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MOLECULAR SYSTEMATICS OF *CARDAMINE* AND ALLIED GENERA (*BRASSICACEAE*): ITS AND NON-CODING CHLOROPLAST DNA

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Abstract: Representatives of the genera *Cardamine*, *Dentaria*, *Nasturtium*, *Rorippa* and *Armoracia* (*Brassicaceae*) were analyzed to elucidate their phylogenetic relationships based on nuclear (ITS) and non-coding chloroplast (cp) DNA sequences. *Dentaria* seems to be polyphyletic. The two studied *Dentaria* species group with different *Cardamine* clades, and it is argued that *D. bulbifera* is an allopolyploid originating from a hybridization between a *Cardamine* and a *Dentaria* species. In the ITS tree, *Nasturtium* and *Rorippa* form well supported clades but their relationship to *Cardamine* and *Armoracia* remains unresolved. In the cpDNA tree, *Nasturtium* groups together with *Cardamine*. Hybridization events apparently played a role in the evolution of *Nasturtium*. The *Cardamine*/*Nasturtium* clade is separated from a clade placing *Rorippa* and *Armoracia* together. *Armoracia* is closely related to *Rorippa*. Analyses of the 19 *Cardamine* species studied revealed three main groupings, a northern hemispheric and two southern hemispheric groups. Within the northern hemisphere taxa the *C. pratensis* complex forms a well supported clade which seems to be closely related to *C. amara*, *C. raphanifolia* and *C. flexuosa*. The positions of *C. hirsuta* and *C. impatiens* are uncertain. The two southern hemisphere clades consist of New Guinean species and south-eastern Australian/Tasmanian and subantarctic species, respectively. They may reflect migration routes from the northern to the southern hemisphere, but further studies are necessary to fully understand the evolution of the bihemispheric distribution pattern of *Cardamine*.

INTRODUCTION

The genera *Cardamine* L., *Dentaria* L., *Nasturtium* R. BR., *Rorippa* SCOP., and *Armoracia* P. GAERTN. et al. of the family *Brassicaceae* are thought to be closely related (HAYEK 1911, JANCHEN 1942, AL-SHEHBAZ 1988, ZUNK 1994) and are placed together in the tribe *Arabideae* DC. by the first three authors. SCHULZ (1936), however, separated *Armoracia* from *Cardamine*, *Rorippa* and *Nasturtium* and placed it in the tribe *Drabeae* O.E. SCHULZ. Phylogenetic relationships between and among these genera are still unsettled and continue to be disputed.

The genus *Cardamine* is of worldwide distribution and comprises about 150 species. SCHULZ (1903, 1936) divided the genus into 13 sections. The largest section is *Cardamine* (= "*Eucardamine*" O.E. SCHULZ) with about 60 species. Six out of the remaining 12 sections are monotypic. AL-SHEHBAZ (1988) criticized this taxonomic treatment as artificial and not reflecting natural groups due to overemphasizing a few characters. The bulk of species show a northern hemispheric distribution but southern hemisphere continents have indigenous species as well, in addition to some cosmopolitan weedy species. The southern hemispheric species are largely restricted to high montane or alpine habitats. An issue of high controversy

is the taxonomic treatment of the genus *Dentaria*. DETLING (1936) for instance, and some authors of modern local floras classify *Dentaria* and *Cardamine* as separate genera, whereas AKERROYD (1993) in Flora Europaea treats *Dentaria* as a subgenus of *Cardamine*, and SCHULZ (1903, 1936) as a section within *Cardamine*. *Nasturtium* (watercress) is recognized as a distinct genus by most European authors (MARKGRAF 1986). There has been some confusion in distinguishing watercress taxa from South American *Rorippa* species due to the lack of exclusive morphological characters (AL-SHEHBAZ 1988, AL-SHEHBAZ & PRICE 1998). *Rorippa* is a genus of 70–80 species of world-wide distribution. The bulk of species are grouped together in the section *Rorippa*. The genus *Armoracia* has four species distributed in eastern and south-eastern Europe, in Siberia and in North America. Early authors placed *Armoracia* as a section within the genus *Cochlearia* L. (e.g. CANDOLLE 1821). HAYEK (1911) treated it as a genus on its own and did not recognize closer affinities to *Cochlearia*.

The aim of this study is to elucidate both, the relationships among taxa described above and within *Cardamine*, based on DNA sequences. We chose to use markers from two different genomes, nuclear (nr) DNA and chloroplast (cp) DNA, because of the fact that polyploidy (auto- and allopolyploidy) has played a major role in the evolution of *Arabideae*. A possible way to detect hybridization events which may lead to polyploidization is to compare phylogenies inferred from nuclear and chloroplast markers (SOLTIS & KUZOFF 1995). The ITS regions of the nuclear ribosomal DNA are an often used tool in angiosperm phylogeny at lower taxonomic levels (reviewed in BALDWIN et al. 1995). Two non-coding regions from the chloroplast genome, recently introduced as a source of evidence on phylogeny at the generic level (GIELLY & TABERLET 1994a, 1994b, 1996, BÖHLE et al. 1994, VAN HAM et al. 1994), were used as chloroplast markers. The two regions are located in the large single copy region of the chloroplast genome; one is an intergenic spacer between the *trn*T (UGU) and the *trn*L (UAA) 5′exon, and the second the *trn*L (UAA) intron.

In the present study we analyzed three representatives of the largest section *Rorippa* from the genus *Rorippa*, two *Nasturtium* species, *Armoracia rusticana*, two *Dentaria* species and 19 *Cardamine* species of which six are southern hemisphere taxa. The analyzed *Cardamine* species are from the sections *Cardamine* and *Cardaminella* PRANTL.

MATERIAL AND METHODS

Plant material

Seed samples, living plants and herbarium specimens were collected from natural populations. Species analyzed are given in Tab. 1. *Capsella bursa-pastoris* served as an outgroup. Voucher specimens are deposited in the Herbarium of the Botany Department of the University of Osnabrück (OSBU).

DNA extraction, amplification, and sequencing

Total DNA was extracted using the CTAB (hexadecyltrimethylammonium bromide) method of DOYLE & DOYLE (1987). We modified the extraction for microcentrifuge tubes. Fresh and herbarium material were treated in the same way. PCR amplification of the ITS1 and ITS2 region, *trn*T/*trn*L spacer, and *trn*L intron were performed in 50 µl volumes of 30 µl sterile water, 125 ng DNA, 150 µl dNTP (Eurogentec, Searing, Belgium), 0.04 µM of each primer, 0.5 units Goldstar-DNA polymerase (Eurogentec, Searing, Belgium), 1.5 mM $MgCl_2$ and 5 µl polymerase reaction buffer (Eurogentec, Searing, Belgium). For ITS regions the primers

Table 1. List of taxa, chromosome numbers*, collection data, sources of plant material and GenBank accession numbers.

Taxa	2n	Country	Locality	Collector/origin	GenBank accession number ITS 1/2	*trn*T/L spacer	*trn*L intron
Armoracia							
Armoracia rusticana P. GAERTN., B. MEY. et SCHERB.	(32)[1]	Germany: Niedersachsen	Bad Bentheim	leg. M. KOCH	AF078031 AF078518	AF078032 AF079350	AF078517
Northern hemisphere *Cardamine* and *Dentaria* taxa							
C. amara L.	16	Switzerland: Uri	Urnerboden	leg. E. LANDOLT et al.	AF077993 AF078486	AF077994 AF079334	AF078485
C. flexuosa WITH.	32	Germany: Niedersachsen	Osnabruck	leg. R. KOHRT	AF077999 AF078492	AF078000 AF079337	AF078491
C. hirsuta L.	16	Germany: Rheinland-Pfalz	Mainz	leg. R. KOHRT	AF077997 AF078490	AF077998 AF079336	AF078489
C. impatiens L.	16	Germany. Nordrhein--Westfalen	Rheine	leg. R. KOHRT	AF078015 AF078508	AF078016 AF079345	AF078507
***C. pratensis* s.l.**							
- *C. crassifolia* POURR	(16)[2]	Spain: Guadalajara	Orea: Rio de la Hoz Seca	leg. A. FRANZKE	AF077983 AF078476	AF077984 AF079329	AF078475
- *C. dentata* SCHULT.	> 50	Germany: Mecklenburg--Vorpommern	Greifswald	leg. W. BLEEKER, M. MANTEY	AF077989 AF078482	AF077990 AF079332	AF078481
- *C. matthioli* MORETTI	16[3]	Slovakia	Brezno	leg. A. FRANZKE	AF077985 AF078478	AF077986 AF079330	AF078477
- *C. majovskii* MARHOLD & ZÁBORSKÝ	32	Slovakia: Bukovské vrchy	Ulič	leg. A. FRANZKE	AF077987 AF077988	AF078479 AF078480	AF079331
- *C. nymanii* GAND.	(64–96)[1]	Norway	Dovrefjell	leg. H. HURKA, B. NEUFFER	AF077991 AF078484	AF007992 AF079333	AF078483
- *C. pratensis* L.	16	Germany: Baden--Württemberg	Tettnang	leg. A. FRANZKE	AF077977 AF078470	AF077978 AF079326	AF078469
- *C. rivularis* auct.	16[4]	Austria: Kärnten	Koralpe	leg W. BLEEKER, A. FRANZKE, M. KOCH	AF077979 AF078472	AF077980 AF079327	AF078471
- *C. rivularis* SCHUR	16[5]	Romania: Munţii Arpaşului	Valea Arpaşului	leg. K. MARHOLD	AF077981 AF078474	AF077982 AF079328	AF078473
C. raphanifolia POURR.	(48)[6]	Spain: León	Puerto de San Isidro	leg. A. FRANZKE	AF077995 AF078488	AF077996 AF079335	AF078487
D. bulbifera L.	(96)[1]	Germany: Niedersachsen	Osnabrück	Botanical Garden	AF078017 AF078510	AF078018 AF079346	AF078509
D. pentaphyllos L.	(48)[1]	Switzerland: Bern	Schynige Platte	Specimen from OSBU, leg. B. NEUFFER	AF078019 AF078512	AF078020 AF079347	AF078511
Southern hemisphere *Cardamine* taxa							
C. africana L.	(16)[7]	New Guinea	Mt. Wilhelm	Specimen from CANB, No. 678	AF078009 AF078502	AF078010 AF079342	AF078501
C. altigena SCHLECHTER	?	New Guinea	Mt. Wilhelm	Specimen from CANB No. 6261 leg. J.F. VELDKAMP	AF078011 AF078504	AF078012 AF079343	AF078503
C. corymbosa HOOK. f.	(48)[8]	Australia	Melbourne	leg. H. HURKA, I.R. THOMPSON	AF078003 AF078496	AF078004 AF079339	AF078495
C. aff. *flexuosa*	32	Australia: Victoria	Mt. Beauty	leg. H. HURKA	AF078001 AF078494	AF078002 AF079338	AF078493

Taxa	2n	Country	Locality	Collector/origin	GenBank accession number ITS 1/2	*trn*T/L spacer	*trn*L intron
C. keysseri O. E. SCHULZ	?	New Guinea	Mt. Wilhelm	Specimen from CANB No. 15340 leg. J.M.B. SMITH	AF078013 AF078506	AF078014 AF079344	AF078505
C. lilacina HOOK.	48	Australia: Tasmania	Mt. Wellington	leg. H. HURKA	AF078005 AF078498	AF078006 AF079340	AF078497
C. lilacina HOOK.	48	Australia: New South Wales	Mt. Kosciusko	leg. H. HURKA	AF078007 AF078500	AF078008 AF079341	AF078499
***Nasturtium* taxa**							
N. officinale R. BR.	32	Germany: Hamburg	Moorwerder	Botanical Garden Univ. Hamburg	AF078027 AF078514	AF078028 AF079348	AF078513
N. microphyllum (BOENN.) RCHB.	64	Denmark: Jütland	Helgenaes	Botanical Institute Risskov	AF078029 AF078516	AF078030 AF079349	AF078515
***Rorippa* taxa**							
Rorippa palustris (L.) BESSER	32	Germany: Niedersachsen	Osnabrück	leg. W. BLEEKER	AF078021 AF078520	AF078022 AF079351	AF078519
Rorippa amphibia (L.) BESSER	16	Germany: Nordrhein--Westfalen	Köln	leg. M. KOCH	AF078025 AF078524	AF078026 AF079353	AF078523
Rorippa sylvestris (L.) BESSER	48	Germany: Niedersachsen	Bad Rothenfelde	leg. W. BLEEKER	AF078023 AF078522	AF078024 AF079352	AF078521
Outgroup taxon							
Capsella bursa-pastoris (L.) MEDIK.	32	USA: California	Loleta	leg. H. HURKA	AF078033 AF078526	AF078034 AF079387	AF078525

* Note: Chromosome numbers are according own analyses and to the literature, in the latter case source of information is cited. Numbers that do not refer to particular localities are given in brackets. 1) JALAS & SUOMINEN 1994; 2) RICO (pers. comm.); 3) MARHOLD 1984; 4) MARHOLD 1995; 5) MARHOLD 1994a; 6) ANCHEV (pers. comm.); 7) JONSELL 1976; 8) PRITCHARD 1957.

ITS4 and ITS5 (WHITE et al. 1990), and for cpDNA spacer/intron the primer A, B, C, and D (GIELLY & TABERLET 1994a) were used. The profile for 40 cycles of amplification was: 1 min at 94 °C (first cycle 3 min), 45 s at 50 °C, and 2 min at 72 °C (last cycle 6 min). Successful PCR reactions resulted in a single band in a minigel. Such products were purified using the Quiaquick PCR purification Kit (Quiagen, Hilden, Germany). The purified PCR products were sequenced following a protocol given in MUMMENHOFF et al. (1997a) using the four ITS primers (WHITE et al. 1990) and the primer A, B, C, and D (GIELLY & TABERLET 1994a,b). For cycle sequencing, the reactions were run through the same PCR program used for the amplification.

Data analysis

The ITS sequences and the non-coding cpDNA regions (spacer between *trn*T and the *trn*L 5′ exon; *trn*L intron) were aligned by hand. Regions with ambiguous alignment were eliminated from analysis. Gaps in the alignments (ITS1 and ITS2 combined, cpDNA intron and spacer combined) were treated as missing data. Synapomorphic indels, however, were recoded as additional binary characters. Both matrices were analyzed by Fitch parsimony using the heuristic search option in PAUP (version 3.1.1; SWOFFORD 1993) with MULPARS, TBR (tree

bisection-reconnection) branch swapping, and simple taxon addition. The evolutionary direction of sequence changes was inferred by outgroup comparison. The consistency index (CI) of KLUGE & FARRIS (1969) is presented to estimate the amount of homoplasy in the characters. Parsimony trees with equal length were summarized using the strict consensus method. Clade robustness was analyzed using the bootstrap method (FELSENSTEIN 1985) with the search settings described above and 100 replicates. Pairwise nucleotide differences were computed in PAUP using the DISTANCE MATRIX option. The comparisons were limited to nucleotide positions without gaps or polymorphic states.

This is a joint paper of the group of authors, but particular resposibilities are as follows: Phylogenetic relationships between *Cardamine* and allied genera (A. Franzke, W. Bleeker & H. Hurka); Relationships within *Cardamine* (A. Franzke, K. Pollmann, R. Kohrt & H. Hurka).

RESULTS AND DISCUSSION

General aspects

Nuclear DNA sequences, ITS: Size, divergence and variation

The ITS and cpDNA alignments are not shown but are available from the authors upon request. The sequences were submitted to GenBank. The accession numbers are given in Tab. 1.

The length of the ITS1 and ITS2 regions varied in the taxa under study from 254 bp to 277 bp for ITS1, and 185 bp to 189 bp for ITS2. In all species analyzed in this study, ITS1 is longer than ITS2. This is in accordance with findings reported for other *Brassicaceae* (MUMMENHOFF et al. 1997a,b). The resulting data matrix contains 447 positions after removing regions with alignment ambiguities. Of these, 99 positions (22%) are potentially phylogenetically informative, i.e. with two or more nucleotide states each present in at least two OTUs. 271 sites (61%) are invariant, and 77 sites (17%) are autapomorphic characters. Seven additional binary informative characters (absence/presence of gaps) were coded in the data matrix. Among ingroup accessions, pairwise sequence comparisons indicated ITS sequence divergence ranging from 0 (between *Cardamine lilacina* accessions, between *C. dentata* and *C. nymanii*, between *C. matthioli* and *C. majovskii*) to 13% (between *Nasturtium microphyllum* and *C. rivularis*). The greatest differences between outgroup taxon *Capsella bursa-pastoris* and one ingroup member (*N. microphyllum*) is 17%, the smallest is 12% (between *Capsella* and *Rorippa palustris*). The range of observed sequence divergence in our ingroup taxa (0% to 13%) is typical for congeneric species or closely related genera (BALDWIN et al. 1995).

Chloroplast DNA sequences: Size, divergence and variation

The *trn*T/L spacer and the *trn*L intron were sequenced. The *trn*T/L spacer has a length of approximately 750 bp. It was not possible to sequence the entire fragment. Starting from primer A we could only read about 170 base pairs because an adjacent poly T region prevented accurate further reading. With primer B, we were able to read ca. 300 bp per reaction. After eliminating regions with ambigous alignment, 402 positions from the *trn*L spacer region and 440 positions from the *trn*L intron fragment (ca. 500 bp) were available for phylogenetic analyses. The two sets of sequence data were combined giving a cpDNA data set of 842 positions. 64 positions (8%) were informative, 632 sites (75%) did not vary, and 146 sites (17%) were autapomorphic. In addition, seven informative binary characters (absence/presence of gaps) were coded in the data matrix. The sequence divergence for the

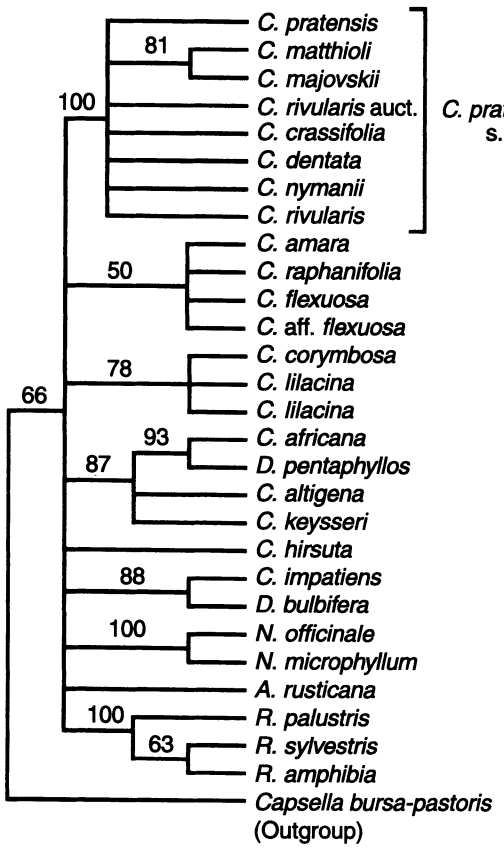

Fig. 1. Strict consensus of the 12 most parsimonious trees based on ITS sequence data. Gaps were treated as missing in the body of the data matrix, but each gap was recoded as an additional presence/absence character. The bootstrap support is shown above branches. Tree length: 265 steps, consistency index (CI): 0.65 (autapomorphies excluded). *Capsella bursa-pastoris* served as outgroup. Only clades with bootstrap support values higher than 50% are shown.

ingroup ranged from 0% (between *Cardamine rivularis* auct. and *C. pratensis*) to 5% (between *Rorippa sylvestris* and *C. hirsuta*). The greatest differences between outgroup taxon *Capsella bursa-pastoris* and one ingroup member (*Cardamine hirsuta*) was 9%, the smallest 6% (between *Capsella* and *Cardamine lilacina* and *C. keysseri*, respectively).

The *trn*T/L spacer region in the taxa studied in this paper is about 700 bp long. This is within the range reported for different taxa in the literature: 658 bp for *Echium* species (BÖHLE et al. 1994) to 833 bp for *Oryza sativa* (TABERLET et al. 1991). The same is true for the *trn*L intron. Sizes from 328 bp (*Gentiana* species, GIELLY & TABERLET 1996) to 527 bp (*Alnus* species, GIELLY & TABERLET 1994a) are known. Our data, with a length of ca. 500 bp, fit well into this range. Sequence divergence varies greatly in different taxa. No interspecific variation was found in the genera *Alnus* and *Fraxinus* (GIELLY & TABERLET 1994a), whereas in *Gentiana* the average divergence between *trn*L intron sequences was 3–4.5% between sections of the genus, and 10.6% over all species (GIELLY & TABERLET 1996). Sequence divergence in our taxa (ingroup) ranged from 0.1% to 6.2%.

Phylogenetic analysis of ITS nucleotide site variation

A parsimony analysis of the ITS data set generated 12 most parsimonious trees with a length of 265 steps and a consistency index of 0.65 (excluding uninformative characters). Fig. 1 shows the strict consensus tree including bootstrap values. Only clades with bootstrap values higher than 50% are shown.

Suitability of ITS sequences for the present study

ITS sequences have become a widely used molecular marker for angiosperm phylogenies at lower taxonomic levels. Despite high copy numbers of the spacer regions, the ITS paralogues

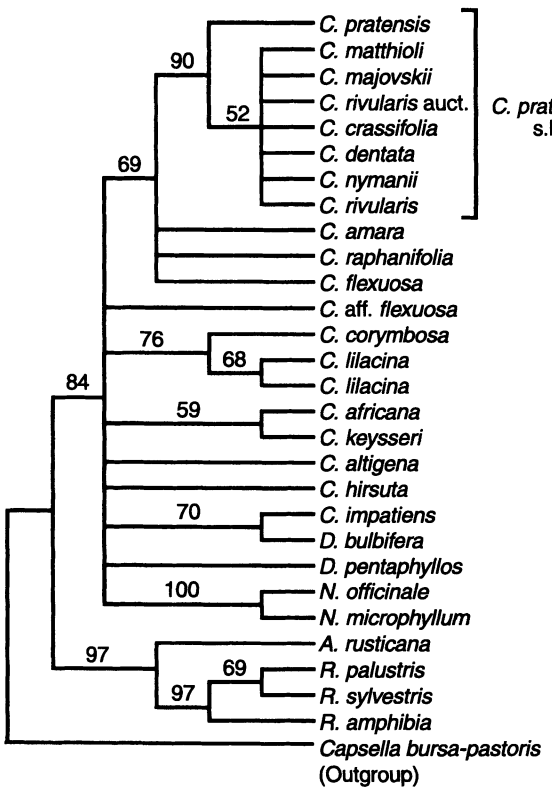

Fig. 2. Strict consensus of the 218 most parsimonious trees based on *trn*T/L spacer-, *trn*L intron-sequence data. Gaps were treated as missing in the body of the data matrix, but each gap was recoded as an additional presence/absence character. The bootstrap support is shown above branches. Tree length: 203 steps, consistency index (CI): 0.68 (autapomorphies excluded). *Capsella bursa-pastoris* served as outgroup. Only clades with bootstrap support values higher than 50% are shown.

are generally uniform due to concerted evolution (BALDWIN et al. 1995). The validity of ITS data for the analysis of alloploids, however, poses a problem and needs to be discussed. Contrasting results have been reported for ITS sequences in allopolyploid species. (i) Both parental ITS sequences have been maintained in the amphiploid hybrid species (e.g. *Arabidopsis suecica* O'KANE et al. 1997; *Paeonia* species, SONG et al. 1995). (ii) Sequences of allopolyploids have been homogenized to that of either diploid parental species (e.g. *Gossypium* species, WENDEL et al. 1995a). (iii) Chimeric (mosaic like) ITS repeat types have been reported for *Microseris* (VAN HOUTEN et al. 1993), *Gossypium gossypoides* (WENDEL et al. 1995b), and for *Microthlaspi* (MUMMENHOFF et al. 1997a). Cases (i) and (iii) are especially problematic when such data are used for cladistic analysis. Our present study includes a number of polyploid *Cardamine* species, some of which are of an apparent autopolyploid origin, as for instance the *C. pratensis* group. Others, however, are presumably allopolyploid although with unknown parentage, and the question arises whether our data are suitable for a cladistic treatment. We have no evidence for two different ITS sequences (case i) in any of the taxa analyzed. The other mentioned problem is whether a single ITS sequence in a polyploid is a recombinant sequence (case (iii) above) or not. If it is, the validity of these ITS sequences for cladistic treatment would be questionable. Direct evidence for a mosaic like ITS type in an allopolyploid species can only be provided if the parent species and their ITS sequences are exactly known. The ancestors of the allopolyploid *Cardamine* species, however, are not definitely known as stated above. Consequently, the occurrence or absence of recombinant ITS sequences can neither be proved nor ruled out. This is a general problem and it is open to question whether recombinant ITS sequences are the rule in polyploids or rather the exception. However, we feel that this problem does not seriously interfere with our *Cardamine*

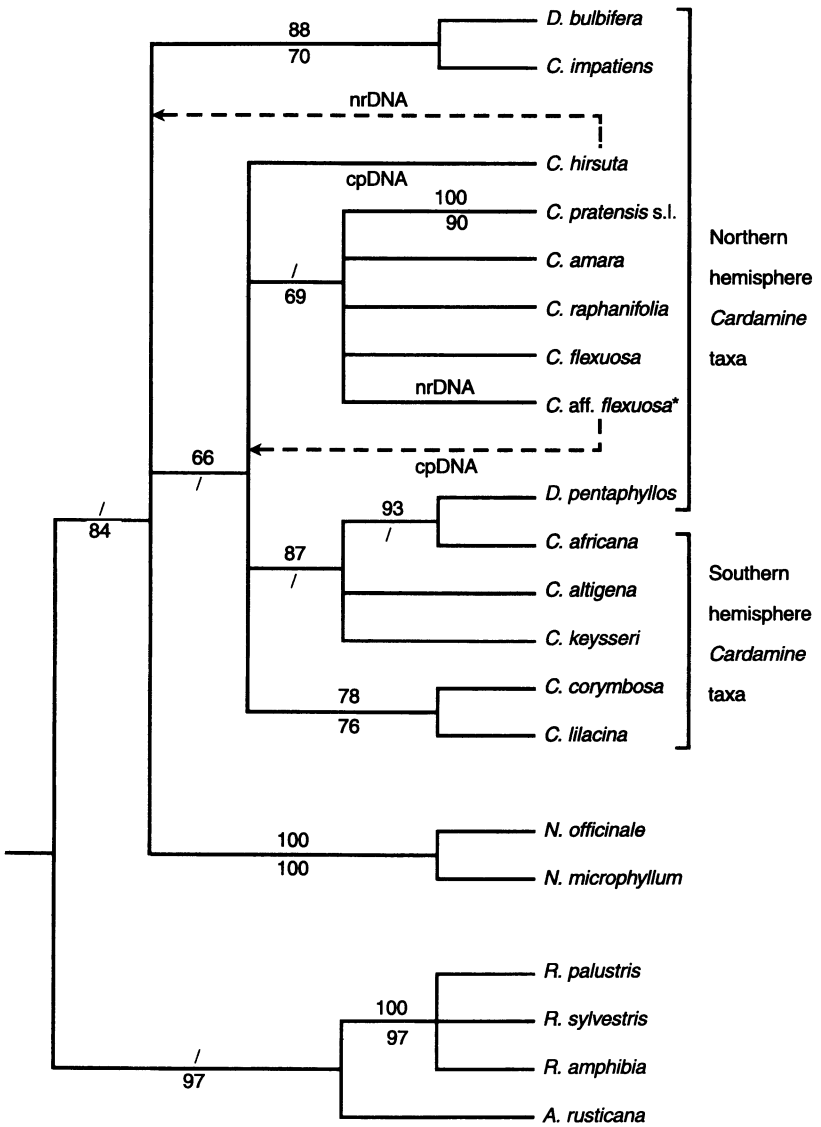

Fig. 3. Synopsis tree of ITS and *trn*T/L spacer-, *trn*L intron-sequence data information. All clades from ITS and cpDNA analyses (Fig. 1 and 2) that are supported with bootstrap values higher than 65% are summarized. The bootstrap values above branches are those from the ITS sequence analysis, bootstrap values below branches refer to the cpDNA data. A slash instead of a bootstrap value means that the refering marker does not support (but in most cases does not contradict) such grouping. Discordances between nuclear and chloroplast phylogenies are indicated by broken lines. * geographical region of origin uncertain.

analysis mainly for the following reason. The examination of biparentally (ITS) and uniparentally (cpDNA) inherited markers in the same species is a sensitive mean for detecting hybridization. Ancient or recent hybridization has been inferred, for instance, from non-concordance between trees based on nuclear and chloroplast markers. Our ITS- and

cpDNA-trees, however, are generally concordant, despite the inclusion of (presumed) allopolyploid species (Fig. 3). There are only two cases of non-concordance, which will be discussed below; one concerns the diploid *Cardamine hirsuta*, the other the tetraploid *C.* aff. *flexuosa* (THOMPSON 1996). To summarize: although recombinant ITS types can not be ruled out with certainty for the reasons given above, the substantial concordance between ITS and cpDNA based trees led us to assume that our ITS data are suitable for the cladistic analysis including polyploid *Cardamine* species.

Phylogenetic analysis of the *trn*T/L spacer- and *trn*L intron-sequences

The heuristic search of PAUP resulted in 218 most parsimonious trees with a length of 203 steps and a consistency index of 0.68 (uninformative characters excluded). Fig. 2 shows the strict consensus tree including bootstrap values. Only clades with bootstrap support values higher than 50% are shown.

Design of a synopsis tree combining information of nrDNA and cpDNA

Fig. 3 shows what we call a synopsis tree. This tree summarizes the information from the ITS and cpDNA tree. In this synoptic tree all clades supported by bootstrap values higher than 65% are shown. The bootstrap values above branches are those from the ITS sequence analysis. Bootstrap values below branches refer to the cpDNA data. A slash instead of a bootstrap value means that the refering marker does not support (but in most cases does not contradict) such a grouping at a 65% bootstrap significance level. The basic idea of the synopsis tree is the presentation of the information available from the different datasets and their respective significance. Concordance and non-concordance between nuclear and chloroplast DNA-based phylogenies can be realized straightforward. The different positions of *Cardamine hirsuta* and *C.* aff. *flexuosa* in the ITS and cpDNA tree (Fig. 1 and 2) are the only two major conflicts between the nuclear and the chloroplast phylogeny and are indicated by broken lines in Fig. 3. The alternative grouping of *Rorippa sylvestris* in the ITS (Fig. 1) and cpDNA tree (Fig. 2) is drawn as a polytomy in the synopsis tree (Fig. 3). The following discussion will mainly refer to the synopsis tree as presented in Fig. 3.

Choice of outgroup

The choice of outgroup taxa within the *Brassicaceae* is often problematic. There is still little knowledge on natural relationships among genera and on higher level phylogeny. In a cpDNA restriction site analysis by ZUNK (1994) *Capsella* appears in the closest related group to a poorly resolved clade consisting of *Rorippa*, *Nasturtium* and *Armoracia*. Therefore, we decided to choose *Capsella* as an outgroup taxon. For both used markers the maximal sequence divergence to ingroup taxa (cpDNA = 9%, ITS = 17%) was at an order of magnitude where multiple hits will not disturb phylogenetic analyses (OLMSTEAD & PALMER 1994).

Phylogenetic relationships between *Cardamine* and allied genera

Cardamine, Nasturtium, Rorippa and *Armoracia*

The generic status of *Nasturtium* has long been a subject of discussion. In some studies *Nasturtium* is maintained as a distinct genus (MARKGRAF 1986, JONSELL 1993, AL-SHEHBAZ & PRICE 1998), in others it is treated as section *Cardaminum* within *Rorippa* (AL-SHEHBAZ 1988, RICH 1991, ROLLINS 1993). *Nasturtium* comprises five species worldwide. The most common and widespread species are *N. officinale* and *N. microphyllum*. The results of our analyses do not support the incorporation of *Nasturtium* within *Rorippa*. In the cpDNA tree,

the two analyzed *Nasturtium* species form a well supported clade together with *Cardamine* taxa. This clade is separated from the *Rorippa* and *Armoracia* clade (Fig. 2). Our findings are in agreement with the *rbc*L data of LES (1994) where *Nasturtium officinale* is grouped together with *Cardamine pensylvanica* WILLD. and was separated from *Rorippa amphibia*, *R. sylvestris* and from *Armoracia*.

In the ITS tree, *Nasturtium* and *Rorippa* form well supported clades, too, but the relationships to *Cardamine* and *Armoracia* remain unresolved due to the lack of internal branchings (Fig. 1 and 3). Isozyme analyses are under progress to investigate the possible role of hybridization events in the evolution of *Nasturtium* taxa.

SCHULZ (1936) placed *Armoracia* distinct from *Rorippa* in the tribe *Drabeae*. Molecular data presented in this study and by ZUNK (1994) do not support this view. *Armoracia rusticana* is at least closely related to *Rorippa* and other genera of the tribe *Arabideae* (Fig. 3), as was previously suggested by HAYEK (1911).

Cardamine and *Dentaria*

The systematic position of *Dentaria* is much debated. Some authors, e.g. DETLING (1936) and authors of local floras treat it as a distinct genus differing from *Cardamine* by its larger flowers, fleshier and larger rhizomes, fewer, often palmately divided cauline leaves, and usually petiolate cotyledons. These characters, however, seem to be unreliable in discriminating the two genera as AL-SHEHBAZ (1988) pointed out. SCHULZ (1903, 1936) treated *Dentaria* as a section of *Cardamine* comprising 16 species distributed mainly in Eurasia from the Pyrenees to the Caucasus, China, Japan, and eastern North America. Two *Dentaria* species were included in our analyses, *D. bulbifera* of central European distribution and *D. pentaphyllos* distributed in the Pyrenees, Central Massive and Alps. It appeared that *Dentaria* is polyphyletic. *Dentaria bulbifera* groups with *Cardamine impatiens* and is well supported by bootstrap values (88% for ITS, 70% for cpDNA, Fig. 3), whereas *D. pentaphyllos* is nested in a clade of southern hemisphere *Cardamine* taxa including *C. africana*. This strong affinity to southern hemisphere *Cardamine* is highly surprising and needs further investigation. The grouping of *Dentaria bulbifera* with *C. impatiens* was unexpected as well. It has been suggested (ERNST 1918, SCHWARZENBACH 1922) that the dodecaploid *D. bulbifera* that very rarely set seeds, is of hybrid origin. It was speculated that other *Dentaria* taxa (e.g. *D. pentaphyllos*, *D. enneaphyllos*, both hexaploid) might be involved as ancestral parents (ERNST 1918), but there have been no serious efforts to substantiate the origin of polyploid *D. bulbifera*. It would appear from our results that *Dentaria bulbifera* might be an allopolyploid hybrid between *Cardamine* and *Dentaria* species.

Relationships within *Cardamine*

General aspects

The genus *Cardamine* comprises some 150 species depending on the author. SJÖSTEDT (1975), for instance, reduced to five the 37 *Cardamine* species recognized by SCHULZ (1903) in Central and South America. SCHULZ (1903, 1936) divided *Cardamine* s.l. (*Dentaria* included) into 13 sections. Six of them are monotypic. AL-SHEHBAZ (1988) questioned this sectional arrangement as not reflecting phylogenetic relationships. About 60% of the *Cardamine* species are polyploids. Most of them have a basic chromosome number of n=8. Both auto- and allopolyploidization events are involved, and hybridization seems to be a phenomenon of considerable importance (e.g. URBANSKA et al. 1997, NEUFFER & JAHNCKE

1997). Phylogenetic relationships within the genus are poorly understood. This is especially true when considering biogeographical aspects. The pleistocene glaciation may have had a tremendous influence on speciation in *Cardamine*, particularly in the northern hemisphere. However, little is known about relationships between *Cardamine* species of the northern and southern hemispheres and about the history of this bihemispheric distribution pattern.

Relationships of northern hemisphere sect *Cardamine* taxa

The largest section within *Cardamine* is sect. *Cardamine* with about 60 species (SCHULZ 1903, 1936). Species of this section are of worldwide distribution and prefer cold to temperate climates. The *Cardamine pratensis* complex is a very complicated and poorly understood group. It is a northern hemisphere complex of about ten taxa of different ploidy levels. Of the *C. pratensis* group we analyzed *C. crassifolia*, *C. matthioli*, *C. pratensis* s. str., *C. rivularis* (cf. MARHOLD 1994b), and *C. rivularis* auct. (cf. MARHOLD 1995) (diploids); *C. majovskii* (tetraploid); *C. dentata* and *C. nymanii* (high polyploids). In our studies, neither nrDNA nor cpDNA markers resolved this group into reliable clades except for a grouping of *C. matthioli* (diploid) and *C. majovskii* (tetraploid) in the ITS tree (Fig. 1). This is consistent with the suggestion of MARHOLD (1996) that *C. majovskii* is an autotetraploid derivative of *C. matthioli*. The lack of significant variations in the ITS- and cpDNA sequences does not support the idea of LÖVKVIST (1956) that *C. crassifolia* and *C. nymanii* are much older than the rest of the group. One might expect clear differences in DNA sequences between these two and the remaining members of the complex in the case of different phylogenetic ages.

Closely related to *Cardamine pratensis* s.l. is a group consisting of *C. amara, C. raphanifolia* and *C. flexuosa*. *Cardamine* aff. *flexuosa* groups together with these taxa in the ITS tree but not in the cpDNA tree (Fig. 1 and 2). *Cardamine hirsuta* and *C. impatiens*, both also members of section *Cardamine*, appear on the phylogenetic trees only distantly related to the *C. pratensis* complex and *C. amara* (Fig. 3). This agrees with the crossing experiments of LÖVKVIST (1956). In these *C. hirsuta* and *C. impatiens* appeared to be well isolated genetically from the *C. pratensis* group whereas crossings between *C. flexuosa*, *C. raphanifolia*, and certain members of the *C. pratensis* complex were successful.

It is noteworthy that the positions of *C. hirsuta* (diploid), *C. impatiens* (diploid) and *C. flexuosa* (tetraploid) (Fig. 3) do not argue for one or both of the diploids as ancestor of the tetraploid *C. flexuosa*. BANACH (in SKALIŃSKA 1950) considered *C. flexuosa* as an autotetraploid species derived from *C. hirsuta*, whereas ELLIS & JONES (1969) think of *C. flexuosa* as an allotetraploid species, *C. hirsuta* and *C. impatiens* being the parent species. It should be kept in mind, however, that the position of *C. hirsuta* on the trees (Fig. 3) might be influenced by "long branch attraction" which can cause an artificial deep branching due to unequal rates of molecular evolution (HUSS & KRANZ 1992). *Cardamine hirsuta* had a high number of autapomorphies in our analyses (23 for ITS, and 28 for cpDNA as compared to a maximum of 13 and 8, respectively, for the other taxa studied). This might be interpreted in terms of a faster rate of molecular evolution in the species. In other words, the distant position of *C. hirsuta* from *C. flexuosa* in our phylogenetic analyses does not necessarily exclude *C. hirsuta* from further discussions on the origin of *C. flexuosa*. Isozyme and RAPD studies are in progress to unravel the phylogeny of *C. flexuosa*.

We included in our studies a tetraploid taxon collected in Australia (Tab. 1) which keyed out as *Cardamine* aff. *flexuosa* when following THOMPSON (1996) in the Flora of Victoria. This plant is identified as *C. debilis* D. DON when using a key given in ROLLINS (1993). *Cardamine debilis* was treated by SCHULZ (1903) as a member of the variable *C. flexuosa*

complex, and it would appear that our *C.* aff. *flexuosa* does indeed show close affinities to *C. flexuosa* as shown in the ITS tree (Fig. 1). Chloroplast DNA data, however, place *C.* aff. *flexuosa* away from *C. flexuosa* (Fig. 2). This discordance between nrDNA and cpDNA phylogenies argues for a hybridization event giving rise to *C.* aff. *flexuosa.* Due to morphological similarities between these species and due to the position on the ITS tree we suggest that *C. flexuosa* is involved as one of the parent species of *C.* aff. *flexuosa.*

Affinities between northern and southern hemisphere *Cardamine* species

The genus *Cardamine* has a "bihemispheric" distribution pattern: species of *Cardamine* are indigenous in the northern hemisphere as well as in the southern hemisphere. We included some southern hemispheric species from New Guinea, South-East Australia, Tasmania and subantarctic islands in our analyses (Tab. 1) to elucidate affinities between northern and southern hemisphere *Cardamine* species.

In Australia, a small number of endemic *Cardamine* species are currently recognized, the majority of which are confined to south-eastern Australia and Tasmania. They occur in lowland wet areas, forests, sub-alpine woodlands and alpine habitats. Plants from the high mountains were first described by HOOKER (*Comp. Bot. Mag.* 1: 273, 1835) as *C. lilacina* HOOK., and later by SCHULZ (1936) as *C. finitima* f. *lilacina* (HOOK.) O.E. SCHULZ. They belong to a complex of terrestial, glabrous perennials with petals more than 4 mm long and treated as the *Cardamine gunnii-lilacina* complex by HEWSON (1982). HEWSON identified some informal variants of *C. gunnii* HEWSON and of *C. lilacina* (see also THURLING 1968). Taxa within this complex grow predominantly in higher altitudes of south-eastern Australia and Tasmania, and were the subject of a phenetic analysis of morphological variation resulting in a revision of the *Cardamine gunnii-lilacina* complex by THOMPSON & LADIGES (1996). Three new species were identified, and the hitherto confusing taxonomy was at least partly resolved (see also THOMPSON 1996).

Cardamine lilacina was considered by HOOKER (1855–1857) to belong to the northern hemisphere *C. pratensis* complex and consequently named as *C. pratensis* var. *lilacina* (HOOK.) HOOK. f. SCHULZ (1936), too, suggested a close relationship of *C. lilacina* to the *C. pratensis* complex.

Our data do not support such a close affinity. Neither nrDNA nor cpDNA data group *C. lilacina* with *C. pratensis* s.l. (Fig. 1, 2). *Cardamine lilacina* is closer to *C. corymbosa* than to any other *Cardamine* species analyzed so far. *Cardamine corymbosa* is a subantarctic species naturally occurring in the southern part of the South Island of New Zealand, Auckland, Campbell, and Macquarie islands. The northern hemisphere *Cardamine pratensis* complex groups together with other northern hemisphere taxa including *C. amara*, *C. raphanifolia*, and *C. flexuosa*. This clade is separated from the rest of the taxa (Fig. 3).

In our synopsis tree (Fig. 3) an additional southern hemisphere clade, apart from the *C. lilacina*/*C. corymbosa* group, is obvious. It consists of *C. africana*, *C. altigena* and *C. keysseri* (the position of *Dentaria pentaphyllos* within this group has already been discussed above). *Cardamine altigena* and *C. keysseri* are high mountain endemics of New Guinea. The analyzed specimen of *C. africana* is also of New Guinean origin. This pantropical species, however, is not confined to New Guinea. It is a highly polymorphic taxon awaiting further study.

In the light of our findings of two southern hemisphere clades, questions on the origin of the bihemispheric distribution pattern arise.

Most *Cardamine* species are of northern hemisphere distribution and show ecological preference for cool temperate climates. This, and the fact that indigenous *Cardamine* species in warmer and tropical climatic regions are mainly confined to mountain and alpine habitats, led SCHULZ (1903) to argue that the origin of *Cardamine* is in the cooler boreal regions of the northern hemisphere. This conforms with current ideas on the history of *Brassicaceae* in the southern hemisphere in general, and in Australasia in particular.

The phytogeographic composition of the Australian vegetation consists of two basic elements, the Gondwanan Element and the Intrusive Element (BARLOW 1981, NELSON 1981). The Gondwanan Element comprises two sub-elements, a Relict Sub-element and a derived Autochthonous Sub-element. The Intrusive Element, comprising plants which have entered Australia subsequent to its separation from Gondwanaland, is a composite of several different sub-elements, a Tropical Sub-element, a Cosmopolitan Sub-element, and a Neoaustral Sub-element (summarized in BARLOW 1994). The Cosmopolitan Sub-element is significant in the arid zone vegetation and includes genera of the *Brassicaceae* endemic to Australia. The Neoaustral Sub-element of the Intrusive Element comprises mainly temperate species of northern hemisphere derivation. It would appear that *Cardamine* belongs to the Neoaustral Sub-element.

The alpine flora of Australia is relatively young (Quaternary) and probably not older than those of New Zealand and New Guinea (BARLOW 1994). Two processes have been involved in the differentiation of an alpine flora. The newly emergent alpine habitats have been colonized, in part, by plants from the communities of the surrounding lowlands (Autochthonous Sub-element). They have also been colonized by long distance dispersal from remote alpine or cool temperate communities (Cosmopolitan and Neoaustral Sub-elements). Whereas SMITH (1986) concluded that the Gondwanan Element was not of major significance, BARLOW (1989 and 1994) argued for a greater contribution of lowland Australian plants to the evolution of the existing alpine flora (see also WILLIAMS & COSTIN 1994, HOPE 1994). Although many questions about the origins of the Australian mainland and Tasmanian alpine flora remain open, long distance dispersal appears to be a necessary requirement for those alpine species which have their nearest relatives in remote alpine or cool temperate habitats.

With regard to *Cardamine*, long distance dispersal in the southern hemisphere is strongly indicated. Two migration routes appear feasible: the Malayan Route via the array of mountains in the Malayan Archipelago and New Guinea ("mountain hopping"), and the Andean Route using the Andean Cordillera as an interhemispheric corridor and from there via wind or bird dispersal to Australia and New Zealand. These two principal migration routes are discussed for *Lepidium* (MUMMENHOFF et al. 1992) and for many other taxa (BARLOW 1981, 1994, RAVEN 1973, SMITH 1986). BARLOW (1994) suggested that long distance dispersal around the southern latitudes may have been more significant than the route across Malaya/New Guinea. This was also argued by SMITH (1986) even in cases of bihemispheric genera with northern origins.

The two southern hemispheric *Cardamine* clades linking together pantropical and New Guinean species in one clade, and Australian/Tasmanian and subantarctic species in the other clade (Fig. 3), may reflect the two migration routes. This conclusion, however, may be premature. In all most parsimonious trees generated from the ITS data, the two southern hemisphere clades are grouped together though with only poor bootstrap support (33%) and therefore are not shown in Fig. 1. We think that neither the first hypothesis (Malayan and Andean Route) nor the second (Malayan Route only) should be rejected at the moment.

Further analyses of southern hemisphere *Cardamine* species, especially from South America, will help our understanding of the evolution of the bihemispheric distribution pattern.

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