tuscany, Arezzo, Villa Fiorita; leg. G. Falkner & C.M. Brandstetter, 2005; SMNS ZI 0071856; GenbankNo. GQ145569.
L506: *Lehmannia marginata*; Sweden, Dalsland; leg. R. Heim, 2001; NMLU 14457; GenbankNo. FJ606455.
L523, L524: *Limax giustii* n. sp.; IT, Capraia (31); leg. F. Giusti, 2005; IZSI 36444/1; IZSI 36444/2; GenbankNo. GQ145582, GQ145581.
L604: *Limax* sp. (Cap Corse B); FR, Corsica, Nonza (18); leg. M. & G. Falkner, 2006; ZSM Mol 20071692; GenbankNo. GQ145570.
L605: *Limax* sp. (Cap Corse A); FR, Corsica, Vallée de la Meria (17); leg. M. & G. Falkner, 2006; ZSM Mol 20071694; GenbankNo. GQ145580.
L607: *Limax* cf. *corsicus* s. str.; FR, Corsica, Étang de Sperono, near Bonifacio, Golfcourse (30); leg. M. & G. Falkner, 2006; ZSM Mol 20071693; GenbankNo. GQ145571.
L765: *Limax brandstetteri* (Falkner, 2008); IT, Abruzzo, Maiella; leg. C.M. Brandstetter, 2005; SMNS ZI 0066222-1 ; GenbankNo. GQ145572.
L811: *Limax ianninii* (Giusti, 1973); IT, Latium, Monti Reatini, Monte Terminillo; leg. C.M. Brandstetter, 2006; SMNS 0071857-1; GenbankNo. GQ145573.
L898: *Limacus flavus*; DE, Goch; leg. S. Henssen, 2006; ZSM Mol 20071629; FJ606456.
L903: *Limax maximus*; CH, Grisons, Chur; leg. B. Nitz & U. Schneppat, 2006; ZSM Mol 20071620; GenbankNo. FJ606467.
L991: *Limax maximus*; IT, Campania, Roccamonfina; leg. C. & L. Cavegu, 2006; ZSM Mol 20071654; GenbankNo. GQ145574.
L1016: *Limax senensis* Lessona & Pollonera, 1882); IT, Tuscany, Siena; leg. M. & G. Falkner, F1; ZSM Mol 20071699; GenbankNo. GQ145575.
L1125: *Limax cinereoniger*; DE, Oberkrumbach; leg. E. Klee, A. Klee & B. Nitz, 2006; ZSM Mol 20071618; GenbankNo. FJ606460.
L1410: *Limacus flavus*; UK, Surrey, Banstead; leg. J. Hutchinson, 2007; ZSM Mol 20071630; GenbankNo. FJ606457.
L1464: *Vitrina pellucida*; DE, Kolbingen; leg. B. Hausdorf, 2006; ZMH 51046; GenbankNo. FJ606454.
L1600: *Limax* sp. (Luogosanto); IT, Sardinia, Luogosanto; leg. B. Ruthensteiner, 2007; ZSM Mol 20071695; GenbankNo. GQ145576.
L1612: *Limax* sp. (Populonia A); IT, Tuscany, Populonia; leg. J. Spelda, 2007; ZSM Mol 20071696; GenbankNo. GQ145577.
L1613: *Limax* sp. (Populonia B); IT, Tuscany, Populonia; leg. J. Spelda, 2007; ZSM Mol 20071697; GenbankNo. GQ145578.
L1749: *Limax* sp. (Cutigliano); IT, Tuscany, Cutigliano-Melo; leg. G. Bertagni, 2007; ZSM Mol 20071698; GenbankNo. GQ145579.
L1778: *Limax cinereoniger*; DE, Oberkrumbach; leg. E. Klee, A. Klee & B. Nitz, 2007; ZSM Mol 20071619; GenbankNo. FJ606463.
W002: *Limax wohlberedti*; HR, Dalmatia; leg. A. Wiktor, 1999; MNHW, Coll. A. Wiktor 3004; GenbankNo. FJ606481.

Appendix: Two New Species and One New Name of Peri-Tyrrhenian *Limax*

Gerhard Falkner and Barbara Nitz

In this appendix, we introduce names for two hitherto unrecognized species originally revealed by COI barcoding and replace a preoccupied name for a well defined species.

Limax specimens from the Tuscan islands Elba and Capraia have been described by Giusti (1969; 1976) and Giusti and Mazzini (1971) and were thought to belong to *Limax corsicus* s. str. (see also Pollonera 1905). However, our Bayesian tree reconstruction of 615 nucleotides of the cytochrome *c* oxidase subunit I gene (COI) grouped specimens of these two islands in two distinct clades with high support values (PP 100% for Elba specimens and 95% for Capraia specimens), placing *L. corsicus* from the type locality in a different group. These findings reveal the anatomical differences (especially in the internal structure of the penis) found by Giusti in a new light. In line with Code Art. 13.1.2 (ICZN 1999), we base the new names on the existing excellent descriptions. The necessary (partly unpublished) information about the type material was kindly provided by the author.

Limax giustii n. sp.

Description: Giusti 1969: Genital apparatus (Fig. 12); Giusti and Mazzini 1971: Internal structure of the penis (Fig. 13).

Derivatio nominis: Named in honor of our distinguished colleague and friend Prof. Dr. Folco Giusti di Massa, whose valuable *Limax*-studies began on his beloved island of Capraia.

Holotype: The specimen represented in Fig. 13 (Giusti and Mazzini 1971); collected in the Capraian site (very close to the village and to the locality called "La Grotta") which is called "San Leonardo"; leg. F. Giusti 14.04.1968 (1966 is a misprint – Giusti, personal communication). Body length in ethanol (after drowning) ca. 6.2 cm, width ca. 1 cm. Length of penis ca. 10.5 cm. Preparation in Giusti collection, IZSI 22001.

Paratypes: Four specimens collected in the Capraian site of "San Rocco"; leg. F. Giusti 31.10.2005. Maximum length in ethanol ca. 8 cm, width ca. 1.5 cm. The back of these specimens is predominantly uniformly dark or with a whitish band corresponding to the keel line. Preparations in Giusti collection, IZSI 36444; Tissue samples SMNS ZI 0071865 (two specimens have been sequenced: L523 and L524, DNA elutions stored in ZSM DNA-bank).

Remarks: According to Giusti's personal observations, the Capraia specimens are actually slightly smaller than other members of the so-called *L. corsicus* ("but this has not a sure relevance, due to the possibility of an insular dwarfism phenomenon"). Estimated from memory, they reach alive a length of ca. 10 cm when fully

extended. Judging from photographs, there are mainly the following colour morphs: dark brown to blackish, medium brown with contrasting yellowish-white lateral bands and keel line, and medium brown with irregular blotchy dark lateral bands which are separated from the darker back by brighter zones, sometimes the mantle shield is spotted. The reddish colouration of the sole is normally not very intense.

Several copulations were observed and photographed by Giusti in spring 1983 and 1985. The basis of comparison is not yet sufficient to draw definite taxonomical conclusions. The action follows the general scheme of the *Corsicus*-group as described for the first time by Gerhardt (1937) for Ischia. Some special features are: the penial combs are quite weakly developed, the penial bases are not in close contact [as, for example, in *L.* sp. (Bonifatu)], the bases of the bursa copulatrix are not everted [as, for example, in *L.* sp. (Tuani) and specimens from Marmuccio], in the contraction phase dense white foam is excreted (which is not the case in the Endemic *Corsicus*-group, but present in the Cape Corse/Tuscany-group), the maximum extension of the penes is between 26 and 30 cm (see Figs. 5 and 6).

The new species is endemic for the Tuscan Island of Capraia.

Limax ilvensis n. sp.

Description: Giusti 1976: Discussion of characters and internal structure of the penis (Fig. 21).

Derivatio nominis: An adjective formed from Ilva, the Roman name for Elba.

Holotype: The specimen represented in Fig. 21 (Giusti 1976); collected at the site "Portoferraio: il Forte", of the Island of Elba; leg. F. Giusti 18.02.74. Length in ethanol (after drowning) ca. 7 cm; width ca. 1.25 cm. Length of penis ca. 12 cm. Preparation in Giusti collection, IZSI 11977.

Paratypes: 1 specimen (L180), Elba, Monte Perone, ca. 600 m, biotope with chestnut and pine trees; leg. E. SCHWABE & J. BOHN 19.10.2004. ZSM Mol 20071687. – 2 specimens (L188 and L189), Elba, Capoliveri, ca. 250 m; ruderalised resting place; leg. E. SCHWABE & J. BOHN 20.10.2004. ZSM Mol 20071688 and 20071689.

Remarks: The paratypes and additional live specimens (ZSM) comprised brown and dark brown colour morphs with reddish sole, the latter characteristic for the *Corsicus*-group.

According to Giusti, the preparation of the holotype has been discoloured by ethanol, but nevertheless its colour is clearly paler than that of the Capraia specimens. The holotype shows a narrow whitish band on both sides which is bordered by interrupted blackish bands similarly narrow. The back shows a pale-brownish colour with more or less large darker lobate spots. The clypeus has a paler, almost whitish basic colour with large darker lobate spots. The lower part of the sides is similarly whitish with small, brownish lobate spots. The bleached sole is whitish throughout.

For two Elba collections with quite well preserved colours in the NMW (no. 39559, leg. Holdhaus 1904; no. 45114, leg. Paganetti 1908) the following

observations have been noted: dark to nearly black morphs are dominant, juveniles nearly black, subadults brighter with diffuse brown lateral bands and slightly darker back; in the second collection, some specimens were deep black with contrasting narrow white lateral bands. The soles were slightly reddish to yellow.

The new species is endemic for the Tuscan Island of Elba.

Limax vizzavonensis n. nom.

This new replacement name is herewith introduced for *Limax* (*Eulimax*) *cinereoniger* var. *minimus* Pollonera, 1896, which is preoccupied by *Limax minimus* Forsskål, 1775.

Nomenclatural history: Although for its time aptly described, *Limax minimus* Pollonera, 1896, was largely neglected. The name was only used by Taylor (1903) and Hesse (1926) for an infrasubspecific entity, by Caziot (1903) and Alzona (1971) at subspecific rank, and by Falkner et al. (2002) at species rank.

Following Hesse (1924), Falkner et al. overlooked the fact that the name is preoccupied by the name of a sea slug. Suppression of the older homonym *Limax minimus* Forsskål, 1775, was proposed by Lemche (1964: 37), who considered the species as unrecognisable, in order to avoid confusion. Accordingly *Limax minimus* Forsskål, 1775, was by Opinion 773 (ICZN 1966) "suppressed for the purposes of the Law of Priority but not for those of the Law of Homonymy." The consequence of the latter is that it continues to preclude the validation of its younger primary homonym which therefore must be replaced. The existing replacement name *L. obscurus* Simroth, 1900, cannot be used as it is itself preoccupied by *L. maximus* var. *obscurus* Moquin-Tandot, 1855.

The results of our foregoing morphological and genetic studies have shown that it is necessary to dispose of a valid name for this distinguishable biological entity which has already been invalidly named twice. The new name *vizzavonensis* is derived from the type locality.

Remarks: The type locality which was given by Pollonera simply as "Vizzavona" is stated more precisely by Caziot (1903), who collected the holotype, as "la Foce, près Vizzavona, à l'altitude de 1,000 m." The sequenced animal collected near Cascade des Anglais (L038) was found only about 200 m away from the type locality.

Despite the fact that the present solution of the nomenclatural problem is provided, a thorough redescription still remains a desideratum for future research.

Additional References

Alzona C (1971) Malacofauna italica. Catalogo e bibliografia dei Molluschi viventi, terrestri e d'acqua dolce. Atti Soc ital Sci nat Mus civ Stor nat Milano 111:433

Caziot E (1903) Étude sur la faune des Mollusques vivants terrestres et fluviatiles de l'Île de Corse. Bull Soc Sci hist nat Corse 22:354

Giusti F (1969) Notulae Malacologicae V. Le Isole di Gorgona, Capraia e Giglio. Atti Soc tosc Sci nat, Mem (B) 75:265–324

Giusti F (1976) Notulae Malacologicae XXIII. I Molluschi terrestri, salmastri e di acqua dolce dell'Elba, Giannutri e scogli minori dell'Arcipelago Toscano. Conclusioni generali sul popolamento malacologico dell'Arcipelago toscano e descrizione di una nuova specie. (Studi sulla riserva naturale dell'Isola di Montecristo. IV. Lav Soc ital Biogeogr (NS) 5:99–355

Hesse P (1924) Kritische Fragmente, XXIX. Aenderungsbedürftige Nacktschnecken-Namen. Arch Mollkunde 56:228–230

Hesse P (1926) Die Nacktschnecken der palaearktischen Region. Abh Arch Mollkunde 2:1–152

ICZN (1966) Opinion 773. *Tergipes* Cuvier, 1805 (Gastropoda): Validated under the plenary powers. Bull zool Nomencl 23:84–86

ICZN (1999) International Code of Zoological Nomenclature. 4th edn, International trust for zoological nomenclature, London

Lemche H (1964) Proposed use of the plenary powers to grant precedence to the family-group name Cuthonidae over Tergipedidae and to stabilize some specific names in the genus known as *Eubranchus* Forbes, 1838 (Class Gastropoda). Bull zool Nomencl 21:35–39

Pollonera C (1905) Note malacologiche II. Molluschi terrestri e fluviatili delle Isole d'Elba e Pianosa. Boll Mus Zool Anat comp r Univ Torino 20:3–9

Taylor JW (1902–1907) Monograph of the Land- and Freshwater Mollusca of the British Isles. Vol 2. Taylor Brothers, Leeds

Palaeogeography or Sexual Selection: Which Factors Promoted Cretan Land Snail Radiations?

Jan Sauer and Bernhard Hausdorf

Abstract The high land snail diversity of Crete is the result of a few radiations. It has been suggested that these radiations were triggered by the fragmentation of Crete in the Neogene. Contrary to the predictions of this model, the ranges of the endemic species are not clustered and their diversity is not higher in the areas of the Neogene palaeo-islands. We investigated the radiation of the helicoid genus *Xerocrassa* in detail. The asymmetry between the range sizes of sister species and clades of *Xerocrassa* indicates that peripatric speciation was the predominant speciation mode. Coalescent simulations show that the differences in the genitalia of the *Xerocrassa* species cannot be explained by genetic drift. They can also not be explained by natural selection against hybrids, because they are not larger between geographically overlapping groups than between allopatric groups. The evolution of differences in flagellum length is concentrated towards the tips of the tree indicating that sexual selection might have facilitated the radiation. If speciation is driven by sexual selection, niches may remain conserved and non-adaptive radiation may result.

1 Introduction

The land snail fauna of Crete is extraordinarily rich. Approximately 140 land snail species are known from Crete. This number is much higher than would be expected considering the area of Crete, if it were compared with other Aegean islands (Welter-Schultes and Williams 1999). The high land snail diversity on Crete is mainly the result of a few radiations, namely of *Mastus* (Maassen 1995; Parmakelis et al. 2005), *Orculella* (Gittenberger and Hausdorf 2004), *Albinaria* (Schilthuizen

J. Sauer and B. Hausdorf (✉)
Zoological Museum, University of Hamburg, Martin-Luther-King-Platz 3, 20146 Hamburg, Germany
e-mail: hausdorf@zoologie.uni-hamburg.de

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_20,

and Gittenberger 1996; Douris et al. 1998; Welter-Schultes 2000a, b; Nordsieck 2004; Schilthuizen et al. 2004), and *Xerocrassa* (Hausdorf and Sauer 2009).

Gittenberger (1991) noted that the *Albinaria* species occupy more or less the same or only a narrow range of habitats. Thus, he suggested that the *Albinaria* radiation on Crete is a non-adaptive radiation. Parmakelis et al. (2005) could not find evidence for differential adaptation in the Aegean *Mastus* species and also classified the *Mastus* radiation as non-adaptive. Which processes resulted in the land snail radiations on Crete, if they were not caused by divergent natural selection and differential adaptation? Welter-Schultes and Williams (1999) suggested that the land snail radiations on Crete were the result of the fragmentation of the region of present-day Crete into several palaeo-islands from the lower Tortonian (11 million years ago) until the late Pliocene (2–3 million years ago) (Dermitzakis 1990; Welter-Schultes and Williams 1999; Welter-Schultes 2000a, b; Fassoulas 2001). Parmakelis et al. (2005) supposed that the radiation of *Mastus* was triggered by a diversification of the spermatophore morphology in allopatry, perhaps by sexual selection.

Considering these hypotheses, we investigated the systematics, the ecology, the biogeography and the potential role of sexual selection in a so far only insufficiently known radiation on Crete, that of the helicoid genus *Xerocrassa*.

2 Systematics of the *Xerocrassa* Radiation on Crete

In the nineteenth century, ten nominal species belonging to *Xerocrassa* were described from Crete, since when this group has not been revised. Because tests of evolutionary hypotheses require robust taxonomic and phylogenetic hypotheses, we have revised the Cretan *Xerocrassa* species based on morphological characters, AFLP markers, and mitochondrial *cox1* sequences.

Eleven native Cretan *Xerocrassa* species can be delimited based on morphological characters of the shell and the genitalia (Hausdorf and Sauer 2009). With the exception of *X. cretica*, which is widespread in the eastern Mediterranean, all species are endemic to Crete. All species except the species pair *X. siderensis* and *X. amphiconus* differ in characters of the genitalia.

The morphological classification is largely supported by multi-locus AFLP data. Of the 11 morphologically defined species, 9 are monophyletic in the neighbor-joining tree based on Jaccard distances between AFLP data of 151 *Xerocrassa* specimens (Fig. 1). Only *X. amphiconus* and *X. siderensis*, that are sympatric, but rarely syntopic, cannot be distinguished based on the AFLP data.

In contrast, the mitochondrial haplotypes of only 6 of the 11 morphologically defined species form monophyletic groups in a maximum likelihood tree of 122 partial *cox1* sequences (634 bp) of Cretan *Xerocrassa* species (Fig. 2), whereas the sequences of the other 5 species do not form distinct clades. In particular, sequences of the widespread *X. mesostena* are paraphyletic with respect to most of the other endemic *Xerocrassa* species. These results confirm other studies (e.g., Sota and

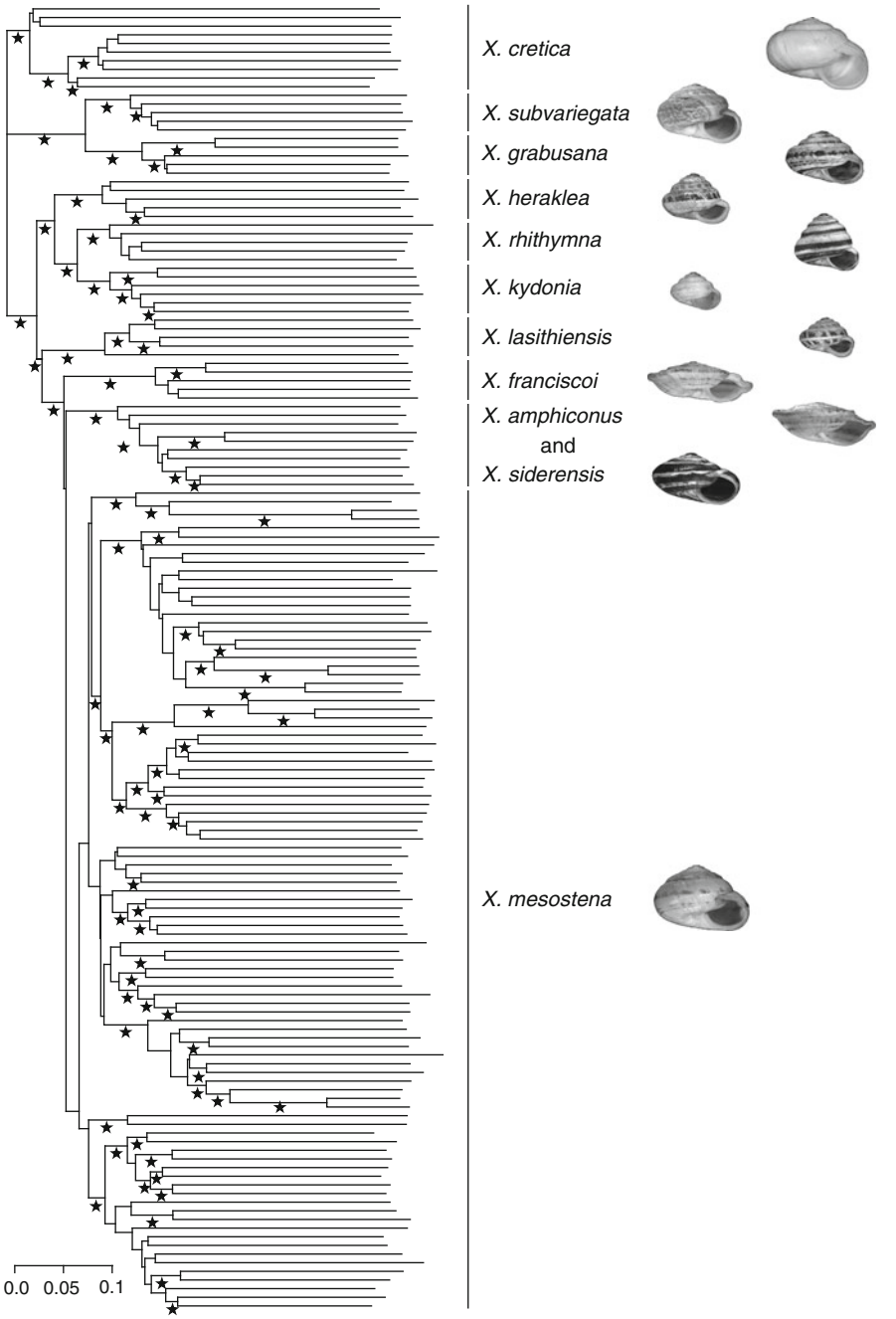


Fig. 1 Neighbor-joining tree based on Jaccard distances between AFLP data of Cretan *Xerocrassa*. Bootstrap support values larger than 70% are indicated by an *asterisk* below the branches

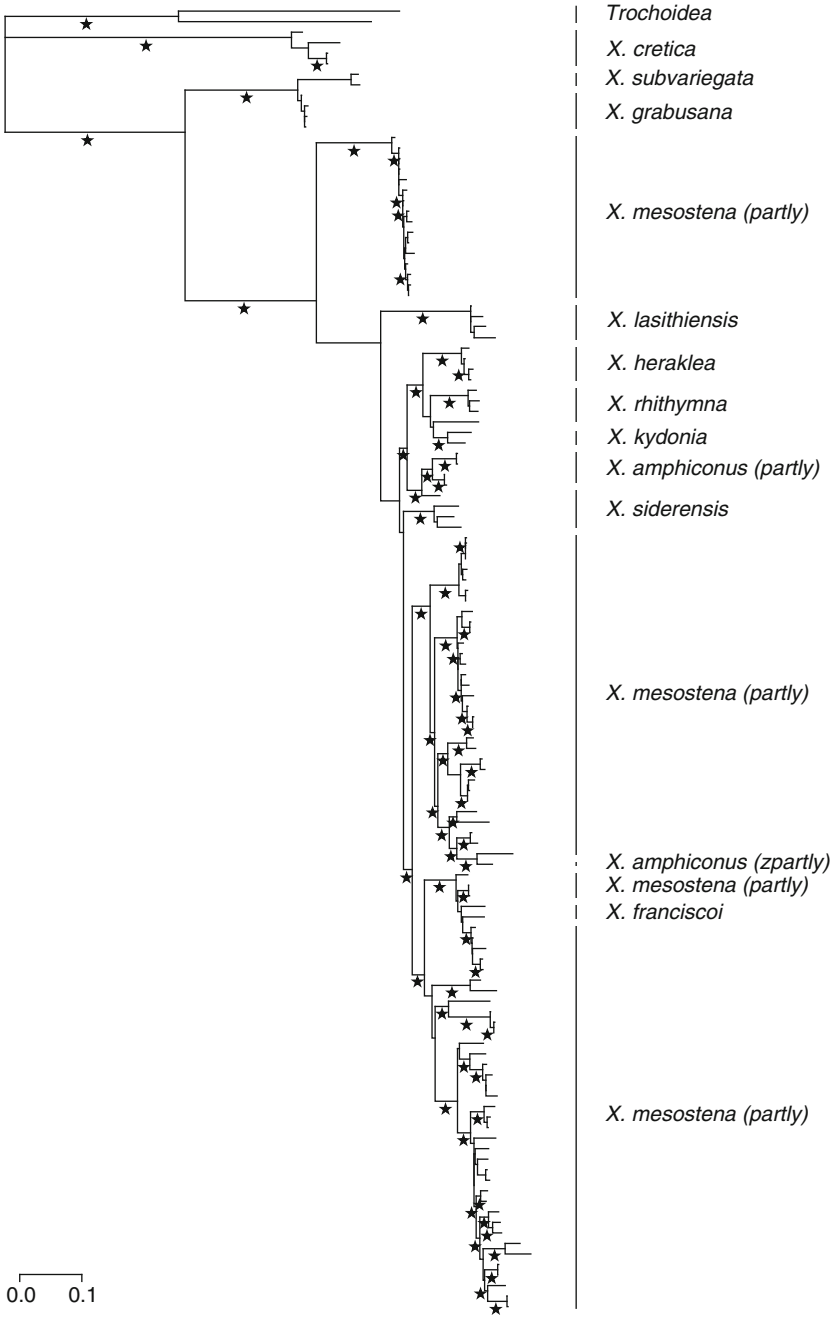


Fig. 2 Maximum likelihood tree of 122 partial *cox1* sequences of Cretan *Xerocrassa* species and two *Trochoidea* species. Bootstrap support values larger than 70% are indicated by *asterisks* below the branches

Vogler 2001; Shaw 2002; Machado and Hey 2003; Weisrock et al. 2006) that have reported large distinctions between mitochondrial gene trees and trees based on nuclear markers and/or species classifications based on morphology. If sets of sequences for which each sequence in a set has at least one other sequence within a 3% threshold distance are considered as approximations for species following Hebert et al. (2003), the 122 *cox1* sequences would represent 50 species. This excessive splitting is at least partly the result of high substitution rates of mitochondrial DNA in helicoid land snails (Thomaz et al. 1996; Chiba 1999; Hayashi and Chiba 2000; van Riel et al. 2005). This result challenges the generality and applicability of approaches to identify and delimit putative species on single signature sequences (e.g., Floyd et al. 2002; Blaxter et al. 2003, 2005; Hebert et al. 2003).

3 Ecological Differentiation of the Cretan *Xerocrassa* Species

All *Xerocrassa* species are xerophilic and live in open, dry habitats. During the summer, they aestivate under stones and bushes or, more rarely, attached to the vegetation. They are generalists feeding on decaying plants. There are no obvious adaptations to different habitats or lifestyles. Usually, only one or two *Xerocrassa* species live in the same place, just as in *Albinaria* (Gittenberger 1991). The only species that regularly co-occurs with other species is the widespread *X. cretica* that is much larger than all other Cretan *Xerocrassa* species. In the few cases in which two of the other species co-occur, there are also conspicuous differences in body size. Thus, we have investigated whether a differentiation in body size might have triggered the radiation. First, we tested whether the differentiation in body size might be the result of competition between the species. If this were the case, we would expect that the differences are larger between species with geographically overlapping ranges than between species that are not in contact or have only slightly overlapping ranges. Following Barraclough et al. (1999), we tested this prediction by randomizing phylogenetic independent contrasts in body size (measured as shell volume) among nodes, holding the degree of overlap fixed for every node. Standardized phylogenetic independent contrasts were calculated based on a species tree derived from the *cox1* gene tree. The AFLP tree could not be used, because it did not provide meaningful branch length. First, we made the *cox1* gene tree ultrametric with the penalized likelihood method (Sanderson 2002) and then we constructed the species tree using a modification of the shallowest divergence clustering method (Maddison and Knowles 2006). Randomization tests based on the resulting tree (*X. cretica*,((*X. subvariegata*, *X. grabusana*),(*X. lasithiensis*, ((*X. heraklea*,(*X. kydonia*, *X. rhithymna*)),(*X. amphiconus*+*X. siderensis*,(*X. franciscoi*, *X. mesostena*))))))) showed that contrasts in body size are significantly larger between geographically overlapping clades in the Cretan *Xerocrassa* radiation than expected under the null model of no association between morphological change and geographical overlap (Table 1). This indicates that competition between

Table 1 Positive associations between standardized contrasts in characters and the degree of geographical overlap and associations between standardized contrasts in characters and relative node age in the Cretan *Xerocrassa* radiation according to randomization tests. Positive signs of association of contrasts in characters with relative node age suggest that changes are concentrated towards the root and negative signs suggest that changes occur near the tips

	Geographical overlap	Node age	
	p	Sign	*p*
Log body size	0.021	+	0.372
Log penis:log body size	0.134	–	0.270
Log epiphallus:log body size	0.775	–	0.092
Log flagellum:log body size	0.458	–	<0.002
Log vagina:log body size	0.598	–	0.102
Log bursa copulatrix:log body size	0.192	–	0.122

co-occurring species resulted in ecological character displacement with regard to body size.

In the next step, we tested whether the changes in body size were associated with speciation. If this were the case, recently split species should display greater divergence in body size than expected under a null model of no association of body size changes with cladogenesis. In contrast, if differences in body size promote persistence and/or subsequent radiation, divergence should be greater between more distantly related lineages. Hence, we tested for a concentration of changes towards either the tips or the root of the species tree by randomly shuffling phylogenetic independent contrasts among branches of the tree and recording where changes occur on the tree in each trial, as proposed by Barraclough et al. (1999). This test showed that the distribution of the contrasts in body size across the species tree does not differ from random distributions (Table 1) indicating that body sizes changes have not triggered speciation.

4 Geographic Mode of Speciation

Organisms with low vagility like land snails are more promising as model groups in studies investigating the geographical modes of speciation than more mobile groups, because frequent range shifts will obscure the geographical pattern of speciation (Barraclough and Vogler 2000).

To test whether the fragmentation of the region of present-day Crete into several palaeo-islands in the late Miocene and Pliocene might have triggered the land snail radiations on Crete as suggested by Welter-Schultes and Williams (1999), we first investigated whether speciation was predominantly allopatric. If this were the case, recently diverged sister species are expected to display little or no overlap in geographic ranges (Barraclough and Vogler 2000). Alternatively, if speciation is predominantly sympatric, one range of recently diverged sister species is expected to enclose the other entirely. The intercept of a regression of the degree of sympatry

between sister clades, the ratio of the area of overlap and the range size of the clade with the smaller range, against node age as approximated by the branch lengths in the ultrametric species tree indicates the predominant geographic mode of speciation. It is expected to be close to 0, if speciation is predominantly allopatric, and close to 1, if speciation is predominantly sympatric. The intercept of the linear regression of the arcsine transformed degree of sympatry against branch length in species tree as approximation for node age is 0.202 (±0.176) (Fig. 3a) indicating that allopatric speciation was at least predominant.

If the land snail radiations on Crete were the result of the fragmentation of Crete into several palaeo-islands from the late Miocene until the Pliocene as hypothesized by Welter-Schultes and Williams (1999), we would expect that the ranges of the species are clustered and that the ranges are centered on the positions of the Neogene palaeo-islands. However, the distribution areas of the 74 endemic land snail species belonging to genera with at least two endemic species are not significantly clustered ($p = 0.311$) according to the test for clustering proposed by Hausdorf and Hennig (2003). The number of occurrences of the endemic land snail species in 10 km UTM grids that are located in areas formerly belonging to palaeo-islands, was not significantly greater ($p = 0.595$) for the real data (742) than for 1,000 datasets obtained by Monte Carlo simulations (mean 723.21, range 431–1,156) as described by Hausdorf and Sauer (2009). These results indicate that there is no evidence for the hypothesis that the land snail radiations on Crete are a result of the fragmentation of Crete in the late Miocene and Pliocene.

Peripatric speciation in small isolated populations at the periphery of a range is an alternative to vicariance. Following Barraclough and Vogler (2000), we used range symmetry as indicator for peripatric speciation. It can be predicted that the geographic ranges of recently split sister species tend to display asymmetry of range size, if peripatric speciation is the predominant diversification mode. The two null models proposed by Barraclough and Vogler (2000) were used to produce random

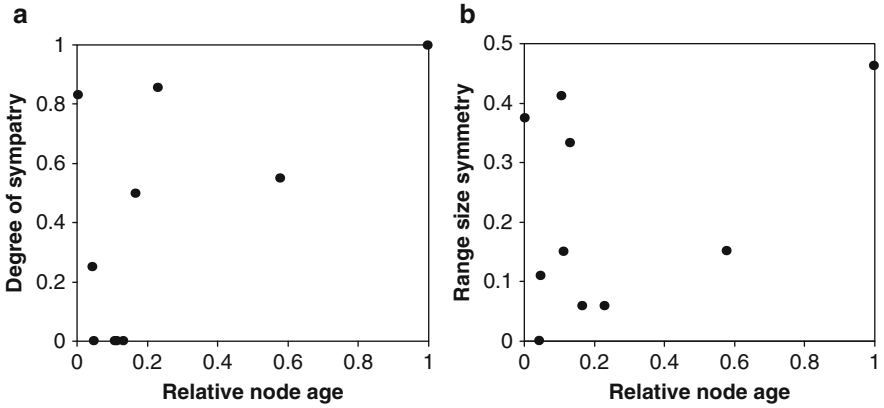


Fig. 3 (**a**) Plot of the arcsine transformed degree of sympatry of *Xerocrassa* clades against relative node age based on the ultrametric species tree constructed from the *cox1* gene tree (see p. 440). (**b**) Plot of range size symmetry of *Xerocrassa* clades against relative node age

range fragments. According to the phylogenetic broken stick model, the range of an ancestral species was split successively into two randomly sized fragments according to the phylogeny for each group. This model produces an even distribution of range size symmetry immediately after speciation that ranges from 0.0 to 0.5 with a mean value of 0.25. Lower values would suggest a tendency towards range size asymmetry indicating the possible importance of peripatric speciation. Alternatively, we also used the simultaneous broken stick model according to which a stick of given length is broken at $n-1$ uniform random points to produce n randomly sized pieces.

The intercept of the linear regression of the doubled and arcsine transformed degree of symmetry of the Cretan *Xerocrassa* species against relative node age (Fig. 3b) is significantly smaller than expected under the phylogenetic broken stick model ($p = 0.043$) as well as under the simultaneous broken stick model ($p = 0.004$). This result indicates that peripatric speciation was the predominant geographic speciation mode in the *Xerocrassa* radiation on Crete.

5 Evolution of Genitalia by Genetic Drift Versus Selection

As already noted, the Cretan *Xerocrassa* species do not differ by adaptation to different niches. Which factors might have triggered the radiation, if not differential adaptation by natural selection? Several theories assume that random genetic drift plays an important role for speciation in peripheral isolates (Mayr 1963, 1982; Carson and Templeton 1984; Slatkin 1996; Templeton 1996). Most of the Cretan *Xerocrassa* species differ in the proportions of different parts of the genitalia. We tested whether the phenotypic differences between the more recently diverged species can be explained by genetic drift using the coalescent simulation approach proposed by Masta and Maddison (2002) based on the *cox1* tree (Fig. 2). According to this approach, the observed phenotypic differentiation is unlikely ($p = 0.016$) to have arisen under neutrality, assuming the fixed phenotypic differences among species reflect fixed differences in underlying nuclear genes (Sauer and Hausdorf 2009). Thus, the evolution of the genitalia is better explained by divergent selection.

6 Lock-and-Key Hypothesis Versus Sexual Selection

Differences in genitalia may result from natural selection against hybrids as supposed by the lock-and-key hypothesis (Shapiro and Porter 1989) or from sexual selection (Eberhard 1985, 2001; Arnqvist 1998; Hosken and Stockley 2004).

If differences in the genitalia are the result of natural selection against hybrids, we would expect that the differences to be larger between species with geographically overlapping ranges than between species that are not in contact or have only slightly overlapping ranges, because selection against hybrids can happen only where two

species co-occur. We test this prediction by randomizing phylogenetic independent contrasts among nodes, holding the degree of overlap fixed for every node as described above for body size (see p. 440). This test showed that independent contrasts in the length of penis, epiphallus, flagellum, vagina, and bursa copulatrix standardized by body size are not larger between geographically overlapping clades in the Cretan *Xerocrassa* radiation than expected under the null model of no association between differences in the genitalia and geographical overlap (Table 1; Sauer and Hausdorf 2009). Thus, there is no evidence for selection against hybrids that resulted in larger contrasts between geographically overlapping species.

7 Influence of Evolution of Genitalia on Speciation in *Xerocrassa*

If changes in the genitalia were associated with speciation, recently split species should display greater divergence than expected under a null model of no association of changes in the genitalia with cladogenesis. We tested this prediction using the approach proposed by Barraclough et al. (1999) (see also p. 440). The tests showed that the distributions of the contrasts in the length of penis, epiphallus, vagina, and bursa copulatrix standardized by body size across the species tree do not differ from random distributions (Table 1; Sauer and Hausdorf 2009). On the contrary, changes in the length of the flagellum standardized by body size are significantly concentrated towards the tips of the tree indicating that the evolution of differences in flagellum length facilitated speciation in the Cretan *Xerocrassa* radiation (Sauer and Hausdorf 2009).

If a lock-and-key mechanism (Shapiro and Porter 1989) triggered the radiation, we would expect that changes in those parts of the genitalia that directly interact during copulation, namely penis and vagina, are concentrated towards the tips of the tree. This is not the case. Rather, speciation in the Cretan *Xerocrassa* radiation has been facilitated by the evolution of differences in flagellum length. The flagellum forms the tail of the spermatophore. During copulation, spermatophores are exchanged and transferred into the partner's bursa copulatrix, the female gametolytic organ. In the bursa copulatrix, the spermatophore of the mating partner is digested. Sperm have to actively swim out via the tail of the spermatophore formed by the flagellum to avoid digestion (Lind 1973). Sperm are most successful at reaching the spermathecae when the tail of the spermatophore is protruding into the vagina. Thus, a lengthening of the flagellum might increase paternity success. Koene and Schulenburg (2005) found correlations between the length of the flagellum and the spermatophore-receiving organ in helicoids land snails indicating counter-adaptation. We also found a positive scaling of male spermatophore-producing organs and female spermatophore-receiving organs in the Cretan *Xerocrassa* species indicating sexual co-evolution. The co-evolution of male spermatophore-producing organs and female spermatophore-receiving organs might be the result of an evolutionary arms race over the control of fertilization, i.e., of

sexual conflict (Chapman et al. 2003; Arnqvist and Rowe 2005). Alternatively, changes in the length of the spermatophore-producing organs might be the result of cryptic female choice (Eberhard 1985, 2001) for sperm that are better in escaping sperm digestion, or for larger spermatophores as nutritional nuptial gifts (Gwynne 1984; Vahed 1998), or as signals of donor quality or condition (Anthes et al. 2008), and the co-evolution might be the result of the necessity to process larger spermatophores (Anthes et al. 2008). Natural selection might counter increasing spermatophore length because of increasing predation or desiccation risk resulting from long copulation times required for the transfer of long spermatophores. Divergence in size and shape aspects of spermatophore morphology in allopatry might also have triggered the radiation in the land snail genus *Mastus* (Parmakelis et al. 2005).

8 Sexual Selection and Non-adaptive Radiation

Our analyses show that speciation in *Xerocrassa* on Crete was associated with and perhaps driven by sexual selection. This result is in accordance with other studies that have shown that sexual selection can promote speciation (Darwin 1871; West-Eberhard 1983; Dominey 1984; Gavrilets 2000; Gray and Cade 2000; Panhuis et al. 2001; Masta and Maddison 2002; Ritchie 2007). This hypothesis has received previous support from comparative studies that found correlations between species diversity and indices of sexual selection in birds (Barraclough et al. 1995; Møller and Cuervo 1998; Owens et al. 1999; Seddon et al. 2008), lizards (Stuart-Fox and Owens 2003), and insects (Arnqvist et al. 2000; Katzourakis et al. 2001). However, similar comparative studies of mammals, butterflies, and spiders (Gage et al. 2002; Isaac et al. 2005) and birds (Morrow et al. 2003) did not find significant correlations between species diversity and indices of sexual selection, and challenge the generality of the importance of sexual selection in speciation and diversification. One reason for the heterogeneous results may be that the coarse taxonomic scale at which most studies were performed may result in comparisons of taxa with very different ecology and biogeographic history so that the effect of sexual selection on speciation may be obscured by such factors (Seddon et al. 2008). Thus, analyses of groups of closely related species considering their phylogenetic relationships as done in our study might be a more powerful approach to investigate the generality and importance of sexual selection in speciation.

In the Cretan *Xerocrassa* radiation, sexual selection seems to be the initial mechanism resulting in speciation. In contrast, ecological differentiation of the lineages as indicated by different body sizes is generally not associated with recent speciation (lineage splitting close to the tips of the tree), but has been achieved when clades came into contact. This is consistent with the pattern found in some Nicaraguan crater lake cichlids in which sexual selection contributes more strongly or earlier during speciation than ecological separation (Wilson et al. 2000), and with the results of the comparative analysis of Barraclough et al. (1999) who also did not find evidence for an association of speciation with ecological disparity in tiger

beetles. However, separation of lineages as a result of sexual selection does not always precede ecological differentiation in radiations. Based on the distribution of ecological and morphological characteristics across the phylogeny of the cichlid fishes of Lake Malawi, Danley and Kocher (2001) suggested that this radiation has proceeded in three major bursts of cladogenesis of which the first two episodes resulted in adaptation to different niches, whereas the third episode was associated with differentiation of male nuptial coloration, most likely in response to divergent sexual selection. Also, studies of other taxa suggest that ecological divergence is common in the early stages of a radiation (Schluter and McPhail 1993; Losos et al. 1998; Sturmbauer 1998; Schluter 2000a, b, 2001). Although it is plausible that niche space might be subdivided early in the history of a radiation, it is unclear why the importance of sexual selection should vary in the history of a radiation. An alternative explanation of the observed patterns might be that an appreciable fraction of the speciation events is always the result of sexual selection, but that lineages that became adapted to different niches during or after speciation have a higher chance of persistence. As the geographical pattern of body size differences in the *Xerocrassa* radiation indicates, differentiation in ecologically important properties is associated with sympatry. Lineages that do not differ in adaptive characteristics may become more easily extinct if they become sympatric. The differential extinction of lineages that differ only in non-adaptive characteristics will result in an apparently almost exclusive adaptive phase in the early history of a radiation and more frequent cases of speciation as a result of sexual selection towards the present.

Without evidence that the observed phenotypic differences reflects adaptation to different niches in *Xerocrassa*, as with other land snail radiations on Crete (Gittenberger 1991; Parmakelis et al. 2005), our results suggest that the *Xerocrassa* radiation was facilitated by sexual selection rather than by adaptation. If speciation is facilitated by sexual selection, niches may remain conserved (Peterson et al. 1999; Wiens 2004; Wiens and Graham 2005) and non-adaptive radiation (Dominey 1984; Gittenberger 1991; Cameron et al. 1996; Turgeon and McPeek. 2002; Parmakelis et al. 2005; Kozak et al. 2006; Kozak and Wiens 2006) may result.

Acknowledgments For the loan or donation of material, we are grateful to E. Clauss, Z. Fehér, E. Gittenberger, M. Glaubrecht, J. Hemmen, G. Lindner, W.J.M. Maassen, T. von Proschwitz, P. Subai, W.G. Ullrich, F.W. Welter-Schultes, and V. Wiese. We thank T. Barraclough for help with the randomization analyses and B. Rudolph and F. Herder for advice concerning AFLP. This study was funded by the priority program "Radiations - Origins of Biological Diversity" of the Deutsche Forschungsgemeinschaft (HA 2763/3-1,2).

References

Anthes N, Schulenburg H, Michiels NK (2008) Evolutionary links between reproductive morphology, ecology and mating behavior in opisthobranch gastropods. Evolution 62:900–916

Arnqvist G (1998) Comparative evidence for the evolution of genitalia by sexual selection. Nature 393:784–786

Arnqvist G, Rowe L (2005) Sexual conflict. Princeton University Press, Princeton, NJ

Arnqvist G, Edvardsson M, Friberg U, Nilsson T (2000) Sexual conflict promotes speciation in insects. Proc Natl Acad Sci USA 97:10460–10464
Barraclough TG, Vogler AP (2000) Detecting the geographical pattern of speciation from species-level phylogenies. Am Nat 155:419–434
Barraclough TG, Harvey PH, Nee S (1995) Sexual selection and taxonomic diversity in passerine birds. Proc R Soc Lond B 259:211–215
Barraclough TG, Hogan JE, Vogler AP (1999) Testing whether ecological factors promote cladogenesis in a group of tiger beetles (Coleoptera: Cicindelidae). Proc R Soc Lond B 266:1061–1067
Blaxter M, Elsworth B, Daub J (2003) DNA taxonomy of a neglected animal phylum: an unexpected diversity of tardigrades. Biol Lett 271:S189–S192
Blaxter M, Mann J, Chapman T, Thomas F, Whitton C, Floyd R, Abebe E (2005) Defining operational taxonomic units using DNA barcode data. Philos Trans R Soc Lond B 360:1935–1943
Cameron RAD, Cook LM, Hallows JD (1996) Land snails on Porto Santo: adaptive and non-adaptive radiation. Philos Trans R Soc Lond B 351:309–327
Carson HL, Templeton AR (1984) Genetic revolutions in relation to speciation phenomena: the founding of new populations. Annu Rev Ecol Syst 15:97–131
Chapman T, Arnqvist G, Bangham J, Rowe L (2003) Sexual conflict. Trends Ecol Evol 18:41–47
Chiba S (1999) Accelerated evolution of land snails *Mandarina* in the oceanic Bonin Islands: evidence from mitochondrial DNA sequences. Evolution 53:460–471
Danley PD, Kocher TD (2001) Speciation in rapidly diverging systems: lessons from Lake Malawi. Mol Ecol 10:1075–1086
Darwin C (1871) The descent of man, and selection in relation to sex. John Murray, London
Dermitzakis MD (1990) Paleogeography, geodynamic processes and event stratigraphy during the late Cenozoic of the Aegean area. Atti Convegni Lincei 85:263–288
Dominey WJ (1984) Effects of sexual selection and life history on speciation: species flocks in African cichlids and Hawaiian *Drosophila*. In: Echelle AA, Kornfield I (eds) Evolution of fish species flocks. University of Maine Press, Orono, pp 231–249
Douris V, Cameron RAD, Rodakis GC, Lecanidou R (1998) Mitochondrial phylogeography of the land snail *Albinaria* in Crete: long-term geological and short-term vicariance effects. Evolution 52:116–125
Eberhard WG (1985) Sexual selection and animal genitalia. Harvard University Press, Cambridge, MA
Eberhard WG (2001) Species-specific genitalic copulatory courtship in sepsid flies (Diptera, Sepsidae, *Microsepsis*) and theories of genitalic evolution. Evolution 55:93–102
Fassoulas CG (2001) Field guide to the geology of Crete, 2nd edn. Natural History Museum of Crete, Heraklio
Floyd R, Abebe E, Papert A, Blaxter M (2002) Molecular barcodes for soil nematode identification. Mol Ecol 11:839–850
Gage MJG, Parker GA, Nylin S, Wiklund C (2002) Sexual selection and speciation in mammals, butterflies and spiders. Proc R Soc Lond B 269:2309–2316
Gavrilets S (2000) Rapid evolution of reproductive barriers driven by sexual conflict. Nature 403:886–889
Gittenberger E (1991) What about non-adaptive radiation? Biol J Linn Soc 43:263–272
Gittenberger E, Hausdorf B (2004) The *Orculella* species of the South Aegean island arc, a neglected radiation (Gastropoda, Pulmonata, Orculidae). Basteria 68:93–124
Gray DA, Cade WH (2000) Sexual selection and speciation in field crickets. Proc Natl Acad Sci USA 97:14449–14454
Gwynne DT (1984) Courtship feeding increases female reproductive success in bush crickets. Nature 307:361–363
Hausdorf B, Hennig C (2003) Biotic element analysis in biogeography. Syst Biol 52:717–723
Hausdorf B, Sauer J (2009) Revision of the Helicellinae of Crete (Gastropoda: Hygromiidae). Zool J Linn Soc 157:373–419

Hayashi M, Chiba S (2000) Intraspecific diversity of mitochondrial DNA in the land snail *Euhadra peliomphala* (Bradybaenidae). Biol J Linn Soc 70:391–401
Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. Proc R Soc Lond B 270:313–321
Hosken DJ, Stockley P (2004) Sexual selection and genital evolution. Trends Ecol Evol 19:87–93
Isaac NJB, Jones KE, Gittleman JL, Purvis A (2005) Correlates of species richness in mammals: body size, life history and ecology. Am Nat 165:600–607
Katzourakis A, Purvis A, Azmeh S, Rotheray G, Gilbert F (2001) Macroevolution of hoverflies (Diptera: Syrphidae): the effect of using higher-level taxa in studies of biodiversity, and correlates of species richness. J Evol Biol 14:219–227
Koene JM, Schulenburg H (2005) Shooting darts: co-evolution and counter-adaptation in hermaphroditic snails. BMC Evol Biol 5:25
Kozak KH, Wiens JJ (2006) Does niche conservatism promote speciation? A case study in North American salamanders. Evolution 60:2604–2621
Kozak KH, Weisrock DW, Larson A (2006) Rapid lineage accumulation in a non-adaptive radiation: phylogenetic analysis of diversification rates in eastern North American woodland salamanders (Plethodontidae: *Plethodon*). Proc R Soc Lond B 273:539–546
Lind H (1973) The functional significance of the spermatophore and the fate of the spermatozoa in the genital tract of *Helix pomatia* (Gastropoda: Stylommatophora). J Zool 169:39–64
Losos JB, Jackman TR, Larson A, de Queiroz K, Rodríguez-Schettino L (1998) Contingency and determinism in replicated adaptive radiations of island lizards. Science 279:2115–2118
Maassen WJM (1995) Observations on the genus *Mastus* from Crete (Greece), with descriptions of twelve new species (Gastropoda Pulmonata: Buliminidae). Basteria 59:31–64
Machado CA, Hey J (2003) The causes of phylogenetic conflict in a classic *Drosophila* species group. Proc R Soc Lond B 270:1193–1202
Maddison WP, Knowles LL (2006) Inferring phylogeny despite incomplete lineage sorting. Syst Biol 55:21–30
Masta SE, Maddison WP (2002) Sexual selection driving diversification in jumping spiders. Proc Natl Acad Sci USA 99:4442–4447
Mayr E (1963) Animal species and evolution. Belknap, Cambridge, MA
Mayr E (1982) Processes of speciation in animals. In: Barigozzi C (ed) Mechanisms of speciation. Liss, New York, pp 1–19
Møller AP, Cuervo JJ (1998) Speciation and feather ornamentation in birds. Evolution 52:859–869
Morrow EH, Pitcher TE, Arnqvist G (2003) No evidence that sexual selection is an 'engine of speciation' in birds. Ecol Lett 6:228–234
Nordsieck H (2004) *Albinaria cretensis* group: definition of the species and subspecies, with the description of new taxa (Gastropoda, Pulmonata, Clausiliidae). Basteria 68:51–70
Owens IPF, Bennett PM, Harvey PH (1999) Species richness among birds: body size, life-history, sexual selection or ecology? Proc R Soc Lond B 266:933–939
Panhuis TM, Butlin R, Zuk M, Tregenza T (2001) Sexual selection and speciation. Trends Ecol Evol 16:364–371
Parmakelis A, Pfenninger M, Spanos L, Papagiannakis G, Louis C, Mylonas M (2005) Inference of a radiation in *Mastus* (Gastropoda, Pulmonata, Enidae) on the island of Crete. Evolution 59:991–1005
Peterson AT, Soberón J, Sánchez-Cordero V (1999) Conservatism of ecological niches in evolutionary time. Science 285:1265–1267
Ritchie MG (2007) Sexual selection and speciation. Annu Rev Ecol Syst 38:79–102
Sanderson MJ (2002) Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. Mol Biol Evol 19:101–109
Sauer J, Hausdorf B (2009) Sexual selection is involved in speciation in a land snail radiation on Crete. Evolution 63:2535–2546
Schilthuizen M, Gittenberger E (1996) Allozyme variation in some Cretan *Albinaria* (Gastropoda): paraphyletic species as natural phenomena. In: Taylor J (ed) Origin and evolutionary radiation of the Mollusca. Oxford University Press, Oxford, pp 301–311

Schilthuizen M, Gutteling E, van Moorsel CHM, Welter-Schultes FW, Haase M, Gittenberger E (2004) Phylogeography of the land snail *Albinaria hippolyti* (Pulmonata: Clausiliidae) from Crete, inferred from ITS-1 sequences. Biol J Linn Soc 83:317–326

Schluter D (2000a) The ecology of adaptive radiation. Oxford University Press, Oxford, UK

Schluter D (2000b) Ecological character displacement in adaptive radiation. Am Nat 156(Supplement):S4–S16

Schluter D (2001) Ecology and the origin of species. Trends Ecol Evol 16:372–380

Schluter D, McPhail JD (1993) Character displacement and replicate adaptive radiation. Trends Ecol Evol 8:197–200

Seddon N, Merrill RM, Tobias JA (2008) Sexually selected traits predict patterns of species richness in a diverse clade of suboscine birds. Am Nat 171:620–631

Shapiro AM, Porter AH (1989) The lock-and-key hypothesis: evolutionary and biosystematic interpretation of insect genitalia. Annu Rev Entomol 34:231–245

Shaw KL (2002) Conflict between nuclear and mitochondrial DNA phylogenies of a recent species radiation: what mtDNA reveals and conceals about modes of speciation in Hawaiian crickets. Proc Natl Acad Sci USA 99:16122–16127

Slatkin M (1996) In defense of founder-flush theories of speciation. Am Nat 147:493–505

Sota T, Vogler AP (2001) Incongruence between mitochondrial and nuclear gene trees in the carabid beetles *Ohomopterus*. Syst Biol 50:39–59

Stuart-Fox D, Owens IPF (2003) Species richness in agamid lizards: chance, body size, sexual selection or ecology? J Evol Biol 16:659–669

Sturmbauer C (1998) Explosive speciation in cichlid fishes of the African Great Lakes a dynamic model of adaptive radiation. J Fish Biol 53:18–36

Templeton AR (1996) Experimental evidence for the genetic-transilience model of speciation. Evolution 50:909–915

Thomaz D, Guiller A, Clarke B (1996) Extreme divergence of mitochondrial DNA within species of pulmonate land snails. Proc R Soc Lond B 263:363–368

Turgeon J, McPeek MA (2002) Phylogeographic analysis of a recent radiation of *Enallagma* damselflies (Odonata: Coenagrionidae). Mol Ecol 11:1989–2001

Vahed K (1998) The function of nuptial feeding in insects: a review of empirical studies. Biol Rev 73:43–78

van Riel P, Jordaens K, van Houtte N, Frias Martins AM, Verhagen R, Backeljau T (2005) Molecular systematics of the endemic Leptaxini (Gastropoda: Pulmonata) on the Azores islands. Mol Phylogenet Evol 37:132–143

Weisrock DW, Shaffer HB, Storz BL, Storz SR, Voss SR (2006) Multiple nuclear gene sequences identify phylogenetic species boundaries in the rapidly radiating clade of Mexican ambystomatid salamanders. Mol Ecol 15:2489–2503

Welter-Schultes FW (2000a) Approaching the genus *Albinaria* in Crete from an evolutionary point of view (Pulmonata: Clausiliidae). Schriften Malakozool 16:1–208

Welter-Schultes FW (2000b) The paleogeography of late Neogene central Crete inferred from the sedimentary record combined with *Albinaria* land snail biogeography. Palaeogeogr Palaeoclimatol Palaeoecol 157:27–44

Welter-Schultes FW, Williams MR (1999) History, island area and habitat availability determine land snail species richness of Aegean islands. J Biogeogr 26:239–249

West-Eberhard MJ (1983) Sexual selection, social competition and speciation. Q Rev Biol 58:155–183

Wiens JJ (2004) Speciation and ecology revisited: phylogenetic niche conservatism and the origin of species. Evolution 58:193–197

Wiens JJ, Graham CH (2005) Niche conservatism: integrating evolution, ecology, and conservation biology. Annu Rev Ecol Evol Syst 36:519–539

Wilson AB, Noack-Kunnmann K, Meyer A (2000) Incipient speciation in sympatric Nicaraguan crater lake cichlid fishes: sexual selection versus ecological diversification. Proc R Soc Lond B 267:2133–2141

Non-Ecological Radiations in Acoustically Communicating Grasshoppers?

Frieder Mayer, Dirk Berger, Brigitte Gottsberger, and Wolfram Schulze

We dedicate this chapter to Professor Dr. Otto von Helversen, who studied the evolution of acoustic communication in grasshoppers for more than 30 years and who tragically died during drafting this review.

Abstract The analysis of mechanisms of how populations differentiate and new species arise is fundamental for understanding the evolution of biological diversity. Mating preferences and sexually selected characters can rapidly diverge between populations, and this can probably lead to premating reproductive isolation and hence the evolution of new species. We have investigated the role of a complex bidirectional acoustic communication system for the radiation of grasshoppers of the subfamily Gomphocerinae. Species are characterized by species-specific songs, which result from complex stridulatory movement patterns of both hind legs. A molecular phylogeny indicates that within the genera *Chorthippus* and *Stenobothrus* several species-rich taxa diverged recently and thus represent independent radiations. Divergence in allopatry and hybridization after secondary contact are two mechanisms that led to new song types and female preferences and thus may have contributed to rapid speciation by the evolution of premating reproductive isolation.

F. Mayer (✉)
Museum für Naturkunde – Leibniz Institute for Research on Evolution and Biodiversity at the Humboldt University Berlin, Invalidenstraße 43, 10115 Berlin, Germany
e-mail: frieder.mayer@mfn-berlin.de

D. Berger
Senckenberg Natural History Collections Dresden, Museum of Zoology, Königsbrücker Landstraße 159, 01109 Dresden, Germany

B. Gottsberger
Department of Population Ecology, University of Vienna, Rennweg 14, 1030 Vienna, Austria

W. Schulze
Animal Physiology, University of Bayreuth, Universitätsstraße, 30, 95440 Bayreuth, Germany

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_21,

1 Introduction

Molecular phylogenetics provides a powerful approach to estimate and compare speciation rates between taxa. Meanwhile, rapid diversification and speciation in some but not in other phylogenetic lineages are well documented in numerous taxa. Despite these advances, the evolutionary mechanisms leading to population divergence and speciation are less clear and require detailed studies of characters involved in reproductive isolation. Such studies have usually concentrated on traits that are directly linked to the ecology of species. A substantial number of studies show how closely related species adapted to different feeding habitats and environments, often after colonization of new areas or the evolution of key innovations. This led to adaptive radiations in numerous taxa. Case studies and evolutionary mechanisms that can cause adaptive radiations were reviewed by Schluter (2000) and Coyne and Orr (2004).

In recent years, it became more and more evident that sexual selection and sexual conflict led to the rapid evolution of prezygotic or premating reproductive isolation (see recent reviews by Ritchie 2007; Snook et al. 2009). This raises two major questions. First, does sexual selection or sexual conflict initiate the evolution of hybridization barriers that could lead to reproductive isolation? Second, does reproductive isolation evolve without a primary role of environmental factors like habitat or food? A positive answer to both questions would support the hypothesis that radiations can occur without a primary role of the environment, i.e., non-ecological radiations.

Grasshoppers of the subfamily Gomphocerinae represent a perfectly suited taxon to test the possibility of non-ecological radiations by rapid divergence of sexually selected traits. Within the family Acrididae only gomphocerine grasshoppers evolved a complex bidirectional acoustic communication system. They produce acoustic signals by rubbing their hind femur against the forewing (e.g., Jacobs 1953; von Helversen and Elsner 1977; von Helversen and von Helversen 1997). The male songs as well as the female acoustic preferences are species-specific and genetically inherited, which resulted in effective premating hybridization barriers (e.g., Perdeck 1958; Stumpner and von Helversen 1994). Several breeding and hybridization experiments among closely related species have shown that premating reproductive isolation evolved prior to obvious postzygotic isolation mechanisms (von Helversen and von Helversen 1975a; Saldamando et al. 2005a, b; Vedenina et al. 2007; Gottsberger and Mayer 2007).

A few lineages of gomphocerine grasshoppers have evolved elaborate acoustic signals. These songs comprise various miscellaneous audible elements, and, in some species, males additionally perform complex visual cues (some examples are given in Figs. 1 and 2). These signals are used in several different behavioral situations, which are all related to mating (for details, see Faber 1953; Jacobs 1953; Alexander 1960; Otte 1970, 1974; Elsner 1974; Ewing 1984; von Helversen 1986). In some species, males produce rivalry songs and respond to each other's songs. The most common songs are male calling songs to which conspecific females respond acoustically when they are willing to mate. The female response song allows the male to locate and approach the female

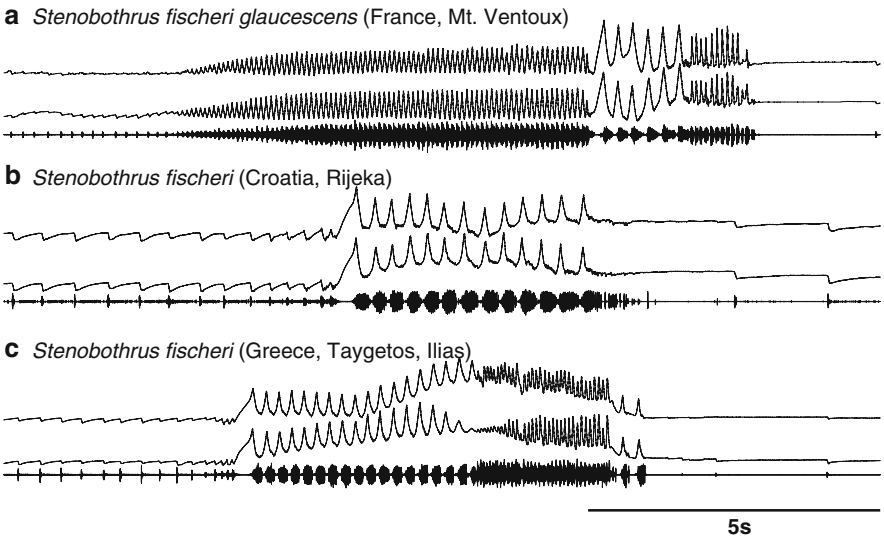


Fig. 1 Courtship songs of *Stenobothrus fischeri* males of three allopatric populations. Simultaneous recordings of stridulatory movements of the two hind legs (*upper traces*) using an optoelectronic device (von Helversen and Elsner 1977) and the airborne sound (*lower traces*)

phonotactically (von Helversen 1997). Once they met, the male tries to copulate but a not-excited female can effectively prevent copulations by kicking off the male with the hind legs. Males perform courtship behavior in front of the female to overcome her mating resistance. Therefore, the complex bidirectional communication system of gomphocerine grasshoppers has three major functions: species recognition (von Helversen and von Helversen 1983), mate localization (von Helversen 1997), and sexual selection by female choice (Kriegbaum 1989, Kriegbaum and von Helversen 1992, Klappert and Reinhold 2003).

In this review, we focus on the role of bidirectional acoustic communication for speciation of grasshoppers in the subfamily Gomphocerinae. Female song preferences and co-evolving acoustic mating signals represent intrinsic factors that probably evolve rather independently from the environment. Therefore, ecological factors may not play a crucial role in speciation in acoustically communicating grasshoppers.

2 Acoustic Communication and Speciation

2.1 Species Diversity and Acoustic Communication

A total of 290 species were listed in a recent survey of grasshoppers of the family Acrididae in Europe (Heller et al. 1998). They are currently classified in seven subfamilies of which the subfamily Gomphocerinae is the most species-rich.

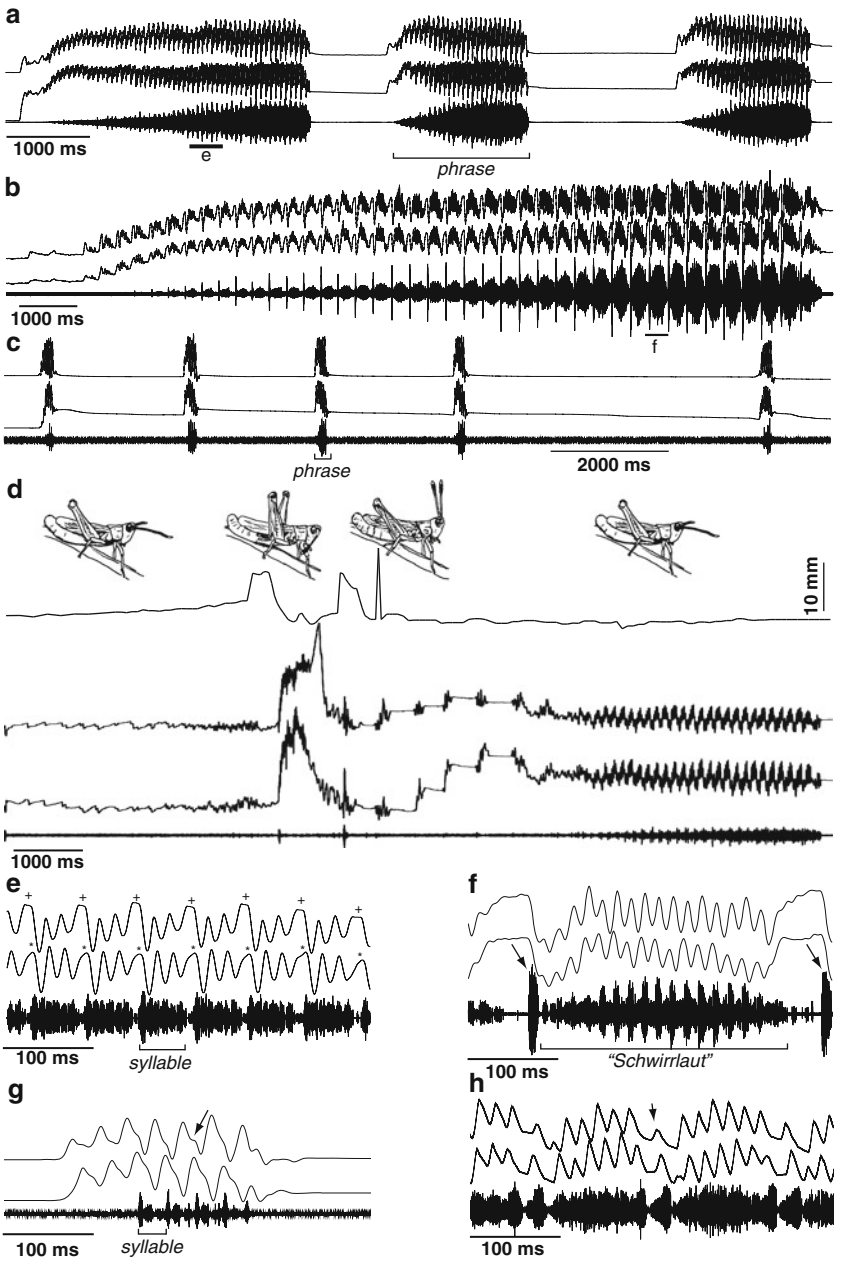


Fig. 2 Calling songs of *Chorthippus biguttulus* (**a**, **e**), *C. mollis ignifer* (**b**, **f**), *C. brunneus* (**c**, **g**) and *Gomphocerippus rufus* (**d**, **h**). Registration of stridulatory movements of the two hind legs (*upper traces*) and oscillograms of simultaneously recorded airborne sound (*lower traces*) are shown. Recording temperature was 31 ± 2°C. *Bars* "**e**" and "**f**" indicate the details shown in (**e**) and (**f**). *Arrows* in **f**, **g** and **h** mark distinctive parts (for details, see text) of the songs. (**a**) Male

It comprises almost half the described species (48%). Within the subfamily Gomphocerinae, species diversity is highest in the genera *Chorthippus* and *Stenobothrus* with approximately 45 and 20 species in Europe, respectively (Heller et al. 1998). Both genera comprise a number of species that show highly derived and complex courtship behavior. Such complex courtship behaviors can consist of multiple distinct song elements, which are produced by specific leg movements such as, for example, in *Stenobothrus fischeri* (Fig. 1). In addition, movements of the body, hind legs or antennae can generate visual signals (e.g., Fig. 2; Jacobs 1953; Vedenina and von Helversen 2003).

Highest species diversities in the acoustically communicating subfamily Gomphocerinae and in genera that comprise species with complex songs are first indications for a link between acoustic communication and signal complexity with speciation. Unfortunately, analyzing species diversity in different taxonomic groups does not tell one much about the evolutionary time frame in which species diversity evolved. However, a molecular phylogenetic approach allows one to infer speciation rates and to compare them between phylogenetic lineages.

2.2 Molecular Phylogeny

The molecular phylogenetic analysis based on the protein coding mitochondrial gene *co1* (subunit 1 of cytochrome oxidase) revealed three species-rich lineages (Fig. 3). In all three cases, a long branch leads to a large group of species with similar mitochondrial DNA sequences. One lineage comprises numerous species of the genus *Chorthippus*. The two other lineages include primarily species of the genera *Stenobothrus* and *Omocestus*. The genetic similarity among species (illustrated by short branches in Fig. 3) within these three groups suggests recent and rapid diversification and possibly occasional inter-specific gene flow by hybridization. So far, the incomplete taxonomic sampling prevents calculation of reasonable speciation rates. Nevertheless, the three species-rich lineages comprise species with complex calling or courtship songs (Faber 1953; Jacobs 1953; Ragge and Reynolds

Fig. 2 (continued) calling song of *C. biguttulus*. (**b**) Complete *C. mollis ignifer* calling song. (**c**) Calling song of *C. brunneus*. (**d**) Courtship behavior of a *Gomphocerippus rufus* male; in addition to sound (*lower trace*) and leg movements (*middle traces*) the position of the left antenna (*topmost trace*) and characteristic postures (*drawings*) of this species are given. (**e**) Detail of the *C. biguttulus* song shown in (**a**). The two hind legs are out of phase and perform different movement patterns: one leg (*top trace*) is leading and performs pattern I (+) during the whole phrase; the other leg (*second trace*) is phase-delayed and performs pattern II (*). (**f**) Detail of the *C. mollis ignifer* song shown in (**b**). *Arrows* indicate the characteristic pulses of high amplitude at the end of the pause between two "Schwirrlaute". (**g**) One phrase of the *C. brunneus* song shown in (**c**). The *arrow* indicates the typical step during the down stroke. (**h**) Detail of the *Gomphocerippus rufus* song shown in (**d**).

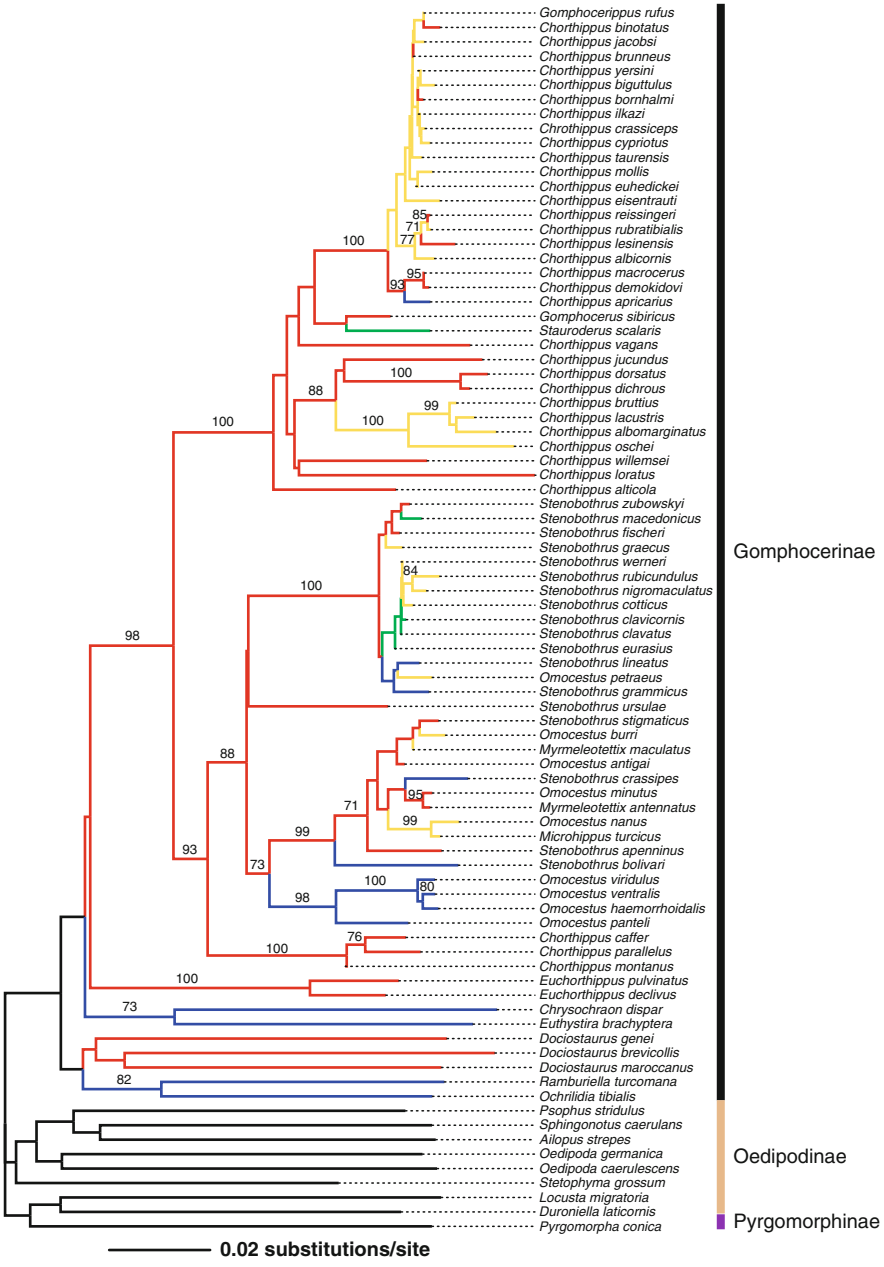


Fig. 3 Molecular phylogeny of the grasshopper subfamilies Oedipodinae and Gomphocerinae. *Pyrgomorpha conica* (Pyrgomorphinae) and Oedipodinae were used as outgroup. The tree was generated with the Neighbor-joining algorithm and Kimura-2-parameter distances using a 915-bp dataset of the mitochondrial gene *cytochrome oxidase* subunit 1 (*co1*). *Numbers* above *branches*

1998; Berger 2008), which supports the hypothesis that song complexity facilities the divergence of species. This is intuitively not surprising; higher song complexity in terms of complex leg movements, multiple song elements, or visual mating signals in addition to acoustic signals may allow more signal patterns and diverse multimodal signals. Two examples of complex courtship in two distinct lineages are shown in Figs. 1 and 2.

2.3 *Evolution of Leg Movement Patterns*

Gomphocerine grasshoppers reach a high degree of song complexity by the tegmino-femural stridulating mechanism. They typically generate sound by rubbing a row of pegs on the inner side of the femur of each hind leg against a vein of the forewing. Each peg being moved across the forewing causes a very short impulse. A series of impacts, caused by an uninterrupted partial or full up or down movement of a hind leg results in a sound pulse. Pulses are usually separated by pauses. They occur either when the hind leg stops during an up or a down stroke, or when it is at its reversal point from an up to a down stroke or from a down to an up stroke. Series of pulses produced by the right and left hind leg in a specific temporal pattern allow the composition of a great diversity of songs. The increasing complexity can be recognised on different levels, e.g., in structures and substructures of syllables, phrases, and the whole song repertoire (compare Faber 1953; Jacobs 1953; Otte 1970; von Helversen and von Helversen 1994).

Elsner (1983) provided the first contribution to the phylogeny of stridulatory motor patterns, and von Helversen and von Helversen (1994) proposed a successive increase of complexity in leg movements. Basal lineages of gomphocerine grasshoppers (Fig. 3) produce series of simple (uninterrupted) up and down strokes of both hind legs. Such a presumably plesiomorphic leg movement pattern is found, for example, in the genera *Ochrilidia*, *Euthystira*, and *Chrysochraon*. Sound could be emitted during up and down strokes or only during down strokes (e.g., Fig. 4). It is conceivable that these simple leg movements descend from locomotion or defence movements (Faber 1953; Jacobs 1953). A number of genera including *Dociostaurus*, *Omocestus*, *Stenobothrus*, and *Chorthippus* generate characteristic song elements (often called syllables; e.g., Ragge and Reynolds 1998) by leg movements with straight up and stepped down strokes (compare Stumpner and von Helversen 1994; Ragge and Reynolds 1998; Berger 2008). This suggests that, early in song evolution of gomphocerine grasshoppers, the continuous down stroke

Fig. 3 (continued) refer to bootstrap support values of at least 70. Poor resolution close to the root is likely the result of homoplasy due to relatively high mutation rates of the mitochondrial genome. In contrast, the *short branches* close to the *tips* suggest recent divergence and thus rapid speciation (radiations) in some but not other lineages (for details, see text)

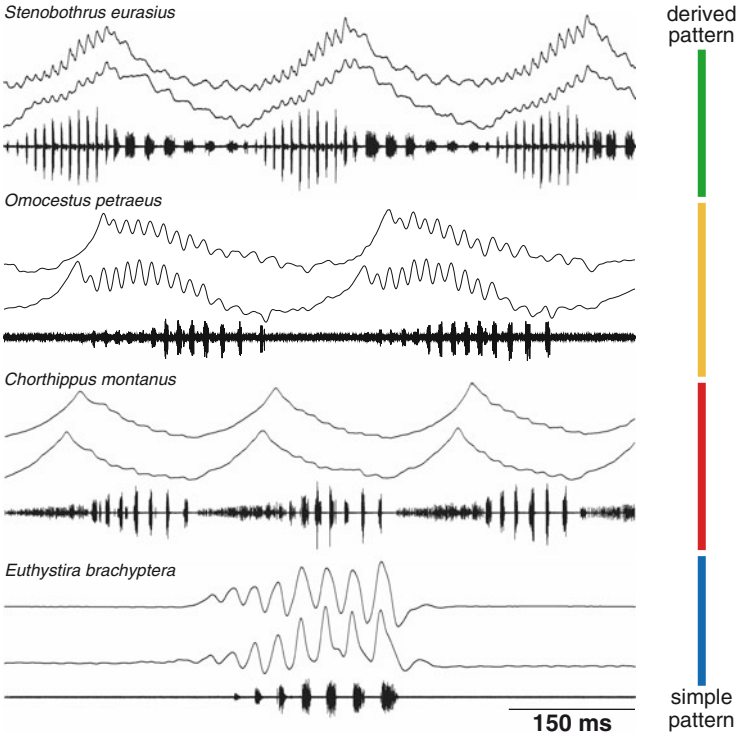


Fig. 4 Four levels of leg movement complexity (modified from von Helversen and von Helversen 1994). Straight up and down strokes (*Euthystira brachyptera*-pattern), straight up and stepped down strokes (*Chorthippus montanus*-pattern), straight up and vibratory down strokes (*Omocestus petraeus*-pattern) and stepped up and stepped down strokes (*Stenobothrus eurasius*-pattern). For each recording leg movements (*upper traces*) and oscillogram of the emitted sound (*lower trace*) are given. The *colors* correspond to those in the phylogenetic tree shown in Fig. 3

became interrupted by short stops during the down movement. This results in series of short pulses during the down stroke, whereas during the up stroke one longer pulse is emitted (e.g., *C. montanus*; Fig. 4). The stepped down stroke was presumably an important initial development that led to the impressive diversity of complex songs. In many species, the stops during the down stroke evolved into a short up movement. This resulted in high frequency vibratory movements during the down stroke and series of short pulses arose. *Omocestus petraeus* (Fig. 4), *Stenobothrus nigromaculatus* and *Myrmeleotettix* spp. (Elsner and Popov 1978; Berger 2008) are nice examples for this trend. In *Stenobothrus eurasius*, complexity increased by the adoption of faster vibrations, also during the up stroke, which resulted in shorter pulses during the up stroke and longer pulses during the down stroke (Fig. 4).

2.4 Song Evolution in Closely Related Species

In order to illustrate the evolution of strikingly different songs within a group of closely related species, we selected the well-studied species group of *Chorthippus biguttulus*. The three species, *Chorthippus biguttulus*, *C. brunneus* and *C. mollis*, occur in sympatry across wide areas in Europe (Ragge et al. 1990). They closely resemble each other in their external morphology suggesting a close relationship. This is supported by the similarity of mitochondrial DNA sequences (Mason et al. 1995; see above).

The male calling song of *Chorthippus biguttulus* (Fig. 2a, e) is a well-known example of the asynchronous co-ordination of both hind legs. The phase shift between legs masks pauses: while one hind leg generates a pulse, the other leg is at its turning point. The resulting prolonged sound elements are often referred to as syllables (Jacobs 1953; von Helversen 1972; von Helversen and von Helversen 1997). The proportion of syllable duration to the pause between the syllables is the most important factor for the innate releasing mechanism of the females (von Helversen 1972; Gottsberger 2007). The syllables are repeated many times resulting in a higher-level song element, often referred to as a phrase (e.g., von Helversen and von Helversen 1997). As with other song elements, phrases are repeated several times forming a song (Fig. 2a).

In contrast to *C. biguttulus*, *C. mollis* produces whirr-like elements called "Schwirrlaute" (Fig. 2f). These "Schwirrlaute" are repeated many times (up to more than 100) to form a song (Fig. 2b). Within the pause between two "Schwirrlaute", a strong down stroke of at least one leg causes an isolated prominent pulse (see arrows in Fig. 2f). Hybridization experiments between *C. biguttulus* and *C. mollis* indicate that "Schwirrlaute" are not homologous to "*C. biguttulus*-syllables" (for details, see von Helversen and von Helversen 1975a).

The calling song of *Chorthippus brunneus* has a characteristic phrase structure that differs conspicuously from those of *C. biguttulus* and *C. mollis* (Fig. 2c). The 5–14 phrases last only about 180 ms and each phrase is usually generated by five leg movement cycles (compare Fig. 2g), each of them producing a syllable. Each syllable is generated by three step leg movement cycle: i.e., one continuous up stroke and a two-step down stroke (Gottsberger and Mayer 2007).

The analysis of mitochondrial DNA sequences suggests that *Gomphocerippus rufus* is also closely related to the species mentioned above. The male performs a conspicuous courtship display in front of the female (Fig. 2d). It starts with primarily visible elements like head waggling, antennal movements, high amplitude leg movements, and two short acoustic pulses. The clubbed antennae with high-contrast tips, the light mouthparts, and reddish hind legs are certainly associated with this behavior. Thereafter, the major sound element follows. It resembles the calling song and comprises two elements performed in alternation as in *C. mollis* calling songs. The first element is a relatively loud pulse caused by a down stroke of both hind legs (see arrow in Fig. 2h). Thereafter, a vibratory element of about six leg movement cycles follows. All in all, the leg movement patterns of

all these four closely related species resemble highly complex stridulation patterns that result in very different songs.

3 Mechanisms of Speciation

The phylogenetic approach and detailed song analyses do not allow one to infer and distinguish between different mechanisms of speciation. Therefore, the question remains how populations diverge and how this can lead to reproductive isolation. At least two mechanisms contribute to population divergence and the evolution of new songs.

3.1 Divergence in Allopatry

The recent radiation of Gomphocerinae is supposed to be a result of divergent evolution of partial populations by geographic separation induced by climatic oscillations during the Pleistocene. Thermophilic species – as are most Gomphocerinae – were restricted to southern refuges during glacial stadials, in Europe, the Iberian Peninsula, the Apennine Peninsula, and the Balkans (Taberlet et al. 1998; Hewitt 1999). During the warm interstadials, they expanded their ranges northwards. In contrast, cold-adapted species, mainly montane, alpine, and boreo-alpine species, survived glacial maxima in lower altitudes and show currently disjunctive distributions (Ramme 1951; Berger et al., 2010).

Many widespread species with a continuous distribution throughout Europe show no or little differences in their calling and courtship songs, e.g., *Chorthippus parallelus*, *C. vagans*, *C. montanus*, *Omocestus ventralis*, *O. haemorrhoidalis, Stenobothrus stigmaticus*, and *S. lineatus* (Ragge and Reynolds 1998; own unpublished data). In contrast to these species, *Stenobothrus fischeri* is an example for a gomphocerine species with a fragmented distribution over a wide range of southern Europe (Harz 1975). Across the whole distribution range, calling songs do not show obvious differences. The song consists of short phrases of about 2–4 s and syllables are produced by leg movement patterns, which are very similar to those of *Chorthippus montanus* (Fig. 4; Berger 2008). In contrast to calling song, the courtship song consists of up to four different song elements as in western populations of *Stenobothrus fischeri* (Fig. 1a; Berger 2008): a series of "ticking" pulses prelude the courtship song. The second element resembles a prolonged calling song phrase. The third element consists of a series of syllables that are produced by highly elevated leg movements. The highly elevated movements of the hind legs (which are conspicuously marked by darkened knees) likely represent a visual stimulus. The fourth element (ritualised pre-mounting jumping strokes) is produced by highly excited males at the very end of a courtship by a series of fast leg strokes that result in sharp pulses.

In contrast to the western populations of *S. fischeri*, the populations from Croatia and Greece lack the second (the calling song-like) element (Fig. 1; for further details, see Berger 2008). These obvious differences in courtship songs of geographically separated eastern and western populations of *Stenobothrus fischeri* populations suggest that the ancestral *S. fischeri* already produced multi-component courtship songs, comprising all four elements. *Stenobothrus fischeri* illustrates that complex songs in terms of multiple song elements and/or multimodal courtship can lead to rapid divergence in geographically separated populations. Nevertheless, it is unclear to which degree selection, novel mutations, or genetic drift contribute to this divergence.

3.2 Evolution of New Songs by Hybridization

Interspecific hybrids and more or less wide hybrid zones were reported from a number of taxa particularly in the genus *Chorthippus*. These hybrids were commonly recognized according to their characteristic and unique calling songs. The analysis of hybrid songs often revealed intermediate song parameters in hybrids. Songs of *Chorthippus p. parallelus* and *C. p. erythropus* differ mainly in the duration of syllables within a phrase. Male hybrids between the two taxa produced songs with an intermediate phrase duration, probably formed by a simple introgression of characters (Butlin and Hewitt 1985a, b). Songs of hybrids between *C. brunneus* and *C. jacobsi* were also intermediate for the characters phrase duration (called "echeme"), syllable duration (called "phrase"), and pulse duration (called "syllable" in Saldamando et al. 2005a). In contrast, the analysis of *C. biguttulus* and *C. mollis* hybrids showed that songs of F1 hybrid males were generated by more complex leg movement patterns than observed in both parental species because hybrids produced leg movement patterns of both species (von Helversen and von Helversen 1975a). Meanwhile, natural hybrids between *C. biguttulus* and *C. mollis* were found, which also showed the combination of parental song parameters of both parental species (own unpublished data). A similar observation of increased complexity in hybrid songs was made in hybrids between *C. albomarginatus* and *C. oschei*. These two parental species have elaborate songs with a conspicuous visual display. The hybrids show more complexity in songs, and moreover even include novel elements in their courtship displays (Vedenina et al. 2007). Different leg movement patterns could possibly be combined in hybrids if two independent neuronal pattern generators are responsible for the different leg movement patterns. Such a neuronal control may allow for both networks being expressed in hybrids and lead to a common final output (von Helversen and von Helversen 1975b; von Helversen and Elsner 1977). Novel songs were also observed in hybrids between *C. biguttulus* and *C. brunneus*. But these hybrids showed more simple songs than the parental taxa. The distinct temporal patterns of syllables and pulses within phrases of the parental species got lost and resulted in uniformly performed up and down movements of legs in the hybrid songs (Gottsberger and

Mayer 2007). This indicates that the neuronal networks of *C. biguttulus* and *C. brunneus* cannot be formed in parallel. These studies of currently hybridizing taxa show that hybridization can rapidly lead to novel songs. Thus, hybridization could have been one factor for speciation in grasshoppers.

4 Conclusions

In gomphocerine grasshoppers, the bidirectional communication system plays a major role in reproductive isolation between species, since other mechanisms (e.g., complex genital apertures or obvious post-mating hybridization barriers) are not developed among recently diverged species. The willingness of females to mate depends on the male's calling and courtship behavior. The evolution of complex species-specific acoustic signals that were complemented by visual signals in several lineages contributed to independent radiations in different clades. Although the underlying evolutionary mechanisms are not well understood, ethological differentiation at least during geographic separation and hybridization after secondary contact of populations has contributed to new song patterns. It is unknown to what degree ecological factors may have influenced species diversity. Songs may also have been influenced among sympatrically occurring grasshopper species (Bukhvalova and Zhantiev 1993; Safi et al. 2006). Nevertheless, rapid evolution of effective premating barriers in gomphocerine grasshoppers in terms of strong female preferences for conspecific males' courtship behavior represents a promising candidate for non-ecological radiations.

Acknowledgements We thank Jana Ustinova and Klaus-Gerhard Heller for fruitful discussions and important suggestions for the manuscript. The Deutsche Forschungsgemeinschaft (DFG) and the Staedtler-Foundation supported our work financially.

References

Alexander RD (1960) Sound communication in Orthoptera and Cicadidae. In: Lanyon WE, Travolga WN (eds) Animal sounds and communication. American Institute of Biological Sciences, Washington, pp 38–92

Berger D (2008) The evolution of complex courtship songs in the genus *Stenobothrus* Fischer, 1853 (Orthoptera, Caelifera, Gomphocerinae). PhD Thesis, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen

Berger D, Chobanov DP, Mayer F (2010) Interglacial refugia and range shifts of the alpine grasshopper *Stenobothrus cotticus* (Orthoptera: Acrididae: Gomphocerinae). Org Div Evol 10:123–133

Bukhvalova MA, Zhantiev RD (1993) Acoustic signals in grasshopper communities (Orthoptera, Acrididae, Gomphocerinae). Zool Zh 72:47–62

Butlin R, Hewitt GM (1985a) A hybrid zone between *Chorthippus parallelus parallelus* and *Chorthippus parallelus erythropus* (Orthoptera: Acrididae): morphological and electrophoretic characters. Biol J Linn Soc 26:269–285

Butlin RK, Hewitt GM (1985b) A hybrid zone between *Chorthippus parallelus parallelus* and *Chorthippus parallelus erythropus* (Orthoptera: Acrididae): behavioural characters. Biol J Linn Soc 26:287–299

Coyne JA, Orr HA (2004) Speciation. Sinauer, Sunderland

Elsner N (1974) Neuroethology of sound production in gomphocerine grasshoppers (Orthoptera: Acrididae) I. Song patterns and stridulatory movements. J Comp Physiol 88:67–102

Elsner N (1983) A neuroethological approach to the phylogeny of leg stridulation in gomphocerine grasshoppers. In: Huber F, Markl H (eds) Neuroethology and behavioural physiology. Springer, Berlin, pp 54–68

Elsner N, Popov AV (1978) Neuroethology of acoustic communication. Adv Insect Physiol 13:229–355

Ewing AW (1984) Acoustic signals in insect sexual behaviour. In: Lewis T (ed) Insect communication 12th symposium of the Royal Entomological Society of London. Academic, London, pp 223–240

Faber A (1953) Laut- und Gebärdensprache bei Insekten (Orthoptera). Mitt Staatl Mus Nat Stuttgart:1–198

Gottsberger B (2007) Interspecific hybridization between the grasshoppers *Chorthippus biguttulus* and *C. brunneus* (Acrididae; Gomphocerinae). PhD Thesis, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen

Gottsberger B, Mayer F (2007) Behavioral sterility of hybrid males in acoustically communicating grasshoppers (Acrididae, Gomphocerinae). J Comp Physiol 193:703–714

Harz K (1975) Die orthopteren europas II. Junk, The Hague

Heller KG, Korsunovskaya O, Ragge DR, Vedenina V, Willemse F, Zhantiev RD, Frantsevich L (1998) Check-list of European Orthoptera. Articulata 7:1–61

Hewitt GM (1999) Post-glacial re-colonization of European biota. Biol J Linn Soc 68:87–112

Jacobs W (1953) Verhaltensbiologische Studien an Feldheuschrecken. Z Tierpsychol 1:1–228

Klappert K, Reinhold K (2003) Acoustic preference functions and sexual selection on the male calling song in the grasshopper *Chorthippus biguttulus*. Anim Behav 65:225–233

Kriegbaum H (1989) Female choice in the grasshopper *Chorthippus biguttulus*. Mating success is related to song characteristics of the male. Naturwissenschaften 76:81–82

Kriegbaum H, von Helversen O (1992) Influence of male songs on female mating behavior in the grasshopper *Chorthippus biguttulus* (Orthoptera: Acrididae). Ethology 91:248–254

Mason DJ, Butlin RK, Gacesa P (1995) An unusual mitochondrial DNA polymorphism in the *Chorthippus biguttulus* species group (Orthoptera: Acrididae). Mol Ecol 4:121–126

Otte D (1970) A comparative study of communicative behaviour in grasshoppers. Misc Publ Mus Zool Univ Mich 141:1–168

Otte D (1974) Effects and functions in the evolution of signaling systems. Annu Rev Entomol 5:385–417

Perdeck AC (1958) The isolating value of specific song patterns in two sibling species of grasshoppers (*Chorthippus brunneus* Thunb. and *C. biguttulus* L.). Behav 12:1–75

Ragge DR, Reynolds WJ (1998) The songs of the grasshoppers and crickets of Western Europe. Harley, Colchester

Ragge DR, Reynolds WJ, Willemse F (1990) The songs of the European grasshoppers of the *Chorthippus biguttulus* group in relation to their taxonomy, speciation and biogeography (Orthoptera: Acrididae). Bol Sanidad Vegetal (Fuera de serie) 20:239–245

Ramme W (1951) Zur Systematik, Faunistik und Biologie der Orthopteren von Südost- Europa und Vorderasien. Mitt Zool Mus Berlin 27:1–431

Ritchie MG (2007) Sexual selection and speciation. Annu Rev Ecol Evol Syst 38:79–102

Safi K, Heinzle J, Reinhold K (2006) Species recognition influences female mate preferences in the common European grasshopper (*Chorthippus biguttulus* Linnaeus, 1758). Ethology 112:1225–1230

Saldamando CI, Miyaguchi S, Tatsuta H, Kishino H, Bridle JR, Butlin RK (2005a) Inheritance of song and stridulatory peg number divergence between *Chorthippus brunneus* and *C. jacobsi*, two naturally hybridizing grasshopper species (Orthoptera: Acrididae). J Evol Biol 18:703–712

Saldamando CI, Tatsuta H, Butlin RK (2005b) Hybrids between *Chorthippus brunneus* and *C. jacobsi* (Orthoptera: Acrididae) do not show endogenous postzygotic isolation. Biol J Linn Soc 84:195–203

Schluter D (2000) The ecology of adaptive Radiation. Oxford University Press, Oxford

Snook RR, Chapman T, More PJ, Wedell N, Crudgington HS (2009) Interactions between the sexes: new perspectives on sexual selection and reproductive isolation. Evol Ecol 23:71–91

Stumpner A, von Helversen O (1994) Song production and song recognition in a group of sibling grasshopper species *(Chorthippus dorsatus*, *Ch. dichrous* and *Ch. loratus*: Orthoptera, Acrididae). Bioacoustics 6:1–23

Taberlet P, Fumagalli L, Wust-Sauc AG, Cosson JF (1998) Comparative phylogeography and postglacial colonization routes in Europe. Mol Ecol 7:453–464

Vedenina VY, von Helversen O (2003) Complex courtship in a bimodal grasshopper hybrid zone. Behav Ecol Sociobiol 54:44–54

Vedenina VY, Panyutin AK, von Helversen O (2007) The unusual inheritance pattern of the courtship songs in closely related grasshopper species of the *Chorthippus albomarginatus*-group (Orthoptera: Gomphocerinae). J Evol Biol 20:260–277

von Helversen D (1972) Gesang des Männchens und Lautschema des Weibchens bei der Feldheuschrecke *Chorthippus biguttulus* (Orthoptera, Acrididae). J Comp Physiol 81:381–422

von Helversen D (1997) Acoustic communication and orientation in grasshoppers. In: Lehrer M (ed) Orientation and communication in arthropods. Birkhäuser, Basel, pp 301–341

von Helversen D, von Helversen O (1975a) Verhaltensgenetische Untersuchungen am akustischen Kommunikationssystem der Feldheuschrecken (Orthoptera, Acrididae) I. Der Gesang von Artbastarden zwischen *Chorthippus biguttulus* und *Ch. mollis*. J Comp Physiol 104:273–299

von Helversen D, von Helversen O (1975b) Verhaltensgenetische Untersuchungen am akustischen Kommunikationssystem der Feldheuschrecken (Orthoptera, Acrididae). II. Das Lautschema von Artbastarden zwischen *Chorthippus biguttulus* und *Ch. mollis*. J Comp Physiol 104: 301–323

von Helversen D, von Helversen O (1997) Recognition of sex in the acoustic communication of the grasshopper *Chorthippus biguttulus* (Orthoptera, Acrididae). J Comp Physiol 180:375–386

von Helversen O (1986) Gesang und Balz bei Feldheuschrecken der *Chorthippus albomarginatus*-Gruppe (Orthoptera: Acrididae). Zool Jahrb Syst 113:319–342

von Helversen O, Elsner N (1977) The stridulatory movements of acridid grasshoppers recorded with an opto-electronic device. J Comp Physiol 122:53–64

von Helversen O, von Helversen D (1983) Species recognition and acoustic localization in acridid grasshoppers: a behavioral approach. In: Huber F, Markl H (eds) Neuroethology and behavioral physiology. Springer, Berlin, pp 95–107

von Helversen O, von Helversen D (1994) Forces driving coevolution of song and song recognition in grasshoppers. Fortschr Zool 39:253–284

Beyond Sympatric Speciation: Radiation of Sailfin Silverside Fishes in the Malili Lakes (Sulawesi)

Fabian Herder and Ulrich K. Schliewen

Abstract Adaptive radiations of plants and animals play an important role as model systems in speciation research. Rapid emergence of biological diversity provides opportunities to study adaptive and non-adaptive factors leading to speciation, including the role of spatial factors and ecological, behavioral and genetic mechanisms potentially driving speciation processes. The radiation of sailfin silversides (Atheriniformes: Telmatherinidae) endemic to "Wallace's Dreamponds", i.e., the Malili Lakes in Central Sulawesi (Indonesia), allows for testing hypotheses of speciation processes under different geographic settings. Compared with other well-known freshwater fish radiations, the Telmatherinid system is of intermediate size in terms of both, geographical size and organismic diversity. Phylogenetic analyses provide evidence for multiple clades that were connected secondarily through reticulate evolution, but combined analyses support an ancient monophyletic origin of all Telmatherinidae clades in Lake Matano. The consensus view is that the lake contains two reciprocally monophyletic groups of sailfin silversides, highly diverse "sharpfins" heavily introgressed by stream populations, and less diverse "roundfins" not affected by allochthonous introgression. Genetic, morphological, habitat-utilization, trophic, and mate-choice data demonstrate that the most plausible hypothesis for the origin of roundfins is by sympatric speciation. Substantial but not absolute restrictions in gene flow coupled with morphological and behavioral adaptations to distinct ecological niches support the hypothesis that natural selection coupled with assortative mating drives speciation processes in

F. Herder (✉)
Sektion Ichthyologie, Zoologisches Forschungsmuseum Alexander Koenig, Adenauerallee 160, 53113 Bonn, Germany
Department of Ichthyology, Bavarian State Collection of Zoology (ZSM), Münchhausenstr. 21, 81247 München, Germany
e-mail: f.herder.zfmk@uni-bonn.de

U.K. Schliewen
Department of Ichthyology, Bavarian State Collection of Zoology (ZSM), Münchhausenstr. 21, 81247 München, Germany

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_22,

roundfins. In contrast, discrete male color polymorphisms can be explained by sexual selection, but appear not to be associated with population divergence. In line with evidence for adaptive radiation in sharpfins, natural selection for distinct resources is certainly a major force shaping diversity in this lacustrine radiation. However, the role of divergent sexual selection on divergence of ecologically diverse sharpfins remains to be tested.

Keywords Adaptive radiation · Ancient lakes · Color polymorphism · Lake Matano · Telmatherina · Telmatherinidae

1 Introduction

The question if speciation essentially depends on the isolating effect of strict geographical barriers or not has initiated and recurrently refuelled intense debates among evolutionary biologists (Bolnick 2004; Coyne 2007; Dieckmann and Doebeli 1999; Jiggins 2006; Mallet 2001). These discussions have severe implications for our understanding of evolutionary processes, as they are directly connected to the mechanisms driving speciation. Based on the geographical distribution of evolving populations, three major concepts have directed speciation research in the last decades (Coyne and Orr 2004). Mayr (1942) introduced the framework of allopatric speciation, which assumes absence of gene flow due to a strict geographical barrier. Reproductive isolation separating species after secondary contact is expected to emerge as a by-product of geographic isolation, which in turn is thought to result from selection or drift. Based on its intuitive plausibility, allopatric speciation has generally been accepted as the norm, and is often still used as a null hypothesis for other geographic speciation scenarios (Coyne and Orr 2004; but see Bolnick and Fitzpatrick 2007 for a critical view).

In contrast to Mayr's concept of extrinsic separation, sympatric speciation assumes the evolution of restrictions in gene flow by intrinsic factors. Divergent selection coupled with assortative mating and intraspecific competition is thought to split a single ancestral population into two or more sister species under sympatric conditions. The third scenario, parapatric speciation, combines elements of sym- and allopatric speciation. Diverging populations are expected to have contact but no overlap areas, thereby facilitating local adaptation, which is less likely to evolve in sympatry (Gavrilets and Vose 2005; Gavrilets et al. 2000). However, unambiguous cases for the parapatric origin of species remain to be detected in nature (Gavrilets et al. 2000; Coyne and Orr 2004).

The idea that speciation can proceed without extrinsic separation preventing homogenization of diverging gene pools has raised substantial scepticism, especially regarding the likelihood of divergence in the face of ongoing gene flow (Coyne and Orr 2004; Mayr 1963). However, recent theoretical work shows that even moderately strong divergent selection may overcome very high rates of gene flow (Bolnick and Fitzpatrick 2007; Doebeli and Dieckmann 2000; Gavrilets and

Vose 2005; see Gavrilets 2004 and Fitzpatrick et al. 2008a for discussion). Indeed, a growing number of field studies support the view that species can remain distinct despite of substantial gene flow, or even diverge under such conditions (Arnold 2006; Danley et al. 2000; Fitzpatrick et al. 2008b; Samonte et al. 2007; Schilthuizen et al. 2005). According to this view, individual members of diverging populations represent genomic mosaics consisting of comparatively few divergently selected loci and a remaining majority of the genome remaining functionally neutral and undifferentiated (Templeton 1981; Via and West 2008; Wu 2001). Understanding the genomic details of divergence processes leading to speciation remains a major challenge, but the rapidly evolving genomic techniques nowadays allow the tackling of population genomic approaches in non-model organisms (Luikart et al. 2003; Nosil et al. 2009).

2 Speciation Research in Adaptive Radiations

Ecologically isolated host races, evolving species pairs or emerging radiations are prime objects for testing hypotheses of the mechanisms promoting early stages of speciation (Berlocher and Feder 2002; Coyne and Orr 2004; Via and West 2008). Especially, island radiations such as Darwin's finches (Grant and Grant 2003), Antillean lizards (Thorpe et al. 2008) or the Hawaiian silverswords (Baldwin and Sanderson 1998) have the advantage of allowing for restricting geographic scenarios involved in speciation processes (Emerson 2002). Analogously, freshwater fish endemic to "reverse islands", i.e., isolated freshwater lakes, rank among the most exciting models for speciation research. These include well-known systems like northern sticklebacks (Schluter 2000) and lake whitefish (Rogers and Bernatchez 2007), cichlid radiations of the East African rift lakes (Kocher 2004), and radiations confined to tiny lakes or small crater lakes or lagoons (Schliewen and Klee 2004; Schliewen et al. 1994, 2001; Strecker et al. 1996). The adaptive character of most of these radiations suggests that natural selection is a major force shaping their diversity; however, sexual selection, drift, hybridization and isolation scenarios are among the possible complementary or alternative explanations (Danley and Kocher 2001; Dieckmann et al. 2004; Mallet 2007). Recent findings of sensory drive speciation uncovered in rock dwelling Lake Voctoria cichlids (Seehausen et al. 2008) suggests that, in line with evidence for reticulate evolution in species flocks (Schliewen and Klee 2004; Seehausen 2004), at least some of these alternatives may contribute significantly to divergence in species flocks.

3 Sailfin Silversides in the Malili Lakes

"Wallace's dreamponds", the Malili Lakes system in the highlands of Central Sulawesi (Indonesia), constitute, with lacustrine radiations or endemic lineages of snails, crustaceans, and fish (Kottelat 1990a, 1990b, 1991; von Rintelen and Cai 2009;

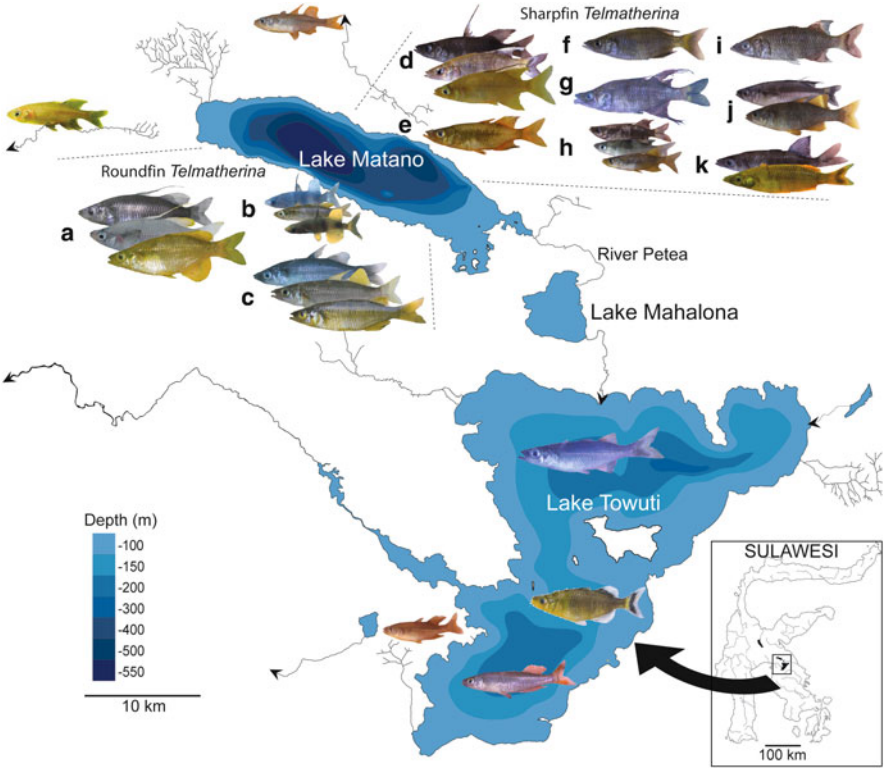


Fig. 1 The Malili Lakes system and its endemic sailfin silversides radiation, with focus on Lake Matano's *Telmatherina*. "Roundfins": (**a**) *Telmatherina antoniae* "large", (**b**) *T. antoniae* "small", (**c**) *T. prognatha*. "Sharpfins": (**d**) *T. sarasinorum*, (**e**) *T. sarasinorum* "bigmouth", (**f**) *T. sarasinorum* "largehead", (**g**) *T.* sp. "thicklip", (**h**) *T. opudi*, (**i**) *T. abendanoni*, (**j**) *T. wahjui*, (**k**) *T.* sp. "elongated". Fish pictures inside Lake Towuti represent major groups of lacustrine groups shared with L. Mahalona; those besides streams visualize parts of the stream sailfin silverside diversity. All pictures show males, with color polymorphisms (typically yellow or blue, in some cases also blue-yellow) present in most Malili Lakes system Telmatherinidae. See Herder et al. 2006a for sailfin silverside diversity; map by T. von Rintelen, modified (with permission)

T. von Rintelen et al. 2004, 2007b; K. von Rintelen et al., in press; Schubart and Ng 2008; Schubart et al. 2008), a hotspot of freshwater diversity. The system consists of three major lakes interconnected by steep rivers, and two additional satellite lakes (Fig. 1). Ancient graben-lake Matano covers an area of approx. 32 × 6 km, and is the uppermost lake of the system (Ahmad 1977; Brooks 1950). It is with 590 m depth extraordinary deep, has mostly steep walls and no major intra-lake barriers above approx. 400 m depth (Haffner et al. 2001). Recent limnological investigations demonstrated that it is anoxic below 100 m depth (Crowe et al. 2008a, b). Lake Matano is drained by the extremely steep River Petea to the comparatively small and shallow Lake Mahalona, which in turn is connected by River Tominanga to the largest lake of the system, Lake Towuti. Despite its size of approx. 560 km^2, L. Towuti is less than

half as deep as L. Matano. The waters of L. Towuti drain from a bay at its western shore to the sea at the Gulf of Bone.

The radiation of sailfin silversides (Telmatherinidae) endemic to the Malili Lakes has received substantial interest as a new model system for studying speciation processes and the evolution of color polymorphisms. Sailfin silversides are small, atheriniform (Teleostei: Atheriniformes) freshwater fishes, which are sexually dimorphic, show conspicuous male polychromatism, and are easy to observe in the crystal clear waters of the oligotrophic lakes. Local endemism to single or some of the lakes or streams in combination with intermediate dimensions in both diversity and geographical size provide excellent preconditions for testing hypotheses regarding most of the factors actually discussed as potentially driving speciation processes.

Based on the taxonomic work by Kottelat in the early 1990s (Kottelat 1990a, 1991), the exploration of sailfin silverside diversity in lakes and streams of the area (Herder et al. 2006a) was the essential first step towards establishing the system as a model for speciation research. Surveys of the rivers and most of the permanent streams of the Malili drainage system resulted in the discovery of several new stream-dwelling sailfin silverside populations, which showed indications for local differentiation (Herder et al. 2006a). Likewise, additional lake-dwelling sailfin silversides or previously unknown color morphs were discovered. However, the major patterns of distribution in lake Telmatherinidae confirmed previous records (Kottelat 1990a, 1991; Fig. 1), with most species endemic to either L. Matano or Lakes Towuti and Mahalona (Herder et al. 2006a). Descriptions of mating behavior by Gray and McKinnon (2006) provided an important baseline for later behavioral studies focusing on evolutionary ecology and the maintenance of color polymorphisms.

4 Patterns of Hybridization

Based on individualized samples of lake- and stream-dwelling sailfin silversides, mitochondrial DNA (mtDNA) and amplified fragment length polymorphism (AFLP) markers were applied to reconstruct the phylogenetic history of the Telmatherinidae (Herder et al. 2006b). Robust phylogenies based on dense taxon sampling are a prerequisite for testing hypotheses on the evolution of radiations, and are especially important for identifying monophyletic clades which are suited as candidates for speciation studies. However, phylogenetic reconstructions of young or evolving species flocks are challenging, especially due to the possible effects of reticulate evolution (hybridization) and incomplete lineage sorting. Indeed, maternally inherited mtDNA marker showed only limited congruence to morphological concepts of Telmatherinidae (Herder et al. 2006a), and mtDNA data covering the whole flock indicated several cases of hybridization between lake- and stream-dwelling sailfin silversides (Herder et al. 2006b). In the case of Lake Matano, morphologically well-defined “roundfins” were clearly identified as a monophyletic group, whereas the highly diverse “sharpfins” carry two very distinct

groups of haplotypes – one forming the sisterclade of roundfins, the other forming a separate clade comprising haplotypes shared with stream-dwelling *Telmatherina*.

The parallel analysis of numerous independent AFLP markers scattered randomly across the genome (Vos et al. 1995) is especially suited for the analysis of recent phylogenetic signals (Albertson et al. 1999; Schliewen and Klee 2004). Here, AFLPs served as a tool for critically evaluating phylogenetic signals derived from mtDNA, and for testing hypotheses on introgressive hybridization as deduced from inconsistencies between mtDNA and morphological data. Based on individuals of the mtDNA dataset described above, the nuclear multilocus AFLP-markers strongly supported most of the morphologically well-defined groups of sailfin silversides as monophyletic (Herder et al. 2006b; Fig. 2). In Lake Matano, AFLPs resolved "roundfins" and "sharpfins" (Figs. 1 and 2) as two monophyletic clades, sharply contrasting the pattern of three mitochondrial haplotype groups. However, monophyly of Lake Matano's *Telmatherina* in toto was not significant given the whole AFLP dataset (but see below).

In summary, nuclear, mitochondrial and morphological data suggested that stream-dwelling sailfin silversides most likely hybridize with lake populations. Subsequent ordination statistics and bootstrap homoplasy excess tests (Seehausen 2004) demonstrated that phylogenetic signal shared by sharpfins and stream populations is highly significant in the multilocus dataset, which provides evidence for massive hybridization. In turn, roundfins and sharpfins of Lake Matano were identified as an ancient monophylum, which is masked in the AFLP and mtDNA data due to introgression from riverine invaders (Herder et al. 2006b).

Introgression of L. Matano's sharpfins by stream populations induced some problems in studies relying solely on mitochondrial haplotype groups. Based on the assumption that Lake Matano's *Telmatherina* radiation is physically isolated from all remaining sailfin silversides, Roy et al. (2004) did not incorporate outgroup samples from the other lakes or rivers and streams of the lakes system into phylogenetic analyses. Hence, the three mtDNA haplotype clades present in Lake Matano's *Telmatherina* were discussed as three major lineages of sailfin silversides evolved in Lake Matano, rather than identifying the introgressed character of sharpfins and clearly monophyletic roundfins. Accordingly, results and predictions derived from a set of studies (Roy et al. 2004, 2007a, b) will have to be carefully linked to concepts based on nuclear monophyly [1].

Pronounced phenotypic, and apparently also ecological differences between and among roundfins and sharpfins, led to the conclusion that sailfin silversides of Lake Matano most likely represent an adaptive radiation (Herder et al. 2006b).

[1]Inferences of evolutionary history of radiations from phylogenetic data require an adequate taxon sampling, including the relevant outgroups (see Herder et al. (2006a, 2006c), Schliewen et al. (2006) and Schwarzer et al. (2008) for discussion with respect to publications on fish radiations in Sulawesi (Roy et al. 2004, 2007a, b) and in Nicaraguan crater lakes (Barluenga et al. 2006). In addition, quality control of a posteriori inferences critically depends on the availability of "individualized" voucher material, in order to enable cross-checking of results. This is especially important for studies of incipient speciation, where phenotypic variation is not necessarily discrete and unambigous assignment of individuals to (emerging) species is not always possible.

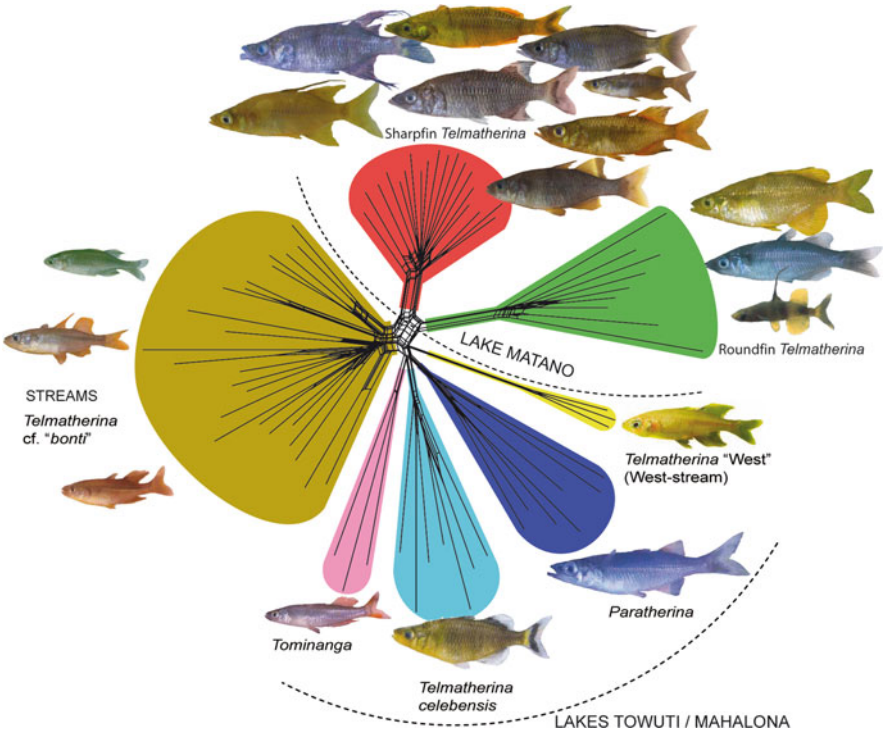


Fig. 2 Distance-based phylogenetic Neighbor-Net network, reconstructed using 1,327 polymorphic AFLP fragments (redrawn from Herder et al. 2006b). In contrast to mitochondrial haplotype data, nuclear AFLPs support monophyletic "sharpfins" and "roundfins" in Lake Matano, and likewise distinguish *Paratherina*, *Tominanga,* and *Telmatherina celebensis* from Lakes Towuti and Mahalona from stream-dwelling *Telmatherina* cf. "*bonti*" populations. The multilocus data contain strong and significant signal for the ancient monophyly of L. Matano's *Telmatherina* in toto (sharpfins + roundfins). However, this signal is confounded by alleles deriving from introgression by allochthonous stream populations into L. Matano's sharpfins (Herder et al. 2006b)

Conspicuously, introgressed sharpfins are more diverse in terms of body shape than the clearly non-introgressed sympatric roundfins. This led to the idea that introgression might force the evolution of adaptive novelties (Nolte et al. 2005) – ideas that are the object of further, ongoing focal studies critically testing for correlations between increased phenotypic diversity and reticulate evolution (Herder and Schliewen, in preperation).

5 A Key Role of River Petea?

The *Telmatherina* population inhabiting River Petea – the single outlet of L. Matano – is the only known population of river-dwelling sharpfins (Herder et al. 2006a, b). Carrying the "introgressed" mitochondrial haplotype, this

population at the "bottleneck" area separating the L. Matano flock from the downstream diversity of Telmatherinidae is especially suited for testing if the introgression detected in sharpfins is a past event, or possibly a still ongoing phenomenon. Evaluating potential gene flow along River Petea (Fig. 1) also appeared appropriate given the fact that Roy et al. (2004) based speciation scenarios on the assumption that this river constitutes a major barrier preventing gene flow between L. Matano and the rest of the Malili Lakes system. Hence, a focus study targeted genotypic and phenotypic variation along this remarkable river, losing 72 m altitude along its only approx. 9 km length (Schwarzer et al. 2008). The datasets consisting of population-level AFLPs, geometric morphometric shape analyses, and additional measurements suggest high levels of gene flow between both, upper and lower stretches of R. Petea, as well as between *Telmatherina* sp. "Petea" and lacustrine sharpfins. Phenotypic and genotypic similarity decreases with distance along this short geographic range, indicating genetic exchange along the river and between lacustrine and stream-dwelling sharpfins. In line with these results, field work along River Petea demonstrated that the major waterfall supposed to serve as strict barrier preventing dispersal (Roy et al. 2004, 2006) is absent.

In summary, the studies available support two well-separated clades of sailfin silversides within Lake Matano, which constitute together an ancient monophylum. Morphologically highly diverse "sharpfins" are introgressed by stream populations, a process which appears ongoing in the light of results showing gene flow between L. Matano and River Petea. In contrast, sympatric roundfins show no indications of introgression, neither in terms of mitochondrial haplotypes, nor in AFLP signatures. Widely lacking phylogenetic species-level resolution within both clades indicates that speciation processes are ongoing in sharpfins and roundfins, which in turn is a prime prerequisite for analyzing mechanisms shaping divergence (Dieckmann et al. 2004; Nosil et al. 2009; Via and West 2008).

6 Sympatric Speciation in Lake Matano

Indications for adaptive speciation within the confined space of ancient Lake Matano, coupled with evidence for their monophyly, renders both clades of Lake Matano's *Telmatherina* possible candidates for sympatric speciation. Evidently not introgressed, with only three morphospecies of limited phenotypic diversity, and characterized by conspicuous male color polymorphisms, roundfins were the first monophyletic clade of sailfin silversides to be analyzed in detail (Herder et al. 2008). The three roundfin morphospecies are large and deep-bodied *Telmatherina antoniae* "large," small and slender *T. antoniae* "small," and large, slender *T. prognatha* (Fig. 1a–c). Males of all three morphospecies occur in either yellow, blue, or blue–yellow courtship coloration, whereas females are dusky gray (Herder et al. 2006a). Morphometric analyses confirmed that the morphospecies are distinct according to size and shape of head and body, but did not support any differentiation among the conspicuous color morphs (Herder et al. 2008; Fig. 3).

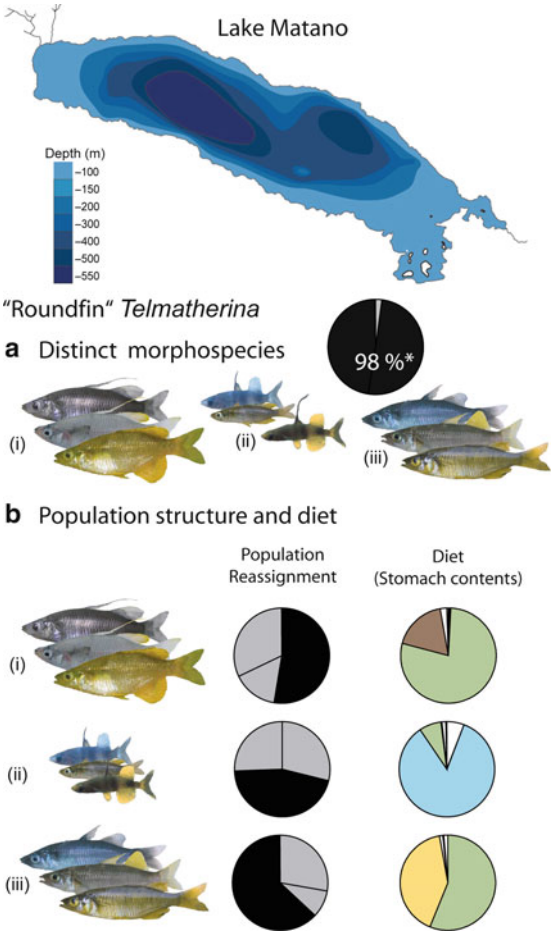


Fig. 3 Endemic "roundfin" *Telmatherina* from ancient and extraordinary deep Lake Matano. (**a**) The three polychromatic morphospecies (*i*) *Telmatherina antoniae* "large", (*ii*) *Telmatherina antoniae* "small" and (*iii*) *Telmatherina prognatha* are distinct according to body shape (*∼98% correct and significant assignments when leaving out 10–30% of individuals in Jackknife estimates of assignment performance; pictures show adult males). (**b**) Population structure of roundfins significantly reflects the three morphospecies, but shows no indications for strict reproductive isolation (data shown are reassignment results based on AFLP data; *black* morphospecies-specific reassignment, *gray* assignment to another morphospecies). Morphospecies show different patterns of trophic resource use (food importance from stomach contents; *green* terrestrial arthropods, *brown* molluscs, *blue* copepods, *yellow* fish, *white* remaining food items). Data from Herder et al. (2008); map by T. von Rintelen, modified (with permission)

All roundfin phenotypes occurred in full sympatry at six sampling locations distributed around the lake, but showed differences in abundance and habitat use. *Telmatherina antoniae* "small" is by far the most abundant morphospecies, courting and spawning in great numbers mainly in the morning hours at open soft-bottom

beach habitats. However, these fish leave spawning areas later in the day, but not towards other benthic inshore areas. Records of *T. antoniae* "small" in non-breeding mood in the offshore area supported the hypothesis that inshore habitats serve this morphospecies predominantly as courting and spawning grounds, whereas they feed predominantly in the pelagic offshore area. In contrast to the pelagic ecology of *T. antoniae* "small, *T. antoniae* "large" inhabit inshore areas. Habitats of these sailfin silversides are typically steep sites characterized by gravel or rock bottom. The rare *Telmatherina prognatha* occur most frequently at similar sites, but mainly in shallower areas providing shelter by dense canopy of overhanging vegetation and structured by submerged vegetation.

Trophic ecology of roundfins fits predictions of body shape and habitat use (Herder et al. 2008). Stomachs of *Telmatherina antoniae* "small" mainly contained copepods, which are likely to be available offshore. In contrast, "large" *T. antoniae* mainly contained small molluscs and terrestrial arthropods like winged ants, corresponding to inshore feeding. *Telmatherina prognatha*, characterized by a predator-like appearance, indeed contained remains of small fish in addition to terrestrial arthropods. In summary, ecological data suggest that the three roundfin morphospecies are fully sympatric in Lake Matano, sharing spawning grounds, but inhabiting distinct ecological niches.

Population-level AFLP genotyping supported substantial but incomplete reproductive isolation of the three morphospecies, but did not indicate strict barriers for gene flow between color morphs – neither in the "small", nor in the "large" morphospecies of *T. antoniae*. Assignment tests showed strong but by far not complete consistency of genotype groups defined by morphology, which supports the hypothesis that differences between morphospecies are significant but not unequivocally distinct in each individual. Focusing on single AFLP loci, only small proportions of the nuclear multilocus dataset turned out to be significantly differentiated, which in turn suggests that footprints of selection are restricted to only small parts of the genome – a result fitting recent ideas about initial stages of ecological speciation (Fitzpatrick et al. 2008b; Via and West 2008; Wu 2001; reviewed by Nosil et al. 2009). In line with significant but incomplete genetic differentiation among roundfin morphospecies, observational transect data highlight very strong but also not absolute morphospecies-assortative mating. Interestingly, significant intrapopulation structure detected between sampling sites in *T. antoniae* "large" clearly does not increase with intralake distances. This indicates that these predominantly benthic populations are spatially structured, possibly as a result of low dispersal and shoaling. However, para- or allopatric differentiation, which serves as null hypothesis for sympatric modes of speciation, would predict differentiation to increase with geographical distance or the presence of barriers. As neither was supported, this null hypothesis is rejected.

Consistent with evidence for a predominant pelagic ecology derived from transect data and offshore sampling, AFLP data did not detect restrictions of gene flow among benthic sample sites in the small morph of *T. antoniae* (Herder et al. 2008). A recent study based on microsatellite loci focusing on dispersal in *T. antoniae* confirmed absence of spatial structure (Walter et al. 2009a). Population

clusters detected in that study which do not coincide with sample sites may result from different roundfin morphospecies, which were not explicitly accounted for in this study (Walter et al. 2009a). Similar levels of genetic structure detected [F_{ST} = 0.03 (microsatellites; Walter et al. 2009a), F_{ST} = 0.019 (AFLPs; Herder et al. 2008)] support this interpretation. Likewise, a second microsatellite study focusing on color polymorphisms (Walter et al. 2009b) also confirmed absence of differentiation among yellow and blue male color morphs in *T. antoniae* "small". The latter study also provided first indications for a lack of color morph–specific pattern in male-male competition of *T. antoniae* "small".

Combined morphological, ecological, behavioral and genetic data are consistent with a sympatric mode of speciation in Lake Matano's roundfins, according to the criteria proposed by Coyne and Orr (2004). Roundfins are clearly also sympatric on the micorgeographic level, as all phenotypes regularly encounter each other directly. Based on genetic data and mate choice observations, their reproductive isolation is substantial but only affects small parts of the genome, and allo- or parapatric scenarios are highly unlikely. The criterion of sister group relationship (Coyne and Orr 2004) appears inadequate in the present case, as speciation is obviously not di- but trichotomus in the roundfin flock. However, the intention of this criterion is claiming evidence for divergence within a monophyletic group, which is clearly provided in case of L. Matano's roundfins.

7 On the Mechanisms Driving Speciation Processes

Answering the question about the geographic scenarios allowing or even promoting speciation processes is important, but remains only an initial step towards understanding the mechanisms driving processes of divergence (Fitzpatrick et al. 2008a). Studies like that of L. Matano's roundfins demonstrate, in line with several others (Feder et al. 2005; Filchak et al. 2000; Noakes 2008; Rolan-Alvarez 2007; Savolainen et al. 2006; Schliewen et al. 1994, 2001; Steinfartz et al. 2007), that speciation does not depend essentially on the isolating effect of extrinsic barriers, and draw attention to the question how population specific differential adaptation helps to overcome the homogenizing effect of sexual reproduction among incipient species (Coyne 2007; Bolnick and Fitzpatrick 2007; Jiggins 2006).

There are strong indications that response to ecological selection is the causal root of speciation in L. Matano's *Telmatherina* (Herder et al. 2006b). Habitat use and trophic ecology concordantly support fine-scaled niche differences in roundfins (Herder et al. 2008), which correspond to morphological traits discussed as adaptive in fish radiations, i.e., body depth and head morphology (Albertson et al. 2003; Kassam et al. 2003; Rüber and Adams 2001). Additional support for adaptation comes from geometric morphometric analyses comparing body shape between roundfins, sharpfins and stream-dwelling populations most likely involved in introgressive hybridization (Herder et al. 2006b). Multivariate axes explaining most of the shape variation demonstrated substantial segregation in body shape among

all three groups, which most likely reflects differential adaptation to stream and lake habitats. Complementary to these two lines of evidence supporting ecological selection acting as a major force in speciation of *Telmatherina* in Lake Matano, a focus study on "trait utility" in sharpfins provides further substantial support for ecological adaptation (Pfaender et al., in press). Trait utility, the performance of traits in terms of fitness, is a central criterion for the recognition of adaptive radiation (Schluter 2000), and can serve as evidence for adaptation due to ecological selection pressure. Pfaender et al. (in press) related expression of potential key traits such as shapes of upper and lower jaw bones, pharyngeal jaws, body shape, gill raker counts and body size to stomach contents, and found surprisingly fine-scaled patterns of morphological differentiation among groups of sharpfins defined by stomach contents. Fish-, shrimp- and egg-feeders were most distinct, with trait expression being widely consistent to expectations derived from other fish radiations.

Morphological adaptations outlined above and distinct patterns of habitat- and mate-choice evident in roundfins (Herder et al. 2008) strongly suggest that divergence in *Telmatherina* may also affect behavior. Indeed, a series of studies conducted by the group of Gray and McKinnon (Gray and McKinnon 2006; Gray et al. 2007, 2008a) demonstrated in line with our own focus study (Cerwenka et al., in review) highly derived mating and foraging behavior in *Telmatherina sarasinorum*, a color polymorphic sharpfin species (Fig. 1d) feeding mainly on eggs of con- and heterospecific *Telmatherina* (Gray and McKinnon 2006; Kottelat 1991). Sailfin silverside eggs are by far the dominating food source of *T. sarasinorum* at spawning grounds of the roundfin sailfin silverside *Telmatherina antoniae* "small" (Fig. 1b), and are obtained using two different behavioral strategies correlated with host density (Cerwenka et al., in review). This also affects the egg-feeding species itself, with filial cannibalism correlating with the numbers of cuckolders involved (Gray et al. 2007). A spectacular example of behavioral egg-feeding adaptation in *T. sarasinorum* has been reported just recently: male *T. sarasinorum* have been observed courting and enticing female *T. antoniae* to spawn, and then eating the eggs (Gray et al. 2008a). Gray et al. termed this unique behavioral tactic "sneaky eating," and suggested that it might have evolved as a possible extension of conspecific egg-feeding in the low resource environment of Lake Matano.

8 Sexual Selection and the Evolution of Colour Polymorphisms

Theory suggests that disruptive sexual selection may promote color polymorphisms (Chunco et al. 2007; Gray and McKinnon 2007) and possibly speciation processes (Kawata et al. 2007), but empirical evidence for speciation triggered by sexual selection is restricted to only a very few cases (Seehausen et al. 2008). The spectacular male color polymorphisms of several sailfin silverside species (Herder et al. 2006a) appeared highly promising for testing hypotheses linking male coloration and speciation processes. However, population-level AFLP or microsatellite

data did not support restrictions in gene flow between the conspicuous male color morphs in roundfins, either in *T. antoniae* "small" or in *T. antoniae* "large" (Herder et al. 2008; Walter et al. 2009b). Likewise, morphometric data did not indicate any difference in body shape between roundfin color morphs, which might be hypothesized to accumulate under restricted gene flow. Surprisingly, however, explorative analyses of the multilocus AFLP data set revealed significant signal for yellow male coloration across all roundfins included, which suggests heritability of color traits. This heritability is, however, clearly not coupled with significant population structure. Stable color polymorphisms maintained by fluctuating or heterogeneous environments, like lighting conditions changing with daytime or season, or differing with habitat structure, may provide an appropriate explanation for this phenomenon (Chunco et al. 2007; Gray and McKinnon 2007). Recent support for the hypothesis that spatial heterogeneity of the visual environment can influence sexual selection on male coloration comes from observational studies on color polymorphic *T. sarasinorum* (Gray et al. 2008b; Fig. 1d). Blue and yellow males each turned out to have significantly increased reproductive fitness in one of two alternative habitats tested, which are characterized by opposing lighting conditions corresponding to likewise increased contrast of coloration with the background. Hence, sexual selection decoupled from speciation processes can explain the existence of color polymorphic sailfin silversides; however, widespread presence of different kinds of color polymorphisms in the Malili Lakes radiation, including river- and stream-dwelling populations inhabiting very different kinds of habitats, raises the question whether habitat heterogeneity alone is likely to explain the flock-wide pattern. A combination of different external factors generating visual heterogeneity, including daytime and seasonal effects, might explain these patterns.

9 Perspectives

In summary, sailfin silversides of "Wallace's dreamponds" have been successfully established and used as a new model system for speciation research, and for the study of selection maintaining color polymorphisms. The comparatively small to medium size of each of the five Malili Lakes as well as the multifaceted geographical structure of the lakes system allows incorporating sympatrically, parapatrically and allopatrically distributed radiations into comprehensive analyses.

This setting is hardly comparable to any other aquatic model systems, neither to the very large and complex East Africal Great Lakes, nor to the tiny crater lake species assemblages in Cameroon and Nicaragua. Hence, the sailfin silverside system offers great potential for evolutionary biology, especially for speciation research. Based on the initial results reviewed here, four major research topics have emerged as especially promising. First, the adaptive character of both sailfin silverside radiations of Lake Matano offers the chance for testing the idea of selection shaping adaptive fitness landscapes (Gavrilets 2004; Kingsolver and Pfennig 2007) in a spatially confined environment. Linking individual fitness

correlates to morphological character expression and their ecological utility, this approach has the potential for providing deeper insight into processes of adaptive speciation in sympatry. Second, and complementary, massive introgression of known stream-dwelling populations into the dynamically evolving sharpfins of Lake Matano has set the optimal stage for testing hypotheses regarding the role of hybridization and introgression on the generation of increased phenotypic and genotypic diversity (Rieseberg et al. 1999; Seehausen 2004; Stelkens and Seehausen 2009). Third, and probably most important, the Malili system offers unique opportunities for testing hypotheses on different genomic consequences of sympatric versus parapatric or allopatric speciation (Nosil et al. 2009). Adaptive sympatric speciation of L. Matano's roundfins is opposed to parapatric and allopatric settings in the Towuti-Mahalona system, where lakes are connected by a gentle river that may serve as corridor for gene flow. Genome scans applied to L. Matano's roundfins (Herder et al. 2008) have served as a first step towards the genomic analysis of the system. Availability of the complete medaka (*Oryzias latipes*) genome (Kasahara et al. 2007), a species much more closely related to *Telmatherina* than other fully sequenced species like stickleback or zebrafish, may allow insights into the basis of speciation relevant genes. Last, but not least, the dominance of color polymorphisms in the whole flock and genetic data supporting heritability and environment contingent fitness consequences suggest that heterogeneous environments maintain color polymorphism in most telmatherinid species, but its mechanisms and potential implications for speciation remain only partially understood. Although a significant role for population divergence of roundfins based on selective female choice for alternative male color polymorphism is unlikely, this is not necessarily the case for sharpfins of Lake Matano or other sailfin silversides. Speciation through sensory drive (Seehausen et al. 2008) remains a plausible hypothesis for the origin of ecologically divergent species, some of which are polymorphic while others are not.

A major task remaining is to provide a time scale for the sailfin silverside radiation. Preliminary results derived from distance-based divergence estimates support an age of 0.95–1.9 Mya for the split separating roundfin and "non-stream" sharpfin mtDNA haplotypes (Roy et al. 2007b; Clades I and II), which roughly corresponds to the estimated geological age of L. Matano (1–2 mya; cited in von Rintelen et al. 2004). However, application of model-based approaches incorporating all relevant clades of the systems (Stoeger et al. in prepararion) appears appropriate to address this rather fundamental aspect.

The Malili Lakes system does not only harbor several independent fish radiations or endemic lineages. The data now available on the evolution of pachychilid snails (Glaubrecht and von Rintelen 2008; von Rintelen et al. 2004, 2007b, this volume), athyid shrinps (K von Rintelen and Cai 2009; K von Rintelen et al. in press) and gecarcinucid freshwater crabs (Schubart and Ng 2008; Schubart et al. 2008) remain to be assembled with the sailfin silverside data. Such an approach linking patterns of divergence in fundamentally different freshwater organisms restricted to the same area may finally provide insight into major environmental effects shaping the endemic diversity, including barriers for

dispersal, and might allow incorporating potential effects of coevolution (see T. von Rintelen et al. 2004, this volume; von Rintelen et al. 2007a). Wallace´s dreamponds undoubtedly provide a rich environment for evolutionary biologists after Darwin to study the origin of species – beyond "simple" sympatric speciation.

Acknowledgments We thank the Indonesian Institute of Sciences (LIPI) for the permit to conduct research in Indonesia. We are especially grateful to R. K. Hadiaty for strongly supporting our project in Indonesia. PT. INCO provided outstanding logistic support in Sulawesi. A. Cerwenka, S. Chapuis, J. Frommen, R. K. Hadiaty, J. Herder, A. Nolte, J. Pfaender and J. Schwarzer contributed in the field and/or laboratory to the success of the project. W. Wickler (MPG) enabled the study by financing a pilot study. Fieldwork greatly benefited from logistic support in Indonesia by T. von Rintelen. We acknowledge T. von Rintelen for providing access to digitized maps. Comments and suggestions by Jobst Pfaender and two anonymous referees helped improving the manuscript. This study was funded by research grants from the Deutsche Forschungsgemeinschaft to UKS (DFG SCHL 567/2-1, 2, 3) and by a graduate fellowship donated by the Rheinische Friedrich Wilhelms- Universität Bonn to F.H.

References

Ahmad W (1977) Geology along the Matano Fault Zone East Sulawesi, Indonesia. In: Proceedings of the regional conference on the geology and mineral resources of South East Asia, Jakarta:1–15

Albertson RC, Markert JA, Danley PD, Kocher TD (1999) Phylogeny of a rapidly evolving clade: the cichlid fishes of Lake Malawi, East Africa. Proc Natl Acad Sci USA 96:5107–5110

Albertson RC, Streelman JT, Kocher TD (2003) Directional selection has shaped the oral jaws of Lake Malawi cichlid fishes. Proc Natl Acad Sci USA 100:5252–5257

Arnold GM (2006) Evolution through genetic exchange. Oxford University Press, New York

Barluenga M, Stölting KN, Salzburger W, Muschick M, Meyer A (2006) Sympatric speciation in crater lake cichlid fish. Nature 439:719–723

Baldwin BG, Sanderson MJ (1998) Age and rate of diversification of the Hawaiian silversword alliance (Compositae). Proc Natl Acad Sci USA 95:9402–9406

Berlocher SH, Feder JL (2002) Sympatric speciation in phytophagous insects: moving beyond controversy? Annu Rev Entomol 47:773–815

Bolnick DL (2004) Waiting for sympatric speciation. Evolution 58:895–899

Bolnick DL, Fitzpatrick BM (2007) Sympatric speciation: models and empirical evidence. Annu Rev Ecol Evol Syst 38:459–487

Brooks JL (1950) Speciation in ancient lakes (concluded). Q Rev Biol 25:131–176

Cerwenka AF, Schliewen UK, Herder F Egg-feeding in Lake Matano's adaptive sailfin silversides radiation. (in review)

Chunco AJ, McKinnon JS, Servedio MR (2007) Microhabitat variation and sexual selection can maintain male color polymorphisms. Evolution 61:2504–2515

Coyne JA (2007) Sympatric speciation. Curr Biol 17:R787–R788

Coyne JA, Orr HA (2004) Speciation. Sinauer, Sunderland

Crowe SA, O'Neill AH, Katsev S, Hehanussa P, Haffner GD, Sundby B, Mucci A, Fowle DA (2008a) The biogeochemistry of tropical lakes: a case study from Lake Matano, Indonesia. Limnol Oceanogr 53:319–331

Crowe SA, Jones CA, Katsev S, Magen C, O'Neil AH, Sturm A, Canfield DE, Haffner GD, Mucci A, Sundby B, Fowle DA (2008b) Photoferrotrophs thrive in an Archean Ocean analogue. Proc Natl Acad Sci USA 105:15938–15943

Danley PD, Kocher T (2001) Speciation in rapidly diverging systems: lessons from Lake Malawi. Mol Ecol 10:1075–1086
Danley PD, Markert JA, Arnegard ME, Kocher TD (2000) Divergence with gene flow in the rock-dwelling cichlids of Lake Malawi. Evolution 54:1725–1737
Dieckmann U, Doebeli M (1999) On the origin of species by sympatric speciation. Nature 400:354–357
Dieckmann U, Doebeli M, Metz JAJ, Tautz D (2004) Adaptive speciation. Cambridge University Press, Cambridge
Doebeli M, Dieckmann U (2000) Evolutionary branching and sympatric speciation caused by different types of ecological interactions. Am Nat 156:S77–S101
Emerson BC (2002) Evolution on oceanic islands: molecular phylogenetic approaches to understanding pattern and process. Mol Ecol 11:951–966
Feder JL, Xie X, Rull J, Velez S, Forbes A, Leung B, Dambroski H, Filchak KE, Aluja M (2005) Mayr, Dobzhanski, and Bush and the complexities of sympatric speciation in *Rhagoletis*. Proc Natl Acad Sci USA 102:6573–6580
Filchak KE, Roethele JB, Feder JL (2000) Natural selection and sympatric divergence in the apple maggot *Rhagoletis pomonella*. Nature 407:739–742
Fitzpatrick BM, Fordyce JA, Gavrilets S (2008a) What, if anything, is sympatric speciation? J Evol Biol 21:1452–1459
Fitzpatrick BM, Placyk JS, Niemiller ML, Casper GS, Burghardts GM (2008b) Distinctiveness in the face of gene flow: hybridization between specialist and generalist gartersnakes. Mol Ecol 17:4107–4117
Gavrilets S (2004) Fitness landscapes and the origin of species. Princeton University Press, Princeton and Oxford
Gavrilets S, Li H, Vose MD (2000) Patterns of parapatric speciation. Evolution 54:1126–1134
Gavrilets S, Vose A (2005) Dynamic patterns of adaptive radiation. Proc Natl Acad Sci USA 102:18040–18045
Glaubrecht M, von Rintelen T (2008) The species flocks of lacustrine gastropods: *Tylomelania* on Sulawesi as models in speciation and adaptive radiation. Hydrobiologia 615:181–199
Grant BR, Grant PR (2003) What Darwin's finches can teach us about the evolutionary origin and regulation of biodiversity. Bioscience 53:965–975
Gray SM, McKinnon JS (2006) A comparative description of mating behaviour in the endemic telmatherinid fishes of Sulawesi's Malili Lakes. Environ Biol Fish 75:471–482
Gray SM, McKinnon JS (2007) Linking color polymorphism maintenance and speciation. Trends Ecol Evol 22:71–79
Gray SM, Dill LM, McKinnon JS (2007) Cuckoldry incites cannibalism: male fish turn to cannibalism when perceived certainty of paternity decreases. Am Nat 169:258–263
Gray SM, McKinnon JS, Tantu FY, Dill LM (2008a) Sneaky egg-eating in *Telmatherina sarasinorum*, an endemic fish from Sulawesi. J Fish Biol 73:728–731
Gray SM, Dill LM, Tantu FY, Loew ER, Herder F, McKinnon JS (2008b) Environment-contingent sexual selection in a colour polymorphic fish. Proc R Soc Lond B 275:1785–1791
Haffner GD, Hehanussa PE, Hartoto D (2001) The biology and physical processes of large lakes of Indonesia: Lakes Matano and Towuti. In: Munawar M, Hecky RE (eds) The great lakes of the world (GLOW) food web, health and integrity. Backhuis, Leiden, pp 182–192
Herder F, Schwarzer J, Pfaender J, Hadiaty RK, Schliewen UK (2006a) Preliminary checklist of sailfin silversides (Pisces: Telmatherinidae) in the Malili Lakes of Sulawesi (Indonesia), with a synopsis of systematics and threats. Verh Ges Ichthyol 5:139–163
Herder F, Nolte A, Pfaender J, Schwarzer J, Hadiaty RK, Schliewen UK (2006b) Adaptive radiation and hybridization in Wallace's Dreamponds: evidence from sailfin silversides in the Malili Lakes of Sulawesi. Proc R Soc Lond B 273:2209–2217
Herder F, Hadiaty RK, Schliewen UK (2006c) Diversity and evolution of Telmatherinidae in the Malili Lakes system in Sulawesi. In: Proceedings of the international symposium "the ecology and limnology of the Malili Lakes" March 20–22 Bogor, Indonesia, pp 67–72

Herder F, Pfaender J, Schliewen UK (2008) Adaptive sympatric speciation of polychromatic "roundfin" sailfin silverside fish in Lake Matano (Sulawesi). Evolution 62:2178–2195

Jiggins CD (2006) Sympatric speciation: why the controversy? Curr Biol 16:R333–R334

Kasahara M, Naruse K, Sasaki S, Nakatani Y, Qu W, Ahsan B, Yamada T, Nagayasu Y, Doi K, Kasai Y, Jindo T, Kobayashi D, Shimada A, Toyoda A, Kuroki Y, Fujiyama A, Sasaki T, Shimizu A, Asakawa S, Shimizu N, Hashimoto S, Yang J, Lee Y, Matsushima K, Sugano S, Sakaizumi M, Narita T, Ohishi K, Haga S, Ohta F, Nomoto H, Nogata K, Morishita T, Endo T, Shin-I T, Takeda H, Morishita S, Kohara Y (2007) The medaka draft genome and insights into vertebrate genome evolution. Nature 447:714–719

Kassam DD, Adams DC, Ambali AJD, Yamaoka K (2003) Body shape variation in relation to resource partitioning within cichlid trophic guilds coexisting along the rocky shore of Lake Malawi. Anim Biol 53:59–70

Kawata M, Shoji A, Kawamura S, Seehausen O (2007) A genetically explicit model of speciation by sensory drive within a continuous population in aquatic environments. BMC Evol Biol 7:99

Kingsolver JG, Pfennig DW (2007) Patterns and power of phenotypic selection in nature. Bioscience 57:561–572

Kocher TD (2004) Adaptive evolution and explosive speciation: the cichlid fish model. Nat Genet 5:288–298

Kottelat M (1990a) Sailfin silversides (Pisces: Telmatherinidae) of Lakes Towuti, Mahalona and Wawontoa (Sulawesi, Indonesia) with descriptions of two new genera and two new species. Ichthyol Explor Freshwaters 1:227–246

Kottelat M (1990b) The ricefishes (Oryziidae) of the Malili Lakes, Sulawesi, Indonesia, with description of a new species. Ichthyol Explor Freshwaters 1:151–166

Kottelat M (1991) Sailfin silversides (Pisces: Telmatherinidae) of Lake Matano, Sulawesi, Indonesia, with descriptions of six new species. Ichthyol Explor Freshwaters 1:321–344

Luikart G, England PR, Tallmon D, Jordan S, Taberlet P (2003) The power and promise of population genomics: from genotyping to genome typing. Nat Genet 4:981–994

Mallet J (2007) Hybrid speciation. Nature 446:279–283

Mallet J (2001) The speciation revolution - commentary. J Evol Biol 14:887–888

Mayr E (1942) Systematics and the origin of species. Columbia University Press, New York

Mayr E (1963) Animal species and evolution. Belknap, Cambridge, MA

Noakes DLG (2008) Charr truth: sympatric differentiation in *Salvelinus* species. Environ Biol Fish 83:7–15

Nolte A, Freyhof J, Stemshorn K, Tautz D (2005) An invasive lineage of sculpins, *Cottus* sp. (Pisces, Teleostei) in the Rhine with new habitat adaptations has originated from hybridization between old phylogenetic groups. Proc R Soc Lond B 272:2379–2387

Nosil P, Funk DJ, Ortiz-Barrientos D (2009) Divergent selection and heterogeneous genomic divergence. Mol Ecol 18:375–402

Pfaender J, Schliewen UK, Herder F (in press) Trait utility in "sharpfin" sailfin silversides of Lake Matano (Sulawesi) suggests adaptive radiation. Evol Ecol (Online First)

Rieseberg LH, Archer MA, Wayne R (1999) Transgressive segregation, adaptation and speciation. Heredity 83:363–372

Rogers SM, Bernatchez L (2007) The genetic architecture of ecological speciation and the association with signatures of selection in natural lake whitefish (*Coregonus* sp., Salmonidae) species pairs. Mol Biol Evol 24:1423–1438

Rolan-Alvarez E (2007) Sympatric speciation as a by-product of ecological adaptation in the galician *Littorina saxatilis* hybrid zone. J Molluscan Stud 73:1–10

Roy D, Docker MF, Hehanussa PE, Heath DD, Haffner GD (2004) Genetic and morphological data supporting the hypothesis of adaptive radiation in the endemic fish of Lake Matano. J Evol Biol 17:1268–1276

Roy D, Kelly DW, Fransen CHJM, Heath DD, Haffner GD (2006) Evidence of small-scale vicariance in *Caridina lanceolata* (Decapoda: Atyidae) from the Malili Lakes, Sulawesi. Evol Ecol Res 8:1087–1099

Roy D, Docker MF, Haffner GD, Heath DD (2007a) Body shape vs. colour associated initial divergence in the *Telmatherina* radiation in Lake Matano, Sulawesi, Indonesia. J Evol Biol 20:1126–1137

Roy D, Docker MF, Paterson G, Hamilton PB, Heath DD, Haffner GD (2007b) Resource-based adaptive divergence in the freshwater fish *Telmatherina* from Lake Matano, Indonesia. Mol Ecol 16:35–48

Rüber L, Adams DC (2001) Evolutionary convergence of body shape and trophic morphology in cichlids from Lake Tanganyika. J Evol Biol 14:325–332

Samonte IE, Satta Y, Sato AS, Tichy H, Takahata N, Klein J (2007) Gene flow between species of Lake Victoria Haplochromine Fishes. Mol Biol Evol 24:2069–2080

Savolainen V, Anstett M-C, Lexer C, Hutton I, Clarkson JJ, Norup MV, Powell MP, Springate D, Salamin N, Baker WJ (2006) Sympatric speciation in palms on an oceanic island. Nature 441:210–213

Schilthuizen M, Cabanban AS, Haase M (2005) Possible speciation with gene flow in tropical cave snails. J Zool Sys Evol Res 43:133–138

Schliewen UK, Klee B (2004) Reticulate sympatric speciation in Cameroonian crater lake cichlids. Front Zool 1:1–12

Schliewen UK, Tautz D, Pääbo S (1994) Sympatric speciation suggested by monophylie of crater lake cichlids. Nature 368:629–632

Schliewen UK, Rassmann K, Markmann M, Markert J, Kocher T, Tautz D (2001) Genetic and ecological divergence of a monophyletic cichlid species pair under fully sympatric conditions in Lake Ejagham, Cameroon. Mol Ecol 10:1471–1488

Schliewen UK, Kocher TD, McKaye KR, Seehausen O, Tautz D (2006) Evidence for symparic speciation? Nature 444:E12–E13

Schluter D (2000) The ecology of adaptive radiation. Oxford University Press, Oxford

Schubart CD, Ng PKL (2008) A new molluscivore crab from Lake Poso confirms multiple colonization of ancient lakes in Sulawesi by freshwater crabs (Decapoda: Brachyura). Zool J Linn Soc 154:211–221

Schubart CD, Santl T, Koller P (2008) Mitochondrial patterns of intra- and interspecific differentiation among endemic freshwater crabs of ancient lakes in Sulawesi. Contrib Zool 77:83–90

Schwarzer J, Herder F, Misof B, Hadiaty RK, Schliewen UK (2008) Gene flow at the margin of Lake Matano's adaptive sailfin silverside radiation: Telmatherinidae of River Petea in Sulawesi. Hydrobiologia 615:201–213

Seehausen O (2004) Hybridization and adaptive radiation. Trends Ecol Evol 19:198–207

Seehausen O, Terai Y, Magalhaes IS, Carleton KL, Mrosso HDJ, Miyagi R, van der Sluijs I, Schneider MV, Maan ME, Tachida H, Imai H, Okada N (2008) Speciation through sensory drive in cichlid fish. Nature 455:620–626

Strecker U, Meyer CG, Sturmbauer C, Wilkens H (1996) Genetic divergence and speciation in an extremely young species flock in Mexico formed by the genus *Cyprinodon* (Cyprinodontidae, Teleostei). Mol Phyl Evol 6:143–149

Steinfartz S, Weitere M, Tautz D (2007) Tracing the first step to speciation: ecological and genetic differentiation of a salamander population in a small forest. Mol Ecol 16:4550–4561

Stelkens R, Seehausen O (2009) Genetic distance between species predicts novel trait expression in their hybrids. Evolution 63:884–897, DOI: 10.111/j.1558-5646.2008.00595.x

Templeton AR (1981) Mechanisms of speciation - a population genetic approach. Annu Rev Ecol Syst 12:23–48

Thorpe RS, Jones AG, Malhotra A, Surget-Groba Y (2008) Adaptive radiation in Lesser Antillean lizards: molecular phylogenies and species recognition in the Lesser Antillean dwarf gecko complex, *Sphaerodactylus fantasticus*. Mol Ecol 17:1489–1504

Via S, West J (2008) The genetic mosaic suggests a new role for hitchhiking in ecological speciation. Mol Ecol 17:4334–4345

von Rintelen K, Cai Y (2009) Radiation of endemic species flocks in ancient lakes: Systematic revision of the freshwater shrimp *Caridina* H. Milne Edwards, 1837 (Crustacea: Decapoda:

Atyidae) from the ancient lakes of Sulawesi, Indonesia, with the description of eight new species. Raff Bull Zool 57:343–452

von Rintelen K, von Rintelen T, Meixner M, Lüter C, Cai Y, Glaubrecht M (2007a) Freshwater shrimp-sponge association from an ancient lake. Biol Lett 3:262–264

von Rintelen K, Glaubrecht, M, Schubart CD, Wessel A, von Rintelen T. (in press) Adaptive radiation and ecological diversification of Sulawesi's ancient lake shrimps. Evolution

von Rintelen T, Bouchet P, Glaubrecht M (2007b) Ancient lakes as hotspots of diversity: a morphological review of an endemic species flock of *Tylomelania* (Gastropoda: Cerithioidea: Pachychilidae) in the Malili lake system on Sulawesi, Indonesia. Hydrobiologia 592:11–94

von Rintelen T, Wilson AB, Meyer A, Glaubrecht M (2004) Escalation and trophic specialization drive adaptive radiation of freshwater gastropods in acient lakes on Sulawesi, Indonesia. Proc R Soc Lond B 271:2541–2549

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucl Acids Res 23:4407–4414

Walter RP, Haffner GD, Heath DD (2009a) Dispersal and population genetic structure of *Telmatherina antoniae*, an endemic freshwater Sailfin silverside from Sulawesi. Indonesia J Evol Biol 22:314–323

Walter RP, Haffner GD, Heath DD (2009b) No barriers to gene flow among sympatric polychromatic 'small' *Telmatherina antoniae* from Lake Matano, Indonesia. J Fish Biol 74:1804–1815

Wu CI (2001) The genic view of the process of speciation. J Evol Biol 14:851–865

The Species Flocks of the Viviparous Freshwater Gastropod *Tylomelania* (Mollusca: Cerithioidea: Pachychilidae) in the Ancient Lakes of Sulawesi, Indonesia: The Role of Geography, Trophic Morphology and Color as Driving Forces in Adaptive Radiation

Thomas von Rintelen, Kristina von Rintelen, and Matthias Glaubrecht

Abstract The study of species flocks in island settings such as ancient lakes can contribute significantly to our understanding of fundamental evolutionary processes, such as speciation, radiation, adaptation, and coevolution. We here use endemic freshwater gastropods in ancient lakes on the Indonesian island of Sulawesi as models to gain insights into patterns and mechanisms of lacustrine diversification. In two central lake systems on the island, i.e., Lake Poso and the five connected Malili lakes, the pachychilid freshwater snail genus *Tylomelania* has radiated into a diverse endemic assemblage of c. 53 species of morphologically distinct viviparous gastropods. Molecular phylogenetic data revealed that independent and multiple colonizations of the two lake systems by fluviatile ancestors have led to four morphologically and ecologically diverse adaptive radiations, one in Lake Poso and three in the Malili system. The evolution of habitat preferences and trophic specialization most likely drive diversification in both systems, while geography, i.e., allopatric speciation, is a major factor in the spatially strongly structured Malili system. Highly characteristic lacustrine shell forms have evolved in all lakes through escalation, i.e., coevolution with molluscivorous crabs. The role of the conspicuous body coloration present mainly in Lake Poso species remains to be elucidated, specifically its potential role in diversification processes. Finally, the setting of the Sulawesi snail species flocks with two independent radiations under almost identical extrinsic conditions offers insights into parallel patterns of adaptive evolution. However, a major conservation effort will be required to preserve the lakes' spectacular species for future research.

Keywords Speciation · Malili lake system · Lake Poso · Molecular phylogeny · Colonization · Co-evolution · Trophic specialization · Conservation

T. von Rintelen, K. von Rintelen, and M. Glaubrecht (✉)
Museum für Naturkunde, Leibniz-Institut für Evolutions- und Biodiversitätsforschung an der Humboldt-Universität zu Berlin, Invalidenstraße 43, 10115 Berlin, Germany
e-mail: matthias.glaubrecht@mfn-berlin.de

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_23,

1 Introduction

1.1 Ancient lakes as "Natural Laboratories"

Evolution on islands has been a major focus of organismic biology ever since Darwin (1859). The isolated setting of islands offers the chance to study evolutionary phenomena and processes in a confined "natural laboratory" (Whittaker and Fernández-Palacios 2007). Adaptive radiations on islands provide an ideal context for research looking into the origin of biological diversity in general (see, e.g., Grant 1998). Islands in an evolutionary sense also comprise island-like habitats such as ancient lakes, which are known to occasionally host spectacular radiations, for instance the species flocks of East African cichlids (see, e.g., Barlow 2000; Kornfield and Smith 2000; Kocher 2004). Species flocks in so-called "ancient lakes" (often regarded as long-lived lakes that have existed for more than 100,000 years (Gorthner et al. 1994; but see also Albrecht and Wilke 2008), often much longer, such as Lake Tanganyika and Lake Baikal, have featured prominently in evolutionary biology since Brooks' (1950) seminal paper, and these "ecological theatres" (Hutchinson 1965) are regarded as hotspots of diversification (for a general overview, see, e.g., Martens 1997; Rossiter and Kawanabe 2000). Organismic ancient lake research has yielded important insights into general patterns of speciation and radiation (see, e.g., Streelman and Danley 2003; Kocher 2004), which were mostly gained from studies on fishes. Recently, an increasing number of studies on invertebrates seeks to extend and test this framework by exploring their often very distinct intrinsic properties, such as widely different dispersal abilities and most likely a less prominent influence of sexual selection; for recent examples, see, e.g., for molluscs, Glaubrecht (1996, 2008), Rintelen et al. (2004, 2007a), Wilson et al. (2004) and Albrecht et al. (2006), and for crustaceans, Marijnissen et al. (2006) and Rintelen et al. (2007b). Among invertebrates, freshwater gastropods with their wide range of reproductive strategies are ideally suited for addressing questions, e.g., in biogeography and biodiversity research (see, e.g., Glaubrecht 1996, 2000, 2004). We use freshwater gastropods as models for the investigation of patterns and mechanisms of diversification by studying species flocks of the pachychilid genus *Tylomelania* Sarasin and Sarasin 1897, which has radiated into more than 50 morphologically diverse species in ancient lakes on the Indonesian island Sulawesi (Appendix 1).

1.2 The Gastropods in the "Ancient Lakes" on Sulawesi

The genus *Tylomelania* is endemic to Sulawesi where it is widely distributed with currently c. 75 known species (Fig. 1; Appendix 1). The species flocks in the two ancient lake systems situated in the central mountains of the island, viz. Lake Poso and the five Malili lakes (Box 1, Figs. 1–4), were discovered by the Swiss

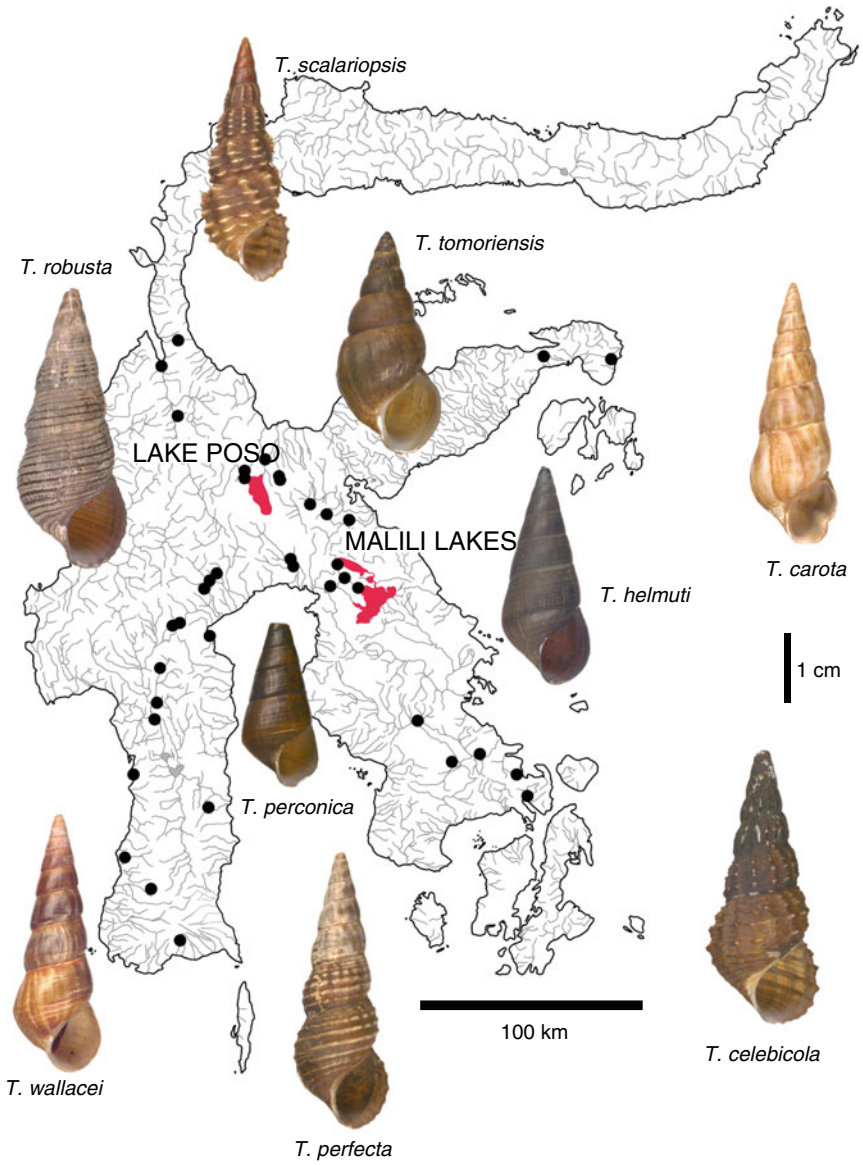


Fig. 1 Sulawesi, riverine sampling stations (1999–2007, closely neighboring sample sites have been reduced to just single *dots*) and all described riverine species. The two ancient lake systems are highlighted in *red*

naturalists Paul and Fritz Sarasin during their extensive travels on the entire island (Sarasin and Sarasin 1905). They described a radiation of 16 species of peculiar gastropods with large and frequently strongly sculptured shells in both lake systems (Sarasin and Sarasin 1897, 1898). More than a decade later, the Dutch geologist

E.C. Abendanon sampled the lakes in 1909–1910, increasing the number of known endemic lake taxa to a total of 24 species (Kruimel 1913). This early taxonomic activity was followed by a century of neglect, with only cursory mentions by Woltereck (1931, 1941), Wesenberg-Lund (1939) and Davis (1982). Interest in the Sulawesi gastropods was only revived in the 1990s through a first sampling campaign by the French malacologist P. Bouchet (Bouchet et al. 1995; Marwoto 1997) leading to the current research activities by the authors since 1999 (Rintelen and Glaubrecht 2003, 2005; Rintelen et al. 2004, 2007a; Glaubrecht and Rintelen 2008).

Traditionally, the species in the lakes were assigned to the pantropical Cerithioidean freshwater family Thiaridae, and it has just recently been recognized that they belong to the Pachychilidae (Glaubrecht and Rintelen 2003; Köhler et al. 2004; Rintelen and Glaubrecht 2005), and that all pachychilid species from Sulawesi belong to one single endemic genus, i.e., *Tylomelania* (Rintelen and Glaubrecht 2005), which was initially only erected for three endemic Lake Poso species (Sarasin and Sarasin 1897; see Rintelen and Glaubrecht 2005 for details on the taxonomic history of the Sulawesi pachychilids). Pachychilid freshwater snails have an apparent Gondwana distribution, with representatives occurring in South America (*Doryssa, Pachychilus*), Africa (*Potadoma*), Madagascar (*Madagasikara*), south and southeast Asia (*Adamietta, Brotia, Jagora, Paracrostoma, Sulcospira, Tylomelania*), and the Torres Strait Islands (*Pseudopotamis*) at the Australian margin (Glaubrecht 2000; Köhler et al. 2004; Köhler and Glaubrecht 2007, 2010). Viviparity, which has long been assumed as an important intrinsic factor in radiations of limnic gastropods (see discussion, e.g., in Glaubrecht 1996, 1999, 2006), is confined to several genera in Asia and in the geographically restricted *Pseudopotamis* only. All other pachychilids are oviparous (Glaubrecht and Rintelen 2003; Köhler et al. 2004). *Tylomelania* shares a unique reproductive anatomy and strategy, i.e., brooding its young in the uterus, with its sister group *Pseudopotamis* (Glaubrecht and Rintelen 2003; Rintelen and Glaubrecht 2005).

1.3 Scope and Aims

Being speciose, morphologically diverse, and brooding, the endemic species flocks of pachychilid gastropods in the ancient lakes of Sulawesi provide an excellent opportunity to study mechanisms of speciation and diversification in general. We explicitly test whether the radiation of *Tylomelania* is a truly *adaptive* radiation sensu Schluter (2000) by employing the four distinctive criteria he provides, viz. (fairly recent) common origin and rapid speciation (as phylogenetic criteria leading to radiation) as well as phenotype–environment correlation and trait utility (as ecological criteria resulting in adaptation). For mollusc radiations in particular, a rigorous approach along these lines is often lacking. Based on analyses of ecological, morphological, and molecular data on various hierarchical levels, the role of extrinsic and intrinsic factors for speciation and radiation is investigated for the

endemic radiation of these lacustrine snails. A particular focus of our work has been on the role of geographic versus ecological factors in speciation.

Here, we summarize our current knowledge on the ecological and phylogenetic patterns that have emerged so far from this model system. We discuss evolutionary hypotheses stemming from these data and we will outline future research perspectives. Ongoing complementary studies on other organisms in the Sulawesi lakes (crustaceans: Rintelen et al. 2007 b, in press; Schubart and Ng 2008; Schubart et al. 2008; fishes: Herder et al. 2006a, b, 2008, this volume; Schwarzer et al. 2008) will offer the opportunity of placing our results on the gastropods into a wider perspective, and first comparisons can already be made in this paper. We also suggest priorities in conservation in order to offer future generations the chance to study this unique "submerged Galapagos" or "Wallace's dreamponds" (Herder et al. 2006a) as well.

Our studies are based on comprehensive sampling in Lake Poso and all five Malili lakes as well as in the connecting and adjacent rivers (Figs. 2 and 3) during nine field campaigns. The lakes were visited in 1999 (dry season, M.G. and T.v.R.), 2000 (wet season, T.v.R.), 2002 (dry season, T.v.R. and K.v.R.), 2003 (dry season, T.v.R. and M.G.), 2004 (three trips, dry and wet season, T.v.R., K.v.R., and M.G.), 2005 (dry season, T.v.R., K.v.R., and M.G.) and 2007 (dry season, T.v.R. and M.G.). Samples were obtained by snorkeling and SCUBA diving.

2 The Species Flocks of *Tylomelania* in Lakes on Sulawesi: Species Diversity and Endemism

Currently, 35 species of *Tylomelania* have been described from both ancient lake systems of Sulawesi (Fig. 2 and 3; Appendix 1; Glaubrecht and Rintelen 2008; Rintelen and Glaubrecht 2008). All species are endemic to their respective limnic system. While 28 species are recognized in the Malili system (Fig. 3; Rintelen et al. 2007a, b; Rintelen and Glaubrecht 2008), only seven taxa have so far been described from Lake Poso and Poso River (Fig. 2; Glaubrecht and Rintelen 2008). Diversity in Lake Poso is expected to be much higher; currently 25 morphospecies can be distinguished (Rintelen et al., unpublished data; Marwoto, personal communication), which would yield a total of 53 species in both ancient lake systems (Appendix 1). The Malili lakes probably also harbor between 1 and 5 yet undescribed taxa, depending on the outcome of ongoing research on the fine-scale differentiation of genetics and morphology in that system (compare Fig. 7; Rintelen et al., unpublished data).

In the Malili system, endemism is not restricted to the lake system as a whole, however, but 24 species (86%) are endemic to a single lake (Fig. 5; Glaubrecht and Rintelen 2008; Rintelen and Glaubrecht 2008). Lake Towuti is most species-rich with 10 species, six of which are endemic, while Lake Mahalona harbours nine species (six endemics) and Lake Matano seven (six endemics). Tominanga River between Lake Mahalona and Towuti hosts four species, only one of which is

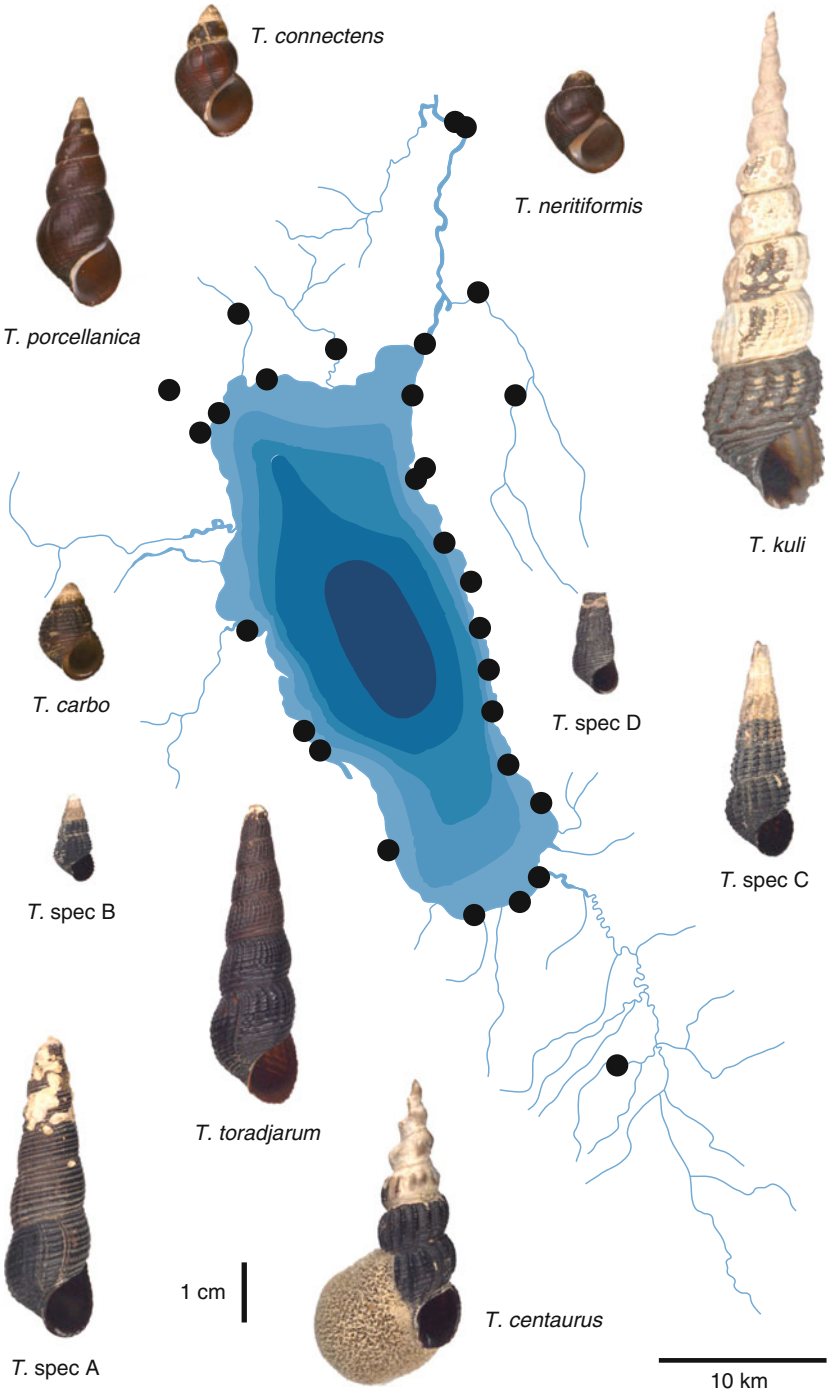


Fig. 2 Lake Poso with sampling stations and endemic species of *Tylomelania*. All described species and a selection of yet undescribed taxa are shown

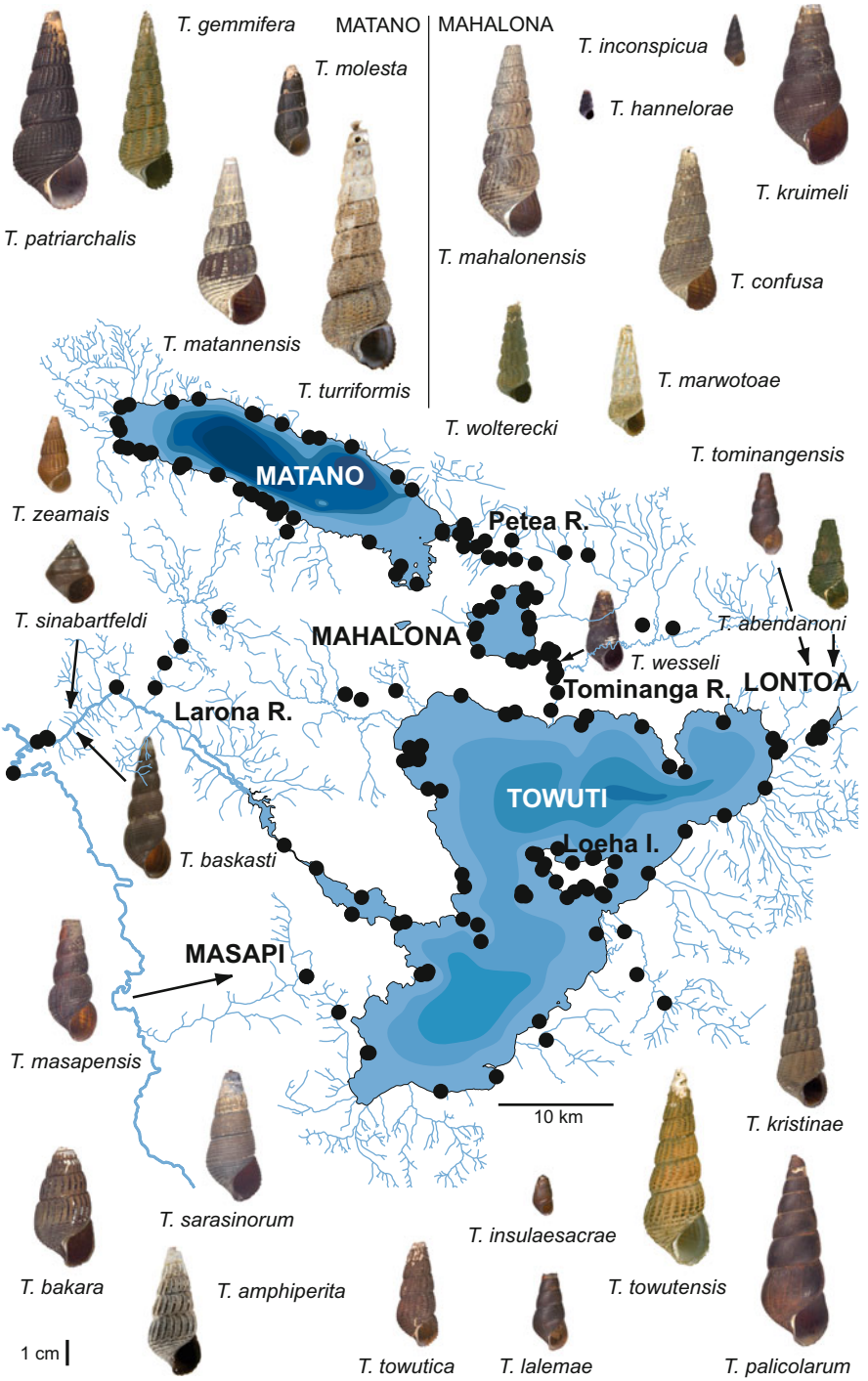


Fig. 3 Malili lake system with sampling stations and the endemic species of *Tylomelania*. Lake names are printed in *capital letters*. *R* River, *I* island

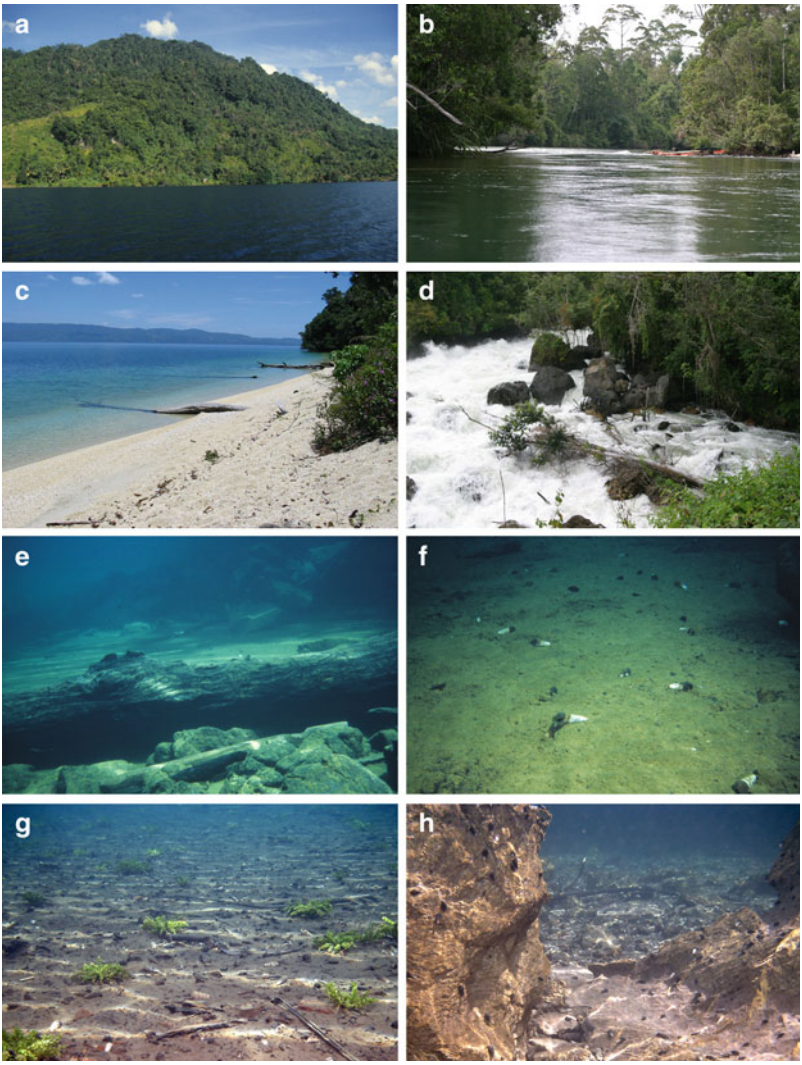


Fig. 4 Characteristic habitats in the ancient lakes of Sulawesi. (**a**,**b**) Malili system. (**a**) Lake Matano, shore with limestone hills; (**b**) rapids in Tominanga River. (**c,d**) Lake Poso; (**c**) typical sand beach; (**d**) Poso River, Sulewana Rapids. (**e–h**) Underwater habitats in the Malili system. (**e**) Lake Towuti, typical shallow water shelf zone; (**f**) Lake Towuti, mud bottom; (**g**) Lake Matano, soft bottom with *Otelia*; (**h**) Lake Matano, limestone rocks

endemic. The smaller satellite lakes only harbor one (Lake Masapi) or two (Lake Lontoa) species, respectively. Two more endemic species occur in Larona River, which drains the entire system.

In Lake Poso, the majority of the 14 morphospecies in the lake is distributed widely where the appropriate substrates are available. While the ongoing process of

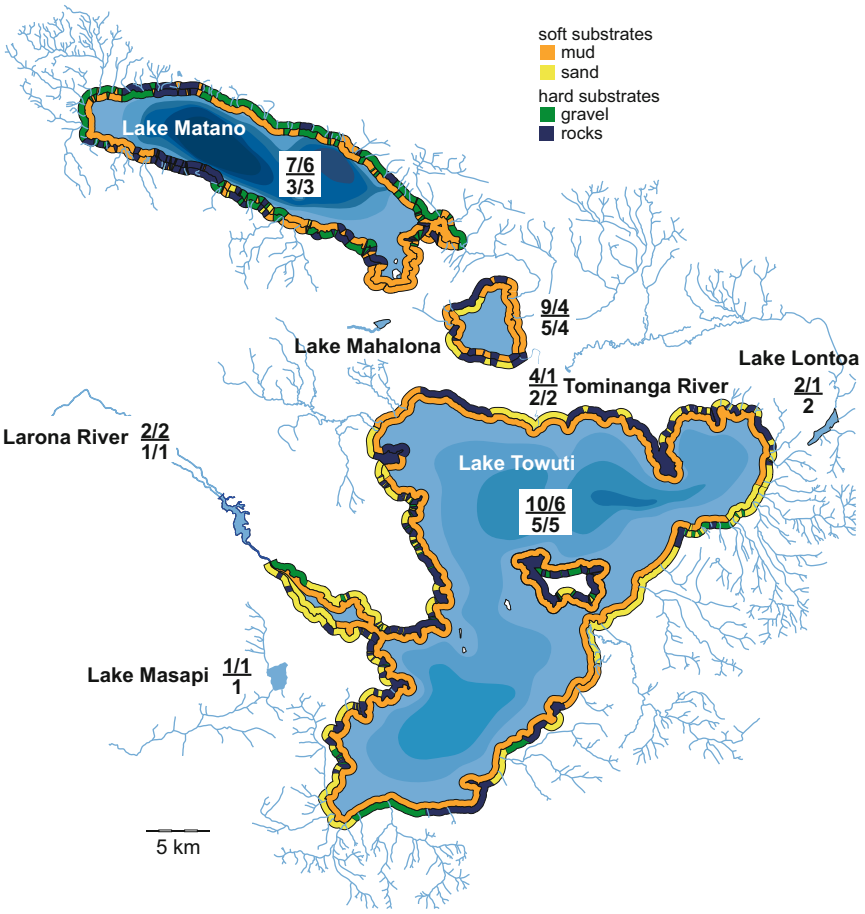


Fig. 5 Malili lake system, substrates and species diversity. Habitats have been mapped separately for shallow water (0–3 m; *outer line*) and deep water (3–20 m; *inner line*). *Top numbers* are total number of species per lake (*left*) and endemic species (*right*), *bottom numbers* are soft substrate dwellers (*left*) and hard substrate dwellers (*right*)

species delimitation does not allow us to yet provide definite numbers, there are probably only around three to four local endemics within the lake, but it cannot be excluded that for a few deep water forms this rather represents a sampling artefact. All 11 morphospecies found in Poso River are endemic there and only five lake species also occur in the river, mostly in a calm sidearm close to the lake.

The high amount of endemism in each lake or river is a striking feature of the Malili system, which suggests a strong influence of geographic factors in species divergence, i.e., allopatric speciation, which is perhaps not entirely unexpected given the spatial structure of the system (Fig. 3; see also Fig. 10). Only a few taxa also show a highly localized occurrence within single lakes, e.g., in Lake Towuti *T. bakara*, which is apparently confined to one cape in Lake Towuti (Rintelen et al.

2007a; Glaubrecht and Rintelen 2008) or *T. hannelorae* at a rocky outcrop in Lake Mahalona (Rintelen and Glaubrecht 2008). The available data do not offer an easy explanation for these restricted distribution ranges, since these taxa are by no means specialized to substrates that only occur locally and nor are there any obvious obstacles to dispersal.

The lack of obvious geographic structure in Lake Poso apparently limits the degree of local endemism. The number of 25 morphospecies for the entire system is at first glance much higher than in the Malili system where 28 species occur in five lakes of together roughly twice the area. However, if the actual number of species confined to the lakes is compared (without the connecting or draining rivers), the resulting figures of 14 species in Lake Poso and 25 in the Malili system seem less of a paradox. The high proportion of river endemics in Poso River is rather surprising and stands in stark contrast to the situation in the Malili system. Potential factors accounting for this difference are a more pronounced structuring of habitats in Poso River, the loss of species in Malili's Larona River through habitat destruction (three hydroelectric dams operate along the course of the river), and poor sampling of the river floor in Larona River, which yielded five of the endemic species in Poso River.

The high species diversity in the lakes and their connecting or draining rivers contrasts strongly with a low number of described species in the rivers of Sulawesi in general. Only nine riverine taxa (Fig. 1) have been described so far, five of which also occur in the vicinity of the ancient lakes. However, we anticipate that species numbers are bound to increase considerably here as well (compare Appendix 1). A preliminary assessment of riverine diversity around the Malili lakes has revealed the occurrence of nine or ten species in this area (Rintelen et al., unpublished data), only one of which had been described when the study was started and one more by the authors (Rintelen and Glaubrecht 2003). The catchment of Lake Poso probably hosts at least four as yet undescribed species (Rintelen et al., unpublished data).

3 A Molecular Phylogeny of *Tylomelania*: Lake Colonization, Species, and Introgression

A molecular phylogeny based on two gene fragments (COI and 16S) of mitochondrial DNA, with a total of 1,535 bp, reveals four strongly supported clades within the lakes: one clade in Lake Poso and three clades in the Malili system (Fig. 6; Rintelen et al. 2004; Rintelen et al., unpublished data). Riverine taxa are identified as sister groups to three lacustrine clades, the Poso and two of the Malili clades. The mitochondrial data suggest four independent colonization events in the lakes, three of these in the Malili lakes alone (Rintelen et al. 2004). A separate invasion of Lake Poso and the Malili lakes is an expected result given that the two lake systems were never connected. However, it appears surprising that colonization took place independently in different ancestral lineages in the three major lakes of the Malili system, which are directly connected by rivers.

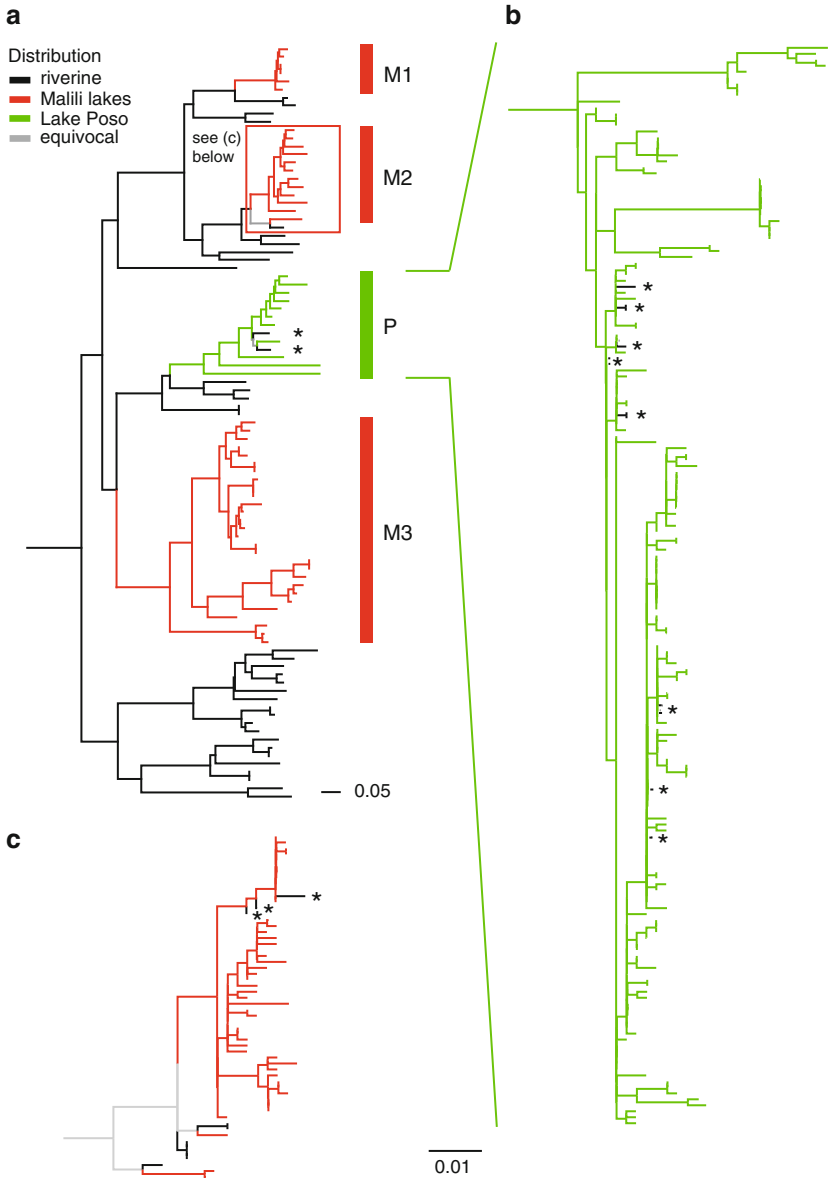


Fig. 6 Molecular phylogeny of *Tylomelania* by Bayesian inference (BI) based on mtDNA sequences. *Asterisks* indicate riverine taxa in a terminal position within lacustrine clades. (**a**) BI phylogram based on a 1,535 bp combined alignment of 16S and COI. Lacustrine taxa are highlighted in *red* (Malili lakes) and *green* (Lake Poso), riverine species in *black*. The four *bars* on the *right* mark the four lake clades representing four independent lake colonization events. M1–M3 refer to the three Malili clades. Modified from Rintelen et al. 2004. (**b**,**c**) Detail sections of BI phylogram based on 660 bp of COI; (**b**) Poso clade; (**c**) Malili 2 clade

The molecular data provide a successful test of the two phylogenetic criteria among Schluter's (2000) four criteria for the recognition of an adaptive radiation, viz. common ancestry and rapid radiation, as each lake clade is monophyletic and rapid cladogenesis can be inferred from short branch lengths between many basal nodes within each of the four clades. As outlined below in detail, each colonization event was followed by diversification into an array of morphologically distinct and ecologically specialized species, thus providing evidence for the fulfilment of the remaining two, ecological criteria for an adaptive radiation sensu Schluter (2000) as well. The occurrence of actually four independent adaptive radiations under identical (Malili system) or very similar (Poso) conditions offers the unique opportunity to study patterns of parallel evolution. These adaptive radiations differ considerably in the extent of their diversification, with species numbers and accordingly morphological "types" ranging from 3 to 25 (Malili 1: 13 spp.; Malili 2: 12 spp.; Malili 3: 3 spp.; Poso: 25 spp.).

The mitochondrial phylogeny provides a rather clear-cut picture at a higher level, whereas there is virtually no resolution at the species level for *Tylomelania*. All lacustrine morphospecies of which more than one specimen or population has been sequenced appear polyphyletic in the molecular phylogeny (Rintelen et al. 2004, 2007a; Glaubrecht and Rintelen 2008). For a more general discussion of species tree-gene tree issues and DNA taxonomy we refer, e.g., to Funk and Omland (2003), Sites and Marshall (2004), Will and Rubinoff (2004), and Vogler and Monaghan (2006). This lack of resolution is even more remarkable since there is no lack of genetic structure per se in the data, i.e., there are several well supported subclades within three of the four major lake clades.

Several phenomena might account for this problem, such as incomplete lineage sorting, i.e., the maintenance of ancestral genetic polymorphism across species boundaries, or introgressive hybridization. We particularly suspect a pivotal role of hybridization here, as concluded from several significant mismatches between observed morphological features of species and their placement in the molecular phylogeny, which cannot be parsimoniously explained by other hypotheses (for details, see Glaubrecht and Rintelen 2008). Data gained from nuclear amplified fragment length polymorphism (AFLP) genotyping for the species from one of the Malili lakes (Lake Mahalona) reveals a considerably better fit of genetic groups to morphological delimited taxa than the mtDNA-based phylogeny (Fig. 7) and thus provides some support for the hypothesis of intralacustrine gene flow, i.e., introgressive hybridization.

In addition, in both the Malili system (clades Malili 2 and 3) and Lake Poso, riverine species appear in terminal positions within the lacustrine mtDNA clades (Fig. 6), suggesting the occurrence of hybridization between lacustrine and riverine species. While this hypothesis needs to be tested using nuclear markers, it might provide the second case of widespread introgression of fluviatile haplotypes in lake populations or, vice versa, in the ancient lakes of Sulawesi, which has already been shown to be a major factor in Malili telmatherinid fishes (Herder et al. 2006a; Schwarzer et al. 2008) and is also suspected for the atyid shrimp species flock in Lake Poso (Rintelen et al. 2007b).

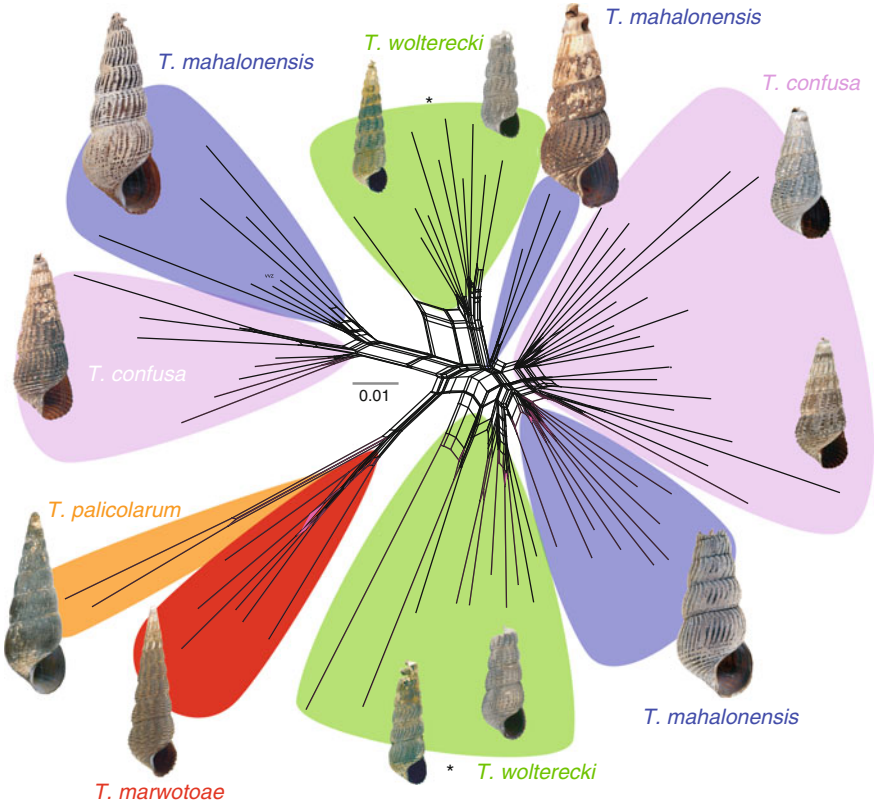


Fig. 7 AFLP Neighbor Network of the endemic Lake Mahalona species of *Tylomelania* constructed with Splitstree (Huson and Bryant 2006). Analysis based on 372 loci from six primer combinations. Species are color-coded. *Asterixes* indicate the two different radula morphs of *T. wolterecki* (see text for details)

4 Coevolution with Crabs

Dramatic changes in shell morphology are associated with lake colonization in both ancient lake systems. With the exception of clade Malili 1 and parts of the Poso flock, the species in each lacustrine clade share characteristic shell features, such as the presence of axial ribs, providing additional support for the independent colonization particularly of the Malili lake system (Rintelen et al. 2004). Species can be distinguished by their characteristic shells, though intraspecific variability is rather high (Rintelen and Glaubrecht 2003; Rintelen et al. 2007a). In each lacustrine clade, convergent evolution of thicker shells relative to riverine species has occurred in almost all cases (Fig. 8a). Shell thickness can be regarded as indicative of shell strength (Fig. 8d) and is used here as an estimator of resistance to crab predation. These findings coincide with the occurrence of one species of molluscivorous crabs

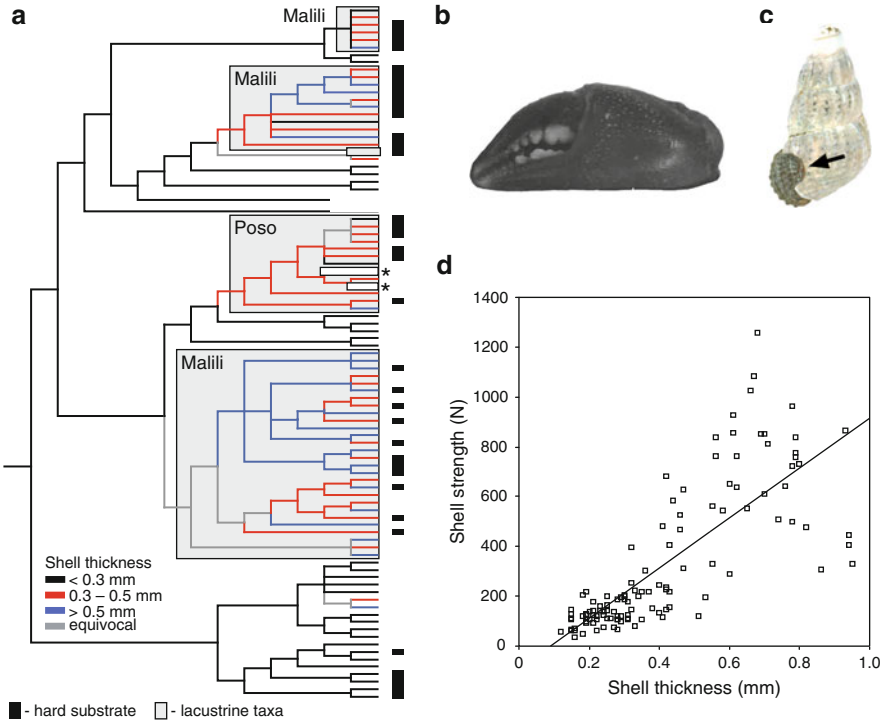


Fig. 8 Shell strength of lacustrine *Tylomelania* species and crab predation. (**a**) Maximum parsimony tree based on 1,535 bp of mtDNA (16S and COI) with colors corresponding to shell thickness; (**b**) Left claw of molluscivorous lacustrine crab species (*Syntripsa matannensis* (Schenkel, 1902)); (**c**) shell with repair scar (*T. zeamais*, Lake Matano). Scale bar 1 cm; (**d**) scatter plot showing correlation between shell thickness and shell strength. Modified from Rintelen et al. (2004)

of the Sundatelphusidae and Parathelphusidae in each of the lakes (Fig. 8b; Schubart and Ng 2008), which possess pronounced dentition on their chelae enabling them to crack shells. These data on lacustrine gastropod shell strength, structure and also the frequent occurrence of shell repair in lacustrine *Tylomelania* (Fig. 8c), in combination with the occurrence of large molluscivorous crabs, suggests that evolution in the face of crab predation is a driving factor in initial shell divergence upon colonization of the lakes (Rintelen et al. 2004). The exclusive presence of massive and dentitioned chelae in just the molluscivorous crab species makes it is very likely that this is an example of true coevolution, i.e., an evolutionary response also on the crab side to the development of stronger gastropod shells.

5 Adaptive Radiation Through Trophic Specialization

A striking pattern found in all lacustrine lineages is the high variety of radula (rasping tongue) morphology in both lake systems (Fig. 9), which contrasts sharply with the situation among the riverine species, where the vast majority of taxa

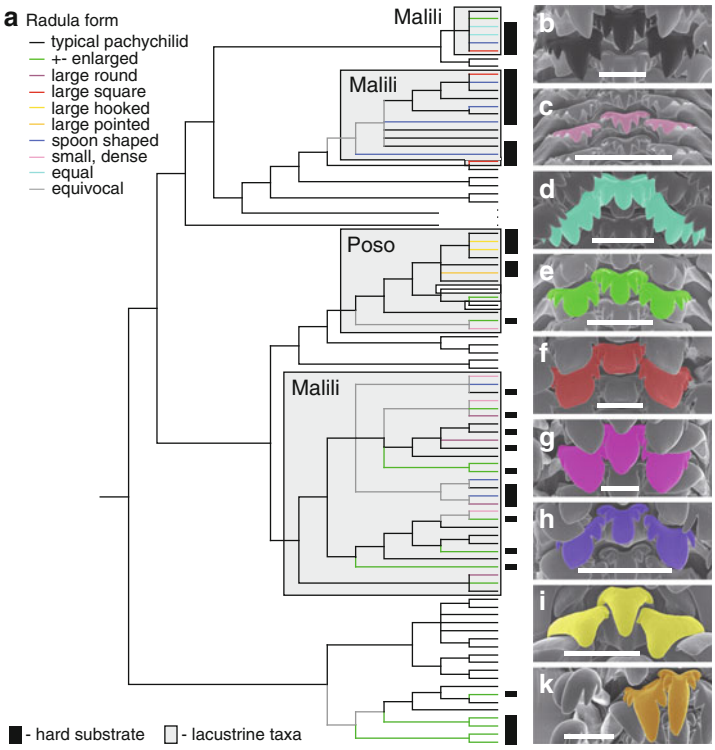


Fig. 9 Radula diversification in *Tylomelania* species. (**a**) Maximum parsimony tree based on 1,535 bp of mtDNA (16S and COI) with colors corresponding to the radula form shown in (**b–k**); **b–k**, major radula types from Malili and Lake Poso Tylomelania; (**b**) typical pachychilid (riverine), *T. perfecta*; (**c**) short with dense dentation, *T. gemmif*era; (**d**) equal sized denticles, *T. kruimeli*;(**e**) enlarged major denticles. *T. towutica*; (**f**) large, square major denticles, *T. sarasinorum*; (**g**) large, round major denticles, *T. matannensis*; (**h**) small, spoon-shaped major denticles, *T. insulaesacrae*; (**i**) single, hooked denticles, *T. carbo*; (**k**) long, pointed major denticles, *T. spec Poso 2*. *Scale bar* 100 μm. Modified from Glaubrecht and Rintelen (2008)

possess almost identical radulae (Fig. 9a; Rintelen et al. 2004). The radula is a pivotal part of the trophic system in gastropods, and radular morphological differences have been demonstrated to be indicative of food and substrate preferences (Hawkins et al. 1989). The molluscan radula is generally considered to be a conservative character with little variation at the species level; but see Padilla (1998), Reid and Mak (1999), and Reid (2000). In contrast, at least nine conspicuously different species-specific radula morphologies distinguished by the shape and relative size of their denticulation are present within the ancient lake clades (Fig. 9), with three to six phenotypes found in each clade of the Malili system (Rintelen et al. 2004) and at least six phenotypes in Lake Poso (Rintelen et al., unpublished data). These major radula types are complemented by even more subtypes, i.e., minor variations for instance in the size and number of denticles. While frequently rather

subtle, these differences can also serve to differentiate species or populations. The two subgroups of *T. wolterecki* revealed by the AFLP data (Fig. 7), for example, differ consistently in the number of marginal teeth denticles (3 vs 4+).

Trophic morphology and substrate were supposed to be highly correlated in all clades (Fig. 9a; see also Rintelen et al. 2004). Substrate mapping in all three major lakes of the Malili system showed a rather heterogeneous distribution of hard and soft substrates in the lakes (Fig. 5), particularly in shallow water close to the shore line (compare also Box 1). This habitat heterogeneity is reflected in the substrate preferences found in the lacustrine species of *Tylomelania*. All species in the major lakes of the Malili system and Lake Poso for which we have data on substrate preferences available are specialized on either soft (mud, sand) or hard (rock, sunken wood) substrates, with about 50% of species occurring on either substrate category (Rintelen et al. 2007a). Further specializations occur particularly within hard substrates, e.g., some species particularly in Lake Poso prefer either rocks or wood (Rintelen et al., unpublished data). This correlation between radula morphology and substrate preferences clearly suggests a phenotype–environment correlation, which is one of the two ecological criteria among the four criteria proposed by Schluter (2000) to recognize an adaptive radiation (see Introduction). The substrate-dependent usefulness of radula differences or trait utility, which is the second ecological criterion of Schluter, still remains to be tested for *Tylomelania*.

Soft substrate species mostly have identical or very similar radulae to those found in riverine taxa (Fig. 9b), while hard substrate taxa particularly from rocks often have strongly enlarged teeth in a variety of shapes (Fig. 9c–k). This tight correlation between enlargement of radula denticles and hard substrate described by Rintelen et al. (2004) is generally supported. However, more detailed radula descriptions have revealed a rather complex pattern, as evident from cases of extensive radula polymorphism in many species both from hard and soft substrates (Rintelen et al. 2007a). The assumption of Rintelen et al. (2004) that radula (i.e., trophic) diversification plays an important role in the ecological diversification of the lake species remains unchallenged. It is further supported by the parallel occurrence of accordingly modified radulae in soft and hard substrate dwellers in both ancient lake systems on Sulawesi (all radula forms shown in Fig. 9b–h are shared between the two lake systems) and in the only constantly rock-dwelling riverine taxa found so far from the Maros area in southwest Sulawesi (Rintelen et al., unpublished data). These observations suggest a functional role for the differences found, although a detailed understanding of the underlying mechanisms requires further investigation.

Habitat specialization (substrate and to a lesser degree depth preferences) and radula differentiation in ancient lake *Tylomelania* enables more than five, possibly up to seven, species to coexist at localities with sufficiently structured habitats (Glaubrecht and Rintelen 2008, and unpublished data). This level of single-site diversity was only observed in taxa from Lake Poso and within the Malili system in Lake Mahalona and Lake Towuti. The absolute numbers of syntopic species in the lakes may not be impressive for themselves, but they become more striking when

contrasted with the situation in rivers outside the lake systems, where only a single species is found in almost all cases.

Thus, a strong role for ecological factors in diversification and possibly even speciation itself is suggested by the marked substrate preferences of most species of *Tylomelania*, coupled with a correlated species-specific radula (trophic) morphology and genetic clustering in some taxa. However, detailed studies on selected species and species complexes so far reveal a rather complicated situation in the limnic systems on Sulawesi. The phenomena encountered range from species with highly characteristic specific radulae, irrespective of their occurrence on various substrates, to species where two fundamentally different radula morphologies are found in the same population on the same substrate. All other morphological features, especially the shell, are highly variable in the lake species, but so far we lack evidence that conchological features play a crucial role in the causation of speciation. In addition, the molecular phylogeny based on mitochondrial genes does not correspond to morphologically delimited species (compare above).

6 Speciation Patterns

In a broader perspective, these results from *Tylomelania* suggest a wider applicability of a radiation model proposed for vertebrates by Streelman and Danley (2003), which assumes that trophic specialization and habitat specialization drive the initial stages of adaptive radiation.

The finding of largely species-specific radula types and the correlation between trophic morphology and substrate in *Tylomelania* as well as marked niche differences in species (Rintelen and Glaubrecht 2003; Rintelen et al. 2007a) suggest a strong role for ecological factors in speciation in these snails. In gastropods, studies on intertidal marine species of *Littorina* have indicated differentiation across ecotones and assortative mating between morphs (Johannesson et al. 1995; Kyle and Boulding 1998; Wilding et al. 2001). The limnic gastropods of Sulawesi offer excellent opportunities to test the importance of these factors.

Nevertheless, allopatric speciation (i.e., speciation in geographic separation) is probably the predominant mode of speciation at least in the Malili lakes. Here, different species of single putative species-groups reveal an allopatric distribution pattern in the three major lakes (Fig. 10; compare also Rintelen et al. 2007a), and single lake endemism is high (see above, Fig. 5). However, independent parallel evolution of hard and soft substrate dwellers with associated trophic traits (and size differences) occurred in all three Malili lineages, suggesting an important role for ecology-driven differentiation, even if the initial phase of allopatric speciation was not associated with ecological divergence. Looking beyond the Malili lakes, some striking cases of parallel evolution can be found between both shallow water mud-dwellers (e.g., *T. kuli* vs *T. gemmifera*) and rock-dwellers (e.g., *T.* specB vs *T. zeamais*) in Lake Poso and the Malili system (Figs. 2, 3, and 9c – *T. gemmifera*/*kuli*, 9h – *T.* specB/*zeamais*). Similar patterns might be suspected for several other

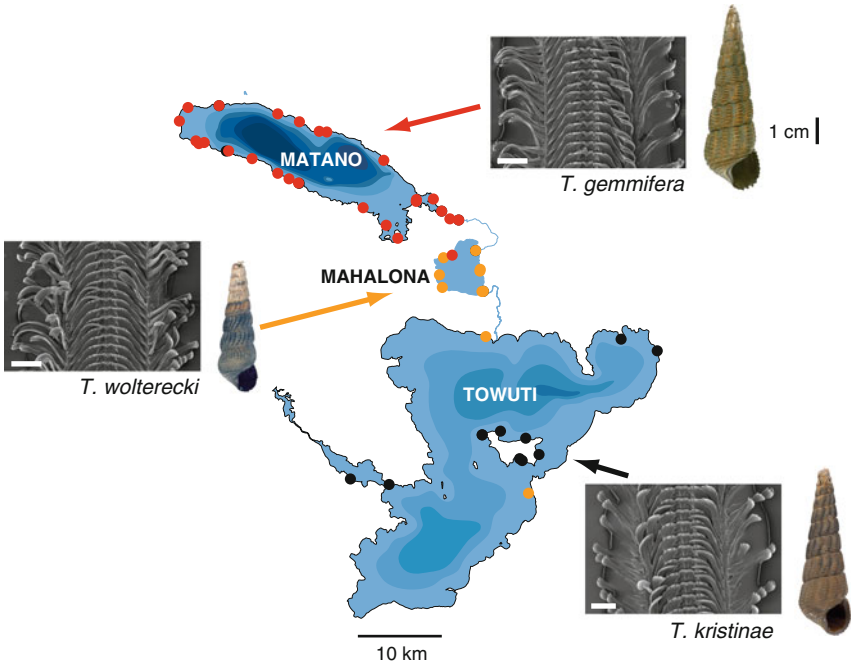


Fig. 10 Allopatric diversification between lakes in the Malili lakes. Radula *scale bars* 0.1 mm

species among the Malili lake clades, but the failure of a simple DNA-taxonomy approach in our system (see above) prohibits a more detailed discussion of convergent evolution for the time being.

7 The Distribution and Role of Body Coloration

A striking feature of at least 10 out of 14 morphospecies in Lake Poso (excluding species from Poso River) is a conspicuous bright body coloration (Fig. 11). This stands in stark contrast not only to the island's riverine species but also to taxa from the Malili lake system, where most species are either black or black with white dots, only five species having yellow or orange dots and tentacles in a varying intensity (see examples in Fig. 11). At present, the causes for the coloration of some species and the strong difference between the lake systems remain largely speculative. Color has been hypothesized to play a role in species recognition and even speciation in other freshwater groups, such as e.g., cichlid fishes (Seehausen et al. 2008) or, in the ancient lakes of Sulawesi, atyid shrimps (Rintelen et al. 2007b, in press). In the Malili Lakes, sailfin silverside radiation, conspicuous male color polymorphisms correlate with mating success in different habitats but are not associated to population divergence (Herder et al. 2008; Gray et al. 2008). Streelman and Danley

Fig. 11 Body colour in *Tylomelania*. (**a**,**b**) Lake Matano soft substrate dwellers; (**a**) *T. patriarchalis*; (**b**) *T. gemmifera*; (**c**–**h**) Lake Poso, *T.* spp. (undescribed); (**c**–**f**) soft substrate dwellers; (**c**,**d**) shallow water species; (**e**,**f**) deep water species; (**g**,**h**) hard substrate dwellers

(2003) have suggested that communication, for example through intensive body coloration, plays a major role in the final stage of radiations leading to a considerable increase in species numbers as compared to radiations lacking this stage, such as, e.g., the Galapagos finches. While this hypothesis remains to be tested for *Tylomelania*, the higher number of species in Lake Poso versus species diversity in any single lake of the Malili system might indicate that color-driven diversification plays a bigger role in the Lake Poso species flock.

8 Patterns of Radiation: Looking Beyond *Tylomelania* and the Sulawesi Lakes

Parallel radiations of gastropods, atyid shrimps (Rintelen et al. 2007b, in press), parathelphusid crabs (Schubart and Ng 2008; Schubart et al. 2008) and telmatherinid fishes (Herder et al. 2006a, b, 2008) in the lakes, particularly the Malili system, offer an outstanding opportunity to compare diversification patterns in organisms differing in fundamental biological properties in the same environmental setting. While a joint in-depth analysis is in preparation, the published results (see citations above) already suggest a number of commonalities between some or all groups. Multiple colonizations, for instance, appear likely in all groups except the sailfin silverside fishes; hybridization is suspected to play a role in all groups, and trophic diversification is likewise considered the driving factor in all adaptive radiations in the lakes. Differences between the taxa might, e.g., concern the role of color in diversification and have been suspected for the age of the lake flocks (Rintelen et al., unpublished data). Future research is expected to provide a further elucidation of common evolutionary strategies which may be universal for adaptive radiations in general, and crucial differences due to the organisms' intrinsic properties.

Focusing on mollusc radiations in ancient lakes, hybridization and several of the other phenomena discussed for the Sulawesi species flocks have also been suggested in other cases, such as, for instance, the diverse riverine assemblage of *Brotia* species in Thailand's Kaek River (Glaubrecht and Köhler 2004), which resembles an ancient lake radiation, at least in pattern if not in setting. The best known radiation in ancient lakes is perhaps the long enigmatic and so-called thalassoid (i.e., marine-like) gastropod radiation in East African's Lake Tanganyika, for which morphological data (Glaubrecht and Strong 2007; Glaubrecht 2008) and molecular data (e.g., Wilson et al. 2004) have recently become available as well. Recently, respective data have also started to become available for the mollusc species flocks in Lake Ohrid (Hauswald et al. 2008; Schultheiß et al. 2008). It should finally be possible to decipher how far different lacustrine model systems and their respective faunal elements are distinctive with respect to the influence of intrinsic factors (e.g., crucial biological features such as viviparity, dispersal, and trophic specialization) versus extrinsic factors (e.g., palaeohydrology, habitat fragmentation), as was initially outlined for limnic Cerithioidea by Glaubrecht (1996). Earlier attempts to evaluate these factors have frequently suffered from a lack of data on the systematics and morphology (i.e., the reproductive biology) of the taxa involved.

9 Conservation

The Sulawesi lakes are a "hotspot" of southeast Asian biodiversity and at the same time a scientific treasure trove for evolutionary biology research. Unfortunately, the lakes' environment is threatened by several factors. At both lake systems, the

growth of the local communities is a general point of concern, since is seems inevitably coupled with pollution and direct habitat destruction. This danger and also the risk posed by the continued activities of illegal loggers which threaten to damage lake habitats by erosion effects is more severe at the Malili lakes, whose shores have been less densely populated and accessible for a long time than Lake Poso. While all of these issues are notoriously difficult to tackle, at least efforts should be made to increase community awareness about the problems, which have the potential to threaten the future of the local people as well.

More specific risks for the lake ecosystems stem from the activities of the nickel mining company P.T. INCO at the Malili lakes, which, in addition to their role in opening up the area, exert a direct impact on the sensitive environment of the lakes. While appreciable efforts have been made by this company to preserve the water quality in the lakes, less attention is paid to the importance of protecting the environment of the lakes and particularly the rivers connecting and draining them. The recently finished construction of a third hydroelectric dam on the Larona River is just one example. A dam has also been constructed on the Poso River just above the Sulewana Rapids, which are a hotspot of endemic species adapted to fast-flowing water. In both cases, the effects on the endemic species in these rivers remains to be evaluated. A new risk has emerged only during the last year, when the collecting of live snails and shrimps for export started to resemble a "gold rush" among pet traders fuelled by the demand from aquarium enthusiasts.

This enumeration of actual and potential risks to the lake biota indicates that their conservation should have a high priority. The risk from pet traders may also have beneficial side effects, though, as it has significantly increased public awareness about the lakes' unique fauna in several countries.

10 Conclusions and Outlook

We have shown that the gastropod species flocks in the ancient lakes of Sulawesi are indeed *adaptive* radiations by testing this assumption employing the four criteria proposed by Schluter (2000). The existence of three separate adaptive radiations in the Malili lakes, e.g., offers, in combination with the Lake Poso flock, an excellent opportunity to study *in parallel* evolutionary processes such as the origin of specialization. The indicated involvement of coevolution at a basal level in this system is unique among radiations in ancient lakes. We believe that, beyond the singular possibility to study coevolution in the context of radiation, the relative simplicity of the Sulawesi system as compared, e.g., to the Great African lakes will especially favor tests of ecological speciation by comparing trait variance under different natural conditions. Recently started work using highly variable nuclear markers (ITS and AFLPs) is expected to yield a highly

Table 1 The ancient lakes of Sulawesi

Lake	Area (km^2)	Max. depth (m)	Transparency (Secchi disk), m
Poso	323.2	450	11
Matano	164.0	590	20
Mahalona	24.4	73	20
Towuti	561.1	203	22
Lontoa	1.6	3	<3
Masapi	2.2	4	<3

Based on data from Abendanon (1915), Giesen et al. (1991), Giesen (1994), Haffner et al. (2001)

resolved phylogeny at the population and species level, and should permit, in conjunction with quantitative ecological data, an elaboration of our hypotheses on ecological speciation sensu Schluter (2000) in the Sulawesi snails. The contradictory patterns found particularly in the trophic specialization in *Tylomelania* as described above certainly caution against simple explanatory models for the evolution of diversity in the Sulawesi lakes' radiations. The suspected importance of introgressive hybridization in the system will be another research focus. In this respect, we are particularly interested in how it contributes to speciation and how species boundaries are maintained despite hybridization, and if our data fit the syngameon hypothesis of adaptive radiation by Seehausen (2004) which, however, is based predominantly on non-allopatric modes of speciation.

The available results from the Sulawesi species flocks now also allow meaningful comparisons with radiations of other organisms in the lakes or with mollusc radiations worldwide. From an even more general perspective, the patterns of diversification found in the species flocks of *Tylomelania* in the Sulawesi lakes are comparable in many respects, ranging from species diversity to trophic morphology variation, to those in other well-studied adaptive radiations, such as, e.g., the Galapagos finches, or Caribbean *Anolis* lizards, and may thus be considered as truly "Darwinian snails."

Box 1 The Ancient Lakes of Sulawesi: Hydrology, Geology, and Limnology

While Lake Poso (Fig. 2; Table 1) is a deep solitary lake, the Malili system situated c. 80 km southwest of Lake Poso comprises five lakes sharing a common drainage (Fig. 3; Table 1). The three larger lakes of that system are directly connected: Lake Matano flows into Lake Mahalona via Petea River, and Lake Mahalona in turn spills into Lake Towuti via Tominanga River. Lake Towuti is drained by the Larona River into the Gulf of Bone

(continued)

(Teluk Bone). Two smaller satellite lakes, Lake Lontoa (also known as Wawontoa) and Lake Masapi, are much less directly connected to the system (see Fig. 3).

Lake Poso and Lake Matano are of tectonic origin, which accounts for their extraordinary depth. They owe their origin to Sulawesi's location in the center of perhaps the most complex geological region of the world, where the Asian and Australian plates converge (see, e.g., van Oosterzee 1997). Lake Matano is situated in a strike-slip fault, the Matano fault, which was formed in the final juxtaposition process of south, southeast and east Sulawesi since the Pliocene (c. 4 Mya) to the present day (Wilson and Moss 1999). The age of Lake Matano has been estimated at 1–2 My (G. Hope, personal communication), estimates for the other lakes are lacking. The major ancient lakes of Sulawesi are oligotrophic, with a very low nutrient and organic content and a high transparency of up to 22 m in Lake Towuti (Giesen et al. 1991; Giesen 1994; Haffner et al. 2001). It has been speculated that the low nutrient content might drive speciation in the Malili lakes (Haffner et al. 2001).

The lakes offer a wide range of habitats ranging from soft-bottom with sand and mud to steep rocky drop-offs (Fig. 4). Typically a shallow shelf zone (2–5 m; Fig. 4c) is followed by a steeper slope with quickly increasing depth. Frequently, hard substrates dominate at the shoreline (0–2 m), with soft substrate in deeper water. Habitat heterogeneity is high in all lakes (see Fig. 5 for a detailed mapping of habitats in the Malili lakes), general differences between the lakes exist for instance in the amount of hard substrate available in deep water (>10 m), which is largely lacking in Lake Mahalona and Lake Towuti. No lake-wide data are available for Lake Poso, but field observations from numerous sampling stations indicate an equally varied habitat structure as in the Malili lakes.

Extensive sand beaches are characteristic for large areas of Lake Poso, while almost entirely lacking in the Malili lakes. In the connecting and draining rivers of both lake systems currents of varying strength form an additional factor determining the limnic environment (Fig. 4b, d).

Acknowledgments We thank Ristiyanti Marwoto (Zoological Museum Bogor) who was very helpful in the preparation of the fieldwork in Indonesia and A. Munandar who provided assistance in the field. We thank LIPI for permits to conduct research in Indonesia. We are most indebted to INCO for its invaluable logistic support at the lakes during our many visits. Claudia Dames, Betina Gregor, Tanja Miethe, Jutta Simonis (Museum für Naturkunde Berlin), Janice Jang (Massachusetts Institute of Technology) and Daniela Franz (Technische Universität Dresden) were of great help in collecting genetic and morphological data from both ancient lake systems. This work was funded by research grants GL 297/1 and GL 297/7 awarded to M.G. from the Deutsche Forschungsgemeinschaft.

Appendix 1. Species of *Tylomelania* described from the ancient lakes of Sulawesi and species diversity estimates for the lakes and the entire island (based on Rintelen and Glaubrecht 2003; Rintelen and Glaubrecht 2008; Rintelen et al. 2007a; unpublished data)

Region/Taxon (*=not endemic)	No. of species
Sulawesi	*N* = 44 + c. 32 undescribed
Malili lake system	*n* = 28
Lake Matano	*n* = 7 (6 endemic)
T. gemmifera Sarasin and Sarasin 1897	
T. matannensis Rintelen et al. 2007a	
T. molesta Sarasin and Sarasin 1897	
**T. palicolarum* Sarasin and Sarasin 1897	
T. patriarchalis Sarasin and Sarasin 1897	
T. turriformis Rintelen et al. 2007a	
T. zeamais Sarasin and Sarasin 1897	
Lake Mahalona	*n* = 9 (4 endemic)
T. confusa Rintelen et al. 2007a	
T. hannelorae Rintelen and Glaubrecht 2008	
T. inconspicua Rintelen et al. 2007a	
**T. insulaesacrae* Sarasin and Sarasin 1897	
T. kruimeli Rintelen and Glaubrecht 2003	
**T. mahalonensis Kruimel* 1913	
**T. marwotoae* Rintelen et al. 2007a	
**T. palicolarum* Sarasin and Sarasin 1897	
**T. wolterecki* Rintelen et al. 2007a	
Tominanga river	*n* = 4 (1 endemic)
**T. tominangensis* Kruimel 1913	
**T. mahalonensis* Kruimel 1913	
**T. towutica* Kruimel 1913	
T. wesseli Rintelen et al. 2007a	
Lake Towuti	*n* = 10 (6 endemic)
T. amphiderita Rintelen et al. 2007a	
T. bakara Rintelen and Glaubrecht 2003	
**T. insulaesacrae* Sarasin and Sarasin 1897	
T. kristinae Rintelen et al. 2007a	
T. lalemae Kruimel 1913	
**T. marwotoae* Rintelen et al. 2007a	
T. sarasinorum Kruimel 1913	
T. towutensis Sarasin and Sarasin 1897	
**T. towutica* Kruimel 1913	
**T. wolterecki* Rintelen et al. 2007a	
Lake Lontoa	*n* = 2 (1 endemic)
T. abendanoni Kruimel 1913	
**T. tominangensis* Kruimel 1913	
Lake Masapi	*n* = 1 (endemic)
T. masapensis Kruimel 1913	
Larona River	*n* = 2 (endemic)
T. baskasti Rintelen and Glaubrecht 2008	
T. sinabartfeldi Rintelen and Glaubrecht 2008	

(*continued*)

Region/Taxon (*=not endemic)	No. of species
Lake Poso	*n* = 7 + c. 18 undescribed
Lake Poso	*n* = 4 + c. 10 undescribed
T. carbo Sarasin and Sarasin 1897	
T. centaurus Sarasin and Sarasin 1898	
T. kuli Sarasin and Sarasin 1898	
T. toradjarum Sarasin and Sarasin 1897	
Poso River	*n* = 3 + c. 8 undescribed
T. connectens Sarasin and Sarasin 1898	
T. neritiformis Sarasin and Sarasin 1897	
T. porcellanica Sarasin and Sarasin 1897	
Rivers	*n* = 9 + c. 14 undescribed
div. *Tylomelania* species (not listed here in detail)	

References

Abendanon EC (1915) Midden-Celebes-Expeditie. Geologische en geographische doorkruisingen van Midden-Celebes (1909–1910), Atlas. E.J. Brill, Leiden

Albrecht C, Trajanovski S, Kuhn K, Streit B, Wilke T (2006) Rapid evolution of an ancient lake species flock: freshwater limpets (Gastropoda: Ancylidae) in the Balkan Lake Ohrid. Organisms, diversity and evolution 6:294–307

Albrecht C, Wilke T (2008) Ancient Lake Ohrid: biodiversity and evolution. Hydrobiologia 615:13–140

Barlow GW (2000) The cichlid fishes: nature's grand experiment in evolution. Perseus, Cambridge, Mass

Bouchet P, Guerra A, Rolán E, Rocha F (1995) A major new mollusc radiation discovered in the ancient lakes of Sulawesi. In: Abstracts of the 12th International Malacological Congress, Vigo 1995, Vigo, pp 14–15

Brooks JL (1950) Speciation in ancient lakes. Q Rev Biol 25:131–176

Darwin C (1859) The origin of species by means of natural selection, or the preservation of favoured races in the struggle for life. Murray, London

Davis GM (1982) Historical and ecological factors in the evolution, adaptive radiation, and biogeography of freshwater mollusks. Am Zool 22:375–395

Funk DJ, Omland KE (2003) Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. Annu Rev Ecol Syst 34:397–423

Giesen W (1994) Indonesia's major freshwater lakes: a review of current knowledge, development processes and threats. Mitt Int Verein Theoret Angew Limnol 24:115–128

Giesen W, Baltzer M, Baruadi R (1991) Integrating conservation with land-use development in wetlands of South Sulawesi. Directorate General of Forest Protection and Nature Conservation, Bogor

Glaubrecht M (1996) Evolutionsökologie und Systematik am Beispiel von Süß- und Brackwasserschnecken (Mollusca: Caenogastropoda: Cerithioidea): Ontogenese-Strategien, paläontologische Befunde und historische Zoogeographie. Backhuys, Leiden

Glaubrecht M (1999) Systematics and the evolution of viviparity in tropical freshwater gastropods (Cerithioidea: Thiaridae sensu lato) – an overview. Courier Forschungs-Institut Senckenberg 203:91–96

Glaubrecht M (2000) A look back in time – Toward an historical biogeography as a synthesis of systematic and geologic patterns outlined with limnic gastropods. Zoology 102:127–147

Glaubrecht M (2004) Leopold von Buch's legacy: treating species as dynamic natural entities, or why geography matters. Am Malacol Bull 19:111–134

Glaubrecht M (2006) Independent evolution of reproductive modes in viviparous freshwater Cerithioidea (Gastropoda, Sorbeoconcha) – a brief review. Basteria 69(suppl 3):32–38
Glaubrecht M (2008) Adaptive radiation of thalassoid Cerithioidean gastropods in Lake Tanganyika, East Africa: morphology and systematization of a paludomid species flock in an ancient lake. Zoosyst Evol 84:69–120
Glaubrecht M, Köhler F (2004) Radiating in a river: systematics, molecular genetics and morphological differentiation of viviparous freshwater gastropods endemic to the Kaek River, central Thailand (Cerithioidea, Pachychilidae). Biol J Linn Soc 82:275–311
Glaubrecht M, Strong EE (2007) Ancestry to an endemic radiation in Lake Tanganyika? Evolution of the viviparous gastropod *Potadomoides* Leloup, 1953 in the Congo River system (Cerithioidea, Paludomidae). Biol J Linn Soc 92:367–401
Glaubrecht M, Rintelen T von (2003) Systematics and zoogeography of the pachychilid gastropod *Pseudopotamis* Martens, 1894 (Mollusca: Gastropoda: Cerithioidea): a limnic relict on the Torres Strait Islands, Australia? Zool Scr 32:415–435
Glaubrecht M, Rintelen T von (2008) The species flocks of lacustrine gastropods: *Tylomelania* on Sulawesi as models in speciation and adaptive radiation. Hydrobiologia 615:181–199
Gorthner A, Martens K, Goddeeris B, Coulter G (1994) What is an ancient lake? In: Martens K, Goddeeris B, Coulter G (eds) Speciation in ancient lakes. Schweizerbart'sche, Stuttgart, pp 97–100
Grant PR (1998) Evolution on islands. Oxford University Press, Oxford
Gray SM, Dill LM, Tantu FY, Loew ER, Herder F, McKinnon JS (2008) Environment-contingent sexual selection in a colour polymorphic fish. Proc R Soc Lond B 275:1785–1791
Haffner GD, Hehanussa PE, Hartoto D (2001) The biology and physical processes of large lakes of Indonesia: Lakes Matano and Towuti. In: Munawar M, Heck RE (eds) The Great Lakes of the world (GLOW): Food-web, health and integrity. Backhuys, Leiden, pp 183–192
Hauswald AK, Albrecht C, Wilke T (2008) Testing two contrasting evolutionary patterns in ancient lakes: species flock versus species scatter in valvatid gastropods of Lake Ohrid. Hydrobiologia 615:169–179
Hawkins SJ, Watson DC, Hill AS, Harding P, Kyriakides MA, Hutchinson S, Norton TA (1989) A comparison of feeding mechanisms in microphageous, herbivorous, intertidal prosobranchs in relation to resource partitioning. J Molluscan Stud 55:151–165
Herder F, Nolte AW, Pfänder J, Schwarzer J, Hadiaty RK, Schliewen UK (2006a) Adaptive radiation and hybridization in Wallace's dreamponds: evidence from sailfin silversides in the Malili lakes of Sulawesi. Proc R Soc Lond B 273:2209–2217
Herder F, Schwarzer J, Pfänder J, Hadiaty RK, Schliewen UK (2006b) Preliminary checklist of sailfin silversides (Telostei: Telmatherinidae) in the Malili Lakes of Sulawesi (Indonesia), with a synopsis of systematics and threats. Verh Ges Ichthyol 5:139–163
Herder F, Pfänder J, Schliewen UK (2008) Adaptive sympatric speciation of polychromatic "roundfin" sailfin silverside fish in Lake Matano (Sulawesi). Evolution 62:2178–2195
Huson DH, Bryant D (2006) Application of phylogenetic networks in evolutionary studies. Mol Biol Evol 23:254–267
Hutchinson GE (1965) The ecological theater and the evolutionary play. Yale University Press, New Haven
Johannesson K, Rolan-Alvarez E, Ekendahl A (1995) Incipient reproductive isolation between two sympatric morphs of the intertidal snail *Littorina saxatilis*. Evolution 49:1180–1190
Kocher TD (2004) Adaptive evolution and explosive speciation: the cichlid fish model. Nature Rev Genet 5:288–298
Köhler F, Glaubrecht M (2007) Out of Asia and into India: on the molecular phylogeny and biogeography of the endemic freshwater pachychilid gastropod *Paracrostoma* Cossmann, 1900 (Caenogastropoda: Pachychilidae). Biol J Linn Soc 91:627–651
Köhler F, Glaubrecht M (2010) Uncovering an overlooked radiation: morphological and mitochondrial DNA differentiation in endemic freshwater snails on Madagascar (Caenogastropoda: Pachychilidae) and their biogeography. Biol J Linn Soc 99:867–894

Köhler F, von Rintelen T von, Meyer A, Glaubrecht M (2004) Multiple origin of viviparity in Southeast Asian gastropods (Cerithioidea: Pachychilidae) and its evolutionary implications. Evolution 58:2215–2226

Kornfield I, Smith PF (2000) African cichlid fishes: model systems for evolutionary biology. Annu Rev Ecol Syst 31:163–196

Kruimel JH (1913) Verzeichnis der von Herrn E.C.Abendanon in Celebes gesammelten Süsswasser-Mollusken. Bijdr Dierk 19:217–235

Kyle CJ, Boulding EG (1998) Molecular genetic evidence for parallel evolution in a marine gastropod, *Littorina subrotundata*. Proc R Soc Lond B 265:303–308

Marijnissen SAE, Michel E, Daniels SR, Erpenbeck D, Menken SBJ, Schram FR (2006) Molecular evidence for recent divergence of Lake Tanganyika endemic crabs (Decapoda: Platythelphusidae). Mol Phylogenet Evol 40:628–634

Martens K (1997) Speciation in ancient lakes. Trends Ecol Evol 12:177–182

Marwoto R (1997) A preliminary study of the biodiversity of the freshwater snail family Thiaridae from Indonesia (Mollusca: Prosobranchia). In: Ulrich H (ed) Tropical biodiversity and systematics. Zoologisches Forschungsinstitut und Museum Alexander Koenig, Bonn, pp 109–112

Padilla DK (1998) Inducible phenotypic plasticity of the radula in *Lacuna* (Gastropoda: Littorinidae). Veliger 4:201–204

Reid DG (2000) The use of the radula in the taxonomy and phylogeny of gastropods: cautionary cases of convergence, intraspecific variation and plasticity. Phuket Mar Biol Center Spec Publ 21:329–345

Reid DG, Mak YM (1999) Indirect evidence for ecophenotypic plasticity in radular dentition of *Littoraria* species (Gastropoda: Littorinidae). J Molluscan Stud 65:355–370

Rintelen T von, Glaubrecht M (2003) New discoveries in old lakes: three new species of *Tylomelania* Sarasin and Sarasin, 1897 (Gastropoda: Cerithioidea: Pachychilidae) from the Malili lake system on Sulawesi, Indonesia. J Molluscan Stud 69:3–17

Rintelen T von, Glaubrecht M (2005) Anatomy of an adaptive radiation: a unique reproductive strategy in the endemic freshwater gastropod *Tylomelania* (Cerithioidea: Pachychilidae) on Sulawesi, Indonesia, and its biogeographic implications. Biol J Linn Soc 85:513–542

Rintelen T von, Glaubrecht M (2008) Three new species of the freshwater snail genus *Tylomelania* (Caenogastropoda: Pachychilidae) from the Malili lake system, Sulawesi, Indonesia. Zootaxa 1852:37–49

Rintelen T von, Wilson AB, Meyer A, Glaubrecht M (2004) Escalation and trophic specialization drive adaptive radiation of viviparous freshwater gastropods in the ancient lakes on Sulawesi, Indonesia. Proc R Soc Lond B 271:2541–2549

Rintelen T von, Bouchet P, Glaubrecht M (2007a) Ancient lakes as hotspots of diversity: a morphological review of an endemic species flock of *Tylomelania* (Gastropoda: Cerithioidea: Pachychilidae) in the Malili lake system on Sulawesi, Indonesia. Hydrobiologia 592:1–94

Rintelen K von, Rintelen T von, Glaubrecht M (2007b) Molecular phylogeny and diversification of freshwater shrimps (Decapoda, Atyidae, *Caridina*) from ancient Lake Poso (Sulawesi, Indonesia) – The importance of being colourful. Mol Phylogenet Evol 45:1033–1041

Rintelen, K von, Glaubrecht M, Schubart CD, Wessel A, Rintelen T von (in press) Adaptive radiation and ecological diversification of Sulawesi's ancient lake shrimps. Evolution

Rossiter A, Kawanabe H (eds) (2000) Ancient lakes: biodiversity, ecology and evolution. Academic, San Diego

Sarasin P, Sarasin F (1897) Über die Molluskenfauna der großen Süßwasser-Seen von Central-Celebes. Zool Anz 539(540):308–320

Sarasin P, Sarasin F (1898) Die Süßwassermollusken von Celebes. Kreidel, Wiesbaden

Sarasin P, Sarasin F (1905) Reisen in Celebes ausgeführt in den Jahren 1893–1896 und 1902–1903. Kreidel, Wiesbaden

Schluter D (2000) The ecology of adaptive radiation. Oxford University Press, Oxford

Schubart CD, Ng PKL (2008) A new molluscivore crab from Lake Poso confirms multiple colonisation of ancient lakes in Sulawesi by freshwater crabs (Decapoda: Brachyura). Zool J Linn Soc 154:211–221

Schubart CD, Santl T, Koller P (2008) Mitochondrial patterns of intra- and interspecific differentiation among endemic freshwater crabs of ancient lakes in Sulawesi. Contr Zool 77:83–90

Schultheiß R, Albrecht C, Bößneck U, Wilke T (2008) The neglected side of speciation in ancient lakes: phylogeography of an inconspicuous mollusc taxon in lakes Ohrid and Prespa. Hydrobiologia 615:141–156

Schwarzer J, Herder F, Misof B, Hadiaty RK, Schliewen UK (2008) Gene flow at the margin of Lake Matano's adaptive sailfin silverside radiation: Telmatherinidae of River Petea in Sulawesi. Hydrobiologia 615:201–213

Seehausen O (2004) Hybridization and adaptive radiation. Trends Ecol Evol 19:198–207

Seehausen O, Terai Y, Magalhaes IS, Carleton KL, Mrosso HDJ, Miyagi R, van der Sluijs I, Schneider MV, Maan ME, Tachida H, Imai H, Okada N (2008) Speciation through sensory drive in cichlid fish. Nature 455:620–626

Sites JW, Marshall JC (2004) Operational criteria for delimiting species. Annu Rev Ecol Syst 35:199–227

Streelman JT, Danley PD (2003) The stages of vertebrate evolutionary radiation. Trends Ecol Evol 18:126–131

van Oosterzee P (1997) Where worlds collide. The Wallace line. Cornell University Press, Ithaca

Vogler AP, Monaghan MT (2006) Recent advances in DNA taxonomy. J Zool Syst Evol Res 45:1–10

Wesenberg-Lund C (1939) Biologie der Süsswassertiere. Wirbellose Tiere. Julius Springer, Wien

Whittaker RJ, Fernández-Palacios JM (2007) Island biogeography. Ecology, evolution, and conservation. Oxford University Press, Oxford

Wilding CS, Butlin RK, Grahame J (2001) Differential gene-exchange between morphs of *Littorina saxatilis* detected using AFLP markers. J Evol Biol 14:611–619

Will KW, Rubinoff D (2004) Myth of the molecule: DNA barcodes for species cannot replace morphology for identification and classification. Cladistics 20:47–55

Wilson MEJ, Moss SJ (1999) Cenozoic palaeogeographic evolution of Sulawesi and Borneo. Palaeogeogr Palaeoclimatol 145:303–337

Wilson AB, Glaubrecht M, Meyer A (2004) Ancient lakes as evolutionary reservoirs: evidence from the thalassoid gastropods of Lake Tanganyika. Proc R Soc Lond B 271:529–536

Woltereck R (1931) Beobachtungen und Versuche zum Fragenkomplex der Artbildung. I. Wie entsteht eine endemische Rasse oder Art? Biol Zentralbl 51:231–253

Woltereck R (1941) Die Seen und Inseln der "Wallacea"-Zwischenregion und ihre endemische Tierwelt. Erster Teil: Vorgeschichte und Aufgabe der Forschungsreise. Int Rev Ges Hydrobiol Hydrogr 41:1–36

Speciation and Radiation in a River: Assessing the Morphological and Genetic Differentiation in a Species Flock of Viviparous Gastropods (Cerithioidea: Pachychilidae)

Frank Köhler, Somsak Panha, and Matthias Glaubrecht

Abstract The Kaek River in central Thailand is unique in harbouring a diverse species assemblage of viviparous gastropods of the genus *Brotia*. A stretch of this river less than 100 km long is inhabited by seven, mostly endemic species that are essentially differentiated by their shell morphology. Earlier, it has been suggested that this species flock fulfils some basic requirements of a radiation (monophyly and phenotype–habitat correlation). However, the present study has shown that there is no strict correlation between radula and shell morphology and the utilisation of substrates, such as rock or sand, thereby refuting the hypothesis that ecological speciation may have played a significant role. Phylogenetic analyses based on mtDNA show that haplotypes cluster together in drainage-specific clades rather than according to the taxonomy. There are also strong indications that introgressive hybridisation has occurred, which may have resulted from secondary contact of previously isolated species due to dispersal or river captures during the Cenozoic. It is assumed that the high species diversity in the Kaek River results from two phenomena that interdigitate. Firstly, the Kaek River fauna may have originated from multiple species invasions from different source areas, while traces of these events may have been obscured by introgression of Kaek River-specific haplotypes. Secondly, waterfalls in the Kaek River seem to affect the directionality and amount of gene flow between local populations within the river and several smaller tributaries. Together with temporally changing water regimes, this highly structured environment may have conserved local genetic differentiation and triggered diversification and speciation in peripheral isolates within relatively short periods of time.

F. Köhler (✉)
Museum für Naturkunde, Invalidenstr. 43, 10115 Berlin, Germany
Australian Museum, 6 College St, Sydney, NSW 2010, Australia
e-mail:frank.koehler@austmus.gov.au
S. Panha
Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
M. Glaubrecht
Museum für Naturkunde, Invalidenstr. 43, 10115 Berlin, Germany

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_24,

1 Introduction

Speciation in the context of (adaptive) radiations is regarded as a key process in creating biological diversity. Like oceanic islands, lakes have been found to provide ideal model systems for elucidating the underlying mechanisms of this evolutionary process. However, not only lacustrine but also riverine species flocks can potentially provide crucial insights into the study of speciation and adaptive radiation (see review of, e.g., Glaubrecht and Köhler 2004). Among invertebrates, limnic gastropods have been found to provide most suitable model organisms for these studies (see, e.g., Glaubrecht 1996, 1999, 2006). In addition to other freshwater gastropod radiations, such as in eastern African lakes (for review and recent literature, see Glaubrecht 2008) or the Indonesian islands of Sulawesi (Glaubrecht and Rintelen 2008; see also, in this volume, Rintelen et al. 2010), a unique and endemic species flock of closely related pachychilid gastropods is found in the Kaek River system in Central Thailand. Here, a total of ten species-level taxa (five species and five subspecies) were originally described from a restricted river stretch of less than 100 km in length, primarily with emphasis on the shell (Brandt 1968, 1974). Two of these species, *Brotia binodosa* and *B. paludiformis*, had been reported earlier by Solem (1966) from the Thung Salaeng waterfall. Subsequently, Brandt (1974) systematically revised the Thai species, but failed to recognise that he was presumably dealing with a radiation of closely related species. He affiliated the species from the Kaek River with either one of two distinct genera, *Brotia* and *Paracrostoma*. Although this treatment transpired the high levels of morphological distinctiveness in the shells of different species, at the same time it obscured the existence of a presumably monophyletic flock of morphologically well-differentiated species for decades. Davis (1982) first noticed the uniqueness of the Kaek River assemblage by stating that "when *Brotia* is found in rivers there is usually one species, two at the most. The exception to this is the small radiation in the Koek Noi River (=Kaek River) (north central Thailand) of the Nan-Chao Phraya drainage."

Our preliminary study of mitochondrial and morphological differentiation hinted at a potentially adaptive radiation in the riverine *Brotia* species from the Kaek River (Glaubrecht and Köhler 2004), very similar to the one found in the lacustrine *Tylomelania* on Sulawesi. In a first step, therefore, we revised the taxonomy of the Kaek River species based on examinations of types and newly collected, alcohol-preserved material. Confirming the existence of a remarkably diverse pachychilid fauna in the Kaek River, we recognised at least seven distinct and endemic *Brotia* species in the river (compared to the original ten species-group taxa). So far, in no other river has a comparable diversity of pachychilid species been found worldwide. Molecular analyses using mitochondrial sequences suggested monophyly of the Kaek River species flock but also revealed a rampant mismatch of the branching pattern of mtDNA-based phylogeny with the delimitation of species by their shell morphology. Morphological analyses and ecological observations suggested that the distribution of shell and radular morphs within the river may be correlated with the usage of certain substrates by the animals (i.e., soft

versus hard substrate dwellers exhibited divergent radular and shell morphologies). Based on these facts (monophyly, local endemism, mismatch between mitochondrial gene and species tree, correlation between phenotypes and environment), Glaubrecht and Köhler (2004) postulated that the Kaek River species flock originated through adaptive radiation possibly triggered by trophic specialisation along the evolutionary trajectories outlined for the confamiliar *Tylomelania* (Glaubrecht and Rintelen 2008; Rintelen et al. 2010).

In contrast to the lacustrine *Tylomelania*, which radiated in situ in the ancient lakes on Sulawesi, the Kaek River species flock has apparently evolved in a riverine environment. Riverine radiations are both rarely known and studied, with only a few known examples for gastropods, such as Asian Triculinae (Davis 1979, 1981), the Stenothyridae in the Mekong (Hoagland and Davis 1979), hydrobioid snails from Tasmania and Eastern Victoria (Ponder et al. 1993; Ponder et al. 1994), and bithyniid snails in West Africa (Brown 1988). In all these cases, however, the exact causes of radiation remained hypothetical.

Therefore, it was the aim of the present study to compare cases of intralacustrine and intrariverine radiations in this group of closely related pachychilid gastropods in order to improve our understanding of the relevance of environmental factors for the evolution of invertebrate species flocks. It has been our goal to unravel the origins of the Kaek River species flock and to reconstruct the spatial and temporal patterns of its evolution, using a combination of molecular and morphological studies. We were also interested in identifying those factors that have been driving the morphological and genetic differentiation of these species. The patterns of morphological and genetic differentiation within and among the Kaek River species were studied with emphasis on possible correlations between morphological traits and environmental factors. Recently, the ecological component of speciation received much attention, with habitat selection, trophic specialisation and sexual selection being identified as key factors promoting speciation in sympatry and, potentially, also adaptive radiation (e.g. Schluter 2000; Streelman and Danley 2003; Gavrilets and Losos 2009). In order to assess whether we are dealing with a truly adaptive radiation driven by ecological speciation in the case of the Kaek River pachychilids, we addressed four of the main criteria, viz. monophyly, rapid speciation, phenotype–environment correlation, and trait utility as suggested by Schluter (2000).

2 The Systematic Framework: Phylogeny of the SE Asian Pachychilidae

Pachychilidae Troschel, 1857 is a group of freshwater gastropods only recently recognised as an independent freshwater radiation within the diverse and otherwise predominantly marine gastropod superfamily Cerithioidea Férussac, 1819 (Glaubrecht 1996; Lydeard et al. 2002; Köhler et al. 2004). Novel studies of

pachychilids provided insights into speciation in the context of adaptive radiation (Glaubrecht and Köhler 2004; Rintelen et al. 2004, 2007; Glaubrecht and Rintelen 2008) as well as evolutionary phenomena, such as the development of parental care in these viviparous snails (Köhler et al. 2004). Within the Pachychilidae, oviparity is considered a plesiomorphic trait (Glaubrecht 1996, 1999, 2006; Köhler et al. 2004), and is found in the African (*Potadoma* Swainson, 1840), Malagasy ("*Melanatria* Bowdich, 1822" [name replaced by *Madagasikara* Köhler and Glaubrecht, 2010]), and Neotropical (*Pachychilus* I. and H.C. Lea, 1850, *Doryssa* Swainson, 1840) taxa (Binder 1959; Grossmann 1967; Starmühlner 1969; Brown 1994; Simone 2001). By contrast, in Southeast Asia – where this gastropod family is particularly diverse – pachychilids are (ovo)viviparous throughout (Brandt 1974; Köhler and Glaubrecht 2001, 2005, 2006, 2007; Glaubrecht and Rintelen 2003; Rintelen and Glaubrecht 2003, 2005; Köhler et al. 2004; ; Rintelen et al. 2007) (Fig. 1). However, in conflict with more traditional assumptions (e.g. Morrison 1954; Brandt 1968, 1974; Glaubrecht 1996), the brooding taxa in South and Southeast Asia do not form a monophyletic group. Instead, three distinct clades have been identified by analyses of morphological and molecular data (Köhler and Glaubrecht 2001; Köhler et al. 2004; Köhler and Dames 2010). In terms of their morphology, these clades are mostly characterised by their reproductive anatomy: Within the genus *Jagora* Köhler and Glaubrecht 2003 (clade 1), females retain yolk-rich eggs in the mantle cavity from which the hatchlings are released. Yolk delivered with the egg capsule represents the only form of nourishment provided by the mother (Köhler and Glaubrecht 2003). In contrast, *Tylomelania* F. & P. Sarasin, 1898 and *Pseudopotamis* Martens, 1894 (clade 2) are eu-viviparous and possess a brood pouch formed from the pallial oviduct. The retained embryos are nourished for a considerable period of time by maternal albumin secreted by the mother (Glaubrecht and Rintelen 2003; Rintelen et al. 2007). Finally, representatives of the third clade possess a subhaemocoelic brood pouch. In these species, nutrients are provided exclusively by the egg capsule; any kind of secretory tissue is absent from the incubatory pouch (Köhler and Glaubrecht 2001).

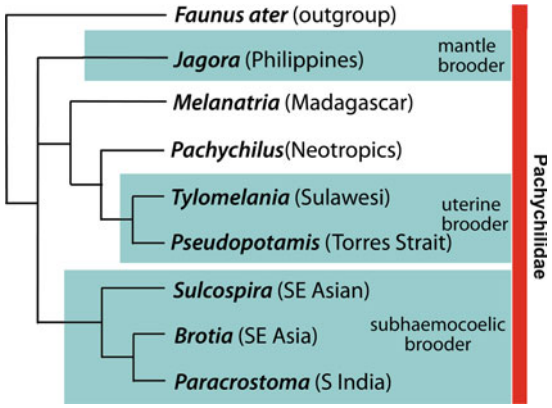


Fig. 1 Backbone tree showing the relationships of pachychilid genera as inferred from analyses of partial 16S sequences by Köhler et al. (2004). Brooding taxa are *shaded*, oviparous taxa are not shaded. The most parsimonious explanation is that the three brooding strategies pursued by different pachychilid clades have evolved independently while oviparity represents a plesiomorphic state within the family (note that *Melanatria* has recently been replaced by *Madagasikara*)

The three Asian clades of brooders are spatially well separated: *Jagora* is endemic to the Philippines, *Tylomelania* and *Pseudopotamis* are restricted to Sulawesi and two Torres Strait islands (northern Australia), respectively, and the subhaemocoelic brooders display an extended Sundaland distribution occurring from India to southern China, including the Malay Peninsula, Sumatra, Java, and Borneo. The latter clade has been referred to as the "Asia mainland clade" (Köhler et al. 2004; Köhler and Glaubrecht 2007; Köhler and Dames 2009). Compared to the two other Asian clades, the subhaemocoelic brooders display a much wider distribution, larger variation with respect to their morphology and in total a higher diversity of species. Various, in part conflicting, generic classifications were suggested for members of this heterogeneous group. Between two and four genera were delimited (*Sulcospira* Troschel, 1858, *Brotia* H. Adams, 1866, *Paracrostoma* Cossmann, 1900, *Adamietta* Brandt, 1974) by various twentieth century authors, such as Thiele (1928, 1929), Morrison (1954), Benthem Jutting (1956), and Brandt (1974). In general, these genera were established on the basis of certain shell, opercula and/or radular features – characters that subsequently proved not to be appropriate at this taxonomic level due to homoplasy (Köhler and Glaubrecht 2001, 2002, 2005, 2006, 2007). For instance, recent studies have shown that shell shape and sculpture often reflect ecological adaptation rather than phylogenetic relationships, with cases of remarkable parallelism being discovered in these pachychilids (Köhler et al. 2008).

Based on a comprehensive taxon sampling that covers the entire range of the group from southern India in the west to southern China in the east and Borneo in the south-east, Köhler and Dames (2009) have addressed the question of monophyly of the nominal genera of mainland Pachychilidae and analysed partial sequences of the mitochondrial genes COI and 16S as well as key morphological characters, notably the female genital anatomy and embryonic shell morphology. They suggested recognition of three genera (*Brotia*, *Paracrostoma*, *Sulcospira*) among the SE Asian subhaemocoelic brooders of mainland SE Asia (Fig. 2). Within *Sulcospira*, which represents the most basal offshoot of the clade of subhaemocoelic brooders, three sub-clades have been recognised that reveal a largely geographical structuring (with lineages each in Borneo–SE Asia mainland, Southern China–Vietnam, and Java–Borneo). All *Sulcospira* species exhibit widely congruent gross morphologies, however, this being the reason to refrain from formally naming these clades. In addition, the molecular phylogeny of the SE Asian mainland Pachychilidae provided evidence for the existence of a monophyletic clade of *Brotia* species in Central Thailand, which contains species that are endemic to the river systems of the Kaek and Kwae Noi River (Nan drainage), the Loei and Pong River (Mekong drainage), and the Pa Sak River (Chao Praya drainage).

Previous analyses of the rates of mitochondrial and morphological differentiation among the Asian Pachychilidae revealed two independent species flocks, which are characterised by (1) monophyly, (2) close relationships between their constituent members, (3) considerable degrees of interspecific morphological differentiation with respect to shell and radula, and (4) rampant mismatch of mtDNA phylogenies and morphology-based species delimitations. Both species flocks,

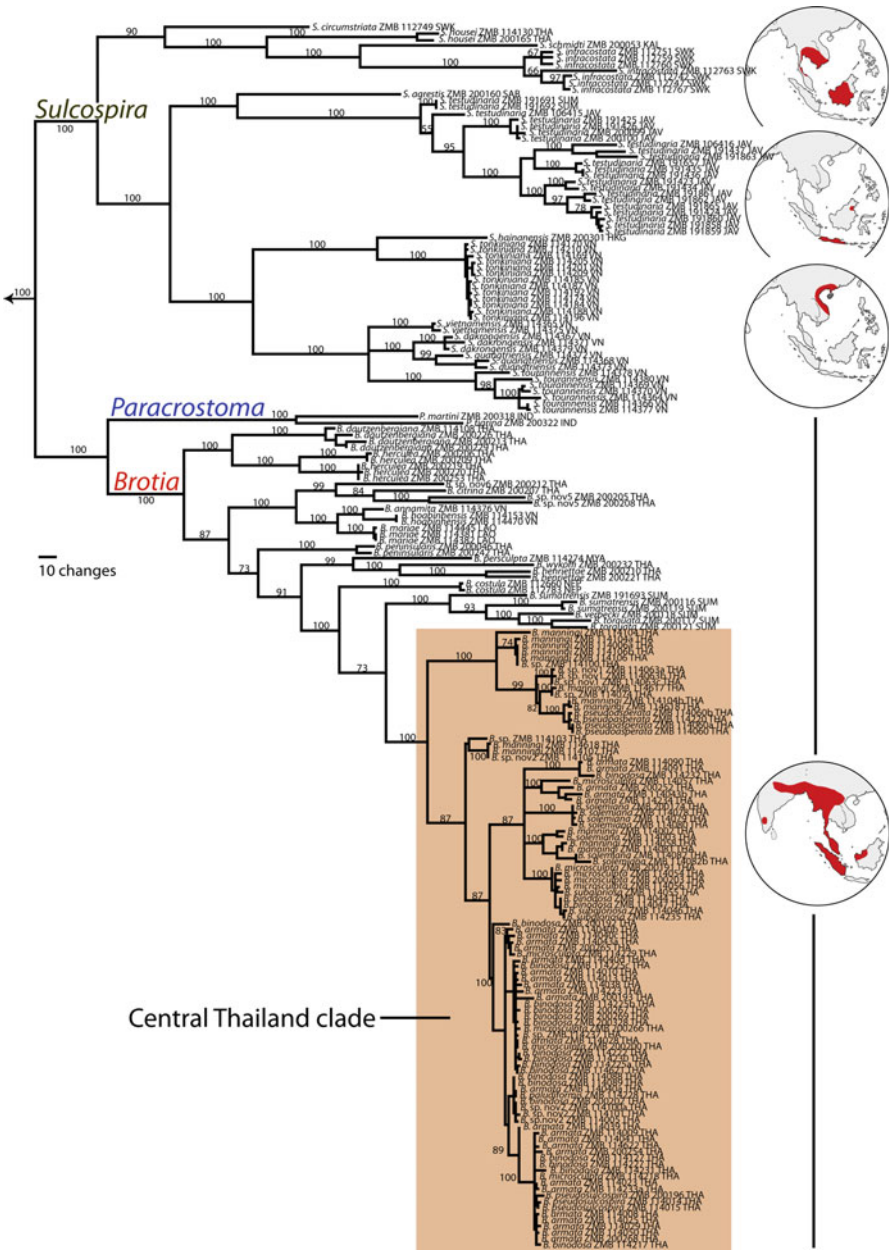


Fig. 2 Phylogenetic relationships within the clade SE Asian mainland clade of subhaemocoelic brooders (genera *Brotia*, *Paracrostoma*, *Sulcospira*) as inferred by analyses of concatenated COI and 16S sequences (Köhler and Dames 2009). The Central Thailand clade of *Brotia* is *shaded* *Area codes*: HGK, Hong Kong; IND, India; JAV, Java; KAL, Kalimantan; LAO, Laos; MYA, Myanmar; NEP, Nepal; SAB, Sabah; SUM, Sumatra; SWK, Sarawak; THA, Thailand; VN, Vietnam

Tylomelania in the Central Lakes of Sulawesi and *Brotia* in the Kaek River, Central Thailand, have been postulated to have resulted from adaptive radiations. The model case of *Tylomelania* on Sulawesi has been extensively studied for a period of almost 10 years, and ongoing work has shown that these endemic freshwater gastropods have radiated extensively in the two ancient lake systems of the island (Rintelen and Glaubrecht 1999, 2005; Rintelen et al. 2004, 2007; Glaubrecht and Rintelen 2008; see also, in this volume, Rintelen et al. 2010).

3 The Kaek River: Geographical and Environmental Settings

Knowledge of the geological history and the current environmental conditions in the Kaek River drainage is relevant for the understanding of the origin of the species flock and the significance of abiotic factors that may have influenced its evolution. The geological and hydrological data presented here has been gathered from various sources, such as topographical maps and online facilities. Note that due to the absence of a generally binding transliteration from Thai to English, locality names as spelt herein may differ from versions used elsewhere. With respect to localities within the Kaek River area, we preferentially refer to names as firstly spelt by Brandt (1968, 1974) for the sake of continuity while otherwise we refer to spellings as used in the current edition of the Times Atlas of the World.

The Kaek River (Maenam Kaek in Thai, also called Klong Talo at its lower reaches; Brandt 1968) flows into the Nan River near the city of Phitsanulok. The Maenam Nan is a first-order tributary of the Chao Praya, which is a broad, moderately fast-flowing river that winds its way through the central plain of Thailand and discharges into the Bay of Bangkok. The Chao Praya basin can be divided into two parts. The lower part is flat at low altitudes and extends towards the north as far as Ang Thong (ca. 15°N). This basin is filled with Quaternary deposits and was flooded for the last time by the South China Sea about 9,000–10,000 years ago when sea levels were ~4 m higher than today. The upper plain extends northwards up to the valleys of the Nan and Ping Rivers. This plain lies at elevations of more than 20 m above sea level and has not been subject to significant tidal flooding in the more recent past. The upper reaches of the watershed are located at ~19°N, in the provinces of Mae Hong Son, Chiang Rai and Chiang Mai.

The Kaek River flows in an E–W direction from the watershed west of Phetchabun towards Phitsanulok. It is located within the transition area between the Nan-Uttaradit suture zone, which is demarked by the Nan River valley between Nan and Saraburi, and the Loei-Phetchabun foldbelt (Cooper et al. 1989). Being situated at higher elevations within the ranges that are part of the Thung Salaeng Luang National Park, the upper and middle part of the river are located at the western fringes of the Loei-Phetachbun foldbelt whereas its lower course

(Klong Talo) between Wang Thong and Phitsanulok reaches the lower and flatter areas within the Nan valley (Chonglakmani and Helmcke 2001). The upper to middle course of the Kaek River has cut a steep-sloped canyon into an area formed essentially by Permian limestone as well as Jurassic sandstones, slate and hardpan across the Thung Salaeng Luang ranges (DNP 2009) between altitudes of 300 and 1,028 m.

For most of its ~150-km-long course east of Wang Tong, the Kaek River is a fast running stream. Its water is clear and relatively cold. The upstream region is characterised by a moderate decline and grounds of gravel and stones. Midstream waters flow swiftly over a rocky bottom with large boulders where they pass a series of rapids and waterfalls on their way west. Between the rocky sections, there are also sections with a more moderate decline in which a reduced flow regime results in the deposition of large amounts of sand and mud that form the main substrate here. But in general, soft substrates are rare in the upper and middle course and may provide only unstable conditions depending on the seasonally variable water regime. On the other hand, in the lower course between Wang Tong and Phitsanulok, only sandy to muddy substrates are found. Pine and bamboo forest as well as mixed species deciduous forest dominate the area surrounding the river, while grassland, lowland scrub and tropical broad-leaved evergreen forest cover smaller areas. Human impact is rather limited (mostly in bathing areas, near settlements), but increases in the downstream region towards Phitsanulok with its expanded farmland. Although the Kaek River is continuously supplied with water, the amount of water changes seasonally. In and shortly after the rainy season from around June through October, significantly more water flows down the river than in the dry season between November and April. During the rainy season, the Kaek is a wild-water stream, while during the dry season, the current is moderate and some of the smaller affluents and headwaters even become entirely dry. Limnological data on rivers and streams in tropical Asia are scarce (Dudgeon 1995). As predicted by the river continuum concept (Vannote et al. 1980), streams and their organismic composition and diversity are characterised by a flowing continuum, with distinct reaches not being delimited by fixed borders. However, in terms of the broadly used geomorphic or physiographical stream classification (Allan 1995; Hauer and Lamberti 1996; Giller and Malmqvist 1998), we interpret the Kaek River herein to represent a medium to large river of third order (with the Chao Praya and Nan River being mainstream rivers). According to the more useful biotic river classification scheme developed by Illies (1961), we classify the Kaek River herein to be a rhithral or middle stream section with its organismic components representing the rhitron. The rhitral is typically characterised by rather cool temperatures, high to moderate dissolved oxygen concentrations (often variable at least seasonally), with water ranging from clear to turbid and oligotrophic to mesotrophic, rather variable medium (semistable) substrates and stronger currents with a comparatively high gradient. The Kaek belongs to the Chao Praya biogeographical region established for freshwater fishes (Yap 2002); for details of zoogeography see Rainboth (1996).

4 River Capture: Paleogeography and Palaeohydrology

In the Cenozoic, SE Asian rivers were affected by two major geological processes: sea level fluctuations and realignments caused by tectonic changes or erosion. Sea level fluctuations have constantly changed coastlines due to the flooding or surfacing of vast areas. For instance, sea levels were apparently higher than today during the Miocene (+150–220 m, at 24–13 mya) and Pliocene (+100 m, at 5.5–4.5 mya) (Woodruff 2003), while they have been considerably lower during the Pleistocene (up to 120 m below today's level; Martinson et al. 1987). Sea levels of +100 m or more would have resulted in a northward extension of the Gulf of Siam and flooding of large parts of the Chao Praya river basin. However, even then, the Kaek River in its current configuration would not have been submerged as it lies at even higher altitudes. It may have, however, lost its connection to other parts of the Chao Praya drainage system.

Changes in drainage configuration of rivers in Central Thailand may have been more relevant in this regard. Gregory (1925) first pointed out that, during the Cenozoic, the major river systems of Central and Southeast Asia underwent dramatic changes due to tectonic processes, such as the uplift of areas and lava flows. The history of these river systems has been described in more detail by Hutchinson (1989) and Rainboth (1996). According to these reconstructions, the Chao Praya lost its headwaters to the growing Mekong in the middle and upper Pleistocene. Until around 2 mya, the Irawaddy, Salween and Mekong drained into the Chao Praya, unless around 1.5 mya volcanic activity separated the Irrawaddy and Salween rivers from this system. Since then, the Mekong changed its river bed repeatedly to successively more easterly directions. After its midstream has been separated from the Salween around 2 mya, its course followed the present course of the Ping River (Chao Praya drainage) until around 1.5 mya. Late Cenozoic faulting diverted the Mekong further eastwards along its present course towards Vientiane until, later in the mid-Pleistocene (∼1 mya), the Mekong once again drained into the Chao Praya, this time via the valley of the Loei and Pa Sak Rivers. Eventually, it changed its course again around 50,000 years ago towards the east, where it has undergone further course changes.

While the details and exact timing of the geological history of the Mekong drainage are not fully understood (Gupta 2008), it is clear that the courses of smaller rivers were also affected by tectonic processes. Some of them even reversed their original direction of flow due to uplifts that affected their upper or mid-streams, such as the Loei River that was once part of the southward-flowing proto-Mekong but today flows in a northward direction, or the Mun River that once drained in a westerly direction into the Chao Praya until it reversed its course towards the east due to the sinking of the Khorat Plateau during the mid-Pleistocene (Hutchinson 1989). On a smaller scale, the details of the geological history of the Kaek River area are difficult to reconstruct. The headwaters of the westward-flowing Kaek River, the southward-flowing Pa Sak River, and the northward-flowing Loei River are in close conjunction, separated by the up to 1,700-m-high mountain ridges of

the Phang Hoei Range, which are of relatively recent (Cenozoic) igneous origin. Prior to the uplift of these mountains approximately during the Pliocene, there was likely a single river flowing in a N–S direction through the beds of the Loei and Pa Sak River (Hutchinson 1989). It can only be speculated as to how the river systems looked like before this period and if the upstream region of the Kaek River was also connected to the proto-Mekong drainage at this time. Later, from the mid-Pleistocene until 0.05 mya, the Mekong flowed through the beds of the present Loei and Pa Sak rivers again and re-connected their once separated faunas. The Kaek River itself has likely not been affected by these more recent reconfigurations of drainage systems as its upstream region was probably already at higher elevations. However, it is clear that hydrological phenomena such as river captures have been effective in the whole area with respect to the connection and separation of drainage systems, which must have also influenced their biota as suggested by Glaubrecht and Köhler (2004).

5 Sampling Design and Collection Sites

In order to account for the possible relevance of ecological factors, we generally collected specimens that occurred on different substrates (rock, wood, sand, mud) separately, and also differentiated between specimens collected at different depths (at levels of 0, 0.5, 1, 1.5 and 2 m water depth). To address the relationships on a larger scale and to reveal the origins of the Kaek River species flock, we collected *Brotia* samples in all adjacent river systems of the Kaek River, viz. the drainages of the Kwae Noi in the north, the Loei in the northeast, the Pa Sak in the southeast and the Pong in the east. The drainages of the Kwae Noi and the Kaek are separated from each other by the southern extensions of the Luang Prabang Range with mountainous ridges reaching elevations of 1,035 m, 1,356 m (at and near the Khao Kho), 1,746 m (Phu Hin Ronkla), and 1,468 m (Phu Khat) (from S to N). Towards the northeast, these ranges separate the catchment areas of these two rivers from the adjacent drainage of the Loei River, which flows northward into the Mekong. The headwaters of the Pong River, which flows to the east via the Mun River into the Lower Mekong, are located in the east of the Phang Hoei Range while the Pa Sak River flows towards the south and discharges into the Chao Praya (Fig. 3).

This material basis was complemented by collections from other parts of Thailand and neighbouring regions of Laos in an attempt to cover all major drainage systems of the region, i.e. the Salween (with its first order tributary Maenam Moei) in western Thailand, the Chao Praya (with its two principal tributaries Nan and Ping) in central Thailand, and the Mekong (with its tributary Mun) in north-western Thailand and Laos (Fig. 4).

The most extensive sampling was undertaken within the Kaek River drainage, however. Not all sectors of the Kaek River were accessible during this study either due to the rugged topology or due to access restrictions in the Thung Salaeng

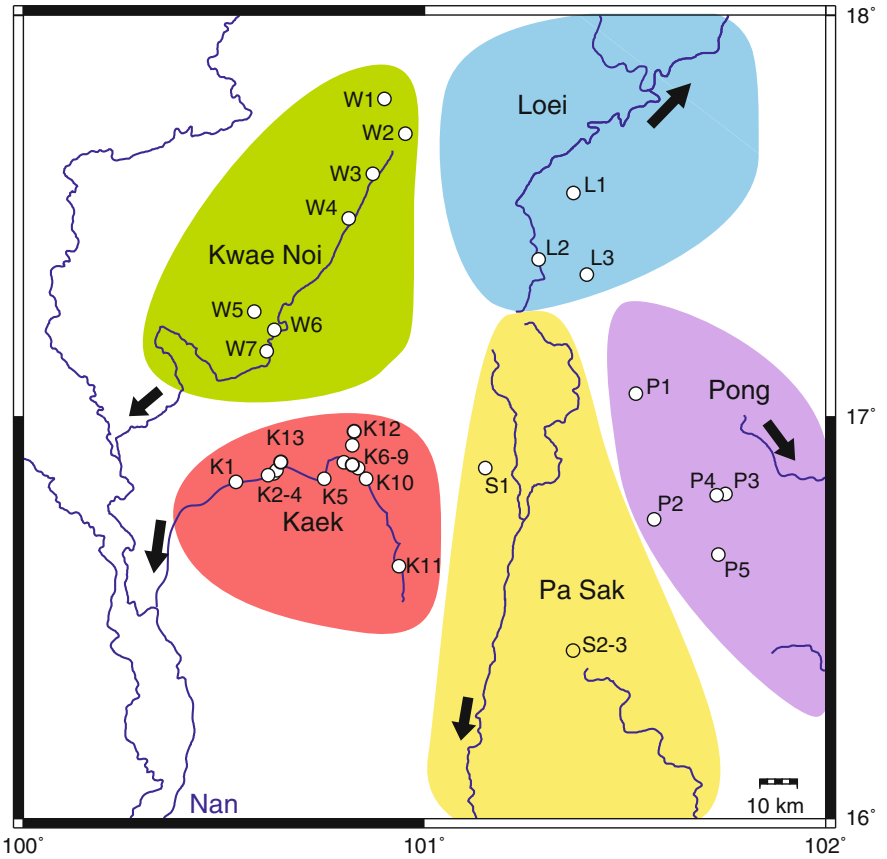


Fig. 3 Collection sites in north-central Thailand and their location within the catchment areas of the five main rivers that drain the Phetchabun Mountains towards the west (Kaek and Kwae Noi River, tributaries of the Nan River), north (Loei River, tributary of the Mekong), east (Pong, tributary of the Mun), and south (Pa Sak River, tributary of the Chao Praya)

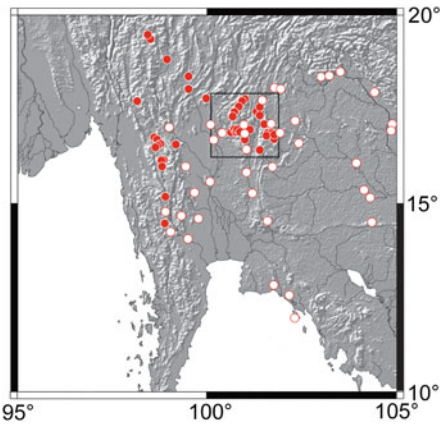


Fig. 4 Topographical map showing the location of collection sites in Thailand and Laos, field work in 2006–07. *Frame in centre* delimits the area depicted in Fig. 05. *Red dots* mark sites where *Brotia* species were found, *white dots* where no *Brotia* species were found. Accordingly, *Brotia* is confined to mountainous regions of NW and W Thailand but absent from the plains in central, south, and east Thailand

National Park or on private properties. Between Wang Tong and the Headquarters of the Thung Salaeng National Park at the Thung Salaeng rapids, the National Road 12 from Phitsanulok to Lom Sak runs parallel to the midstream segment of the river. Alongside this road, there are several areas that are within easy reach, mostly near or at waterfalls and rapids that are signposted as tourist attractions and also used for recreational purposes by local tourists. The material first described by Brandt (1968, 1974) mostly originates from these sites. In his descriptions, Brandt (1974) referred to the road distances of the sampling sites along the highway 12 from Phitsanulok. We continue to refer to these distances to ensure comparability of ours and his data; a reference number is assigned to each of them. Since the Kaek River flows in a westward direction towards Phitsanulok, in the following the sampling sites are listed in an upstream order (Fig. 5). In addition to the sampling sites referred to by Brandt (1968, 1974), our work covers further sites along the river course as well as in permanently water-filled affluents of the Kaek River; some of which were found not to harbour *Brotia* species. Between the Nan River near Phitsanulok and Wang Tong, the Kaek River flows through a plain on muddy to sandy substrates. No *Brotia* species were found in this lower segment. East of Wang Thong, the area ascends steeply to higher elevations, which marks the end of the fast-running midstream region. The first accessible sampling site are the Sakunothayan rapids at km 33 (K1). This spot is followed by a smaller affluent that flows over rocks (km 37), where we collected samples at around 8 km distance from the main stream of the Kaek River (K2). The third spot is an area with unnamed rocky rapids at km 42 of the highway (K3), followed by the Kaeng Song waterfall

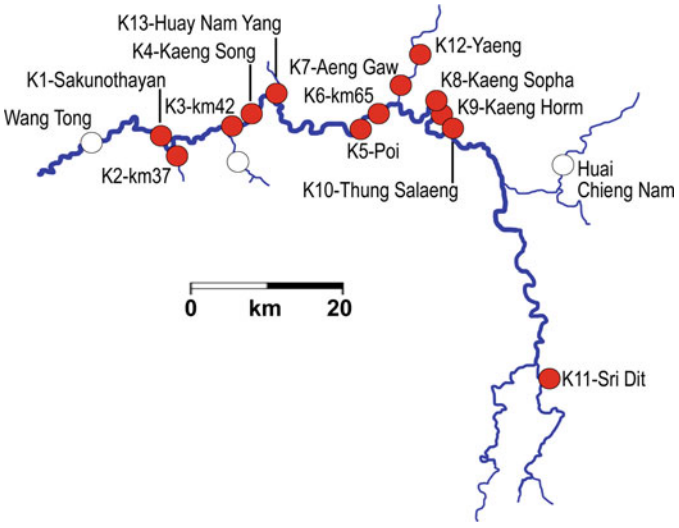


Fig. 5 Location of collecting sites at the Kaek River, field work in 2006 and 2007. *Red dots* mark localities at which *Brotia* samples were found, *white dots* mark localities at which no *Brotia* species was found

at km 45 (K4), a small affluent from the north with rocky substrates, the Huay Nam Yang (K13), the Poi waterfall at km 60 (K5), a further area with unnamed rocky rapids at km 65 (K6), the Aeng Gaw waterfall, situated in an small affluent from the north at km 67 (K7) that flows through the village of Yang (K12), the Kaeng Sopha waterfalls at km 72 (K8), and the Kaeng Horm rapids at km 73 (K9). The Thung Salaeng rapids at km 76 (K10) are the last collection site along the National Road 12. From here, the Kaek River flows through protected and inaccessible areas of the Thung Salaeng National Park. The next and last sampling site is the Sri Dit waterfalls (K11) about 40 km SE of Thung Salaeng. Sites that were found not to harbour *Brotia* species are the lower portion of the Kaek River near Wang Tong, an affluent from the south near km 42, and the affluent Huay Chieng Nam east of Thung Salaeng. The latter creek is the type locality of *Brotia subgloriosa* (Brandt 1974). However, complete deforestation and degradation of the whole area have obviously affected this river, which is now a slow-flowing, muddy creek that is not suitable for *Brotia*.

The configuration of the collection sites differs to a certain degree (Fig. 6). Rocks are the predominant substrate across the entire length of the Kaek River. At waterfalls within the main course of the river (at Kaeng Song, Poi, Kaeng Sopha), the water runs over large steps with heights from 1 to 4 m. At rapids, the water runs swiftly over a broader stretch of rocks and boulders. There, water depth is usually low (less than 0.5 m) and sandy patches are absent. In between the waterfalls and rapids, there are also quieter areas with moderate currents and depths of up to 2.5 m. Here, sandy and muddy substrates are found to cover the rocky bottom of the river bed. Sandy patches were found near Sakunothayan in depths of 0–2 m and muddy patches at Kaeng Song and near km 45 at depths of around 2 m. Smaller areas with sand between larger rock fields were also found at Thung Salaeng and Kaeng Sopha in depths of around 0.5–1 m.

6 Patterns of Shell Variation Among and Within the Kaek River Species

Compared to most regions in SE Asia, the Kaek River harbours an exceptionally diverse pachychilid fauna with respect to the species composition as well as the variability found in the shells of these species. Brandt (1968, 1974) initially delimited ten species-group taxa, of which seven were subsequently also recognised by Glaubrecht and Köhler (2004), all of them being considered as distinct species, essentially discriminated by means of their shell shape and sculpture. Accordingly, among the Kaek River species shells vary from elongate and sculptured (*B. binodosa*) via conical and sculptured (*B. armata*), to globular and smooth (*B. paludiformis*), broadly conical and smooth (*B. pseudosulcospira*), elongately conical and smooth (*B. microsculpta*) or elongately turreted and smooth (*B. subgloriosa*) (Fig. 7).

Fig. 6 Collection sites at the Kaek River (in downstream order). (**a**) Sri Dit waterfall (K11). (**b**) Thung Salaeng rapids (K10). (**c**) Kaeng Sopha waterfall (K8). (**d**) Poi waterfall (K5). (**e**) Aeng Gaw waterfall (K7), end of rainy season, November 2007. (**f**) Aeng Gaw waterfall, end of dry season (K7). (**g**) Kaeng Song waterfall (K4). (**h**) Lower course of Kaek River in Wang Tong. All photos except of (**e**) were taken in February 2006 at the end of the dry season. Specimens were collected above and below the waterfalls

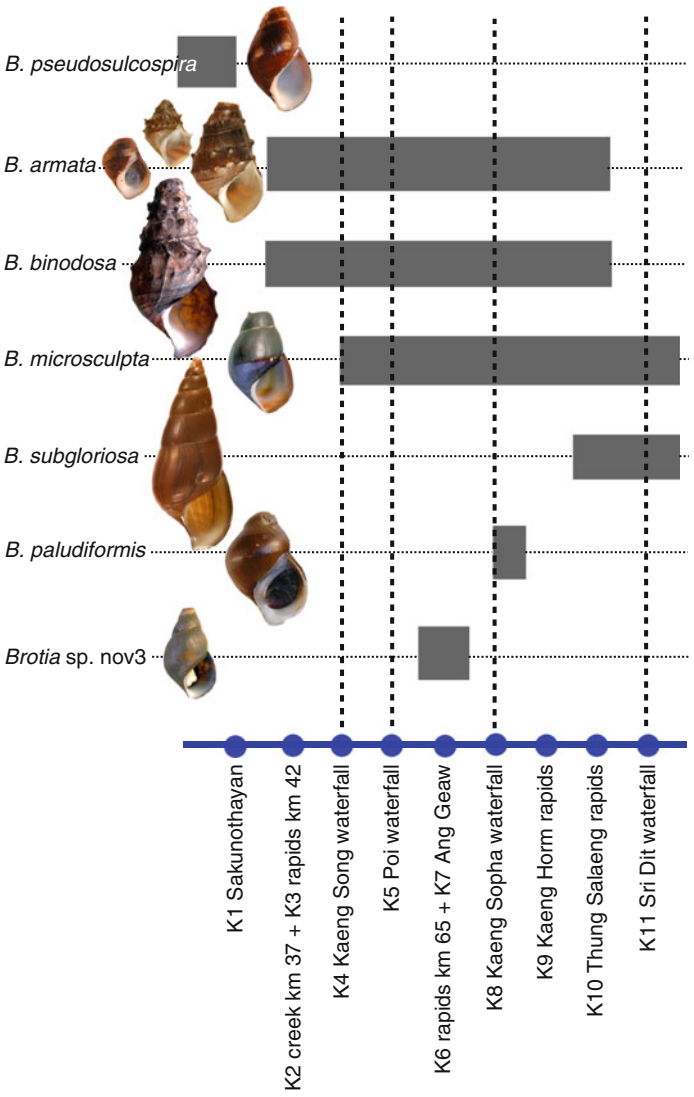


Fig. 7 Distribution of *Brotia* species within the Kaek River. *Vertical lines* indicate barriers in the river course formed by waterfalls. Note that the Aeng Gaw waterfall is not situated directly in the main stream of the Kaek River but part of an affluent creek

The results of the current study are based on the most comprehensive basis of material, which also includes newly collected samples from various localities within and outside the Kaek River drainage that were not available to previous workers. They widely confirmed the taxonomical treatment of the *Brotia* species in the Kaek River by the latest systematic revisions (Glaubrecht and Köhler 2004;

Köhler and Glaubrecht 2006). Only a few details are considered to be in need of revision, as will be outlined in the following. However, we here refrain from a formal taxonomic treatment and instead refer to informal names where considered necessary. Only short diagnoses for species are presented here for the sake of readability of the text; for more comprehensive descriptions, we refer to previous taxonomic treatments, such as Brandt (1968, 1974), Glaubrecht and Köhler (2004), and Köhler and Glaubrecht (2006). See Table 1 for a general comparison of shell parameters.

Brotia pseudosulcospira has an almost limpet-like shell with no more than two whorls. It is generally smooth and thick-shelled and has a wide and ovate aperture as well as a large, oval operculum that almost fits the aperture. The body whorl comprises most of the shell height. It is well rounded in diameter; a slight depression below the upper suture being visible. This species only occurs at the Sakunothayan rapids (K1), and no other congener has been found to co-occur. *Brotia armata* was reported in error from Sakunothayan by Glaubrecht and Köhler (2004).

Brotia armata is widespread, being found in the Kaek River between the Kaeng Song rapids (K3) and the Thung Salaeng rapids (K10), in two creeks that discharge into the river (at km 37 (K2) and Huay Nam Yang (K13)), as well as in the drainage of the Kwae Noi River (W6, W3). It is not only the most widespread but also the most variable species with respect to its shell. Shells are typically sculptured with two to four spiral ridges, of which one or two may support spiral rows of pointed nodules or small spines at the periphery of the whorl. However, some shells are almost entirely smooth. Shells comprise between two and three whorls; the body whorl being inflated and proportionally considerably larger than the preceding whorls. The operculum is oval and almost fits the aperture. Apart from this general pattern, local populations differ considerably in shell shape and sculpture: almost limpet-like specimens with only a single whorl were found at lower reaches of the Kaek River (Kaeng Song, K3), while specimens with up to three whorls were found in upper midstream regions of the Kaek River (between Poi and Thung Salaeng) as well as in the Kwae Noi (W3). Populations within the Kaek River showed mostly a rather weakly developed sculpture whereas specimens collected in an affluent creek at km 37 (K2) exhibited a well-developed sculpture including the presence of spines. For spiny specimens like these, Brandt (1974) introduced the subspecies name '*morissoni*', which has subsequently been considered a synonym of *B. armata* by Glaubrecht and Köhler (2004). This treatment is still considered correct, since we found all transitions from spiny to the complete lack of spines within this population, which is considered as evidence that the occurrence of phenotypes that differ with respect to presence and development of spines is controlled by environmental factors, such as, possibly, water current and predation. *Brotia armata* differs from *B. pseudosulcospira* by its relatively more inflated body whorl, the absence of a sub-sutural depression, and the presence of usually well-developed sculptural elements.

Brotia binodosa occurs in the Kaek River between Kaeng Song and Thung Salaeng, in a tributary at Yaeng (K13), and in the Kwae Noi. Three shell forms can be differentiated, (1) a large, thick-shelled, and broadly conical form with

Table 1 Shell parameters (mm) of Kaek River *Brotia* species (means and standard deviation)

	B. pseudosulcospira	*B. armata*	*B. binodosa* (A)	*B. binodosa* (B + C)	*B. microsculpta*	*B. paludiformis*	*B. subgloriosa*	*B.* sp. nov3
Shell height	27.1 ± 2.5	18.1 ± 4.8	34.5 ± 4.9	27.7 ± 6.8	15.1 ± 3.1	23.2 ± 3.9	37.7 ± 3.0	14.8 ± 3.6
Shell breadth	17.5 ± 1.5	13.3 ± 3.4	22.9 ± 1.9	15.7 ± 3.4	9.8 ± 1.7	18.2 ± 2.6	19.3 ± 1.6	8.6 ± 2.1
Aperture length	17.4 ± 1.8	12.2±2.9	20.3 ± 1.7	14.0 ± 2.9	9.0 ± 2.2	16.0 ± 2.4	15.0 ± 0.9	7.7 ± 1.9
Aperture width	10.8 ± 1.3	7.9 ± 2.0	12.0 ± 1.1	8.5 ± 2.0	5.5 ± 1.2	11.3 ± 2.0	10.1 ± 0.8	4.8 ± 1.3
Body whorl	24.0 ± 2.2	16.3 ± 4.5	28.4 ± 2.5	20.6 ± 4.7	12.4 ± 2.1	21.2 ± 3.5	24.4 ± 1.5	11.5 ± 2.8
Whorls	2.1 ± 0.4	2.0 ± 0.8	2.5 ± 0.7	3.2 ± 0.9	2.6 ± 0.7	1.8 ± 0.3	4.2 ± 0.6	3.2 ± 0.9

pronounced spiral ridges that support up to two spiral rows of rounded nodules, (2) a small form with conical shells and pointed tips with rather flattened whorls and one or two spiral rows of well-developed spines, and (3) a form with more elongated shells that usually exhibit a sub-sutural depression and one or two spiral rows of weakly developed spines or nodules and a well-produced basal lip of the aperture. These three forms are spatially separated: form A occurs in the Kwae Noi drainage (W5-7), form B in an affluent of the Kaek in Yaeng (K13), and form C in the Kaek River. Comparisons of specimens show that specimens of form B are similar to juveniles of form C. In addition, a graphical chart of shell heights and breadths confirms that forms B and C exhibit a congruent correlation between shell height and breadth (Fig. 8). We conclude that both forms are conspecific and that form B represents predominantly juvenile and sub-adult specimens. In contrast, form A displays a different height–breadth ratio. Together with the distinct sculpture, this is most likely indicative of the fact that the forms A and (B + C) represent two distinct species. Shells of form A resemble the type specimens of *Melania binodosa* Blanford, 1903 more closely than the specimens of form B and C. Showing a similar overall shape, the typical form A of *B. binodosa* differs from *B. armata* essentially by its much larger size. The Kaek River form of *B. binodosa*, however, differs with respect to its more elongated shape, the presence of more whorls, and larger size.

Unlike the former species, *B. microsculpta* has a smooth shell that lacks any sculptural elements except for faint growth lines. Shells comprise one to three whorls, of which the body whorl is the largest, being well rounded to slightly flattened in diameter. The body whorl of some specimens is keeled below the periphery; in others, it is rather rounded. The aperture is broadly ovate, laterally rounded to slightly angulated, and narrowly pointed above. The operculum is round and smaller than the aperture. There has been some confusion with regard to the identity of this species due to a possible mix-up of specimens. Glaubrecht and Köhler (2004) depicted a shell of *B. solemiana* allegedly found at the Sri Dit rapids

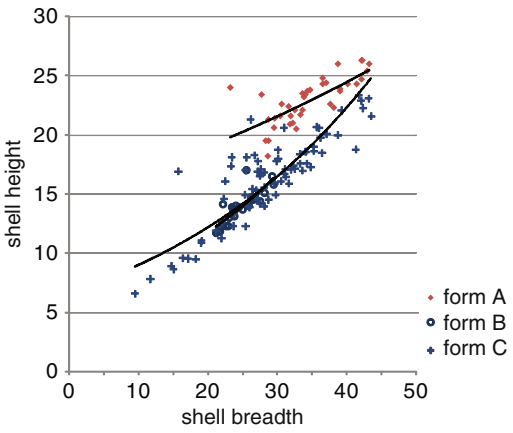


Fig. 8 Diagram showing the relationship of shell height and shell breadth in the three forms of *B. binodosa* recognised by their shape

(K11) and concluded that *B. solemiana* occurs in the upper course of the Kaek River. However, newly collected specimens from the same locality differ from the depicted specimen. They are more similar to the holotype of *B. microsculpta*, which itself is a not very representative specimen of this species due to its exceptionally large size and the rarely found presence of three complete whorls. Here, we correct the former statement that *B. solemiana* occurs in the headwaters of the Kaek River and attribute the relevant specimens from the upper course of the Kaek River at Sri Dit (K111) to *B. microsculpta* instead. This species is found throughout the river between Kaeng Song (K3) and Sri Dit (K11) and also in the affluent creek at km37 (K2).

Each of the three other species with smooth shells, *B. paludiformis*, *B. subgloriosa*, and *Brotia* sp nov3, is only found at a single locality.

Brotia paludiformis has a pronouncedly globular shell that comprises a maximum of two whorls; the second whorl being always much smaller than the body whorl. The aperture is widely oval. The operculum is oval and slightly smaller than the aperture. The species is found only at the Kaeng Sopha waterfalls (K8).

Brotia subgloriosa has been described from the Huai Chieng Nam, a tributary of the Kaek River, where it has not been found since. However, the species also occurs in the upper course of the Kaek River between Sri Dit (K11) and Thung Salaeng (K10). The shell is elongately turreted, relatively large and smooth. The operculum is slightly oval to almost round. *Brotia* sp. nov3 was found at the Aeng Gaw waterfall (K7), which is located near the Kaek River along the course of a small affluent river. Specimens were collected at the end of the rainy season in the flowing water. The creek is not permanent, however, and usually dries out in the dry season. In this period of time, the river is believed to flow only through subterranean cavities in the limestone rocks, where the snails apparently live most of the year. Their body is entirely white (whereas all other known *Brotia* species are brownish or blackish), possibly due to their largely subterranean lifestyle. The shells of this species are rather small, smooth, elongately conical, and comprise three to four well-rounded whorls. The operculum is almost round.

7 Radular Morphology and Substrate Usage

In general, three distinct radula types can be found among the species in the Kaek River (Glaubrecht and Köhler 2004). Type 1 corresponds to the general radular morphology found in a large number of *Brotia* species (see Köhler and Glaubrecht 2006) (Fig. 9a–b, g–h). The radula has a length of around 20–25 mm length (equivalent to about half of the shell height) with about 180–200 rows of teeth (∼9–10 rows/mm). The central teeth have a squarish shape and possess a well-developed glabella and a large main cusp, the lateral teeth have short lateral extensions, and the hooked marginal teeth are moderately long with two cusps – the outer one being broad and ovate in shape, the inner one being much smaller in size. This radula type is found in *B. armata* (Fig. 9a–b), *B. binodosa*,

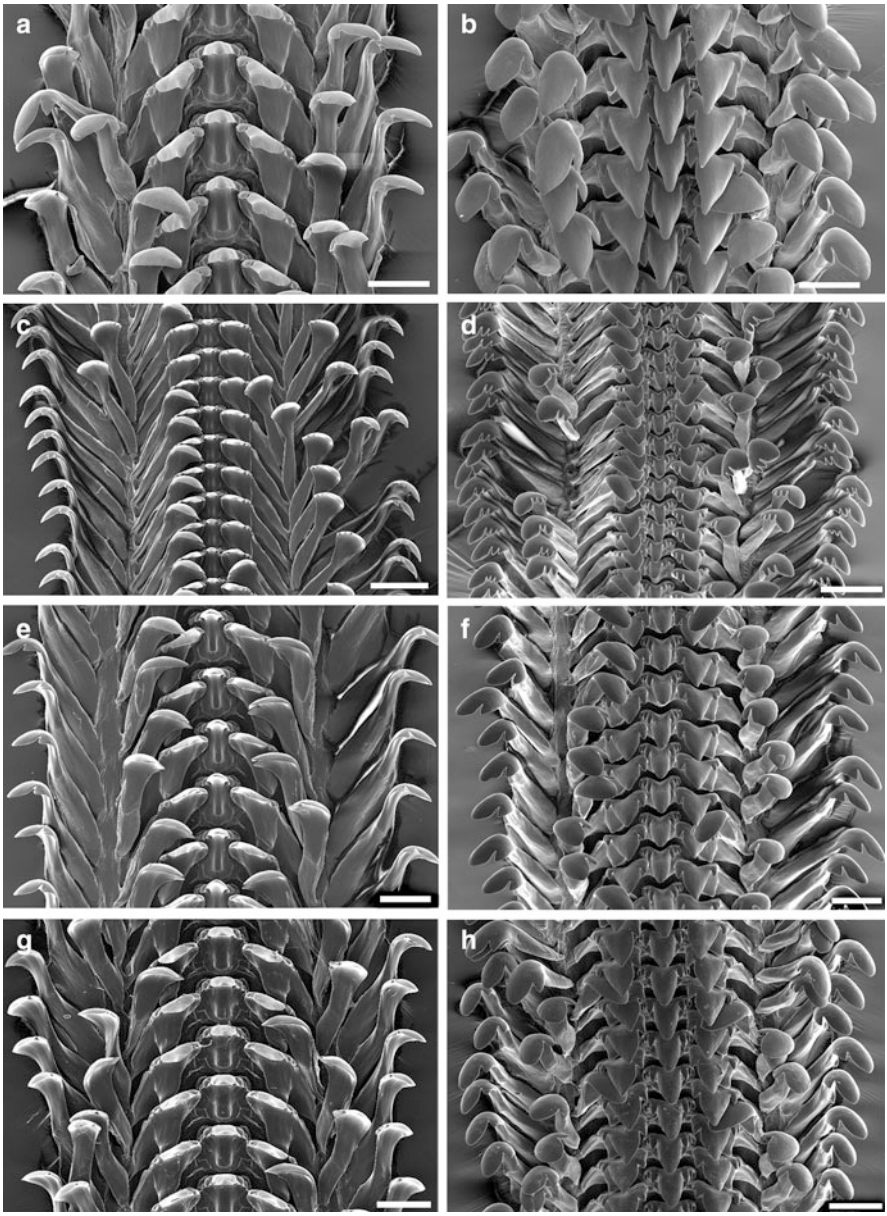


Fig. 9 Representative radulae of *Brotia* species from the Kaek River. Shown are two views of each the same radula segment: *Left*, view from above; *right*, front view at ~45° obliquely from above. (**a**,**b**) *Brotia armata* (ZMB 114.009, Kaeng Song, on rock; radula type 1). (**c**,**d**) *Brotia microsculpta* (ZMB 114.223, Poi, on rock; type 3). (**e**,**f**) *Brotia microsculpta* (ZMB 114.054, Sri Dit, on rock; type 2). (**g**,**h**) *Brotia subgloriosa* (ZMB 114.046, Thung Salaeng, on sand; type 1). *Scale bars* 100 μm

Table 2 Radula types found among *Brotia* species in the Kaek River. Given are means and standard deviations of the length of the radular ribbon (mm), numbers of rows of teeth, and rows per mm of ribbon length

Species	Examined radulae	Radular length	Rows of teeth	Rows per mm
Type 1				
B. armata	31	21.5 (±4.0)	194 (±33)	9.1 (±1.1)
B. binodosa	18	19.6 (±4.7)	189 (±32)	9.8 (±2.0)
B. paludiformis	1	23.2	178	7.7
B. pseudosulcospira	3	25.1 (±3.2)	224 (±28)	8.9 (±0.2)
B. subgloriosa	10	17.6 (±4.1)	185 (±25)	10.8 (±1.9)
Type 2				
B. microsculpta (2)	13	16.7 (±4.1)	182 (±38)	11.2 (±2.1)
Type 3				
B. microsculpta (3)	4	12.6 (±4.7)	220 (±86)	17.7 (±2.2)
B. sp. nov3	2	14.1 (±2.1)	214 (±18)	15.3 (±1.0)

B. paludiformis, *B. pseudosulcospira* and *B. subgloriosa* (Fig. 9g–h) without marked and consistent interspecific differences. Type 2 is similar to the former type but differs by the presence of a narrower and shorter glabella of the central tooth, a slightly shorter ribbon length, and more densely packed rows of teeth (Fig. 9 e–f; Table 2). These conditions have been found in 13 of 17 examined specimens of *B. microsculpta*. Type 3 is rather distinct and differs from the others by a generally much shorter ribbon length (∼15 mm), significantly more densely packed rows (∼15–17 rows/mm), a more weakly developed glabella of the central teeth, lateral teeth with longer lateral extensions, and marginal teeth with a more elongated shape that support two or three accessory inner cusps (Fig. 9c–d). Type 2 has been found in 4 of 17 specimens of *B. microsculpta* and in the two examined specimens of *Brotia*. sp. nov3.

Radulae of the types 1 and 2 are rather similar, and the differences between them are subtle with the deviant shape of the glabella of the central tooth being the only distinctive feature. In addition, some radulae of *B. armata* have been found to also possess relatively short and narrow glabellas representing transitional stages between the two types. Type 2 has also been reported from *B. solemiana* by Glaubrecht and Köhler (2004) both from in and outside the river (but note that this name was erroneously attributed to specimens of *B. microsculpta* from the upper course of the Kaek River). This observation has been confirmed by the present study with respect to *B. solemiana* from the Loei River drainage.

Our recent findings are also in agreement with the observation of Glaubrecht and Köhler (2004) that *B. microsculpta* has the most distinctive radula among the Kaek River species (together with the newly found *Brotia* sp. nov3 from the Aeng Gaw waterfall).

During field work in 2006 and 2007, sandy and muddy areas as well as rock surfaces were systematically searched for specimens. In general, all *Brotia* species were found to graze on rocks irrespective of which radula type they possess, whereas sand and mud flats were as a rule not found to be inhabited by *Brotia*

species. Only exceptionally, single individuals of different species were found on sand (while no *Brotia* was ever found on mud), which suggests that these snails do usually not live on this substrate but occur there by accident. Only at the Thung Salaeng rapids, were specimens of *B. subgloriosa* frequently (but not exclusively) found on small sandy patches within rock holes formed by scouring. Next to *B. subgloriosa*, individuals of *B. binodosa* were also found in these holes. These holes had limited surface areas (usually not more than ~0.5–5 m^2), however, and in order to reach them, snails would have needed to crawl over larger stretches of rock.

In summary, our recent findings do not indicate that there is a correlation between radula morphology and substrate usage in *Brotia* species in the Kaek River. First, there are no obvious differences between type 1 radulae of specimens collected on rock and sand. Second, the species with the most distinctive radulae, *B. microsculpta* and *Brotia* sp. nov3, do not differ from any other species in the way they utilise a certain substrate. In contrast, at various sites, *B. microsculpta* was found to occur syntopically with other rock grazers, such as *B. armata*. This result contradicts earlier assumptions by Glaubrecht and Köhler (2004) that there is a possible correlation between radula phenotypes and environment (substrate) among species of the Kaek River flock, which was based on limited observations.

In order to infer phenotypic responses to changing substrates, we set up aquarium experiments than ran over the period of 1 year between May 2007 and April 2008. In order to test if grazing on different substrates affects the radular morphology, we conducted transplant experiments. Series of 10–12 individuals each of *B. armata*, *B. binodosa*, and *B. microsculpta* collected on rocky surfaces and of *B. binodosa* and *B. subgloriosa* collected on sand were split into two groups. Each group was kept for the entire period in aquaria that provided either only sand or only rocks as substrate. Under both settings, animals were fed with fish food and various kinds of vegetables. After the period of 1 year, the radulae of these animals (and their young) from different aquarium set-ups were compared with each other as well as with specimens collected at the same localities in the wild. The numbers of compared radulae were low as some specimens died during the period of study. Radulae of some individuals that were born and raised in the aquaria were not analysed since their intermediate shell phenotypes suggested that they were of hybrid origin, which might have also affected the radular morphology. In fact, the shell morphology of these specimens that grew up in the aquaria corresponded with *B. armata* or *B. binodosa*, while they had radulae of type 2 normally being found in *B. microsculpta*.

For the low numbers of compared radulae of captive adults ($n = 14$), we refrained from a statistical analysis of our results. However, in general, the specimens raised in aquaria (including both adult animals collected in the field as well as most specimens that were newly born in the aquaria) showed no significant changes in their dentition patterns (by means of the shape of teeth) with respect to specimens collected at the same localities in the wild irrespective of the substrate on which they were kept.

Radulae of captive animals only differed from those collected in the field by having slightly shorter ribbons (in average by 1–2 mm shorter), while the density of

rows did not vary significantly with respect to specimens collected in the wild. Independent of the substrate provided, the habitat conditions in the aquaria certainly differed from those in the native environment. For example, in the field, *Brotia* species were found to graze on the biofilm on hard substrates, while in the aquarium, the main source of food was fish food. Since these altered habitat conditions did not result in significantly changed radular dentition, we conclude that in *Brotia* the phenotypic plasticity of the radula with respect to the source of food or the utilised substrates is rather limited. This finding contrasts with reports of immense intraspecific variation observed in rock-dwelling Littorinidae, which was partly attributed to phenotypic plasticity (Padilla 1998; Reid and Mak 1999). The observed inconsistency underscores the need for further studies that address the plasticity of the radula in different gastropod groups with respect to ecological conditions.

8 Phylogenetic Relationships Inferred by Analyses of Mitochondrial Genes

In order to infer the phylogenetic relationships of the Kaek River species, a partial fragment of the cytochrome c oxidase gene (COI) was analysed by employing Bayesian Inference. Previous studies based on analyses of combined 16S and COI data have already demonstrated the monophyly of a Central Thailand clade of *Brotia*, which comprises all species inhabiting the drainages of the Kaek, Kwae Noi, Loei, Pa Sak, and Pong Rivers in northern Central Thailand (Köhler and Glaubrecht 2006; Köhler and Dames 2009). An earlier study has also suggested the monophyly of the Kaek River species flock based on 16S and COI (Glaubrecht and Köhler 2004). Compared to these previous studies, the phylogeny reconstructed here is based on a significantly more comprehensive basis of data with respect to both taxon sampling and area covered. The previous study of Glaubrecht and Köhler (2004) did not include species from the Pa Sak and Loei drainages and only one sample each from the Pong and Kwae Noi drainage. A comprehensive coverage of the pachychilid fauna of all five rivers, however, is required if we want to understand the evolution of the Kaek River species flock, due to the geological history of the entire area which has seen altered flow regimes of rivers as explained above. The present phylogeny has been computed with *Brotia sumatrensis* used as outgroup because it was found by Köhler and Glaubrecht (2006) to be the sister group of the Central Thailand clade. The outgroup has subsequently been pruned from the tree.

In general, the phylogenetic tree (Fig. 10) has a very flat topology, and species recognised by their morphology fall not into monophyletic clusters but remain widely unresolved. However, the tree contains largely monophyletic, drainage-specific clades. Species from the Kaek River form a huge monophyletic crown-group, which includes admixed sequences of *B. armata*, *B. binodosa* and *Brotia* sp.

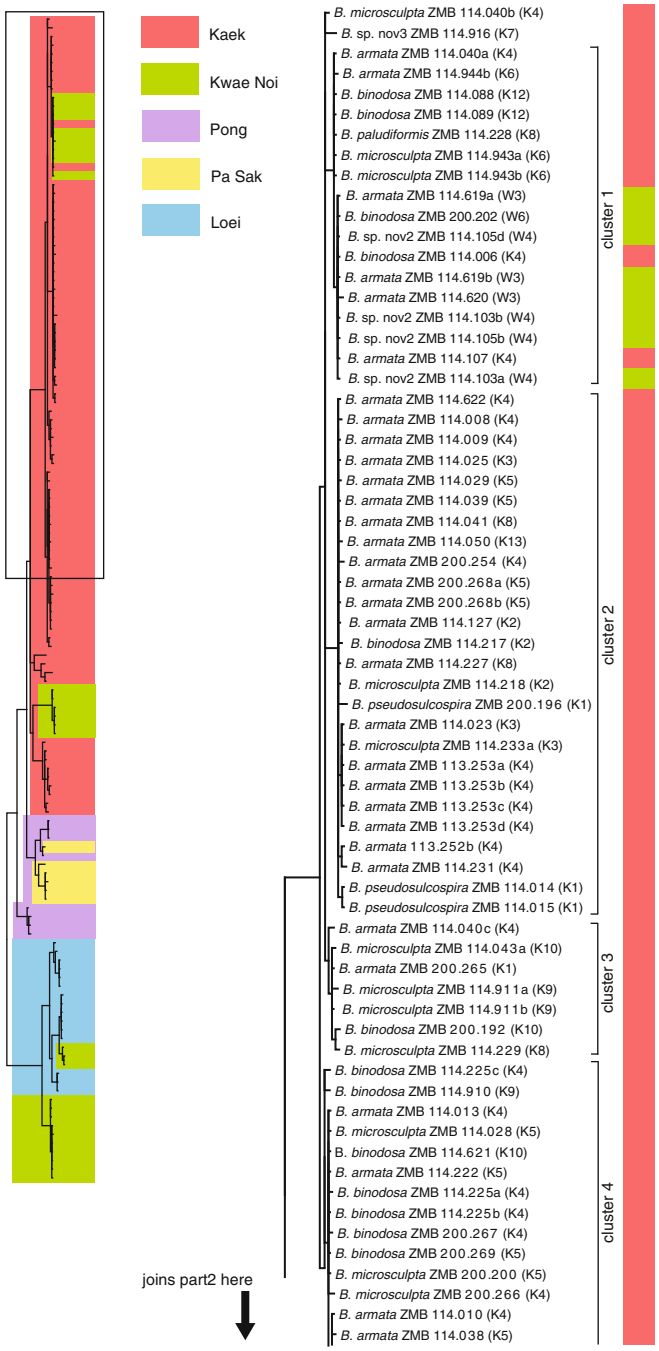


Fig. 10 Phylogenetic tree based on analyses of partial sequences of COI showing the relationships among the *Brotia* species from central Thailand as inferred by Bayesian Inference. Outgroup pruned from the tree (part 2, see former page for continuation) Left hand side: entire tree, right hand side: enlarged portion

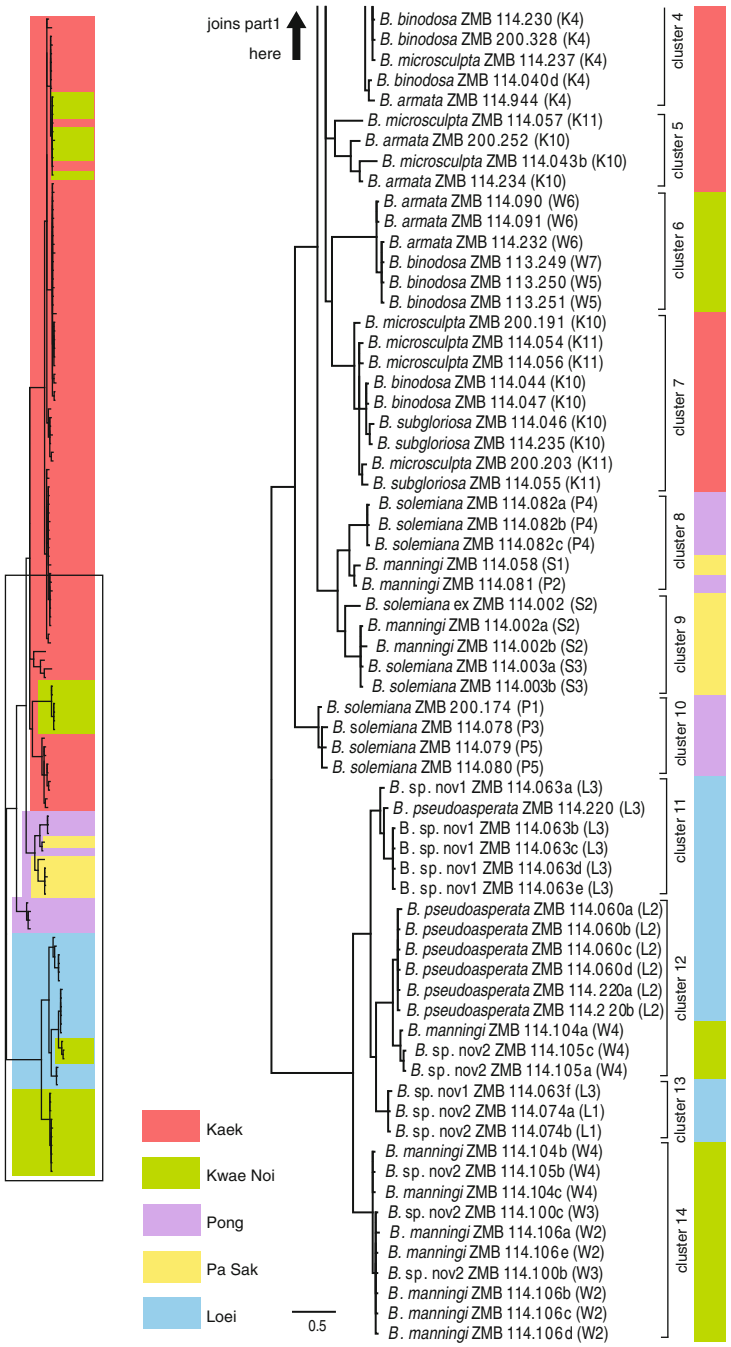


Fig. 10 (continued) (past 2, see forms page for continuation). Left hand side: entire tree, right hand side: enlarged portion

nov2 from the Kwae Noi (Kaek-Kwae Noi clade). Haplotypes from the Pong and Pa Sak River form a second clade (Pa Sak-Pong clade), which is the sister group of the Kaek-Kwae Noi clade. A further clade exclusively contains Pong River-specific haplotypes (Pong clade) and forms the sister group of the two previous clades together (Kaek-Kwae Noi + Pa Sak-Pong). Eventually, at the most basal bifurcation of the tree, a clade containing haplotypes from the Loei and Kwae Noi Rivers (Loei-Kwae Noi clade) forms the sister group of all previously mentioned clades. All aforementioned river clades are well-differentiated from each other by means of average genetic distances between around 5 and 12% (Tables 3 and 4).

The Kaek-Kwae Noi clade comprises seven more or less well-differentiated haplotype clusters as well as two single sequences that do not cluster together with any of the others. These sequences may represent another rare (or rarely sampled) haplotype cluster. The mean sequence divergence within the clusters does not exceed a maximum of 1.5% while the divergence between clusters ranges between 1.3 and 7.5% (Table 3).

The three haplotype clusters at more basal positions of the Kaek-Kwae Noi clade (5–7) are especially well differentiated and contain only sequences of specimens collected at upstream localities (K10–K11) or in the Kwae Noi drainage (W5–W7), while the haplotype clusters at more derived positions (1–4) show little genetic differentiation overall and contain mostly specimens collected at midstream locations (K1–K6), but also admixed sequences from upstream localities (K10–K11) and the Kwae Noi drainage (W3–W6). In addition, there is one well-differentiated

Table 3 Mean sequence divergence within and between the haplotype clusters 1–7 of the Kaek-Kwae Noi clade (in %, Kimura-2-parameters)

Cluster	1	2	3	4	5	6	7
1	0.1						
2	0.2	0.2					
3	1.3	1.7	0.4				
4	1.4	1.8	1.1	0.4			
5	4.1	4.7	4.1	4.4	1.5		
6	6.4	7.5	7.0	6.7	5.8	0.2	
7	4.8	5.6	5.1	4.9	4.3	5.6	0.6

Table 4 Mean sequence divergence within and between haplotype clusters and phylogenetic clades from different drainage systems (in %, Kimura-2-parameters model). Bold frames: sequence divergence within drainage-specific clades

Clade	Kaek- Kwae Noi	Pa Sak-Pong			Pong	Loei-Kwae Noi		Kwae Noi
Cluster	1–7	8	9	10	11	12	13	14
1–7	0.1–7.5							
8	5.8–6.9	1.1						
9	4.9–6.1	3.3	0.9					
10	5.8–11.2	5.7	5.7	0.5				
11	9.5–11.2	9.5	10.7	10.9	0.5			
12	9.6–11.7	9.2	10.1	10.5	3.2	0.4		
13	10.2–12.2	9.5	11.1	11.0	2.6	2.6	0.1	
14	9.1–11.4	9.1	10.0	10.1	4.4	4.1	4.1	0.1

haplotype cluster (6) that exclusively contains sequences of *B. armata* and *B. binodosa* from the Kwae Noi drainage.

Within the Pa-Sak-Pong clade, there are three distinct haplotype clusters that are separated from each other by genetic distances between 3.3 and 5.9%, while the genetic differentiation within the clusters does not exceed 1.1% (Table 4). The Loei-Kwae Noi clade contains two clusters with 2.6% sequence divergence. In addition, there is an additional, well-differentiated river-specific cluster within each of the Pong and Kwae Noi River clades (Fig. 10; Table 4).

Among *Brotia*, interspecific distances in COI between morphologically well-differentiated and allopatric species were frequently found to be as low as 1.4–1.7% (Köhler and Glaubrecht 2006). This value is considered to represent a conservative estimate of a minimum threshold for interspecific rates of sequence divergence in this group. Compared to this threshold, the amount of genetic differentiation within the Central Thailand clade of *Brotia* would be equivalent to the existence of at least 12 distinct (species-specific) gene pools (or 13 if the 2 single sequences are considered that do not cluster together with others). Accordingly, within the Kaek-Kwae Noi clade there are between 5 and 6 such distinct groups (clusters 1 + 2, 3 + 4, 5, 6, 7, single sequences) plus 3 within the Pa Sak-Pong clade, 2 within the Loei-Kwae Noi clade, and 1 each within the Pong and Kwae Noi clades, respectively. This number of genetically well-differentiated groups correlates perfectly with the total number of 13 species as recognised by their morphology (*B. armata*, *B. binodosa*-A, *B. binodosa*-B, *B. manningi*, *B. microsculpta*, *B. paludiformis*, *B. pseudoasperata*, *B. pseudosulcospira*, *B. solemiana*, *Brotia* sp. nov1, *Brotia* sp. nov2, *Brotia* sp. nov3, *B. subgloriosa*) (see Fig. 11 for photographs of living specimens of some of these species). In addition, there is also a good correlation between the numbers of genetically distinct groups and the numbers of recognised "morphospecies" in each river drainage (genetic group/morphospecies): Kaek River (6/7), Kwae Noi (3/4), Loei (3/3), Pong (2/2), Pa Sak (2/2).

However, generally, there is a significant mismatch between the branching of the mitochondrial gene tree into haplotype clusters or groups and the distribution of morphologically recognised species across this topology, which apparently renders all morphospecies as polyphyletic assemblages (Fig. 10). The average infraspecific genetic distances among most of these morphospecies exceed by far the empirical minimum threshold of interspecific differentiation as mentioned above (with values up to 5.8%) and lie well within the range of observed interspecific genetic distances of 1.8–10.7%.

9 Towards an Evolutionary Explanation: Conclusions from Incongruence

Other studies of cerithioidean freshwater gastropods have also revealed in part extensive incongruence between the branching patterns of mitochondrial gene trees and the delineation of (putative) species by use of morphological characters

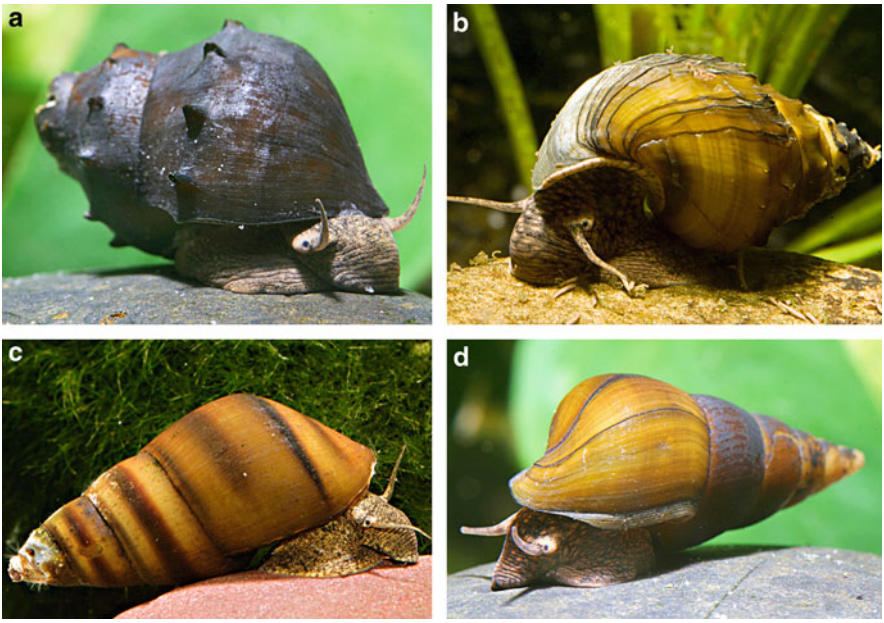


Fig. 11 Photographs of living specimens. (**a**) *Brotia armata*. (**b**) *Brotia binodosa*. (**c**) *Brotia manningi*. (**d**) *Brotia subgloriosa*. Photos (**a**,**d**) courtesy of Chris Lukhaup (Bittenfeld), photos (**b**,**c**) courtesy of Andreas Helmenstein (Gummersbach)

(Lydeard et al. 2002; Minton and Lydeard 2003; Wilson et al. 2004; Köhler and Glaubrecht 2006; Lee et al. 2007; Rintelen et al. 2007; Glaubrecht and Rintelen 2008; Köhler and Dames 2009; Köhler et al. 2009; Strong and Köhler 2009). Workers attributed these discrepancies between gene and species trees to a wide range of phenomena, including incomplete lineage sorting, the existence of cryptic species, taxonomical over-splitting of lineages, and hybridisation. However, strikingly different conclusions were drawn from quite similar observations depending on which actual cause has been postulated. For example, in Korean *Semisulcospira* populations (Semisulcospiridae), both mitochondrial (16S) and nuclear (28S) genes of seven species recognised by their morphology (*S. libertina*, *S. coreana*, *S. forticosta*, *S. gottschei*, *S. multicincta*, *S. nodiperda*, *S. tegulata*) revealed a structure that followed geographical rather than taxonomic trajectories, with haplotypes and genotypes largely clustering into drainage-specific clades, but without resolution with respect to morphologically delineated species (Lee et al. 2007). The authors concluded that pending the demonstration of any reliable differentiation within this complex, all but one species should be synonymised into a single polymorphic species complex – *S. libertina*. In contrast, faced with similar phenomena in the pachychilid taxon *Tylomelania*, Rintelen et al. (2004) and Glaubrecht and Rintelen (2008) suggested that incomplete lineage sorting and introgressive hybridisation caused the mismatch of gene and species trees, and

concluded that mitochondrial gene trees are misleading with respect to the recognition of species. In general, we agree that the pervasiveness of this phenomenon across various freshwater cerithioidean groups, in combination with the specifics of mitochondrial DNA inheritance (Nichols 2001; Funk and Omland 2003; Ballard and Whitlock 2004; White et al. 2008), corroborate the notion that mitochondrial markers may have limited utility in assessing status at the species level, and that a meaningful molecular characterisation of species should make use of a combination of mitochondrial and fast evolving nuclear markers (in addition to morphology, of course). We anticipate that amplified fragment length polymorphism (AFLP) or microsatellite analyses are the methods of choice to address species limits within the present group, since both methods are known as powerful tools to resolve relationships at the population to species-level and to investigate the gene flow between populations (see, e.g., Richard and Thorpe 2001; Albertson et al. 1999; Savekoul et al. 1999). The application of AFLP analyses is part of the present research project; the completion of the analyses is pending.

Nevertheless, even in the absence of comparative analyses of nuclear markers, the patterns of morphological and mitochondrial differentiation provide intriguing insights into the evolution of the Kaek River species flock. As mentioned above, in principle, several phenomena may explain the mismatch between gene and species trees in the present case. We exclude the possibility that the incongruence is caused by nuclear pseudogenes ('numts'), because translation of the analysed COI sequence alignment into amino acid sequences produced a highly conserved alignment that did not contain stop codons or gaps. We also do not consider ancestral polymorphism as a possible explanation for incongruence because the observed rates of sequence divergence of up to 1.5% within and 5.8% between haplotype clusters are considered to be out of the range of infraspecific polymorphism. Ancestral polymorphism may only be considered as an explanation for the unresolved relationships within sub-clades that overall show low rates of genetic differentiation, such as haplotype clusters 1–4. In contrast, the presence of morphologically cryptic species cannot be entirely ruled out as a possible cause for apparently unresolved species limits. The presence of potentially misidentified (=cryptic) species could indeed explain why morphologically similar populations from different river drainages, such as those attributed to *B. manningi* or *Brotia* sp. nov2, appear at different positions in the tree, or why Kaek River species, such as *B. microsculpta*, seem to have two different radula types. However, this explanation is very unlikely to account for genetic admixtures among morphologically distinctive species within a single drainage system, such as *Brotia* sp. nov1 and *B. pseudosulcospira* in the Loei drainage or *B. manningi* and *Brotia* sp. nov2 in the Kwae Noi drainage. In these cases, the low rates of genetic differentiation contradict the presence of further unrecognised species. Furthermore, morphological shell polymorphism (e.g. ecophenotypism) is also unlikely to account for the unresolved species limits. It has been demonstrated that modifications of shell sculptures may occur in freshwater cerithioideans depending on the substrate (Urabe 2000). However, it has also been demonstrated that ecophenotypism is restricted to

relatively small changes while clear differences (as found here) are considered to be genetically controlled (Gittenberger et al. 2004; Haase and Misof 2009).

We are convinced that introgressive hybridisation caused by cross-breeding is the most likely cause for a great deal of the observed incongruence between the mitochondrial gene and the morphological species tree – however, this is a hypothesis that can only be validated by comparative analysis of genetic markers from other linkage groups (i.e. nuclear genes). In fact, various studies of land snails have shown that introgressive hybridisation, though difficult to demonstrate conclusively, accounts for unresolved species limits in mitochondrial gene trees (Thacker and Hadfield 2000; Goodacre and Wade 2001; Haase et al. 2003; Haase and Misof 2009), and similar conclusions were drawn for pachychilid freshwater gastropods (Glaubrecht and Rintelen 2008; Köhler et al. 2009). In most of their range across South and Southeast Asia, *Brotia* species have restricted distributions, being confined to the headwaters of single rivers or creeks but absent from the lower courses of larger streams. There are usually two species at the most that co-occur in a given habitat while the majority of species occurs in allopatry or parapatry. Accordingly, Köhler et al. (2009) suggested that geographical separation is the main factor that drives speciation in pachychilids in the rivers of mainland Asia, and that, when no isolation mechanisms have evolved that prevent species from cross-breeding, secondary contact between originally allopatric populations or species frequently leads to the introgression of neutral markers. In agreement with this hypothesis, the more or less random distribution of morphotypes across drainage-specific haplotype clades is probably best explained by introgression of mitochondrial genes into foreign gene pools due to secondary contact of previously isolated populations or species caused by the translocation of specimens either due to dispersal or vicariance events. Indeed, this assumption agrees well with theoretical considerations, which predict that foreign invasions of already occupied territories lead to massive introgression of neutral genes if interbreeding is not severely prevented between invading and local species. In such cases, introgression occurs almost exclusively from the local to the invading species, especially for populations located far away from the source of the invasion, and this occurs irrespective of the relative densities of the two species (Currat et al. 2008). It has also been argued by the authors that this pattern is strongest in markers experiencing reduced gene flow, which implies that organelle genes are often preferentially introgressed across species boundaries. Such massive introgression has the potential to explain the observed rates of discordance in the COI tree presented here. In addition, we believe that the presence of two different radula types in *B. microsculpta* can be attributed to the existence of species hybrids. *Brotia microsculpta* exhibits a very distinct radular morphology (type 3), while type 2, which is somehow an intermediate form between types 1 and 3, is possibly found in hybrids. Because most *Brotia* species have radulae of the generalised type 1 anyway, their hybrids cannot be recognised by the radula morphology.

10 Dispersal or Vicariance: Genetic Exchange Between River Faunas and the Relevance of River Captures Within the Mekong Drainage System

Above, we have postulated that massive introgression of haplotypes occurred due to extensive faunal exchange across the five river drainages studied herein. We were interested to learn whether there are corresponding patterns in the timing of geological events in the region (i.e. river captures) and the occurrence of major splits in the phylogenetic tree. We performed a likelihood ratio test in order to test whether our sequence data would allow for a molecular clock approach. However, a chi squared test showed that Bayesian trees produced under the conditions of a strict clock resulted in significantly lower likelihood scores compared to an analysis in which branches were allowed to evolve at variable rates. The application of a molecular clock under use of an external calibration as suggested by Wilke (2003) for our COI data was therefore refuted. It was thus not possible to test whether certain splits in the tree fall within the time frame of major tectonic events in Central Thailand.

The fossil record of *Brotia* dates back as far as Middle Miocene (Annandale 1919; Gurung et al. 1997), which is equivalent to a minimum age of the entire group of at least 8–12 Ma. Even though the Central Thailand clade is found at a derived position in the molecular tree, it is plausible to assume that it has originated several million years ago. Accordingly, we postulate that the era from the late Pliocene to the Quaternary was critical for the evolution of the gastropods under study. This was a time when stream captures of various magnitudes impacted river alignments in northern Central Thailand as a result of local tectonic or hydrological processes (see above). Probably the most important event was the realignment of the Mekong River, which between ~1 and 0.05 mya flowed through the Loei-Pa Sak river beds. Because *Brotia* species do usually not inhabit the mid- and upstream regions of larger rivers, changes of the flow direction of the Mekong may have both connected populations in earlier stages of the realignment of stream, when the flow regime has been at a lower magnitude, and separated populations, when the Mekong formed a large stream which was not a suitable habitat for *Brotia*. For instance, it is considered possible that the disjunctive distribution of *B. manningi*, which occurs in both the Pa Sak and Kwai Noi drainage, may have been caused by the realignment of the Mekong. Genetic exchange between river drainages may have occurred either due to the translocation of specimens from one river to the other (dispersal) or due to events related to the geological history of the area (vicariance). The importance of dispersal is difficult to both reject or confirm. However, we believe that tectonic events and processes since the mid-Tertiary have likely influenced the evolution and distribution of species by mediating phases of contact and isolation of faunas through the capture or separation of river systems. Attwood and Johnston (2001) have shown that episodic changes of river catchments have had a significant influence on the distribution and evolution of pomatiopsid snails by separating and reconnecting populations or species. There is little doubt that other freshwater

animals with low dispersal abilities, such as pachychilid gastropods, may also have been affected by these changes. However, while the mtDNA tree provides information on the divergence of clades, it tells us little about gene flows between drainage-specfic clades because introduced foreign haplotypes become quickly replaced by local, drainage-specific haplotypes due to the general directionality of introgression from local to alien species (Currat et al. 2008). Therefore, unlike divergences, events that connected river faunas are difficult to trace in the mtDNA-based phylogeny. Gene flows across the borders of drainage systems can probably be confirmed only if they have occurred more recently, because then the foreign haplotypes may not yet have been completely replaced by local ones. This seems to be the case in the phylogenetically derived Kaek River-specific haplotype cluster 1, which also contains specimens from the Kwae Noi drainage. The low genetic differentiation suggests that the underlying gene flow between the Kaek and Kwae Noi Rivers must have occurred rather recently.

11 Speciation and Radiation of *Brotia* in the Kaek River

The *Brotia* species flock in the Kaek River shows some unique aspects that call for an explanation. The number of *Brotia* species occurring in the river exceeds that found in any other river across SE Asia by at least two times. Additionally, these species live largely in sympatry whereas species in other drainages mostly occur in different sectors or tributaries in complete spatial isolation or with only narrow zones of contact. The dense sampling regime covering the entire region of northern Central Thailand has revealed that all but one species (*B. armata*) are indeed endemic to the Kaek River and, consequently, must have evolved within the drainage system. Glaubrecht and Köhler (2004) argued that the lack of resolution in the molecular phylogeny and its shallow topology indicate the recent origin of the Kaek River species flock and, consequently, a rapid morphological divergence of its constituent species. Preliminary results have suggested that the Kaek River species flock may have evolved as a result of an adaptive radiation and that ecological factors may have driven speciation. It has been the foremost goal of the present study to test this hypothesis. Streelman and Danley (2003) suggested for vertebrates that radiations usually follow similar evolutionary trajectories. Groups diverge along the axes of habitat and trophic morphology as well as communication, often in that order. They argued that divergence with respect to habitat and trophic morphology is likely to follow ecological selection models and that divergence with respect to communication proceeds according to sexual selection models. In agreement with this postulate, studies of the confamiliar gastropod genus *Tylomelania* have shown that indeed substrate choice and trophic specialisation seem to trigger speciation. It remained to be tested if corresponding patterns were to be found in the Kaek River species flock. However, herein we show that the *Brotia* species in the Kaek River do not differ with respect to their preferred substrate or the water depth at which they were found. The radular dentition of

most species is very similar, and only two species differ clearly from all others by possessing a distinct radula type (*B. microsculpta*, *Brotia* sp. nov2). Both findings suggest that habitat-mediated segregation and trophic specialisation have not played a significant role in the evolution of the Kaek River species flock. In addition, it is unlikely that speciation has been triggered by sexual selection due to the likely presence of species hybrids, which hints towards incomplete mechanisms of postzygotic isolation. Consequently, to our current knowledge, there is no evidence in favour of the assumption that ecological speciation has accounted for the diversity of species in the Kaek River. Alternatively, speciation within the Kaek River is currently best explained by geographical isolation. Firstly, it cannot be ruled out that some species originate from outside the Kaek River and that introgressed Kaek River-specific mtDNA has replaced the foreign haplotypes, by obscuring traces of repeated river colonisation (see Currat et al. 2008). Secondly, it is suggested by the mtDNA-based phylogeny that gene flow within the Kaek River occurs predominantly from upstream to downstream areas because basal haplotype clusters belong exclusively to upstream populations whereas derived haplotype clusters contain a mixture of both up- and midstream populations. The Kaek River flows over a series of waterfalls and cascades. Although according to own observations snails are able to crawl above the water line and may thus in principle be able to climb the vertical walls, the waterfalls seem to form barriers that significantly delimit the gene flow across vertical structures against the direction of flow. In addition, the water regime of the Kaek River is rather unstable. The ground, consisting predominantly of sandstone and limestone, is very permeable for water, which may cause large sectors of the river to fall dry during extended periods of drought. Even in regular dry seasons, the water body of the Kaek River is largely reduced and some of its affluents become entirely dry (Fig. 4f).These fluctuations regularly cause local extinctions in restricted stretches of the river and its affluents, which are followed by re-colonisation of the areas in the rainy season. Both factors, river fragmentation by waterfalls and regular local extinctions, may assist the retention of a reticulate genetic structure and the conserving of rates of local genetic differentiation. Moreover, extended periods of drought may have occurred during the Cenozoic and could have triggered speciation in peripheral isolates – a process generally considered as a significant modus of allopatric speciation (Mayr 1963). It has been shown for plants that small populations may differentiate quickly (Ellstrand and Elam 1993), and the limitations for gene flow as described above may have assisted this genetic differentiation to persist. In analogy, it has been confirmed by theoretical considerations that rapid parapatric speciation on the time scale of up to a few thousand generations is plausible even in the presence of moderate genetic exchange between neighbouring subpopulations. Divergent selection for local adaptation is also not required for the evolution of reproductive isolation as a by-product of genetic divergence (Gavrilets et al. 2000). The authors showed that populations or species with small range sizes should have higher speciation rates – circumstances that probably do apply in the present case.

Hence, the present model case of a riverine radiation apparently does not follow the same evolutionary trajectories as recently demonstrated for a number

of lacustrine radiations of various groups of animals, which involve a major ecological component (i.e. ecological speciation, sensu Schluter 2000). By contrast, the flock of *Brotia* species in the Kaek River has more in common with other riverine radiations, such as the Triculinae of the Mekong. It has been demonstrated that speciation and radiation in these freshwater snails were triggered by geological events, such as the uplift of mountain chains, lava flows, and river captures or realignments, as well as waves of local extinctions and re-colonisations (Davis 1979, 1981; Attwood and Johnston, 2001) – all of which have probably inititiated speciation in peripheral isolates. Similar patterns have been observed in the Tasmanian hydrobiid *Beddomeia* (Ponder et al. 1993) and the hydrobiid *Fluvidona* in Victoria, Australia, (Ponder et al. 1994). Both radiations involve small-range species. The mode of speciation is allopatric or parapatric and mainly driven by vicariance due to restriction of ranges resulting in isolation and subsequent differentiation of peripheral populations. This does not exclude secondary sympatry of closely related species, such as in *Fluvidona*, which followed events of speciation in isolation due to restrictions of habitats. In summary, we conclude that, with respect to adaptive radiations of freshwater organisms, long-lived lakes provide unique environmental conditions that may facilitate ecological speciation. In contrast, rivers apparently provide different conditions that favour para- and allopatric models of speciation.

Acknowledgements The authors most gratefully acknowledge the funding by the German Research Foundation (DFG, KO 3381/3-1). In addition, we would like to thank the National Research Council of Thailand (NRCT) for kindly permitting fieldwork in Thailand. We owe very special thanks to Claudia Dames (Berlin) for the great help with all kinds of routine work, such as specimen preparation, SEM, and shell measurements. We are also grateful to Thomas von Rintelen and Robert Schreiber (both Berlin) for helping with laboratory work. Finally, we wish to express our thanks to two reviewers for critically reading a former version of the manuscript. Their constructive comments helped to improve the quality of this paper. Thanks are also due to Rosemary Golding (Sydney) for stylistic improvements of the text.

References

Albertson RCJ, Markert A, Danley PD, Kocher TD (1999) Phylogeny of a rapidly evolving clade: the cichlid fishes of Lake Malawi, East Africa. Proc Nat Acad Sci USA 96:5107–5110

Allan JD (1995) Stream ecology. Structure and function of running waters. Chapman & Hall, London

Annandale N (1919) The gastropod fauna of old lake-beds in upper Burma. Rec Geol Surv India 50:209–240

Attwood SW, Johnston DA (2001) Nucleotide sequence differences reveal genetic variation in *Neotricula aperta* (Gastropoda: Pomatiopsidae), the snail host of schistosomiasis in the lower Mekong basin. Biol J Linn Soc 73:23–41

Ballard JWO, Whitlock MC (2004) The incomplete natural history of mitochondria. Mol Ecol 13:729–744

Benthem Jutting WSSv (1956) Systematic studies on the non-marine Mollusca of the Indo-Australian archipelago. V. Critical revision of the Javanese freshwater gastropods. Treubia 23:259–477

Binder E (1959) Anatomie et systématiques des melaniens d'Afrique occidentale. Rev Suisse Zool 66:735–759
Brandt RAM (1968) Description of new non-marine mollusks from Asia. Arch Molluskenkd 98:213–289
Brandt RAM (1974) The non-marine aquatic Mollusca of Thailand. Arch Molluskenkd 105:1–423
Brown DS (1988) *Sierraia* – rheophilous West-African river snails (Prosobranchia, Bithyniidae). Zool J Linn Soc 93:313–355
Brown DS (1994) Freshwater snails of Africa and their medical importance. Taylor and Francis, London
Chonglakmani C, Helmcke D (2001) Geodynamic evolution of Loei and Phetchabun regions - does the discovery of detrial chromian spinels from the Nam Duk Formation (Permian, north-central Thailand) provide new constraint? Gondwana Res 4:437–442
Cooper M, Herbert A, Hill GS (1989) The structural evolution of Triassic intermontane basins in northeastern Thailand. In: Proceedings of International Symposium lntermontane Basins: Geology and Resources. Chiang Mai, Thailand, pp 231–242
Currat M, Ruedi M, Petit RJ, Excoffier L (2008) The hidden side of invasions: massive introgression by local genes. Evolution 62:1908–1920
Davis GM (1979) The origin and evolution of the gastropod family Pomatiopsidae, with emphasis on the Mekong River Triculinae. Monogr Acad Nat Sci Phila 20:1–120
Davis GM (1981) Different modes of evolution and adaptive radiation in the Pomatiopsidae (Prosobranchia: Mesogastropoda). Malacologia 21:209–262
Davis GM (1982) Historical and ecological factors in the evolution, adaptive radiation, and biogeography of freshwater mollusks. Am Zool 22:375–395
DNP (2009). Homepage of the national park division, Bangkok. http://www.dnp.go.th. Cited 14 Feb 2009
Dudgeon D (1995) The ecology of rivers and streams in tropical Asia. In: Cushing CE, Cummins KW, Minshall GW (eds) Ecosystems of the world, vol 22. Elsevier, Amsterdam
Ellstrand NC, Elam DR (1993) Population genetic consequences of small population size: Implications for plant conservation. Annu Rev Ecol Syst 24:217–242
Funk DJ, Omland KE (2003) Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. Annu Rev Ecol Evol Syst 34: 397–423
Gavrilets S, Losos JB (2009) Adaptive radiation: contrasting theory with data. Science 323: 732–737
Gavrilets S, Li H, Vose MD (2000) Patterns of parapatric speciation. Evolution 54:1126–1134
Giller PS, Malmqvist B (1998) The biology of streams and rivers. Oxford University Press, Oxford
Glaubrecht M (1996) Evolutionsökologie und Systematik am Beispiel von Süß- und Brackwasserschnecken (Mollusca: Caenogastropoda: Cerithioidea): Ontogenese-Strategien, paläontologische Befunde und historische Zoogeographie. Backhuys, Leiden
Glaubrecht M (1999) Systematics and the evolution of viviparity in tropical freshwater gastropods (Cerithioidea: Thiaridae sensu lato) – an overview. Courier Forsch-Inst Senckenberg 215: 91–96
Glaubrecht M (2006) Independent evolution of reproductive modes in viviparous freshwater Cerithioidea (Gastropoda, Sorbeoconcha) – a brief review. Basteria 69(suppl 3):28–32
Glaubrecht M (2008) Adaptive radiation of thalassoid gastropods in Lake Tanganyika, East Africa: morphology and systematization of a paludomid species flock in an ancient lake. Zoosyst Evol 84:71–122
Glaubrecht M, Köhler F (2004) Radiating in a river: systematics, molecular genetics and morphological differentiation of viviparous freshwater gastropods endemic to the Kaek River, central Thailand (Cerithioidea, Pachychilidae). Biol J Linn Soc 82:275–311
Glaubrecht M, von Rintelen T (2003) Systematics and zoogeography of the pachychilid gastropod *Pseudopotamis* Martens, 1894 (Mollusca: Gastropoda: Cerithioidea): a limnic relict on the Torres Strait Islands, Australia? Zool Scr 32:415–435

Glaubrecht M, von Rintelen T (2008) The species flocks of lacustrine gastropods: *Tylomelania* on Sulawesi as models in speciation and adaptive radiation. Hydrobiologia 615:181–199

Gregory JW (1925) The evolution of the river systems of south-eastern Asia. Scott Geogr Mag 41:129–141

Gittenberger E, Piel WH, Groenenberg DSJ (2004) The Pleistocene glaciations and the evolutionary history of the polytypic snail species *Arianta arbustorum* (Gastropoda, Pulmonata, Helicidae). Mol Phylogenet Ecol 30:64–73

Goodacre SL, Wade CM (2001) Patterns of genetic variation in Pacific island land snails: the distribution of cytochorme b lineages among society Island *Partula*. Biol J Linn Soc 73:131–138

Grossmann E (1967) Zur Lebensweise und Fortpflanzungsbiologie von *Melanatria fluminea* (Gmelin) (Gastropoda). Sitzungsber math-naturwiss Abt 176:1–4

Gupta A (2008) The Mekong: morphology, evolution, management. In: Gupta A (ed) Large rivers. Wiley, New York, pp 435–455

Gurung D, Takayasu K, Matsuoka K (1997) Middle Miocene-Pliocene freshwater gastropods of the Churia group, west-central Nepal. Pal Res 1:166–179

Haase M, Misof B (2009) Dynamic gastropods: stable shell polymorphism despite gene flow in the land snail *Arianta arbustorum*. J Zool Syst Evol Res 47:105–114

Haase M, Misof B, Wirth T, Baminger H, Baur B (2003) Mitochondrial differentiation in a polymorphic land snail: evidence for Pleistocene survival within the boundaries of permafrost. J Evol Biol 16:415–428

Hauer FR, Lamberti GA (1996) Methods in stream ecology. Academic, San Diego

Hoagland KE, Davis GM (1979) The stenothyrid radiation of the Mekong River. I. The *Stenothyra mcmulleni* complex (Gastropoda: Prosobranchia). Proc Acad Nat Sci Phila 131:191–230

Hutchinson CS (1989) Geological evolution of South-east Asia. Clarendon, Oxford

Illies J (1961) Versuch einer allgemeinen biozönotischen Gliederung der Fließgewässer. Int Rev Gesamten Hydrobiol 46:205–213

Köhler F, Brinkmann N, Glaubrecht M (2008) Convergence causes confusion: on the systematics of the freshwater gastropod *Sulcospira pisum* (Brot, 1868) (Cerithioidea, Pachychilidae). Malacologia 50:331–339

Köhler F, Dames C (2009) Phylogeny and systematics of the Pachychilidae of mainland Southeast Asia – novel insights from morphology and mitochondrial DNA (Mollusca, Caenogastropoda, Cerithioidea). Zool J Linn Soc 157:679–699

Köhler F, Glaubrecht M (2001) Toward a systematic revision of the Southeast Asian freshwater gastropod *Brotia* H Adams, 1866 (Cerithioidea:Pachychilidae): an account of species from around the South China Sea. J Molluscan Stud 67:281–318

Köhler F, Glaubrecht M (2002) Annotated catalogue of the nominal taxa of Southeast Asian freshwater gastropods, family Pachychilidae Troschel, 1857 (Mollusca, Caenogastropoda, Cerithioidea), with an evaluation of the types. Mitt Mus Naturkd Berl Zool Reihe 78:121–156

Köhler F, Glaubrecht M (2003) Morphology, reproductive biology and molecular genetics of ovoviviparous freshwater gastropods (Cerithioidea, Pachychilidae) from the Philippines, with description of a new genus *Jagora*. Zool Scr 32:35–59

Köhler F, Glaubrecht M (2005) Fallen into oblivion - the systematic affinities of the enigmatic *Sulcospira* Troschel, 1858 (Cerithioidea: Pachychilidae), a genus of viviparous freshwater gastropods from Java. Nautilus 119:15–26

Köhler F, Glaubrecht M (2006) A systematic revision of the Southeast Asian freshwater gastropod *Brotia* (Cerithioidea: Pachychilidae). Malacologia 48:159–251

Köhler F, Glaubrecht M (2007) Out of Asia and into India: on the molecular phylogeny and biogeography of the endemic freshwater gastropod *Paracrostoma* Cossmann, 1900 (Caenogastropoda: Pachychilidae). Biol J Linn Soc 91:627–651

Köhler F, Holford M, Do VT, Ho TH (2009) Exploring a largely unknown fauna: on the diversity of pachychilid freshwater gastropods in Vietnam (Caenogastropoda: Cerithioidea). Molluscan Res 29:121–146

Köhler F, von Rintelen T, Meyer A, Glaubrecht M (2004) Multiple origin of viviparity in Southeast Asian gastropods (Cerithioidea: Pachychilidae) and its evolutionary implications. Evolution 58:2215–2226

Lee T, Hong HC, Kim JJ, O'Foighil D (2007) Phylogenetic and taxonomic incongruence involving nuclear and mitochondrial markers in Korean populations of freshwater snail genus *Semisulcospira* (Cerithioidea: Pleuroceridae). Mol Phylogenet Evol 43:386–397

Lydeard C, Holznagel WE, Glaubrecht M, Ponder WF (2002) Molecular phylogeny of a circumglobal, diverse gastropod superfamily (Cerithioidea: Mollusca: Caenogastropoda): pushing the deepest phylogenetic limits of mitochondrial LSU rDNA sequences. Mol Phylogenet Evol 22:399–406

Martinson DG, Pisias NG, Hays JD, Imbrie J, Moore TC, Shackleton NJ (1987) Age dating and orbital theory of the ice ages: development of a high resolution 0-300 000 year chronostratigraphy. Quart Res 27:1–29

Mayr E (1963) Animal species and evolution. Belknap, Cambridge, MA

Minton RL, Lydeard C (2003) Phylogeny, taxonomy, genetics and global heritage ranks of an imperilled, freshwater snail genus *Lithasia* (Pleuroceridae). Mol Ecol 12:75–87

Morrison JPE (1954) The relationships of old and new world melanians. Proc U S Natl Mus 103:357–394

Nichols R (2001) Gene trees and species trees are not the same. Trends Ecol Evol 16:358–364

Padilla DK (1998) Inducible phenotypic plasticity of the radula in *Lacuna* (Gastropoda: Littorinidae). Veliger 4:201–204

Ponder WF, Clark GA, Miller AC, Toluzzi A (1993) On major radiation of freshwater snails in Tasmania and eastern Victoria: a preliminary overview of the *Beddomeia* group (Mollusca: Gastropoda: Hydrobiidae). Invertebr Taxon 7:501–750

Ponder WF, Colgan DJ, Clark GA, Miller AC, Terzis T (1994) Microgeographic, genetic and morphological differentiation of freshwater snails – the Hydrobiidae of Wilson's Promontory, Victoria, South-eastern Australia. Aust J Zool 42:557–678

Rainboth WJ (1996) The taxonomy, systematics, and zoogeography of *Hypsibarbus*, a new genus of large barbs (Pisces, Cyprinidae) from the rivers of Southeastern Asia. Univ Calif Publ Zool 129:1–199

Reid DG, Mak YM (1999) Indirect evidence for ecophenotypic plasticity in radular dentition of *Littoraria* species (Gastropoda: Littorinidae). J Moll Stud 65:355–370

Richard M, Thorpe RS (2001) Can microsatellites be used to infer phylogenies? Evidence from population affinities of the Western Canary Island lizard (*Gallotia galloti*). Mol Phylogenet Evol 20:351–360

von Rintelen T, Glaubrecht M (1999) On the reproductive anatomy of freshwater gastropods of the genera *Brotia* H Adams, 1866 and Tylomelania Sarasin & Sarasin, 1897 in the central lakes on Sulawesi, Indonesia (Cerithioidea: Melanatriidae). Cour Forschungsinst Senckenb 125:163–170

von Rintelen T, Glaubrecht M (2003) New discoveries in old lakes: three new species of *Tylomelania* Sarasin and Sarasin, 1897 (Gastropoda: Cerithioidea: Pachychilidae) from the Malili lake system on Sulawesi, Indonesia. J Molluscan Stud 69:3–17

von Rintelen T, Glaubrecht M (2005) Anatomy of an adaptive radiation: a unique reproductive strategy in the endemic freshwater gastropod *Tylomelania* (Cerithioidea: Pachychilidae) on Sulawesi, Indonesia, and its biogeographic implications. Biol J Linn Soc 85:513–542

von Rintelen T, Bouchet P, Glaubrecht M (2007) Ancient lakes as hotspots of diversity: a morphological review of an endemic species flock of *Tylomelania* (Caenogastropoda: Cerithioidea: Pachychilidae) in the Malili lake system on Sulawesi, Indonesia. Hydrobiologia 592:11–94

von Rintelen T, von Rintelen K, Glaubrecht M (2010) The species flocks of the viviparous freshwater gastropod *Tylomelania* (Mollusca: Cerithioidea: Pachychilidae) in the ancient lakes of Sulawesi, Indonesia – the role of geography, trophic morphology and colour as driving

force in adaptive radiation. In: Glaubrecht M (ed) Evolution in action, pp 485–483. Springer, Berlin

von Rintelen T, Wilson AB, Meyer A, Glaubrecht M (2004) Escalation and trophic specialization drive adaptive radiation of viviparous freshwater gastropods in the ancient lakes on Sulawesi, Indonesia. Proc R Soc Lond B 271:2541–2549

Savelkoul PH, Aarts HJ, de Haas J, Dijkshoorn L, Duim B, Otsen M, Rademaker JL, Schouls L, Lenstra JA (1999) Amplified-fragment length polymorphism analysis: the state of an art. J Clinic Microbiol 37:3083–3091

Schluter D (2000) The ecology of adaptive radiation. Oxford University Press, Oxford

Simone LRL (2001) Phylogenetic analyses of Cerithioidea (Mollusca, Caenogastropoda) based on comparative morphology. Arq Zool 36:147–263

Solem A (1966) Some non-marine mollusks from Thailand, with notes on classification of the Helicarionidae. Spolia Zool Mus Hauniensis 24:1–110

Starmühlner F (1969) Die Gastropoden der Madagassischen Binnengewässer. Malacologia 8:1–434

Streelman JT, Danley PD (2003) The stages of vertebrate evolutionary radiation. Trends Ecol Evol 18:126–131

Strong EE, Köhler F (2009) A morphological and molecular analysis of "*Melania*" *jacqueti* Dautzenberg and Fischer, 1906: from anonymous orphan to critical basal offshoot of the Semisulcospiridae (Gastropoda: Cerithioidea). Zool Scr 38:483–502

Thacker RW, Hadfield GM (2000) Mitochondrial phylogeny of extant Hawaiian tree snails (Achatinellinae). Mol Phylogenet Evol 16:263–270

Thiele J (1928) Revision des Systems der Hydrobiiden und Melaniiden. Zool Jahrb Abt Syst Ökol Geogr Tiere 55:351–402

Thiele J (1929) Teil 1 Loricata; Gastropoda: Prosobranchia Handbuch der Systematischen Weichtierkunde, vol 1. Gustav Fischer, Jena

Urabe M (2000) Phenotypic modulation by the substratum of shell sculpture in *Semisulcospira reiniana* (Prosobranchia: Pleuroceridae). J Moll Stud 66:53–59

Vannote RL, Mishall GW, Cummins KW, Sedell JR, Cushing CE (1980) The river continuum concept. Can J Fish Aquat Sci 37:130–137

White DJ, Wolff JN, Pierson M, Gemmell NJ (2008) Revealing the hidden complexities of mtDNA inheritance. Mol Ecol 17:4925–4942

Wilke T (2003) *Salenthydrobia* gen. nov. (Rissooidea: Hydrobiidae): a potential relict of the Messinian salinity crisis. Zool J Linn Soc 137:319–336

Wilson AB, Glaubrecht M, Meyer A (2004) Ancient lakes as evolutionary reservoirs: evidence from the thalassoid gastropods of Lake Tanganyika. Proc R Soc Lond B 271:529–536

Woodruff DS (2003) Neogene marine transgressions, palaeogeography and biogeographic transitions on the Thai-Malay Peninsula. J Biogeogr 30:551–567

Yap SY (2002) On the distributional patterns of Southeast-East Asian freshwater fish and their history. J Biogeogr 29:1187–1199

The Neglected Side of the Coin: Non-adaptive Radiations in Spring Snails (*Bythinella* spp.)

Thomas Wilke, Mandy Benke, Martin Brändle, Christian Albrecht, and Jean-Michel Bichain

Abstract Recently, there has been increased scientific interest among evolutionary biologists in both causes and consequences of radiations. Whereas one form of radiation – adaptive radiation – has been studied extensively, another form – non-adaptive radiation – is discussed controversially and is poorly understood. In fact, the concept of non-adaptive radiation (i.e., rapid diversification of species not accompanied by adaptation into various niches and resulting in a group of allopatric taxa) is rejected by some workers.

Therefore, the present paper aims to review patterns and processes of radiation(s) in a model taxon – the stenoecious spring snail genus *Bythinella* – within the theoretical framework of adaptive versus non-adaptive radiations. Based on a taxon-wide phylogeny, several methods for identifying radiations are applied, including a new pragmatic approach based on the species flock concept and a temporal frame of rapid speciation. Then, the criteria of non-adaptive radiations are assessed and the driving forces discussed both in general and specifically for *Bythinella* spp. Based on eight identified radiations as well as ecological, morphological, and distribution data for up to 50 species, the presence of non-adaptive radiations is suggested in this taxon. Driving forces for these radiations might be genetic drift in small sub-divided populations, though natural selection may be involved as well. Moreover, it is shown that adaptive and non-adaptive radiations might not be entirely discrete in space and time. The present study underlines the need for a better understanding of the underlying mechanisms of adaptive and non-adaptive radiations and for a judicious use of these epithets.

T. Wilke (✉), M. Benke, and C. Albrecht
Department of Animal Ecology & Systematics, Justus Liebig University, Giessen, Germany
e-mail: tom.wilke@allzool.bio.uni-giessen.de

M. Brändle
Department of Ecology, Philipps University Marburg, Marburg, Germany

J.-M. Bichain
Muséum National d'Histoire Naturelle, Paris, France

M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_25,

1 Introduction

The study of radiations plays a crucial role in evolutionary biology and, in recent years, there has been an increased interest in both causes and consequences of radiations. Although the term radiation is frequently used in evolutionary biology, it is, however, only vaguely defined.

From a strictly taxonomical point of view, a radiation constitutes a relatively large monophyletic group of taxa (Gittenberger 1991). Schön and Martens (2004), however, suggested considering rate of speciation when discussing radiations, as the term is applied to relatively large monophyletic groups of species that either gradually evolved over long periods of time or are the result of rapid speciation. In fact, some workers even use the term "explosive" radiation or speciation (reviewed in Schön and Martens 2004), emphasizing that in these radiations species evolved within a relatively short period of time (e.g., Gavrilets and Vose 2005).

Another conceptual aspect of radiation research that has puzzled scientists for decades is the differentiation of adaptive versus non-adaptive radiations. Losos and Miles (2002) pointed out that further progress in the study of radiations is impeded by the fact that no reliable quantitative criteria exist to determine whether a certain group is subject to adaptive radiation. In fact, even the term adaptive radiation is discussed highly controversially.

Classical adaptive radiation was suggested by Osborn (1918) and refined by Simpson (1949) as an extreme diversification of a group evolving in different directions permitted by its own potentialities and by the environments encountered. Although the term adaptive radiation is used in a fairly broad sense, the common understanding associates these radiations with rapidly multiplying lineages leading to the evolution of phenotypic diversity and the subsequent filling of new niches (Simpson 1953; Minkoff 1983; Futuyma 1998; Schluter 1998, 2000; Schön and Martens 2004; Seehausen 2004; Rundell and Price 2009).

The famous geneticist Sewall Wright (1929, 1931, 1932) introduced an alternative concept to adaptive radiations; that of "non-adaptive sort" (Wright 1931: p.127) stressing the role of drift ("It appears, however, that the actual differences among natural geographical races and subspecies are to a large extent of the non-adaptive sort expected from random drifting apart," Wright 1931: p.127) in small sub-divided populations ("Complete isolation [of subdivided populations] originates new species differing for the most part in non-adaptive respects ...", Wright 1931: p.158). He even went so far to say that "The principal evolutionary mechanism in the origin of species must thus be an essentially non-adaptive one." (Wright 1932: p.364).

Some 60 years later, Gittenberger (1991) revived the discussion by pointing out that many workers still use the term adaptive radiation but that the epithet "adaptive" suggests that radiations per se do not necessarily imply adaptation. As a consequence, he refined the term "non-adaptive radiation" as a special form of allopatric speciation, which he considered "a kind of diversification not accompanied by adaptation into various significantly different niches and, therefore,

resulting in a group of allopatric species which are isolated because of competitive interactions" (Gittenberger 1991: p.263).

Just two years later, Davis (1993) introduced the term "morphostatic radiation" as a monophyletic radiation of species involving low anatomical diversity, little habitat (niche) diversification, and with species in allopatry. Based on a comparison of adaptive and non-adaptive radiations, he suggested that, in many invertebrate groups (and particularly in molluscs), morphostatic radiations (from here on synonymized with non-adaptive radiations) are not the exception but the rule.

Despite the interesting theoretical and practical concept of non-adaptive radiations, this form of radiation remains largely unstudied as many evolutionary biologists focused on adaptive radiations. However, in his text book on adaptive radiations, Schluter (2000: p.18) pointed out that "adaptive radiation cannot be assumed a priori, and non-adaptive radiation is a logical null hypothesis". He also stated that, in non-adaptive radiations, the evolution of species is not driven by the environment (see also Rundell and Price 2009).

Nevertheless, Sudhaus (2004: pp.127–128) rejected outright "such a thing as non-adaptive radiation" as "non-adaptation cannot be demonstrated, since it is principally impossible to show that a structure has no function". In response, Gittenberger (2004) pointed out that genetic drift and founder effects can result in speciation in the absence of clear niche differentiation, that is, in non-adaptive radiation. Acknowledging that intermediate situations between adaptive and non-adaptive radiations may occur, he suggested that radiations may start with an initial non-adaptive stage and later become adaptive. In contrast, Davis (1993, 1994), who performed intensive studies on non-adaptive radiations in SE Asian freshwater gastropods and North American bivalves, rejected the notion of Gittenberger (1991) and suggested that adaptive and non-adaptive are discrete and well discernable conditions.

Despite the partly different standpoints of Gittenberger (1991, 2004) and Davis (1993), three criteria for non-adaptive radiations can be derived (see also Brooks et al. 1985; Givnish 1997). Within a non-adaptive radiation:

(1) There is no clear niche differentiation,
(2) Species usually have a low degree of phenotypical variation, and
(3) Species usually evolve in allopatry (or peripatry).

Probably because the epithet non-adaptive was re-introduced by malacologists, several non-adaptive radiations have been studied in land snails (e.g., Gittenberger 1991; Cameron et al. 1996; Gittenberger and Hausdorf 2004; Wilke and Duncan 2004; Cook 2008; Sauer and Hausdorf 2009), and particularly in freshwater snails (e.g., Davis et al. 1992; Wilke and Pfenninger 2002; Wilke et al. 2002; Attwood et al. 2003; Clark et al. 2003). In fact, some ground-breaking evolutionary studies (e.g., "What makes a narrow-range taxon?" by Ponder and Colgan 2002) were conducted utilizing presumed non-adaptive radiations of Australian spring snails.

Recently, the concept of non-adaptive radiation has also become more popular in other systematic groups, for example, in fish (Albertson et al. 1999), in amphibians (Kozak et al. 2006; Wake 2006), in reptiles (e.g., Losos and Miles 2002), in

Plathelminthes (Brooks et al. 1985), and even in unicellular organisms (e.g., Leander and Keeling 2003). However, many studies of non-adaptive radiations are restricted to small geographic areas (e.g., ancient lakes or oceanic islands) or to small systematic groups. Therefore, a comprehensive picture of non-adaptive radiations is still missing.

In an excellent review paper, Barraclough and Nee (2001) discussed several general problems in studies of speciation and radiation based on molecular phylogenies. Among others, they stress the need for studying a sufficiently large monophyletic group and a large geographic area, for sampling nearly all representatives of a certain group and a more intensive sampling within species, for using multilocus approaches and calibrating trees in real time (molecular clock approach), and for distinguishing radiation events from regular speciation.

Whereas some progress has been made in resolving statistical and theoretical problems in radiation studies (e.g., Pybus et al. 2002; Gavrilets and Vose 2005; Freckleton and Harvey 2006; Seehausen 2007; Oliver et al. 2009), taxon and sampling problems continue to persist (e.g., Guyer and Slowinski 1993). In particular, continental animal groups have largely been affected by humans either directly or through habitat loss. This makes it extremely challenging to study radiation events in, for example, Europe or North America because one would ideally need a monophyletic group that is distributed throughout the continent, yet not or little affected by humans.

One potential group that might fulfil these criteria is the spring snail genus *Bythinella* (Rissooidea: Bythinellidae, Radoman 1976; see also Ponder et al. 2008). Representatives occur throughout continental Europe from northern Germany to Sicily, and from Spain to Turkey and Ukraine. They are only absent in Scandinavia, in north-eastern Europe (Baltic republics, Belarus and Russia) and, perhaps, Portugal. Except for continental Europe, they occur on some Mediterranean Islands as well as in the Asian part of Turkey. Currently, some 83 European *Bythinella* species and subspecies are recognized (Fauna Europaea Web Service 2004).

In the present paper, we aim to review patterns and processes of radiations in this model taxon within the theoretical framework of adaptive versus non-adaptive radiations. For doing so, we first introduce general ecological and biogeographical characteristics of *Bythinella* spp. and discuss different approaches for identifying radiations within this snail group, including a new approach introduced here. We then test the criteria of putative non-adaptive radiations in this taxon, and finally engage in a discussion of the driving forces of non-adaptive radiations in general and of *Bythinella* radiations in particular, concluding with an attempt to answer the question whether adaptive and non-adaptive radiations constitute distinct processes.

In order to account for the evolutionary nature of radiations, we here apply the term only to radiations that are associated with rapid speciation at the species rather than a higher taxonomic level (sensu Mayr 1963; Sanderson 1998; Schluter 1998, 2000; Gittenberger 2004; Schön and Martens 2004) and specifically relate radiations to ecological opportunities (Simpson 1953; Schluter 1998; Rundell and Price 2009).

2 The Spring Snail Genus *Bythinella*

Spring snail species of the genus *Bythinella* are, as the common name suggests, primarily restricted to springs with relatively low water temperatures (Fig. 1). Occasionally, they also occur in spring outlets and associated creeks, as well as in

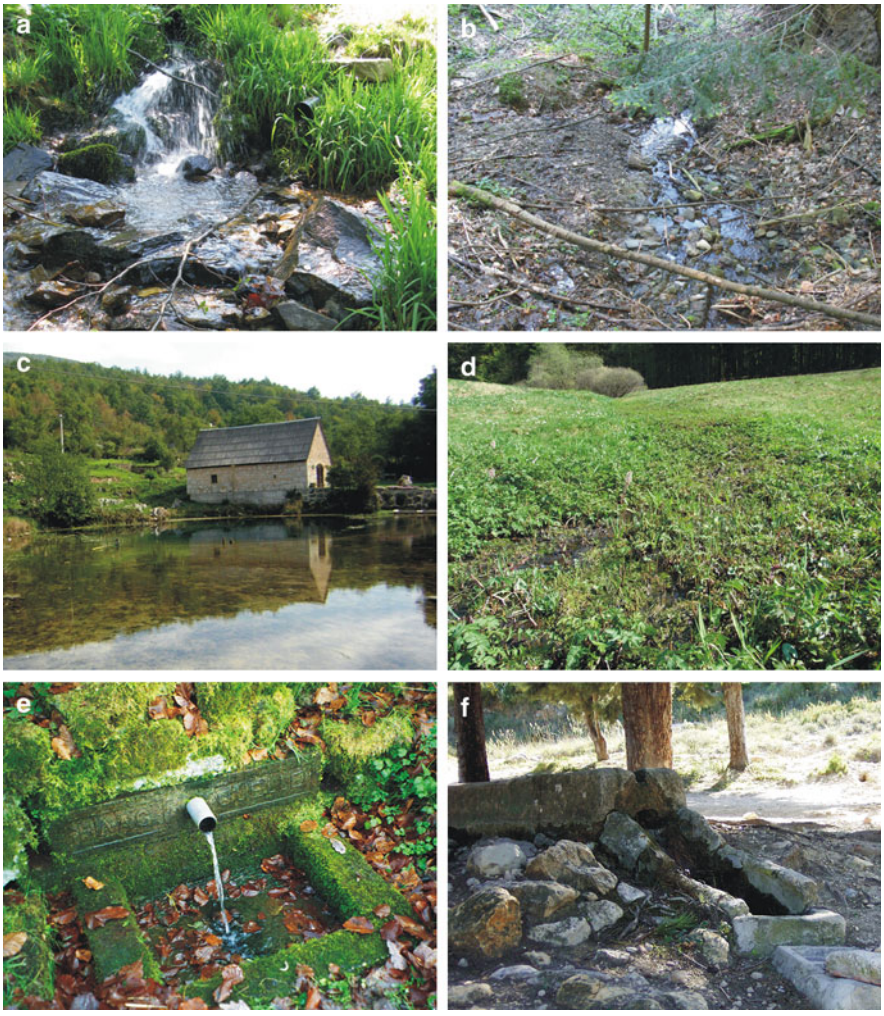


Fig. 1 Selected habitats for *Bythinella* spp. (**a**) Rheocrene spring (Germany, *Black* Forest, near Oberried, *B. badensis*), (**b**) Spring creek (Germany, *Black* Forest, near Badenweiler, *B. badensis*), (**c**) Limnocrene spring (Croatia, Gacko Polje, near Sinac, *B. magna*), (**d**) Helocrene spring (Germany, Hoherodskopf, *B. compressa*), (**e**) Captured spring with *Bythinella* population (Germany, Rhön, near Sinntal-Schwarzenfels, *B. compressa*), (**f**) Captured spring with *Bythinella* population having gone extinct (Spain, near Javea)

hypogean habitats such as caves or groundwaters (e.g., Jungbluth 1972; Boeters 1968; Bichain et al. 2007a). Although springs per se are endangered habitats and some European lowland springs have been destroyed (e.g., Szarowska 2000), *Bythinella* specimens are often very abundant in relatively unaffected mountainous areas from which most of the respective species are described. But even in populated lowland areas, *Bythinella* may be rather abundant, as long as the springs provide the necessary resources.

As pointed out by Radoman (1976: p.134), the ecological characteristics of spring snails make them well suited candidates for speciation and radiation studies: they are monophyletic, species-rich, widely distributed, usually have very restricted habitats, and "they are completely and probably definitely territorially isolated". Although the mechanisms of dispersal in *Bythinella* spp. are still largely unknown, it has been suggested that adult individuals are rather resistant to desiccation, and dispersal by birds or by wind with leaves is presumed (Falniowski 1992). Nonetheless, these and other potential vectors like fish, insects, or rodents are rare in springs (particularly in rheocrenes where spring snails occur most frequently).

In addition to the presumed lack of clear niche differentiation, there is also relatively little anatomical variation in *Bythinella*. The ground plan of these small (2–5 mm shell height) dioecious snails is, as in many other rissooidean genera, simple, and species delimitation, traditionally based on shell morphology and genital anatomy, is often a matter of debate (e.g., Giusti and Pezzoli 1977; Mazan and Szarowska 2000; Haase et al. 2007). However, some shell characters are more variable and have been used to differentiate taxa mostly from presumably different radiations (Fig. 2; see also Radoman 1976). The first genetic studies on restricted groups of *Bythinella* spp. in southern Poland (e.g., Falniowski et al. 1998), southern France (Bichain et al. 2007a, b), Slovakia/Hungary (Szarowska and Wilke 2004), and southern Austria/Slovenia (Haase et al. 2007) indicated that species delimitation should be based on a combination of morphology, anatomy, and genetics.

Given these interesting characteristics, it is not surprising that rissooidean snails restricted to spring habitats have been used for comprehensive evolutionary studies in Australia (e.g., Colgan and Ponder 1994; Ponder et al. 1996; Ponder and Colgan 2002; Perez et al. 2005) and in North America (e.g., O'Brien and Blinn 1999; Liu et al. 2003; Moline et al. 2004). In contrast, spring snail radiations in Europe remained little studied, despite their potential for inferring driving forces triggering bursts of non-adaptive radiations. Possible exceptions include geographically restricted allozyme (Klemm and Schlegel 1989; Falniowski et al. 1998; Szarowska et al. 1998; Mazan and Szarowska 2000; Brändle et al. 2005) and DNA sequencing studies (Szarowska and Wilke 2004; Bichain et al. 2007a; Bichain et al. 2007b; Haase et al. 2007) and the recent taxon-wide genetic study of Benke et al. (2009).

Nonetheless, non-adaptive radiations in Europe (and elsewhere) are poorly studied in general. There are a few cryptic radiations that have been investigated for parts of Europe (e.g., Gittenberger 1991; Pinceel et al. 2004), but we do not know of any large-scale study of continental animal groups that has tried to address the problem of low anatomical and niche differentiation. The lack of relevant studies may, in part, be due to the greatly adverse effects humans have had on

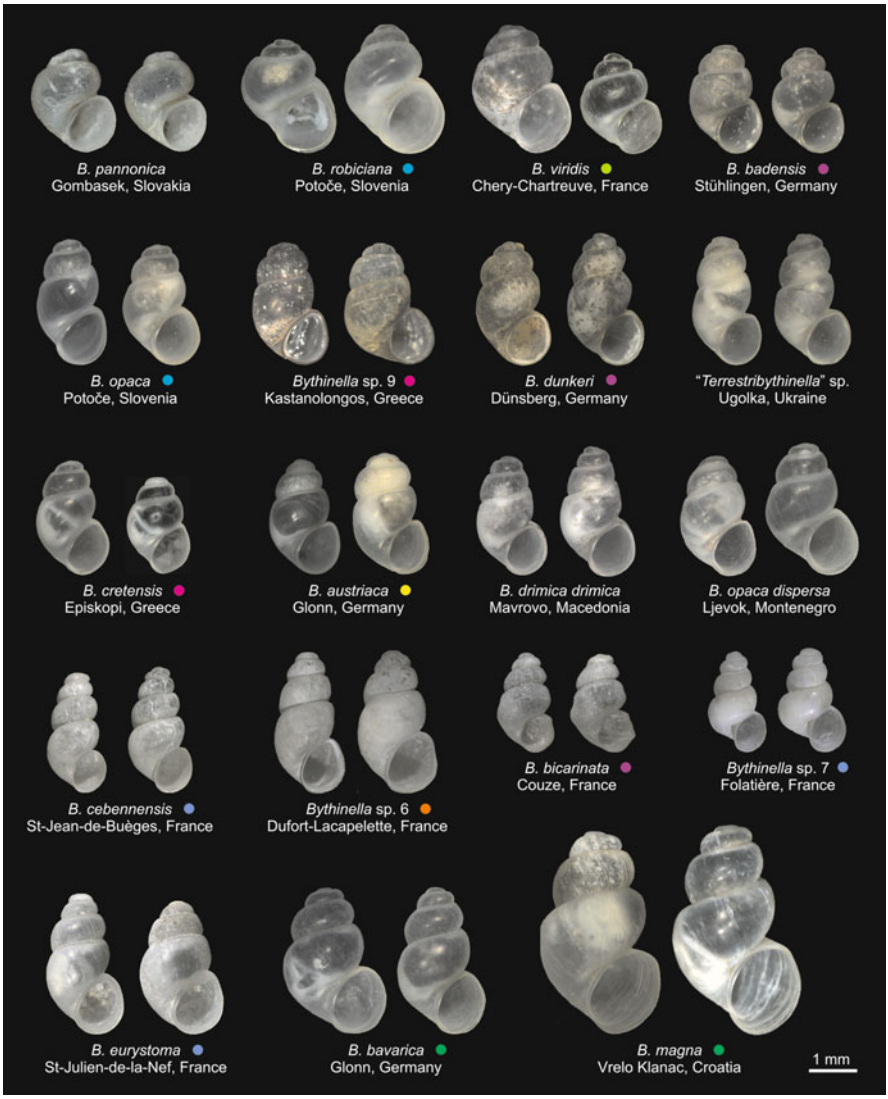


Fig. 2 Variation in shell morphology of *Bythinella* spp. Assignments to respective radiations (see Fig. 3) are given by *colored circles*, if applicable. Note that the figured specimens of *B. bavarica* and *B. austriaca* as well as of *B. opaca* and *B. robiciana* occurred in sympatry. See text for details

most European taxa and their respective habitats, which makes it very difficult to infer radiation patterns in space and time.

The relatively unaffected radiations of the spring snail genus *Bythinella* could therefore constitute a paradigm for studying causes and consequences of radiations in general and of non-adaptive radiations in particular.

3 Identifying Radiations in the Genus *Bythinella*

In order to study the nature of radiations within a given group, it is necessary to first identify individual radiations. If one considers all relatively large monophyletic groups of species that gradually evolved over long periods of time to be radiations, then these radiations could easily be identified from phylogenies: all larger clades would constitute radiations with nested radiations being possible. The whole genus *Bythinella* as well as all larger sub-clades within the genus would thus constitute radiations. If, however, the concept of radiation is considered within the framework of rapid speciation, operational criteria and an appropriate test statistic would be required.

Attempts to estimate differences in diversification rates based on lineage-through-time plots and γ-statistics pointing to the aspect of rapid changes of diversification were carried out by, for example, Pybus and Harvey (2000) and Pybus et al. (2002). The original purpose of this approach, however, was not to identify (individual) radiations but to investigate changes in diversification rates over time in a given taxon.

Explicit approaches for identifying radiations based on differences in tree topology were introduced by Guyer and Slowinski (1993) using a tree-unbalance test, by Purvis et al. (1995) with their relative cladogenesis test (RCT), by Foote (1996) using a morphological disparity approach, and by Chan and Moore (2004) with SymmeTree. Two of these approaches are applied in the present paper (Fig. 3).

The RCT compares two or more ancestral lineages of equal age utilizing the number of their contemporary descendants. If one lineage left considerably more descendants than the other, then a significant event of fast speciation (or reduced extinction) is suggested.

SymmeTree implements topology-based tree tests of diversification rate variation, incorporating information on the relative diversity of all internal nodes of a given tree. The probability of a shift along the lone internal branch is calculated as a function of likelihood ratios and the cumulative probability for each statistic is derived by Monte Carlo simulations.

Applying both approaches to a mitochondrial cytochrome *c* oxidase subunit I (COI)-based phylogeny of 50 nominal species of *Bythinella* (taxonomy according to Fauna Europaea Web Service 2004) resulted in the identification of three potential radiations with SymmeTree (white bars in Fig. 3) and two radiations with the RCT (black bars in Fig. 3).

Though both approaches are based on compelling criteria for indentifying radiations (see above), a comparison of the results of these two tests points to several problems:

(1) Individual radiations identified are greatly different and they also largely differ in depth (whereas in our example SymmeTree inferred few deeper radiations, only younger radiations are found with the RCT),
(2) Both approaches deduced nested radiations making direct comparisons of individual radiations impossible,

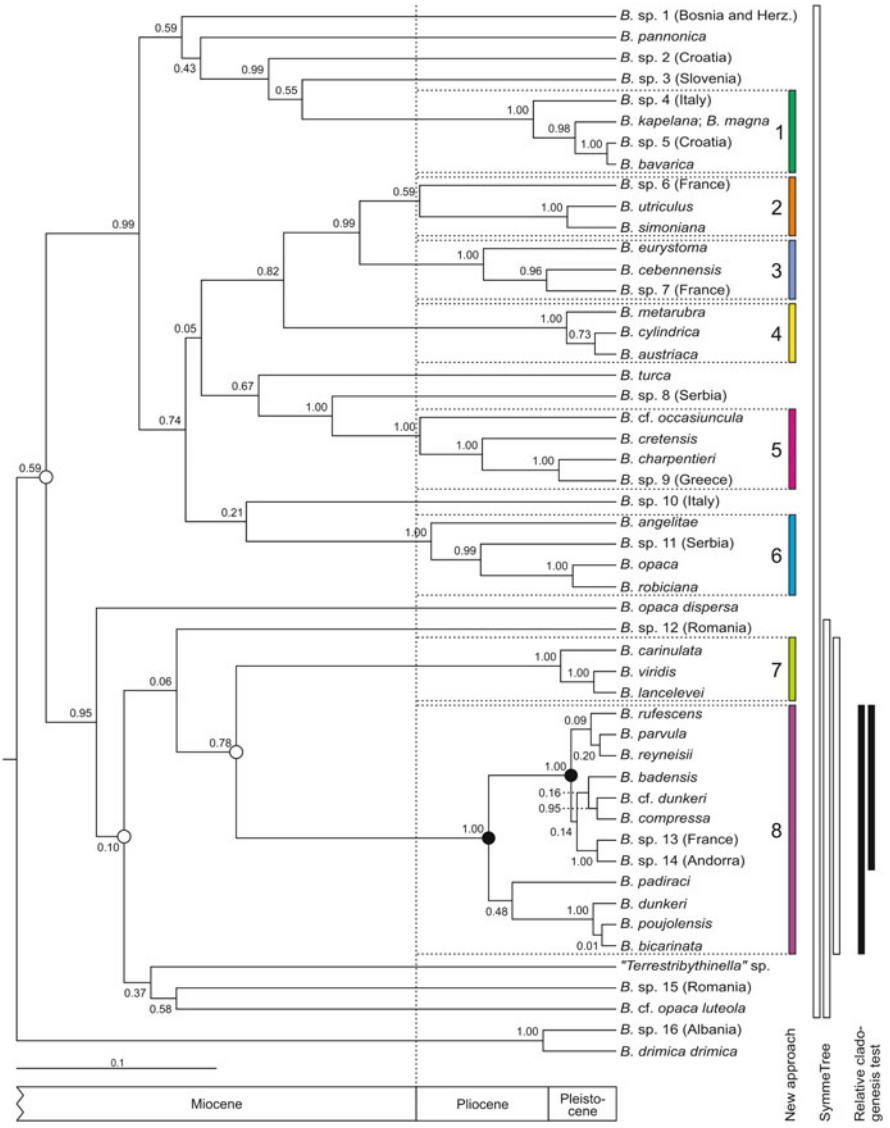


Fig. 3 Radiations identified in the genus *Bythinella* based on three different approaches. *Black circles* at nodes and *black bars* at taxa: relative cladogenesis test ($p < 0.01$), *white circles* at nodes and *white bars* at taxa: SymmeTree, *colored bars* at taxa: new approach introduced in the present paper with a Pliocene/Pleistocene time frame. The clock-enforced consensus Bayesian COI tree was generated from the dataset of 458 *Bythinella* specimens from 142 sites presented in Benke et al. (2009) under the best-fit model of sequence evolution (HKY+I+Γ), pruned to 50 taxa according to Fauna Europaea Web Service (2004) (specimens not assignable to a nominal species were labelled with "sp."). The outgroups were removed a posterior. For the clock analysis, the method of Wilke et al. (2009) was used with the beginning of the Pliocene estimated from a trait- and model-specific clock rate of $1.84 \pm 0.24\%$ My^{-1}

(3) As both approaches are based on shifts in phylogenetic events, poorly supported nodes may severely influence the results (see the poor support of radiations inferred with SymmeTree in Fig. 3),
(4) These shift-based approaches are prone to errors introduces by missing taxa (both extinct or not sampled; see Guyer and Slowinski 1993),
(5) They are also prone to problems with species identification (see Haase et al. 2007 and Bichain et al. 2007a for a discussion on species concepts and associated problems in *Bythinella*).

We do not intend to criticise the performances of the RCT and SymmeTree used above. Both approaches have proved to be useful in a number of evolutionary studies. Rather, we try to raise awareness about general problems associated with identifying radiations. Particularly when studying nature and mechanism of radiations, it might be advantageous to apply more straightforward and robust criteria for identifying radiations. Moreover, considering that the nature of radiations may change over time, such a concept should also incorporate absolute age of radiations, which would allow for distinguishing species-level radiations from higher-level radiations.

A potential solution could come from the (ancient lake) species flock concept in combination with information on the temporal scale of speciation events. According to Greenwood (1984) and Schön and Martens (2004), a species flock is a monophyletic group of at least three species that are typically endemic to the lake. The latter workers also indicated the synonymy of the terms species flock and radiation at the species level when disregarding the criterion endemicity.

The species flock concept, however, does not explicitly address rate of evolution. This is because most species flocks typically do not exceed the age of the respective lake and are often of Pliocene or Pleistocene age (e.g., Schultheiß et al. 2009). Even species flocks in the oldest ancient lake on earth, Lake Baikal, proved to be relatively young (see, for example, the discussion in Wilke 2004). Thus, the limnological age of the respective lake typically provides the time frame of intra-lacustrine evolution.

The situation is different in radiations. Within a larger taxon, there might be radiations that started tens of millions years ago or radiations that are only few hundred thousand years old (e.g., Streelman and Danley 2003). Radiations may occur at the species level or involve higher taxonomic groups. If, however, fundamental evolutionary processes are of concern, then this ideally is done at the species level because speciation events are the actual building blocks of radiations. Thus, if rapid speciation processes are to be studied and individual radiations are to be compared, then an appropriate time frame should be considered that, on average, only comprises a few consecutive speciation events. The actual extent of the time frame may vary from taxon to taxon. Radiation patterns in cichlids, for example, might be deduced within a time frame of only few hundred thousand years (e.g., Seehausen 2002, 2006; Streelman and Danley 2003). For *Bythinella* spp., this time frame may be longer. Benke et al. (2009) showed that most extant species evolved during the Pliocene/Pleistocene, thus providing a potential time frame for the study of radiations in this group.

Combining the species flock concept (Greenwood (1984) with the temporal frame of rapid speciation (e.g., Gittenberger 2004), we here suggest a practical approach for identifying species-level radiations:

A radiation is a monophyletic group of at least three species that evolved within a relative short, taxon-specific period, typically comprising only few consecutive speciation events.

Based on a Pliocene/Pleistocene time frame, eight radiations can be identified in our *Bythinella* phylogeny (coloured bars in Fig. 3). The geographical distribution of these radiations is shown in Fig. 4. Acknowledging that an appropriate time frame may be hard to establish, that the time frame may differ between adaptive and non-adaptive radiations (see below), and that such a time frame requires, for example, molecular clock analyses and detailed knowledge of evolutionary patterns in the group studied, the approach suggested here has a number of advantages over shift-based approaches.

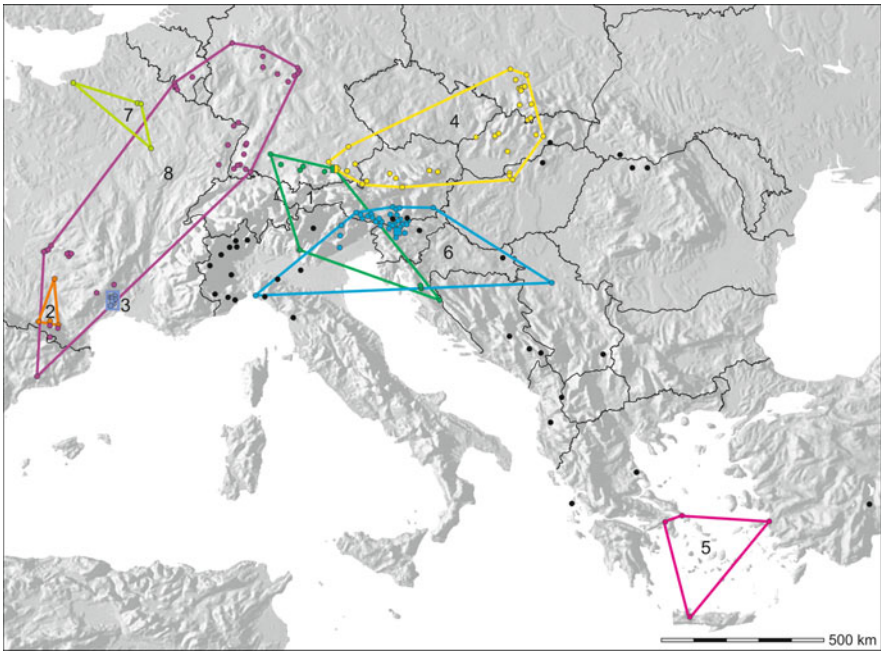


Fig. 4 Localities of populations of *Bythinella* spp. studied in Europe and western Asia, and geographical distribution of radiations inferred. *Colors* and *numbers* refer to the radiations given in Fig. 3. *Black dots* represent localities of species that could not be assigned to radiations. Sympatric populations are indicated by *squares* (*yellow*/*green square*: Glonn Springs in Germany with *B. bavarica* and *B. austriaca*; *blue square*: roadside spring in Potoče, Slovenia with *B. opaca* and *B. robiciana*). Note that the sampling design for some areas, particularly on the Balkans, the eastern Carpathian Mountains, and Asia Minor, is incomplete. Therefore, more radiations might be discovered in the future

Individual radiations are directly comparable because they comprise the same time frame and are not nested. As no shifts are required (and therefore comparisons of at least two monophyletic groups), this approach may be less sensitive:

– To problems associated with poorly supported clades,
– To problems with extinct or unsampled taxa, and
– To artefacts resulting from problems associated with species determination.

4 *Bythinella* spp. and the Criteria for Non-adaptive Radiations

Testing for the nature of radiations, that is, whether they are adaptive, non-adaptive, or a combination of both, remains a challenging task in evolutionary biology. This is because different approaches for identifying radiations may be affected by varying degrees of methodological problems (e.g., missing taxa, taxonomy), and may lead to substantially different results (see Fig. 3). Thus, the underlying identification of radiations already remains problematic and, to some extent, arbitrary. Another major problem is related to testable criteria for distinguishing adaptive from non-adaptive radiations. The principal criteria for non-adaptive radiations, as outlined by Gittenberger (1991, 2004) and Davis (1993), are straightforward: the resulting species usually have a low degree of anatomical variation, there is no clear niche differentiation, and species of the same radiation are usually allopatric or peripatric (see Introduction). These criteria, however, are only applicable to species-level radiations because in higher-level radiations one may encounter either different patterns in different nested radiations or possible transitions between adaptive and non-adaptive radiations as suggested by Gittenberger (2004) and Rundell and Price (2009).

In such cases, the new approach for identifying radiations introduced above might be useful because it provides a temporal constraint.

Based on the *Bythinella* radiations identified from the phylogeny shown in Fig. 3, we here provide selected ecological and morphological data as well as information on allopatric/peripatric versus parapatric/sympatric relationships of *Bythinella* spp. in order to test whether the criteria for non-adaptive radiations are applicable.

4.1 Ecological (Niche) Variation within Bythinella *Radiations*

To test whether species prefer different ecological conditions (niche differentiation) is extremely difficult because diversification may have occurred in niche dimensions unknown to us, not assessable by us, or during life history stages not considered (sensu Schön and Martens 2004). Particularly in radiations with species that are restricted to small geographic areas and with primarily allopatric

distributions, such studies are challenging, if not impossible. This is because geographic separation inevitably causes a strong spatial autocorrelation of, for example, climate conditions and therefore a potential ecological differentiation among species.

To avoid or at least mitigate these problems, we specifically chose the genus *Bythinella* as a paradigm. Their spring habitats are among the best described and most stable continental habitats known (despite the fact that some springs may be prone to desiccation or flooding). Moreover, throughout the distribution range of the genus *Bythinella*, springs are typically characterized by similar habitat characteristics such as low and constant water temperature, low water flow, oligotrophic conditions, lack of limnic vegetation, and apparent lack of competitors (see Sect. 2). Acknowledging that there are different spring types such as rheocrenes, limnocrenes, etc. (Fig. 1), we found that populations of the same species often live in different types of springs (see Fig. 1a, b, and d, e). Thus, these different spring types likely might not reflect niche variations.

Given the wide geographical distribution of *Bythinella* spp. in Europe and western Asia, there are, of course climatic gradients potentially affecting niche differentiation. Springs in southern Europe, for example, typically have slightly higher average water temperatures (Schwoerbel 1999). They are also more prone to summer desiccation. Our analysis of the spatial distribution of radiations (Fig. 4), however, shows that individual *Bythinella* radiations are typically restricted to a narrow latitudinal band. Thus, species within radiations are likely not affected by latitudinal gradients. The only exception is radiation #8, which has a disjunct distribution east of the Alps (northern Spain and southern France) and north of the Alps (Germany, France, Belgium, and Switzerland). As shown by Benke et al. (2009), this disjunct distribution is associated with Pleistocene glaciations (Fig. 6). Today, the hilly habitats in the northern part of the distribution area have climatic conditions comparable to the mountainous habitats in the southern part of the distribution. Therefore, latitudinal gradients can possibly be ignored when considering niche differentiation of *Bythinella* species within individual radiations.

Acknowledging the methodological difficulties in characterizing niche differences outlined above, our studies did not yield any information that would undermine the assumption of lack of clear niche differentiation in *Bythinella* species belonging to the same radiation.

4.2 *Phenotypical Variation among and within* Bythinella *Radiations*

Shell variations among and within individual *Bythinella* radiations were studied utilizing both canonical variates analysis (CVA; Haase et al. 2007) and discriminant function analyses (DFA; current paper). As examples for individual radiations, we here use two contrasting groups; a relatively small radiation with a restricted range

occurring SE of the Alps in Austria and Slovenia (radiation #6 in Fig. 3; see also Fig. 4), and the presumably largest radiation in our phylogeny, occurring W and NW of the Alps from northern Spain to northern Germany (radiation #8 in Fig. 3; see also Fig. 4).

For radiation #6, a CVA of seven quantitative shell measurements from >200 individuals was performed. In the plot of the CVA (see Fig. 5 in Haase et al. 2007) only the population of *B. robiciana* was clearly differentiated from the remaining populations; each of which overlapped at least with three other populations. Nonetheless, a MANOVA highly significantly rejected the null hypothesis of identical samples (Wilk's $\Lambda = 0.02659$, $F_{54,835.7} = 16.04$, $p < 0.0001$). This indicates some differentiation at the population level and points to a common problem in freshwater gastropods – a relatively high variability of shell characters at the population level, potentially masking species-level differences. The fact that *B. robiciana* could be distinguished from the other members of the radiations, however, is not surprising. Whereas most species of this radiation have the typical ovate-conical shell shape of *Bythinella*, the shell of *B. robiciana* is valvatiform (a shell form that is rare in *Bythinella* spp.; see Fig. 2). Qualitatively, the shells of the populations investigated could not be distinguished regarding teleoconch or protoconch structure (see figs. 2–4 in Haase et al. 2007). Moreover, with respect to soft part anatomy (cephalic tentacles, number of gill filaments, osphradium, ctenidium,

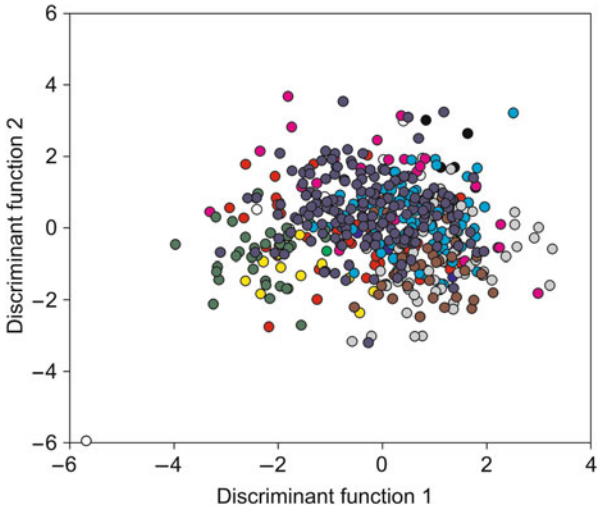


Fig. 5 Discriminant function analysis of four shell measurements for a restricted dataset comprising 495 individuals of 12 species from radiation #8 in Fig. 3 based on the classification of the Fauna Europaea Web Service (*B. badensis* = *black*, *B. bicarinata* = *dark blue*, *B. compressa* = *white*, *B. dunkeri* = *red*, *B.* cf. *dunkeri* = *yellow*, *B. padiraci* = *pink*, *B. parvula* = *grey*, *B. reyneisii* = *cyan*, *B. poujolensis* = *dark green*, *B. rufescens* = *green*, *Bythinella* sp. 13 = *dark red*, *Bythinella* sp. 14 = *blue*). The first function accounts for 52% and the second function for 33% of the morphological variability

kidney, radula, stomach, rectum, ovary, pallial and renal oviduct, albumen gland, seminal receptacle, bursa copulatrix, testis, seminal vesicle, vas deferens, prostate, and penis with accessory gland typical for *Bythinella*), the investigated species were also practically identical (see Figs. 4 and 6 in Haase et al. 2007).

A DFA (all measurements were log10-transformed prior to the analysis) across radiation #8 (see Fig. 5) revealed relatively large variation in shell morphospace within as well as among species, but with substantial overlapping and no distinct grouping of individuals. This suggests that, in this radiation, no strong adaptive shifts in morphology have occurred during speciation.

Moreover, a DFA of 1,331 individuals from 33 different species belonging to eight different radiations also resulted in a poor grouping (data not shown). Thus, with few exceptions, the shell characters used here cannot unequivocally distinguish *Bythinella* spp. either within or between radiations. However, in a second approach, we partitioned the variation in morphology among radiations and among species within radiations (the function ADONIS in the VEGAN package in R, see http://vegan.r-forge.r-project.org/). We found that 32% of the morphological variation is distributed among radiations and 19% among species within radiations, indicating that there is some degree of morphological differentiation, particularly among different radiations.

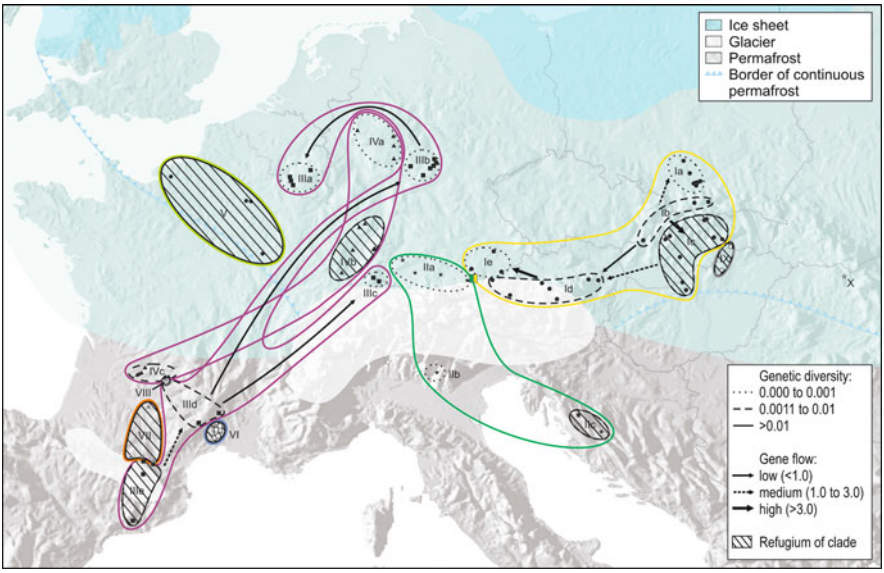


Fig. 6 Pleistocene phylogeography of *Bythinella* spp. (modified from Benke et al. 2009) showing the spatial distribution of major clades. Clades I–X refer to lineages present in the phylogenetic tree of Benke et al. (2009) and are coloured according to the radiations identified in Fig. 3. Note that only clades and radiations are shown that are associated with Pleistocene refugia. Regions being most likely the glacial refugium of a clade based on combined results of phylogenetic analyses are indicated by *hatchings* (see Benke et al. 2009 for details). The sympatric population of *B. bavarica* and *B. austriaca* is indicated by a *yellow/green square*

4.3 *Sympatry versus Allopatry of* Bythinella *spp.*

Of a total of 173 springs and related sites harboring *Bythinella* spp. studied throughout Europe and western Asia (Fig. 4), 171 sites contained single species. Only two sites (Figs. 2 and 4) were inhabited by sympatric species, the Glonn springs in Germany (*B. bavarica* + *B. austriaca*) and a roadside spring in Potoče, Slovenia (*B. opaca* + *B. robiciana*). Though it is likely that more sympatric populations will be found in the future, these figures strongly suggest a largely allopatric occurrence of *Bythinella* spp. In fact, *B. bavarica* and *B. austriaca* from the Glonn springs belong to two different radiations (#1 and #4 in Figs. 3 and 4), and thus do not violate the assumption of allopatric or peripatric relationships of species belonging to the same radiation. Benke et al. (2009) showed that these two radiations are probably subject to secondary contact resulting from range expansion out of glacial refugia (Fig. 6).

The situation is different for *B. opaca* and *B. robiciana* from Slovenia. Both species not only belong to the same radiation (radiation #6 in Figs. 3 and 4), they are also genetically almost identical (Haase et al. 2007), and are located in the approximate geographical centre of their radiation (see Figs. 4 and 6). This would violate the assumption of allopatric occurrence. These two species are, however, morphologically very different. Whereas *B. opaca* has an ovate-conical shell shape, the shell of *B. robiciana* is unusually valvatiform (Fig. 2; see also the information on morphological variation provided above). We do not know whether this atypical shell form has any adaptive value. But the fact that the only sympatric occurrence of *Bythinella* species from the same radiation in our dataset is associated with a significant shift in morphology may hint towards an adaptive sweep, thus actually supporting the assumption of allopatry of species from the same non-adaptive radiation.

All the data provided above largely fulfil the criteria of Gittenberger (1991, 2004) and Davis (1993) suggested for species within non-adaptive radiations. Thus, there is evidence pointing to the existence of this form of radiation in *Bythinella* spp. Nonetheless, at least in respect to morphological characters, occasional adaptive sweeps cannot be excluded.

It should, however, be noted that a conclusive test as to the existence of non-adaptive radiations at this point remains impossible. First of all, as discussed by Schön and Martens (2004), if the Popperian rule is accepted that a hypothesis cannot be proven but only rejected, then the logical null hypothesis for non-adaptive radiations would be adaptive radiations (see also Sudhaus 2004). But even if this null hypothesis is rejected, diversification may have occurred in any niche dimension unknown to us (see above). On the other hand, if the null hypotheses of adaptive radiation cannot be rejected because of distinct morphological/anatomical or niche differences, it is possible that these differences are derived purely by chance and that they do not have any adaptive value. As pointed out by Schluter (2000), the exploitation of different environments should only be related to adaptive radiations when associated with traits that increase the fitness in these environments (see also von Rintelen et al. 2004).

5 Non-adaptive Radiations in *Bythinella* spp.

5.1 What Drives Non-adaptive Radiations?

In order to understand mechanisms and processes that drive non-adaptive radiations in *Bythinella* spp. and other taxa, it might be useful to first look at adaptive radiations. There is an overwhelming body of literature on adaptive radiation (e.g., Gavrilets and Losos, 2009 and references therein) and although the complexity of the matter has led to numerous partly conflicting theories, there is a common understanding that adaptive radiations are typically associated with adaptations to new (ecological) niches. The basic idea is that rapid changes in the environment ("adaptation tracking", Eldredge 2000), the colonization of new or underutilized environments free of competition and predation, or extinction or replacement of competitors ("release from competition", Simpson 1953; Schluter 1998) promote adaptive change through natural selection (see also Schluter 1994). Alternatively, adaptive breakthroughs may enable the utilization of available resources ("key evolutionary innovations", e.g., Nitecki and Nitecki 1990; Guyer and Slowinski 1993; Hodges 1997; Schluter 2000; Ree 2005).

Whereas in many animal (and plant) groups, adaptive radiations can be linked to natural selection, either diversifying or directional selection (Schluter 2000; Eldredge 2003; Eldredge et al. 2005; Gavrilets and Vose 2005), adaptive radiations in some groups might be the result of hybridization (e.g., Seehausen 2004; Stelkens and Seehausen 2009), sexual selection (disruptive or diversifying) or a combination of both (Seehausen et al. 1997, 1999; Seehausen 2000; Van Oppen et al. 2000; Bolnick 2006; Salzburger 2009; Sauer and Hausdorf 2009).

These different scenarios account for partly conflicting patterns observable in adaptive radiations. Naturally, phenotypical (morphological) diversity is high in species resulting from adaptive radiations. Though, physiological (e.g., Montgomery and Givnish 2008), molecular (e.g., Kapralov and Filatov 2006), or behavioral adaption (e.g., Shaw 1995) to new niches are possible as well, not necessarily leading to morphological diversity. On the other hand, it is often difficult to recognize and to assess phenotypical diversity. Thus, (observed) phenotypical diversity alone is not a sufficient indicator for adaptive radiations. The same applies to habitat and niche diversities of species from the same radiation. They typically are high but maybe low in cases where adaptive radiation is associated with, for example, sexual selection.

Geographically, adaptive radiations are often occurring in confined geographical regions with barriers against dispersal, driving differentiation of species in parapatry or sympatry (Schön and Martens 2004; Seehausen 2004; Gavrilets and Vose 2005; Bolnick 2006; Davison and Chiba 2006; but see Sturmbauer et al. 2001 and Dieckmann et al. 2004 for exceptions).

In contrast, non-adaptive radiations are largely related to allopatric or peripatric speciation (Gittenberger 1991; Wiens 2004; Whittaker and Fernández-Palacios 2007; but see Rundell and Price 2009 who invoke allopatry in non-adaptive

radiations, and the note on sexual selection below). Here, subdivision of an ancestral population into isolated but similar habitats through dispersal or vicariance events may cause diversification not accompanied by adaptation into various significantly different niches (Wright 1931; Gittenberger 1991). Eldredge (2000) suggested that environmental change may cause organisms to seek familiar habitats to which they are already adapted ("habitat tracking") rather than to adapt to those changes. In fact, he suggested that this is an inevitable and most expected reaction, and Kozak and Wiens (2006), and Kozak et al. (2006) argue that niche conservatism might be important for maintaining isolation of local populations and thus speciation.

As already mentioned in the Introduction, Wright (1931: p.150) stressed the role of genetic drift, particularly in small sub-divided populations for non-adaptive radiations: "Complete separation of the species into large subspecies should be followed by rather slow more or less closely parallel evolution, if the conditions are similar, or by adaptive radiation, under diverse conditions, while isolation of small groups would be followed by a relatively rapid but more largely non-adaptive radiation". Whittaker and Fernández-Palacios (2007), Gittenberger (2007), and Comes et al. (2008) discussed the implications of founder effects and bottlenecks in small isolated populations, adding to the increasing body of evidence that genetic drift is a major driving force of non-adaptive radiations, particularly under peripatric conditions.

Other mechanisms for non-adaptive radiations reported in the literature include reproductive isolation due to chromosomal architectural changes, gene or genome duplications, hybridization leading to polyploidy, or infection with pathogens conferring cytoplasmic incompatibilities (reviewed in Schön and Martens 2004; see also Givnish 1997).

These mechanisms per se have no effect on phenotype and might thus be responsible for the lack of morphological diversity seen in many species of non-adaptive radiations. But is there always complete morphological stasis in such species? Certainly not. There are a number of circumstances and mechanisms potentially leading to phenotypical (morphological) diversity within and/or divergence between related species, including environmentally controlled expression of phenotypes, sexual dimorphism, life history characteristics, parasitism, mutations, or simply aberrations. These are possibly some of the considerations which led Gittenberger (1991) and Davis (1993) to propose a low degree of anatomical variation in non-adaptive radiations rather than the complete lack thereof. In the end, differences are relative. Small phenotypical variations might, for example, be detectable in a presumably non-adaptive radiation of pyrgulinid freshwater gastropods from the Black Sea; these subtle differences, however, pale into insignificance beside the splendid radiation of pyrgulinid gastropods from ancient Lake Ohrid (see fig. 2 in Wilke et al. 2007).

In fact, the essence of non-adaptive radiation is lack of niche diversification, not lack of phenotypic diversification. Moreover, phenotypical and niche diversity are not necessarily related. Recently, even the splendid diversity of some cichlid groups resulting from sexual selection through assortative mating were categorized into

non-adaptive radiation (Streelman and Danley 2003) as phenotypic divergence is limited to secondary sexual traits that are not related to the environment (Schön and Martens 2004).

5.2 *Potential Mechanisms of Non-adaptive Radiations in* Bythinella *spp.*

The evolutionary data presented above for *Bythinella* spp. indicates the presence of non-adaptive radiations. Comparably low phenotypical diversity, presumably low niche diversity and species mostly occurring in allopatry or peripatry, however, are patterns only and may not fully reflect the underlying mechanisms of non-adaptive radiations such as genetic drift, chromosomal architectural changes, gene or genome duplications, hybridization or mate choice (see above).

Assortative mating has, according to our knowledge, not been reported in rissooidean freshwater snails. Substantial hybridization can probably also be excluded as species largely occur in allopatry or peripatry, and ranges typically do not overlap. Polyploidy or other major chromosomal or genome changes in *Bythinella* spp. have not been reported either. The most probable explanation for the patterns observed is genetic drift in small subdivided and isolated populations. The mostly allopatric and particularly peripatric occurrence of *Bythinella* spp. together with the patchy distribution of spring habitats may support this assumption. Brändle et al. (2005), for example, found in *B. dunkeri* based on allozyme studies a positive correlation between geographic distance and genetic differentiation. Furthermore, patterns of genetic variation within populations suggested that populations may have been faced with founder effects, which would also explain the rather high amount of genetic differentiation between populations. Falniowski et al. (1998: p.611) found very similar results for *Bythinella* populations from southern Poland, indicating a "high level of isolation between the populations". In contrast to Brändle et al. (2005), the workers, however, suggested that founder effects are not responsible for the high degree of polymorphisms but that selection pressures act at loci. Nonetheless, most data for *Bythinella* spp. reviewed here indicate the existence of small isolated subpopulations (in terms of effective population sizes) with relatively little gene flow occurring among populations. Reasons for that are (1) the often isolated occurrence of spring habitats, (2) the typically low dispersal capacities of *Bythinella* spp., and (3) the strong bottlenecks and founder effects that have acted (e.g., Pleistocene glaciations) or still act (e.g., desiccation of springs) on *Bythinella* populations. These are exactly the conditions where genetic drift may play a significant role (sensu Wright 1931, 1932).

Finally, we here provide strong evidence for "habitat tracking" (sensu Eldredge 2000) in *Bythinella* spp. The reconstruction of the Pleistocene phylogeography of major *Bythinella* lineages based on mitochondrial sequence data (Benke et al. 2009; see also Fig. 6) indicates major dispersal and/or vicariance events in *Bythinella* spp.

combined with significant range shifts. Despite the massive geological/ecological/climatic changes associated with Pleistocene glaciations in Europe, we do not, however, know of any *Bythinella* species that has fully adapted to freshwater habitats other than springs and associated water bodies. Moreover, a test for serial independence (TFSI; Abouheif 1999) involving a total of 29 nominal *Bythinella* species showed that phylogenetically closely related taxa did not occupy similar refugia (Benke et al. 2009). This lack of phylogenetic concordance could possibly be explained by the stochasticity of survival and dispersal in spring snails. Thus, "habitat tracking" may have played a major role and significant "adaptive tracking" (sensu Eldredge 2000) can probably be excluded in *Bythinella* spp. Of course, springs are not entirely homogeneous habitats and they might experience severe environmental changes throughout the year (e.g., flooding, desiccation). Species of *Bythinella*, however, appear to be well adapted to these settings, for example due to the ability to aestivate in the substratum. Moreover, as individual *Bythinella* species are frequently found in different types of springs, springs as a whole may provide a single niche. This niche conservatism might not only be responsible for the lack of clear phenotypical divergence among species (despite some possible adaptive sweeps) but also for a relatively uniform selection pressure on all species from the same radiation.

5.3 *Adaptive and Non-adaptive Radiations: Discrete Processes or Not?*

The putative adaptive sweep found in *Bythinella robiciana* (see Haase et al. 2007; and Sect. "Sympatry versus allopatry of *Bythinella* spp." above) raises the question whether adaptive and non-adaptive radiations constitute discrete processes. This question also relates to the dispute between Gittenberger (1991) and Davis (1993) with the former one acknowledging intermediate situations and the latter one explicitly rejecting this notion.

The problem, however, has several dimensions. That is (1) whether there are radiations that are neither adaptive nor non-adaptive, (2) whether adaptive radiations can change into non-adaptive radiations and vice versa over time, and (3) whether adaptive and non-adaptive elements can coexist within a single radiation.

The answers to these questions partly depend on the definitions for adaptive and non-adaptive radiations in terms of underlying mechanisms, taxonomic extant, and rate of evolution. If we here consider only rapid species level events either triggered by drift or natural selection, then Wright (1931: p.158) already provided part of the answer by writing that, in subdivided populations "there is a continually shifting differentiation . . . intensified by local differences in selection . . .". It generally can be assumed that drift and selection do not act exclusively and completely independently. Wright (1931) assumed some degree of natural selection in allopatric speciation processes. Conversely, mostly sympatric and/or parapatric speciation

in adaptive radiations may be largely due to natural selection, but drift resulting from, for example, bottlenecks may act as well. Thus, adaptive and non-adaptive radiations might not be entirely discrete. We, however, do not know of any radiation process where selection and drift act equally, and therefore have doubts whether there is a full range of intermediate states between adaptive and non-adaptive radiations.

As to whether adaptive radiations can change into non-adaptive radiations and vice versa over time, this is certainly possible (e.g., Rundell and Price 2009). The extant biodiversity of freshwater limpets of the genus *Ancylus*, for example, might be the result of non-adaptive radiations. A representative of one of these radiations then invaded ancient Lake Ohrid and rapidly diversified via adaptive intra-lacustrine radiation (Albrecht et al. 2006). In fact, many higher-level radiations may be the product of a series of adaptive and non-adaptive radiations together with regular species processes. As, in such "radiations", patterns typical for adaptive radiations like niche and phenotype diversification accumulate over time, they are usually classified as adaptive radiation. This is one of the reasons why many workers argue that the application of the term radiation should be restricted to cases of multiple speciation events within a relatively short period of time (e.g., Gittenberger 2004).

Another twist is added to this problem when considering sexual selection. Streelman and Danley (2003), for example, introduce their "radiation-in-stages" model in cichlids and parrotfish where they consider a radiation to consist of an adaptive macrohabitat stage followed by an adaptive trophic morphology stage, and a presumably non-adaptive stage of signaling phenotypes.

As for the question whether adaptive and non-adaptive elements can coexist within a single radiation, this can be answered with yes as well. Non-adaptive elements may well be part of an adaptive radiation. There is, for example, a splendid radiation of pyrgulinid gastropods in ancient Lake Ohrid (see above). Within this relatively young monophyletic group, there are, however, three taxa of the genus *Pyrgohydrobia* that are phenotypically very similar. One of them occurs within the lake proper and one each in springs south and north of the lake (see plate X in Radoman 1983 and figs. 8 and 9a in Albrecht and Wilke 2008). Equally, non-adaptive radiations may contain adaptive elements.

For the genus *Bythinella*, we could show that in the only case of sympatry of species from the same radiation in our dataset (*B. opaca* and *B. robiciana*; Fig. 2*)*, character displacement has occurred (see Sect. 4.3 above). Acknowledging that the adaptive value of this phenotypical change remains unclear, it could be seen as an adaptive shift. Similar processes may have played a role in a few other taxa like *B. bicarinata*, which has a very unusual shell with two keels but is very closely related to other congeners (Bichain et al. 2007a; see also radiation #8 in Fig. 3).

Interestingly, there is no evidence in our data indicating that non-adaptive radiations in *Bythinella* changed into adaptive ones or vice versa. Even among species from different non-adaptive radiations, we do not see clear niche or phenotypical differences (see Sect. 4.2), and no substantial sympatry. These patterns, however, would be expected if adaptive elements via natural selection would accumulate over time. This may serve as another indication that *Bythinella*

spp. is an excellent example for studying the other side of the coin – non-adaptive radiations.

5.4 Perspectives in Studying Non-adaptive Radiations

As noted in the Introduction, our current knowledge of non-adaptive radiations is based on a few studies of often restricted taxa. Although our model taxon, *Bythinella* spp., might add important information, we are still far away from a comprehensive understanding of non-adaptive radiations. Therefore, future research should focus on truly comparative studies of non-adaptive radiations in order to identify the evolutionary processes that drive this form of radiation. Of interest could be questions as to the role of genetic drift in non-adaptive radiations and as to the rate of evolution (both in terms of absolute speed of evolution in non-adaptive radiations and in terms of potential rate differences between adaptive and non-adaptive radiations). Finally, understanding habitat requirements of species in non-adaptive radiations may be of uttermost interest. Is "habitat tracking", indeed, responsible for the lack of clear niche differentiation within non-adaptive radiations? And if so, under what conditions does habitat tracking constitute an evolutionary advantage over adaptation to new ecological niches? A possible starting point for assessing habitat requirements could be the question whether non-adaptive radiations are more common in systems with a relatively small degree of habitat diversification, such as limnic systems. It is remarkable that many limnic ecosystems (with the exception of, e.g., ancient lakes) harbor similar species with low morphological diversity (see Hebert 1998 and references therein) compared to, for example, many terrestrial habitats that are often characterized by strong environmental gradients and hence strong habitat diversification. This, finally, would bring the discussion back to the aspect of low degree of phenotypical variation in non-adaptive radiations stressed by Gittenberger (1991) and Davis (1993). The question then would be whether the similar (and often simple) ground plan is a plesiomorphic character or whether similar habitats may lead to extensive homoplasy (e.g., parallelism or reversal) within non-adaptive radiations.

6 Take Home Message

Studies of radiations in general and of adaptive or non-adaptive radiations in particular are compounded by several conceptual, practical, and philosophical difficulties. First of all, there is no universally accepted definition for either term and no common understanding as to the taxonomic extent and evolutionary rate of radiations. Moreover, different types of radiations are mainly distinguished based on extant patterns rather than underlying mechanism and are therefore easily misinterpreted. Then, there are difficulties in establishing operational criteria allowing for

identifying radiations from, for example, phylogenies, and different approaches may recognize sets of vastly different radiations. Finally, there is an almost philosophical debate relative to the role of genetic drift in speciation (and therefore radiation) processes and whether speciation can occur in sympatry or not. If, however, one rejects the notion of sympatric speciation, then adaptive radiation would have to occur in parapatry and/or allopatry, confounding adaptive and non-adaptive radiations. Likewise, if one does not believe in the role of genetic drift, then the concept of non-adaptive radiations would be ill-defined. Interestingly, even Sewall Wright, who was a foremost advocate of the role of genetic drift in non-adaptive radiations (see the various papers cited above) and who was repeatedly criticized for his view (the depreciatory term "Sewall Wright Effect" was introduced by his critics, Fisher and Ford 1950: p.117), later begun to have some doubts. In 1982, he wrote "I emphasize here that while I have attributed great importance to random drift in small local populations ... I have never attributed importance to non-adaptive differentiation of species" (Wright 1982: p.12). Nonetheless, today, it is generally accepted that small subdivided (peripatric) populations is exactly the situation in which genetic drift has such a profound effect on speciation and radiation processes. If non-adaptive radiations, indeed, are more associated with genetic drift and adaptive radiations more with natural selection, then this may lead to another difference between these two types of radiation, that is, rate of evolution. As random drift is assumed to often act slower at loci than natural selection (Haldane 1949), non-adaptive radiations may produce fewer species during the same time frame than adaptive radiations. This might be another reason why non-adaptive radiations are sometimes overlooked.

So, what can be learned from Sewall Wright and subsequent discussions? For a better understanding of radiations, it is necessary to have a better understanding of the underlying mechanisms rather than resulting patterns. The term radiation should be reserved for rapid evolutionary processes at the species level. Differences between adaptive and non-adaptive radiations are often related to drift and selection. As both mechanisms are not mutually completely exclusive, it might not always be possibly to unequivocally differentiate between these two forms of radiation. In such cases, the term radiation should be used without epithet. Finally, more work has to be done to better fit mechanisms of, for example, hybridization, chromosomal architectural changes, genome duplications, and sexual selection into the concept of adaptive versus non-adaptive radiations, particularly in invertebrates. Sexual selection, for example, is per se not associated with niche differentiation and may therefore be associated with non-adaptive radiations. On the other hand, sexual selection is a form of selection (maybe even a form of natural selection) and may therefore be associated with adaptive radiations. Thus, we will likely see more discussions on this topic in the years to come, and maybe workers will decide to establish a third category of radiations – for mechanisms related to sexual selection.

Acknowledgements We sincerely thank V. Anistratenko, H. Blatterer, M. Bodon, U. Bößneck, M. Colling, Z. Erõss, A. Falniowski, Z. Fehér, P. Glöer, M. Haase, B. Hausdorf, H. J. Hirschfelder,

P. Mildner (†), V. Pešić, C. Renker, D. Reum, S. K. Şahin, M. Szarowska, and Z. Yildirim for kindly providing samples of *Bythinella* spp. and two anonymous referees for their comments. E. Gittenberger and G. Davis are acknowledged for inspiring our work on non-adaptive radiations. This study was financially supported by DFG grants WI 1902/5-1 (T. Wilke) and BR 1967/6-1 (M. Brändle), as well as by a stipend of the Fazit-Foundation to M. Benke.

References

Abouheif E (1999) A method for testing the assumption of phylogenetic independence in comparative data. Evol Ecol Res 1:895–909

Albertson RC, Markert JA, Danley PD, Kocher TD (1999) Phylogeny of a rapidly evolving clade: the cichlid fishes of Lake Malawi, East Africa. Proc Natl Acad Sci USA 96:5107–5110

Albrecht C, Wilke T (2008) Lake Ohrid: biodiversity and evolution. Hydrobiologia 615:103–140

Albrecht C, Trajanovski S, Kuhn K, Streit B, Wilke T (2006) Rapid evolution of an ancient lake species flock: freshwater limpets (Gastropoda: Ancylidae) in the Balkan lake Ohrid. Org Divers Evol 6:294–307

Attwood SW, Ambu S, Meng X-H, Upatham ES, Xu F-S, Southgate VR (2003) The phylogenetics of triculine snails (Rissooidea: Pomatiopsidae) from south-east Asia and southern China: historical biogeography and the transmission of human schistosomiasis. J Molluscan Stud 69:263–271

Barraclough TG, Nee S (2001) Phylogenetics and speciation. Trends Ecol Evol 16:391–399

Benke M, Brändle M, Albrecht C, Wilke T (2009) Pleistocene phylogeography and phylogenetic concordance in cold-adapted spring snails (*Bythinella* spp.). Mol Ecol 18:890–903

Bichain J-M, Gaubert P, Samadi S, Boisselier-Dubayle M-C (2007a) A gleam in the dark: phylogenetic species delimitation in the confusing spring-snail genus *Bythinella* Moquin-Tandon, 1856 (Gastropoda: Rissooidea: Amnicolidae). Mol Phylogenet Evol 45:927–941

Bichain J-M, Boisselier-Dubayle M-C, Bouchet P, Samadi S (2007b) Species delimitation in the genus *Bythinella* (Mollusca: Caenogastropoda: Rissooidea): a first attempt combining molecular and morphometrical data. Malacologia 49:293–311

Boeters HD (1968) Die Hydrobiidae Badens, der Schweiz und der benachbarten französischen Departements. Mitt Bad Landesver Naturk Naturschutz Freiburg 9:755–778

Bolnick DI (2006) Multi-species outcomes in a common model of sympatric speciation. J Theor Biol 241:734–744

Brändle M, Westermann I, Brandl R (2005) Gene flow between populations of two invertebrates in springs. Freshw Biol 50:1–9

Brooks DR, O'Grady RT, Glen DR (1985) Phylogenetic analysis of the Digenea (Platyhelminthes; Cercomeria) with comments on their adaptive radiation. Can J Zool 63:411–443

Cameron RAD, Cook LM, Hallows JD (1996) Land snails on Porto Santo: adaptive and non-adaptive radiation. Philos Trans R Soc Lond B 351:309–327

Chan KMA, Moore BR (2004) SymmeTree: whole-tree analysis of differential diversification rates. Bioinformatics 21:1709–1710

Clark SA, Miller AC, Ponder WF (2003) A revision of the snail genus *Austropyrgus* (Gastropoda: Hydrobiidae): a morphostatic radiation of freshwater gastropods in southeastern Australia. Rec Aust Mus 28(Suppl):1–109

Colgan DJ, Ponder WF (1994) The evolutionary consequences of restrictions in gene flow: examples from hydrobiid snails. Nautilus 2(Suppl):25–43

Comes HP, Tribsch A, Bittkau C (2008) Plant speciaton in continental island floras as exemplified by *Nigella* in the Aegean Archipelago. Philos Trans R Soc Lond B 363:3083–3096

Cook LM (2008) Species richness in Madeiran land snails, and its causes. J Biogeogr 35:647–653

Davis GM (1993) Evolution of prosobranch snails transmitting asian *Schistosoma*; coevolution with *Schistosoma*: A Review. Prog Clin Parasitol 3:145–204
Davis GM (1994) Molecular genetics and taxonomic discrimination. Nautilus Suppl 2:3–23
Davis GM, Chen CE, Wu C, Kuang TF, Xing XG, Li L, Liu WJ, Yan YL (1992) The Pomatiopsidae of Hunan, China (Gastropoda: Rissoacea). Malacologia 34:143–342
Davison A, Chiba S (2006) Labile ecotypes accompany rapid cladogenesis in an adaptive radiation of *Mandarina* (Bradybaenidae) land snails. Biol J Linn Soc 88:269–282
Dieckmann U, Doebeli M, Metz JAJ, Tautz D (2004) Adaptive speciation. Cambridge University Press, Cambridge, UK
Eldredge N (2000) Species, speciation and the environment. http://www.actionbioscience.org/evolution/ eldredge.html. Cited 6 Feb 2009
Eldredge N (2003) The sloshing bucket: how the physical realm controls evolution. In: Crutchfield J, Schuster P (eds) Evolutionary dynamics: exploring the interplay of selection, accident, neutrality, and function (SFI Studies in the Sciences of Complexity Series). Oxford University Press, New York, pp 3–32
Eldredge N, Thompson JN, Brakefield PM, Gavrilets S, Jablonski D, Jackson JBC, Lenski RE, Lieberman BS, McPeek MA, Miller W (2005) The dynamics of evolutionary stasis. Paleobiology 31(sp 5):133–145
Falniowski A (1992) Genus *Bythinella* Moquin-Tandon, 1855, in Poland (Gastropoda, Prosobranchia, Hydrobiidae). In: Gittenberger E, Goud J (eds) Proceedings of the ninth international malacological congress, Edinburgh, 1986. Unitas Malacologica, Leiden, pp 135–138
Falniowski A, Szarowska M, Fiałkowski W, Mazan K (1998) Unusual geographic pattern of interpopulation variation in a spring snail *Bythinella* (Gastropoda: Prosobranchia). J Nat Hist 32:605–616
Fauna Europaea Web Service (2004) Fauna Europaea Version 1.1. http://faunaeur.org. Cited 4 Nov 2008
Fisher RA, Ford EB (1950) The "Sewall Wright effect". Heredity 4:117–119
Foote M (1996) Models of morphological diversification. In: Jablonski D, Erwin DH, Lipps JH (eds) Evolutionary paleobiology. University of Chicago Press, Chicago, IL, pp 62–86
Freckleton RP, Harvey PH (2006) Detecting non-Brownian trait evolution in adaptive radiations. PLoS Biol 4:2104–2111
Futuyma D (1998) Evolutionary biology. Sinauer, Massachusetts
Gavrilets S, Losos JB (2009) Adaptive radiation: contrasting theory with data. Science 323:732–737
Gavrilets S, Vose A (2005) Dynamic patterns of adaptive radiation. Proc Natl Acad Sci USA 102:18040–18045
Gittenberger E (1991) What about non-adaptive radiation? Biol J Linn Soc 43:263–272
Gittenberger E (2004) Radiation and adaptation, evolutionary biology and semantics. Org Divers Evol 4:135–136
Gittenberger E (2007) Islands from a snail's perspective. Top Geobiol 29:347–364
Gittenberger E, Hausdorf B (2004) The *Orculella* species of the South Aegean island arc, a neglected radiation (Gastropoda, Pulmonata, Orculidae). Basteria 68:93–124
Giusti F, Pezzoli E (1977) Primo contributo alla revisione del genere *Bythinella* in Italia. Natura Bresciana, Ann Mus Civ St Nat Brescia 14:3–66
Givnish TJ (1997) Adaptive radiation and molecular systematics: issues and approaches. In: Givnish TJ, Sytsma KJ (eds) Molecular evolution and adaptive radiation. Cambridge University Press, New York, pp 1–54
Greenwood PH (1984) What is a species flock? In: Echelle AA, Kornfield I (eds) Evolution of fish species flocks. Orono, Maine, pp 13–19
Guyer C, Slowinski JB (1993) Adaptive radiation and the topology of large phylogenies. Evolution 47:253–263
Haase M, Wilke T, Mildner P (2007) Identifying species of *Bythinella* (Caenogastropoda: Rissooidea): a plea for an integrative approach. Zootaxa 1563:1–16

Haldane JBS (1949) Suggestions as to quantitative measurement of rates of evolution. Evolution 3:51–56

Hebert PDN (1998) Variable environments and evolutionary diversification in inland waters. In: Carvalho GR (ed) Advances in molecular ecology. IOS Press, Amsterdam, pp 175–195

Hodges SA (1997) Rapid radiation due to a key innovation in colombines (Ranunculaceae: *Aquilegia*). In: Givnish TJ, Sytsma KJ (eds) Molecular evolution and adaptive radiation. Cambridge University Press, New York, pp 391–405

Jungbluth JH (1972) Die Verbreitung und Ökologie des Rassenkreises *Bythinella dunkeri* (Frauenfeld, 1856) (Mollusca: Prosobranchia). Arch Hydrobiol 70:230–273

Kapralov MV, Filatov DA (2006) Molecular adaptation during adaptive radiation in the Hawaiian endemic genus *Schiedea*. PLoS ONE 1(1):e8

Klemm M, Schlegel M (1989) Genetic differentiation in *Bythinella dunkeri* and *Bythinella badensis* from Black Forest (SW Germany). (Prosobranchia, Bythinellidae). In: Abstracts of the 10th international malacological congress, Tübingen, 1989. Unitas Malacologica, p 133

Kozak KH, Wiens JJ (2006) Does niche conservatism promote speciation? A case study in North American salamanders. Evolution 60:2604–2621

Kozak KH, Weisrock DW, Larson A (2006) Rapid lineage accumulation in a non-adaptive radiation: phylogeographic analysis of diversification rates in eastern North American woodland salamanders (genus *Plethodon*). Proc R Soc Lond B 273:539–546

Leander BS, Keeling PJ (2003) Morphostasis in alveolate evolution. Trends Ecol Evol 18:395–402

Liu H-P, Hershler R, Clift K (2003) Mitochondrial DNA sequences reveal extensive cryptic diversity within a western American springsnail. Mol Ecol 12:2771–2782

Losos JB, Miles DB (2002) Testing the hypothesis that a clade has adaptively radiated: iguanid lizard clades as a case study. Am Nat 160:147–157

Mayr E (1963) Animal species and evolution. Harvard University Press, Cambridge, Mass

Mazan K, Szarowska M (2000) Morphological and allozymic variation within and between populations of *Bythinella* Moquin-Tandon, 1855 (Gastropoda, Prosobranchia). III. Phylogenetic analysis. Folia Malacologica 8:257–269

Minkoff EC (1983) Evolutionary biology. Addison-Wesley, Reading, MA

Moline AB, Shuster SM, Hendrickson DA, Marks JC (2004) Genetic variation in a desert aquatic snail (*Nymphophilus minckleyi*) from Cuatro Cienegas, Coahuila, Mexico. Hydrobiologia 522:179–192

Montgomery R, Givnish T (2008) Adaptive radiation of photosynthetic physiology in the Hawaiian lobeliads: dynamic photosynthetic responses. Oecologia 155:455–467

Nitecki MH, Nitecki DV (1990) Evolutionary innovations. University of Chicago Press, Chicago

O'Brien C, Blinn DW (1999) The endemic spring snail *Pyrgulopsis montezumensis* in a high CO_2 environment: importance of extreme chemical habitats as refugia. Freshw Biol 42:225–234

Oliver PM, Adams M, Lee MSY, Hutchinson MN, Doughty P (2009) Cryptic diversity in invertebrates: molecular data double estimates of species diversity in a radiation of Australian lizards (Diplodactylus, Gekkota). Proc R Soc Lond B 276:2001–2007

Osborn HF (1918) The origin and evolution of life. Scribner's, New York

Perez KE, Ponder WF, Colgan DJ, Clark SA, Lydeard C (2005) Molecular phylogeny and biogeography of spring-associated hydrobiid snails of the Great Artesian Basin, Australia. Mol Phylogenet Evol 34:545–556

Pinceel J, Jordaens K, Van Houtte N, De Winter AJ, Backeljau T (2004) Molecular and morphological data reveal cryptic taxonomic diversity in the terrestrial slug complex *Arion subfuscus*/*fuscus* (Mollusca, Pulmonata, Arionidae) in continental north-west Europe. Biol J Linn Soc 83:23–38

Ponder WF, Colgan DJ (2002) What makes a narrow-range taxon? Insights from Australian freshwater snails. Invertebr Syst 16:571–582

Ponder WF, Eggler P, Colgan DJ (1996) Genetic differentiation of aquatic snails (Gastropoda: Hydrobiidae) in artesian springs in arid Australia. Biol J Linn Soc 56:553–596

Ponder WF, Wilke T, Zhang W-H, Golding RE, Fukuda H, Mason RAB (2008) *Edgbastonia alanwillsi* n. gen & n. sp. (Tateinae: Hydrobiidae s.l.: Rissooidea: Caenogastropoda); a snail from an artesian spring group in western Queensland, Australia, convergent with some Asian Amnicolidae. Mol Res 28:89–106

Purvis A, Nee S, Harvey PH (1995) Macroevolutionary inferences from primate phylogeny. Proc R Soc Lond B 260:329–333

Pybus OG, Harvey PH (2000) Testing macro-evolutionary models using incomplete molecular phylogenies. Proc R Soc Lond B 267:2267–2272

Pybus OG, Rambaut A, Holmes EC, Harvey PH (2002) New inferences from tree shape: numbers of missing taxa and population growth rates. Syst Biol 51:881–888

Radoman P (1976) Speciation within the family Bythinellidae on the Balkans and Asia Minor. Z Zool Syst Evol 14:130–152

Radoman P (1983) Hydrobioidea a superfamily of Prosobranchia (Gastropoda). I. Systematics. Serbian Academy of Sciences and Arts, Belgrade, Serbia

Ree RH (2005) Detecting the historical signature of key innovations using stochastic models of character evolution and cladogenesis. Evolution 59:257–265

Rundell RJ, Price TD (2009) Adaptive radiation, nonadaptive radiation, ecological speciation and nonecological speciation. Trends Ecol Evol 24:394–399

Salzburger W (2009) The interaction of sexually and naturally selected traits in the adaptive radiations of cichlid fishes. Mol Ecol 18:169–185

Sanderson MJ (1998) Reappraising adaptive radiations. Am J Bot 85:1650–1655

Sauer J, Hausdorf B (2009) Sexual selection is involved in speciation in a land snail radiation on Crete. Evolution 63:2535–2546

Schluter D (1994) Experimental evidence that competition promotes divergence in adaptive radiation. Science 266:798–801

Schluter D (1998) Ecological causes of speciation. In: Howard D, Berlocher S (eds) Endless forms: species and speciation. Oxford University Press, Oxford, pp 114–129

Schluter D (2000) The ecology of adaptive radiation. Oxford University Press, Oxford

Schön I, Martens K (2004) Adaptive, pre-adaptive and non-adaptive components of radiations in ancient lakes: a review. Org Divers Evol 4:137–156

Schultheiß R, Van Bocxlaer B, Wilke T, Albrecht C (2009) Old fossils–young species: evolutionary history of an endemic gastropod assemblage in Lake Malawi. Proc R Soc Lond B 276:2837–2846

Schwoerbel J (1999) Einführung in die Limnologie. Gustav Fischer, Stuttgart

Seehausen O (2000) Explosive speciation rates and unusual species richness in haplochromine cichlid fishes: effects of sexual selection. In: Rossiter A, Kawanabe H (eds) Adv Ecol Res. Ancient lakes: biodiversity, ecology and evolution. Academic, San Diego, pp 237–274

Seehausen O (2002) Patterns in fish radiation are compatible with Pleistocene desiccation of Lake Victoria and 14600 year history for its cichlid species flock. Proc R Soc Lond B 269:491–497

Seehausen O (2004) Hybridization and adaptive radiation. Trends Ecol Evol 19:198–207

Seehausen O (2006) African cichlid fish: a model system in adaptive radiation research. Proc R Soc Lond B 273:1987–1998

Seehausen O (2007) Chance, historical contingency and ecological determinism jointly determine the rate of adaptive radiation. Heredity 99:361–363

Seehausen O, Van Alphen JJM, Witte F (1997) Cichlid fish diversity threatened by eutrophication that curbs sexual selection. Science 277:1808–1811

Seehausen O, Van Alphen JJM, Witte F (1999) Can ancient colour polymorphisms explain why some cichlid lineages speciate rapidly under disruptive sexual selection? Belg J Zool 129:43–60

Shaw KL (1995) Biogeographic patterns of two independent Hawaiian cricket radiations (*Laupala* and *Prognathogryllus*). In: Wagner WL, Funk VA (eds) Hawaiian biogeography: evolution on a hot spot archipelago. Smithsonian Institution Press, Washington, pp 39–56

Simpson GG (1949) The meaning of evolution, a study of the history of life and of its significance for man. Yale University Press, New Haven
Simpson GG (1953) The major features of evolution. Columbia University Press, New York
Stelkens R, Seehausen O (2009) Genetic distance between species predicts novel trait expression in their hybrids. Evolution 63:884–897
Streelman JT, Danley PD (2003) The stages of vertebrate evolutionary radiation. Trends Ecol Evol 18:126–131
Sturmbauer C, Baric S, Salzburger W, Ruber L, Verheyen E (2001) Lake level fluctuations synchronize genetic divergences of cichlid fishes in African lakes. Mol Biol Evol 18:144–154
Sudhaus W (2004) Radiation within the framework of evolutionary ecology. Org Divers Evol 4:127–134
Szarowska M (2000) Environmental threats and stability of *Bythinella* populations in South Poland (Gastropoda: Prosobranchia: Hydrobioidea). Malakol Abh (Dresd) 20:93–986
Szarowska M, Wilke T (2004) *Sadleriana pannonica* (Frauenfeld, 1865): a lithoglyphid, hydrobiid, or amnicolid taxon? J Molluscan Stud 70:49–57
Szarowska M, Falniowski A, Fiałkowski W, Mazan K (1998) Adaptive significance of glucose phosphate isomerase (GPI) allozymes in the spring snail *Bythinella*? J Molluscan Stud 64:257–261
Van Oppen MJH, Turner G, Hewitt GM (2000) Extensive homoplasy, nonstepwise mutations, and shared ancestral polymorphism at a complex microsatellite locus in Lake Malawi cichlids. Mol Biol Evol 17:489–498
von Rintelen T, Wilson AB, Meyer A, Glaubrecht M (2004) Escalation and trophic specialization drive adaptive radiation of freshwater gastropods in ancient lakes on Sulawesi, Indonesia. Proc R Soc Lond B 271:2541–2549
Wake DB (2006) Problems with species: patterns and processes of species formation in salamanders. Ann Mo Bot Gard 93:8–23
Whittaker RJ, Fernández-Palacios JM (2007) Island biogeography: ecology, evolution, and conservation. Oxford University Press, New York
Wiens JJ (2004) Speciation and ecology revisited: phylogenetic niche conservatism and the origin of species. Evolution 58:193–197
Wilke T (2004) How dependable is a non-local molecular clock? A reply to Hausdorf et al. (2003). Mol Phylogenet Evol 30:835–840
Wilke T, Duncan N (2004) Phylogeographical patterns in the American Pacific Northwest: lessons from the arionid slug *Prophysaon coeruleum*. Mol Ecol 13:2303–2315
Wilke T, Pfenninger M (2002) Separating historic events from recurrent processes in cryptic species: phylogeography of mud snails (*Hydrobia* spp.). Mol Ecol 11:1439–1451
Wilke T, Pfenninger M, Davis GM (2002) Anatomical variation in cryptic mudsnail species: statistical discrimination and evolutionary significance. Proc Acad Nat Sci Phila 152:45–66
Wilke T, Albrecht C, Anistratenko VV, Sahin SK, Yildirim MZ (2007) Testing biogeographical hypotheses in space and time: faunal relationships of the putative ancient Lake Egirdir in Asia Minor. J Biogeogr 34:1807–1821
Wilke T, Schultheiß R, Albrecht C (2009) As time goes by: a simple fool's guide to molecular clock approaches in invertebrates. In: Symposium of "Molluscs as models in evolutionary biology: from local speciation to global radiation" presented at the World Congress of Malacology, held from 15 to 20 July 2007 in Antwerp, Belgium. Am Malac Bull 47:25–45
Wright S (1929) The evolution of dominance. Comment on Doctor Fisher's reply. Am Nat 63:556–561
Wright S (1931) Statistical theory of evolution. Am Stat J 26(Suppl):201–208
Wright S (1932) The roles of mutation, inbreeding, crossbreeding and selection in evolution. In: Proceedings of sixth international congress of genetics, pp 356–366
Wright S (1982) The shifting balance theory and macromutation. Ann Rev Genet 16:1–19

Index