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Molecular ecology of rotifers: from population differentiation to speciation

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Abstract

The development of cost-effective molecular tools allowing the amplification of minute amounts of DNA effectively opened the field of molecular ecology for rotifers. Here I review these techniques and the advances they have provided in the understanding of sibling species complexes, clonal structure, resting egg banks, population structure, phylogeographic patterns and phylogenetic relationships in rotifers. Most of the research to date has focused on the rotifer species complex *Brachionus plicatilis*. The use of DNA sequence and microsatellite variation, in the context of the background knowledge of life history, mating behaviour, and temporal population dynamics in these organisms have revolutionised our views into the processes shaping the genetic diversity in aquatic invertebrates. Rotifers have populations with a very high number of clones in genetic equilibrium. In temporary populations clonal selection is effective in eroding the number of clones. Rotifer populations are strongly differentiated genetically for neutral markers, even at small geographical scales, and exhibit deep phylogeographic structure which might reflect the impact of Pleistocene glaciations. Despite the high potential for dispersal afforded by resting eggs, rotifers display persistent historical colonisation effects, with gene flow effective only at a local scale and with marked isolation by distance. Instances of long-distance transcontinental migration resulting in successful colonisation have also been revealed. *B. plicatilis* is composed of a group of several ancient species and sympatry is common. Despite this, the presence of cosmopolitan species in this species complex cannot be discounted. I discuss future priorities and point out the main areas where our knowledge is still insufficient.

Introduction

Molecular ecology concerns the application of molecular techniques to questions in ecology, evolution, behaviour and conservation biology (Carvalho, 1998). Several technical and methodological developments in molecular biology in the 1980s facilitated such application, among these, refinement in DNA extraction protocols, polymerase chain reaction (PCR), design of conserved primers which allowed amplification and sequencing of genes of virtually any organism, and, the publication of protocols for the development of species-specific microsatellite primers. The use of molecular tools has been revolutionary in diverse fields of ecology, evolution and behaviour and has yielded numerous insights into population structure, mating strategies, among many others.

The field of rotifer biology has much to gain from molecular ecology, as several themes have aroused much theoretical debate with little support from empirical data. Some of these long formulated questions include: how many clones are there in rotifer populations? Is clonal selection important in eroding genetic diversity? Are resting egg banks repositories of past and present genetic variation? And in a related question, is sex serving a function other than the way rotifers make resting

eggs? How important are dispersal and gene flow in structuring rotifer populations? Are rotifer taxonomic species complexes of sibling species? Is cosmopolitanism an artefact of poor taxonomy, or are there true cosmopolitans? Is cyclomorphosis mainly determined by genetic replacement of clones or species, or by phenotypic plasticity? As discussed here, although still in its infancy, the molecular ecological approach in rotifers has provided novel insights into these questions and revitalised many areas of rotifer research.

The small body size of rotifers, and the difficulty of laboratory culture for many species, has hindered the application of allozyme electrophoresis to their study (Gómez, 1998). In spite of this, labour-intensive studies using allozyme markers in rotifers have yielded valuable data (King, 1977; King, 1980; Gómez et al., 1995; Ortells et al., 2000). The results based on allozyme markers were often limited due to the reduced amount of polymorphism detected. Therefore, and unlike in cladocerans, molecular ecology did not become a strong field in rotifers immediately. Although rotifers have long been a well-established model system in limnology, ecology and life history, the lack of basic genetic knowledge substantially hampered advances in some of these fields. For example, studies involving the rearing and the comparison of the ecological characteristics (e.g., optimal growth rates, mixis inducing cues) of wild-caught or domesticated clones (for some examples see King, 1980; Snell & Carrillo, 1984; Serra et al., 1994) revealed a strong genetic component in life history trait variation. However, we now know that these studies almost certainly involved comparison of strains belonging to sympatric cryptic species.

Here I will review and discuss (i) methodological advances that have facilitated the application of molecular techniques to rotifers; (ii) recent advances in the field of rotifer molecular ecology; and (iii) future developments and areas which are likely to benefit further from the molecular ecological approach. It must be emphasised that most of the research to date has been performed on the species complex *Brachionus plicatilis*, therefore the results obtained with this taxon will not necessarily apply to other rotifers. For the sake of simplicity however, the name 'rotifers' will be employed throughout the review mostly to refer to this taxon.

Technical and methodological advances in molecular ecology

The number of techniques being applied to molecular ecology is quite substantial and new methods are being added to the molecular ecologist's toolkit virtually every year. For the present purposes I will focus on those techniques crucial for the investigation of basic questions in rotifer ecology and evolution, and which have already provided some progress. In doing this, I have favoured reviews and monographs instead of the primary literature. The interested reader can trace back the relevant literature cited therein. Although allozyme electrophoresis can be considered a molecular technique, it will not be treated here as the methods and its applications have been reviewed elsewhere (Gómez, 1998).

DNA extraction procedures

The small body size of rotifers means that only procedures that involve PCR (polymerase chain reaction) amplification of extracted DNA can be applied to population problems cost-effectively. Techniques originally developed to recover DNA from forensic material, usually small dried blood or semen samples containing little or degraded DNA (Walsh et al., 1991) were adapted to extract DNA from single rotifer females, males and resting eggs (Gómez et al., 1998; Leutbecher, 2000). The advantages of such techniques were immense, as rotifer genetic variation could be examined without the need to culture them in the laboratory. Morevover, the genetic variation in sexual females, males and sediment borne resting eggs could be analysed, allowing wide-scale analysis of population structure, as well as long-term genetic temporal variation.

Polymerase Chain Reaction (PCR) amplification using conserved primers

The minute amounts of DNA recovered from single rotifers must be amplified prior to analysis using PCR (Palumbi, 1996; Birt & Baker, 2000), and this amplification necessitates a pair of oligonuclotides (the so called 'primers') of sufficient sequence

similarity to regions flanking the target organism DNA sequence. Several sets of 'universal' primers, have been designed from conserved gene regions in mitochondrial DNA (mtDNA) and nuclear DNA (nDNA), and used to amplify rotifer DNA successfully. To amplify mtDNA regions, these include cytochrome *c* oxidase I primers developed by Folmer et al. (1994) used by Gómez et al. (2000; 2002b) and Derry et al. (2003), and 16S ribosomal genes developed by Palumbi (1996) used by Derry et al. (2003). Among nuclear genes Gómez et al. (Gómez et al., 2002b) amplified and sequenced the ITS1 ribosomal DNA using primers developed by Palumbi (1996), and Mark Welch & Meselson (2000) described and used several coding gene primers in bdelloids. Sufficient variation at a local or regional scale can often be found in a species when relatively fast-evolving genes are examined, and this is one reason why mtDNA is favoured for phylogeographic investigations and phylogenetics at low taxonomic ranks. Other reasons for the choice of mtDNA are (1) its haploidy, clonality and uniparental mode of inheritance (usually mother to offspring in animals), which reduces to ¼ its effective population size relative to nuclear markers making it more sensitive to demographic and evolutionary relevant events such as bottlenecks and population subdivision (Birky et al., 1989; Birky, 2001), (2) the fact that it occurs in multiple copies per cell, which favours its preservation and retrieval from ancient, poorly preserved or small tissue samples (Wayne et al., 1999). A cautionary note on using mtDNA used on its own is that, being a maternally inherited molecule, introgression and hybridisation may not be detected, therefore calling for the use of nuclear markers to support it. Although hybridisation not been detected in *B. plicatilis*, it might well be present in other monogononts.

Microsatellite loci

Population genetic studies in rotifers have been hampered by the scarcity of known polymorphic genetic markers. Although allozyme loci proved to be useful tools to detect sibling species complexes, little or no genetic variation has been reported within populations (Gómez et al., 1995; Ortells et al., 2000; but see Ortells, 2002). Furthermore, allozyme loci do not allow for the exploration of genetic diversity stored in rotifer dormant egg banks in lake sediments, or in the sexual individuals of the population, and individuals collected in the field need to be cultured in the laboratory to obtain enough biomass, which is often limiting sampling sizes. Analysis of microsatellite loci can help circumvent these problems. Microsatellites are DNA sequences made of short nucleotide motifs (up to six bases long) repeated in tandem, and can reach sizes of 200 bp (Goldstein & Schlotterer, 1999). Microsatellite loci are abundant and ubiquitous in the genome of eukaryotes and so they may provide a nearly unlimited set of markers for the study of clonal and population structure (Jarne & Lagoda, 1996; Li et al., 2002).

Microsatellites are used by molecular ecologists to address questions of population structure, migration and gene flow, mating patterns, parentage, and individual and clonal identification (Jarne & Lagoda, 1996). Several characteristics make microsatellites good markers for these ends, including high mutation rates, a large number of alleles per locus, codominant Mendelian inheritance and selective neutrality.

Microsatellite loci are amplified using PCR primers designed from unique flanking sequences. The main limitation for microsatellite analysis is in fact the availability of such primers, as they tend to be species-specific and have to be developed following a time-consuming protocol. Some degree of conservation across species can be present and cross-amplification of microsatellites has been reported for different animal species of the same genera or even the same family, but this has to be determined empirically on a case-by-case basis (Primmer et al., 1996; Primmer & Merila, 2002). In rotifers, microsatellite markers have been developed only for *Brachionus plicatilis* sensu stricto (Gómez et al., 1998) and, unfortunately, they have failed to cross-amplify even in other species of the complex (Gómez et al., 1998). Therefore, microsatellites might not be the markers of choice for future population studies of rotifers. Although, more promising recently developed methods, often with high-throughput, including AFLP (amplified fragment length polymorphism) (Vos et al., 1995) and SSCP (single stranded conformation polymorphism) (Sunnucks et al., 2000) or SNPs (single nucleotide polymorphisms) (Brumfield et al., 2003) need yet to be tested on these organisms.

Applications to the understanding of rotifer ecology and evolution

Cryptic species complexes and biogeography

Rotifera is considered a relatively minor metazoan phylum with less than 2000 described species (Segers, 2002). Due to their assumed considerable abilities for passive dispersal, rotifers were long considered to comprise mostly cosmopolitan species. However, in his review on the biogeography of rotifers, Dumont (1983) argued that rotifers show some evidence for vicariance and illustrated the levels of endemicity of the group in several continents. However, Segers (1996) noticed that in comparison with other animal groups, rotifer morphospecies display large distribution ranges, which could at least partly be a consequence to widespread dispersal, with vicariance playing a subordinate role. Both Dumont (1983) and Segers (1996) concur in attributing the apparently high proportion of widely distributed taxa to insufficient taxonomic resolution, that is, to the presence of cryptic taxa within the described morphospecies. In addition to increased sampling of poorly known habitats or regions of the world, the description of cryptic or sibling species could contribute substantially to increase our knowledge of rotifer biodiversity. King (cited in Dumont, 1983) suggested that, in addition to plankton nets, rotifer researchers should carry electrophoretic equipment with them. Although allozyme electrophoresis has indeed been used to identify cryptic species (Gómez & Snell, 1996; Ortells et al., 2000; Ortells, 2002), King's advice has not been followed widely and the confusion between cryptic species (compounded by cyclomorphosis, see section *The Proximate Causes of Cyclomorphosis* below) has crippled much of rotifer basic research.

The wealth of ecological information on many rotifer taxa has very little value unless the species used are 'real' biological entities. For example, variation among isolates of the same taxonomic species attributed to cyclomorphosis *sensu stricto*, or to intraspecific ecological variation, led to proposals of models of temporal adaptation of populations which had little connection with the reality of rotifer populations. As King (1980) put it the ''population'' investigated in many limnological studies may be an artifact with closer affinities to griffins, unicorns and mermaids than to the population as a biological unit'. Without a proper analysis of genetic differentiation or mating compatibility, many species have been lumped together and labelled 'generalists' or euryoic, polymorphic and cosmopolitan. In addition to seriously underestimate rotifer biodiversity, the lack of knowledge of cryptic species is preventing rotifer researchers from studying niche partitioning, population dynamics and many other ecological and evolutionary questions.

The taxonomic uncertainty surrounding cryptic species complexes has traditionally been resolved using lengthy and costly experimental approaches. For example, after decades of experimental work in *Brachionus plicatilis*, which suggested hidden species diversity, three species in this taxon were described or redescribed (Ciros-Pérez et al., 2001). In order to discriminate these species morphologically in a consistent way, different genetically characterised clones were grown in the laboratory in the same controlled conditions and a biometric study was performed on scanning electron microscopy photographs of females of the same age (Ciros-Pérez et al., 2001). It is doubtful that the same approach can be employed widely for the whole of the Rotifera. First, not all rotifers can be cultured readily, and, second, the workload involved would be insurmountable, given the human and economic limitations attached to rotifer studies. A second more straightforward and promising approach is to screen populations for one or a few genes in order to identify such cryptic species complexes (see Hebert et al., 2003; Tautz et al., 2003). Sequences obtained from a few genes can often yield the information necessary to conclude that two taxa are good species (see for example Baum & Shaw, 1995). Since the advent of PCR based techniques the number of cryptic species described in a variety of taxa is increasing steadily (Fig. 1) reflecting a tradition of lumping by taxonomists, but also morphological conservatism (for a recent review see Knowlton, 2000). The sequences obtained in molecular assessments can be annotated and deposited in public DNA databases such as GenBank/EMBL and subsequently retrieved by any interested researcher through the internet. Although these ideas are still controversial, it should be possible to base species descriptions on sequence information, while still

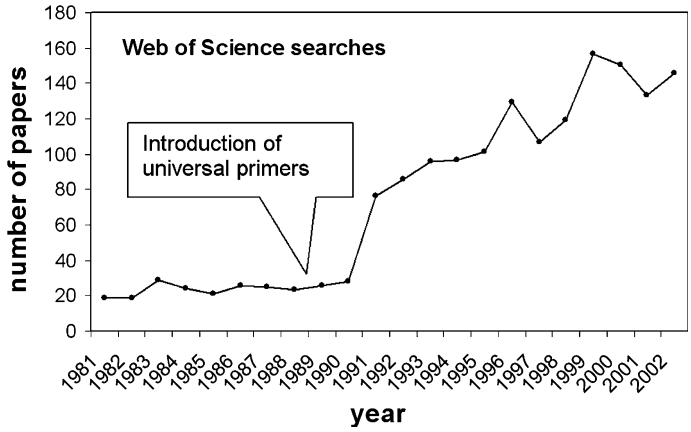


Figure 1. Effect of molecular tools on the recognition of cryptic species. Plot showing *Web of Science* searches on the keywords 'sibling species' or 'cryptic species'. The introduction of universal PCR primers is shown.

maintaining the importance of morphologically based descriptions (Tautz et al., 2003). Voucher specimens and details of collection localities would allow morphological or ecological appraisals *a posteriori*.

B. plicatilis remains by far the best studied monogonont species complex from a genetic point of view. Allozyme studies had previously indicated the occurrence of several species in this taxon, with no evidence of introgression in sympatry (Gómez et al., 1995; Ortells et al., 2000; Ortells, 2002). Using a collection of 57 specimens, including laboratory grown clones of known allozyme profiles, and field-collected resting eggs from 27 Iberian salt lakes and other worldwide locations, including North Africa, North America, Europe and Australia, Gómez et al. (2002b) obtained sequence information from two genes, a mtDNA gene, cytochrome *c* oxidase I (COI) and a nuclear gene, ribosomal internal transcribed spacer (ITS1). Phylogenetic analysis of both genes revealed nine concordant genetically divergent lineages (Fig. 2), six of them present in the Iberian Peninsula. COI evolves faster and therefore was more informative for the shallower branches of the phylogeny, whereas ITS1 was able to resolve deeper splits in the phylogeny. The three main branches of the phylogeny were strikingly concordant with the three described morphologies of the *B. plicatilis* complex, L, M and S (Fig. 2). The level of sequence divergence was well over that commonly found between different species and indicated that cladogenesis had not been a recent event.

Tentative dating of the radiation of the complex using molecular clocks for each gene goes back to 10–27 mya (Miocene) (Gómez et al., 2002b). Several additional lines of evidence support that these genetic lineages are different species or groups of species. First, the previously mentioned lack of hybridisation of these lineages when in sympatry (Gómez et al., 1995; Ortells et al., 2000; Ortells, 2002); second, cross-mating experiments performed between strains belonging to different lineages indicate behavioural reproductive isolation between them (Gómez & Serra, 1995; Gómez & Snell, 1996; Ortells et al., 2000; Berrieman et al., 2004); and third, ecological differences have been found when clones belonging to different lineages have been tested in the laboratory for optimal growth rates and mixis patterns (Gómez et al., 1997). The molecular phylogenetic assessment of Gómez et al. (2002b) was performed on a very patchy sampling of the geographical distribution of the *B. plicatilis* complex – Sub-Saharan Africa, South and Central America and most of Asia were not sampled – therefore it is very likely that several other species are present in this taxon, especially considering its thermophilic character. In addition, information on the geographic, genetic and ecological diversity and mating behaviour of some of these lineages is scarce or absent and therefore the detection of additional species in the already sampled lineages is likely.

A further consequence of this study concerns the issue of cosmopolitanism. Rotifer species show a high propensity towards local and regional

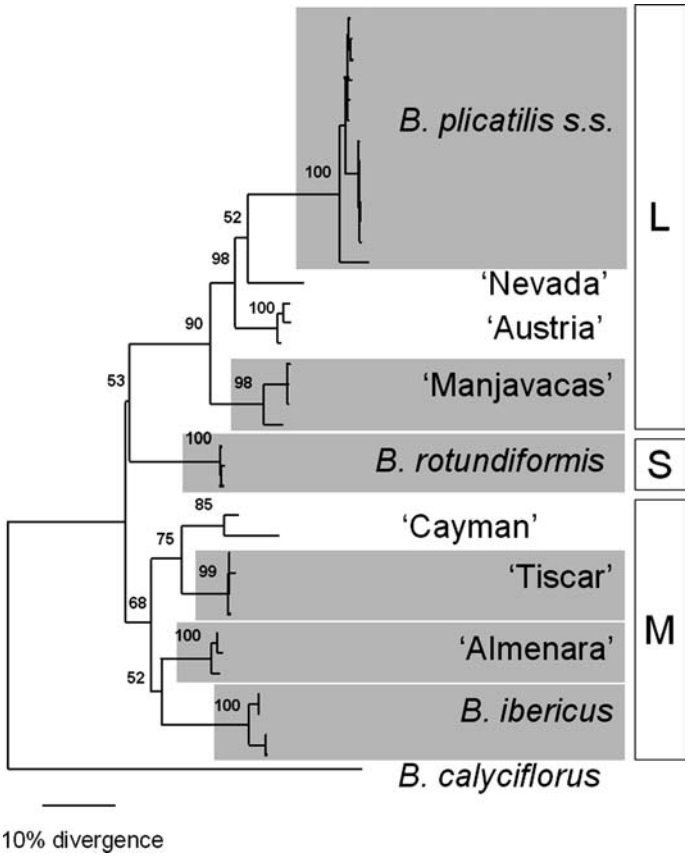


Figure 2. Phylogeny of the *Brachionus plicatilis* complex using *B. calyciflorus* as outgroup. Neighbour Joining tree based on a combined dataset using ITS1 and COI using a matrix of ML distances (see Gómez et al., 2002b for details). Values above branches indicate bootstrap support for nodes. The boxes indicate the size associated with the three main branches of the species complex (small, medium, large). Names boxed in grey indicate species present in the Iberian Peninsula.

genetic differentiation in spite of their capabilities for dispersal. Does the resolution of sibling species complexes in rotifers mean that good species will be regional endemics instead of cosmopolitan species? The answer seems to be: not completely. Undoubtedly, better geographic sampling is needed before strong conclusions can be drawn, but even with the relatively small effort undertaken so far, several of the lineages found are distributed in several continents (see Table 1). Whereas some of these lineages display a pattern of regional differentiation, others show very little genetic differences when clones retrieved from very distant geographical locations are compared, indicating that long-distance (even transcontinental) migration and colonisation was relatively recent. Human induced transportation is an issue to be considered here. Humans have been held responsible directly or indirectly for exotic introductions and range expansion of many organisms in aquatic habitats (Rahel, 2002). However, in contrast to other aquatic habitats (see for example Bailey et al., 2003 for data on several rotifer species transported through ballast water), salt lakes and coastal lagoons are usually remote, isolated from each other and from watercourses, little visited and devoid of any commercial use – other than the extraction of salt in some instances – and therefore it is unlikely that humans are responsible for such cases of species long-distance migrations. Therefore, rotifers from this sibling species complex undergo long-distance, intercontinental migrations and such events seem to be common enough to be detectable even with restricted sampling. A tentative conclusion regarding cosmopolitanism is that due to their high dispersal capabilities and occasional long-distance

Table 1. Geographic distribution of species in the *B. plicatilis* complex

Species	Europe	Asia	North America	North Africa	Australia
B. plicatilis s.s.	x		x		x
B. 'Manjavacas'	x	x		x	
B. 'Austria'	x	x	x		
B. 'Nevada'			x		
B. 'Cayman'		x	x		
B. 'Tiscar'	x				
B. 'Almenara'	x		x		
B. ibericus	x				
B. rotundiformis	x			x	

migration, rotifer species are always in the process of becoming cosmopolitan. A role for some dispersal limitation cannot be discarded, specially for very small and isolated lakes or for very large distances, although evidence for dispersal limitation in zooplankton is still controversial (Jenkins & Buikema Jr., 1998; Shurin, 2000).

A surprising finding of this study which supported previous data was that many species in the complex were often found in sympatry (Gómez et al., 2002b) (Fig. 3). Lakes containing two and three rotifer species were not rare and a lake has been found where four of the species coexist (Ortells, 2002). Due to the strong seasonality of salt lakes and coastal lagoons, the high level of sympatry in rotifers could be due to seasonal succession and temporal niche partitioning and/or different susceptibilities to predators and parasites. In fact, species in the *B. plicatilis* complex can be involved in seasonal succession (Gómez et al., 1995), and this has been attributed to their ecological specialisation to different salinities or temperatures (Serra et al., 1998) and also to their different food preferences (Ciros-Perez et al., 2001). If this is a common pattern, then it should be possible to predict the number of species from the complex likely to be found in a lake, based on the degree of temporal variability of that lake (ideally estimated across several years to account for the interannual variation of the habitats). However, Ortells et al. (2003) found that in some cases two species from the *B. plicatilis* complex coexist throughout most of their presence in the pond, which seems to suggest that factors mediating coexistence (disturbance, predators, etc.) must play a role in facilitating sympatry (Ciros-Perez et al., 2001).

A pattern of common sympatry reflects several processes: each species should (i) reach the lake or pond, (ii) establish populations with positive growth rates, and (iii) persist through time in the face of environmental variation. I have already mentioned that long-distance dispersal seems to be a common phenomenon in this species complex. A better understanding of ecological preferences (including pre-competitive and post-competitive niches) in these species would allow us to further understand the factors contributing to the establishment success of rotifer species when they reach new habitats. It is clear that the fact that these organisms maintain resting egg banks will contribute to the long-term persistence of species in a lake, even if its conditions are unsuitable for a few years (what has been termed a 'storage effect' (Cáceres, 1997). Thus, a combination of effective dispersal and colonisation, successful niche partitioning and occurrence of factors mediating coexistence, and storage-effect allowed by the resting egg bank, seems to be responsible for the high degree of sympatry observed in rotifer species from this complex.

Gómez et al. (2002b) study illustrates how different processes govern species and populations in rotifers. Populations show evidence of very low gene flow due to the monopolisation hypothesis (see the *Population Structure* section below), and as a consequence, a population in a lake contains a reduced proportion of the neutral genetic diversity of its species. In contrast, that very same lake can contain a significant proportion of sibling species,

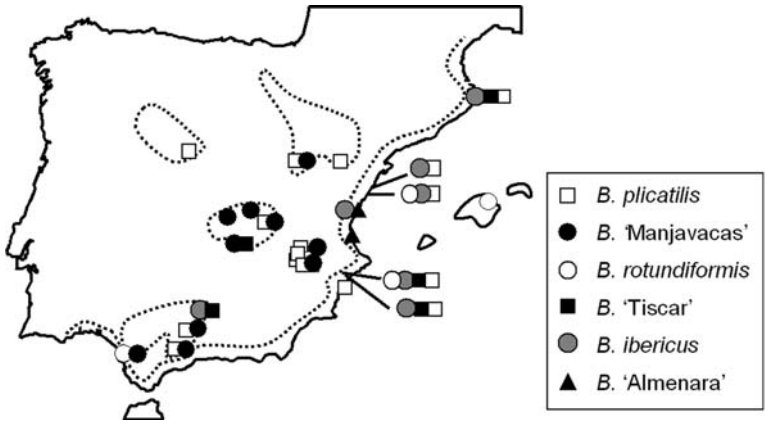


Figure 3. Sympatry in the *B. plicatilis* complex. Map of the Iberian Peninsula showing the distribution of the six species detected so far.

reflecting the colonisation abilities of the different species.

Finally, phenotypic plasticity, but also morphological conservatism, and a pattern of extensive sympatry among cryptic species are factors that might have contributed to the difficulties of recognising good rotifer species and led the average rotifer taxonomist to become a 'lumper'. If the patterns found in *B. plicatilis* are found to be common in other rotifer morphospecies the amount of hidden biodiversity in rotifers could be substantial. Indeed, the presence of cryptic species has been reported in *B. calyciflorus* using mating behaviour and sequence variation data (Gilbert & Walsh, this volume, Part V) and *Keratella* (Derry et al., 2003). The day might come when rotifers are not considered a minor phylum.

Routine species identification

Once the members of a species complex have been described using molecular techniques, a simple method available to a large number of rotifer researchers must be developed so that species can be identified from field samples routinely, or contamination detected in aquaculture facilities. In the case of *B. plicatilis*, several techniques are available, and they could be applied to other rotifer species. First, allozyme electrophoresis, which can be set up relatively cheaply (Gómez, 1998), could provide 'diagnostic loci' (Ortells et al., 2000; Ortells et al., 2003) which could be genotyped in laboratory grown clones. As mentioned before, the application of this technique is restricted to those species of rotifers that reproduce rapidly and can be cultured readily. For those species for which DNA sequence information is available, an approach based on PCR-RFLP could be used. This procedure consists of using PCR to amplify a fragment of DNA of known sequence and then digesting the amplification product with those restriction enzymes yielding a diagnostic restriction profile (restriction fragment length polymorphism, RLFP) when samples are run on a gel. Although this procedure requires the use of a thermocycler and basic electrophoresis tools, such equipment is basic to genetic and evolutionary laboratories of many universities, and the cost of materials could be relatively cheap, even cheaper than allozyme electrophoresis.

A PCR-RFLP method was used by (Berrieman et al., 2005, this volume) to discriminate between wild caught clones of the sympatric and morphologically very similar *Brachionus plicatilis* s.s. and *B.* 'Manjavacas', and between 'northern' and 'southern' *B. plicatilis* s.s. lineages in order to perform mating behaviour experiments.

Rotifer clonal structure and resting egg banks

Monogonont rotifer planktonic populations are made of numerous clones produced by parthenogenetic females that hatched from sexually produced resting eggs in the resting egg bank. In lakes and ponds that undergo periodic drying or freezing over, these planktonic populations are necessarily reconstituted every year from the resting egg bank. In mild years, parthenogenetic populations

might survive several growing seasons with a variable input from the resting egg bank. In at least some cases, species are present in the water column during part of the year, partly reflecting their tolerance ranges or competitive abilities. The clonal composition and dynamics of planktonic rotifer populations and the interplay with the resting egg bank was virtually unknown until recently. Meanwhile, our understanding of clonal structure had advanced to a mature state in the other group of aquatic cyclical parthenogens, cladocerans. Cladoceran researchers working with *Daphnia* and mainly using allozyme markers had produced two models that seemingly accounted for the clonal and genetic structure of populations. In 'intermittent' ponds, where populations were founded every year from sediment banks, investment in sexual reproduction was important and genetic analysis revealed a large number of clones in genetic equilibrium (both Hardy–Weinberg and linkage) (see review in De Meester, 1996). No evidence for clonal selection was found in these ponds. In contrast, in permanent ponds some *Daphnia* populations persisted among years, sexual investment was reduced and there were often a low number of clones which underwent rapid changes in frequencies, indicating clonal selection. Although these models are simplifying and exceptions have been found, they can be used as a framework to help us understand what takes place in rotifer populations.

Gómez & Carvalho (2000) used a set of seven polymorphic microsatellite markers to screen an intermittent population of *Brachionus plicatilis* (Poza Sur, in Prat de Cabanes–Torreblanca Marsh). There were three consecutive planktonic samples along a parthenogenetic phase, a sample from the resting egg bank, and a sample after the re-establishment of populations after the summer drought. The set of seven polymorphic microsatellite loci previously developed for *Brachionus plicatilis* (Gómez et al., 1998) proved to be a useful tool for clonal identification because the probability that two clones produced by separate sexual recombination events (hatching from two different resting eggs) have the same multilocus genotype is very low. Overall, 349 different genotypes were found in the 390 individuals screened. A graph of the number of genotypes found versus sample size did not plateau, indicating that rotifer populations are made of a very large number of clones. Unexpectedly, most samples, including the resting egg bank, were in genetic equilibrium. However, evidence for linkage disequilibrium due to replicate genotypes was found in the planktonic sample at the end of the growth cycle (March), when the effects of clonal selection are expected to be noticeable. Indeed, in this sample 11 genotypes were found more than once, and a simulation revealed evidence of significantly small expected genotypic diversity, probably due to clonal selection. A different analysis on the same dataset (Stenberg et al., 2003) showed that at least four of the repeated multilocus genotypes are likely to be members of the same clone.

This study revealed that, although clonal selection is significant, and actually reduced the genotypic diversity along the parthenogenetic sample, its effects are weaker than might be predicted (King, 1980), as populations at the end of the parthenogenetic phase are still made of a very high number of clones, partly due to the very large number of initial clones. If the sample sizes in the study had been halved, the effects of clonal selection would not have been detected at all. Therefore, clonal selection might be effective in reducing genotypic diversity, but allelic diversity (at least in neutral alleles at significant frequencies) remains virtually the same. At least in the set of loci investigated, the observed genetic diversity is generated every year by recombination (input from the resting egg bank), rather than by mutational input, even for loci with relatively high mutation rates.

The clonal structure of this population resembles the 'incomplete genetic discontinuity model' of King (1977), in which clones might coexist during long periods, their frequencies fluctuating depending on the seasonal conditions of the lake. To further understand to what extent clones are ecological generalists or specialists, a joint investigation of genetic diversity and ecological characteristics should be undertaken. In spite of being a temporary population, the short generation times of rotifers facilitate the detection of clonal selection. In contrast, clonal selection in *Daphnia* has only been detected in permanent populations, in which selection has much longer time to take effect (Gómez & Carvalho, 2000). Recently, further evidence for the importance of directional clonal selection in rotifer populations was found

Figure 4. Phylogeography of species from the *B. plicatilis* complex. (a) Neighbour Joining phylogenetic tree representing the phylogeography of *B. plicatilis* s.s. colleted from Iberian lakes. (b) phylogeography of *B.* 'Manjavacas' lineage 1 collected from Iberian salt lakes. (c) phylogeography of rotifers tentatively classified as *B. plicatilis* s.s. collected in Wood Buffalo National Park (Canada) (tree produced from sequences deposited in GenBank, AF499054–AF499069, and published in Derry et al., 2003). Different symbols on each map and corresponding tree indicate geographically concordant lineages.

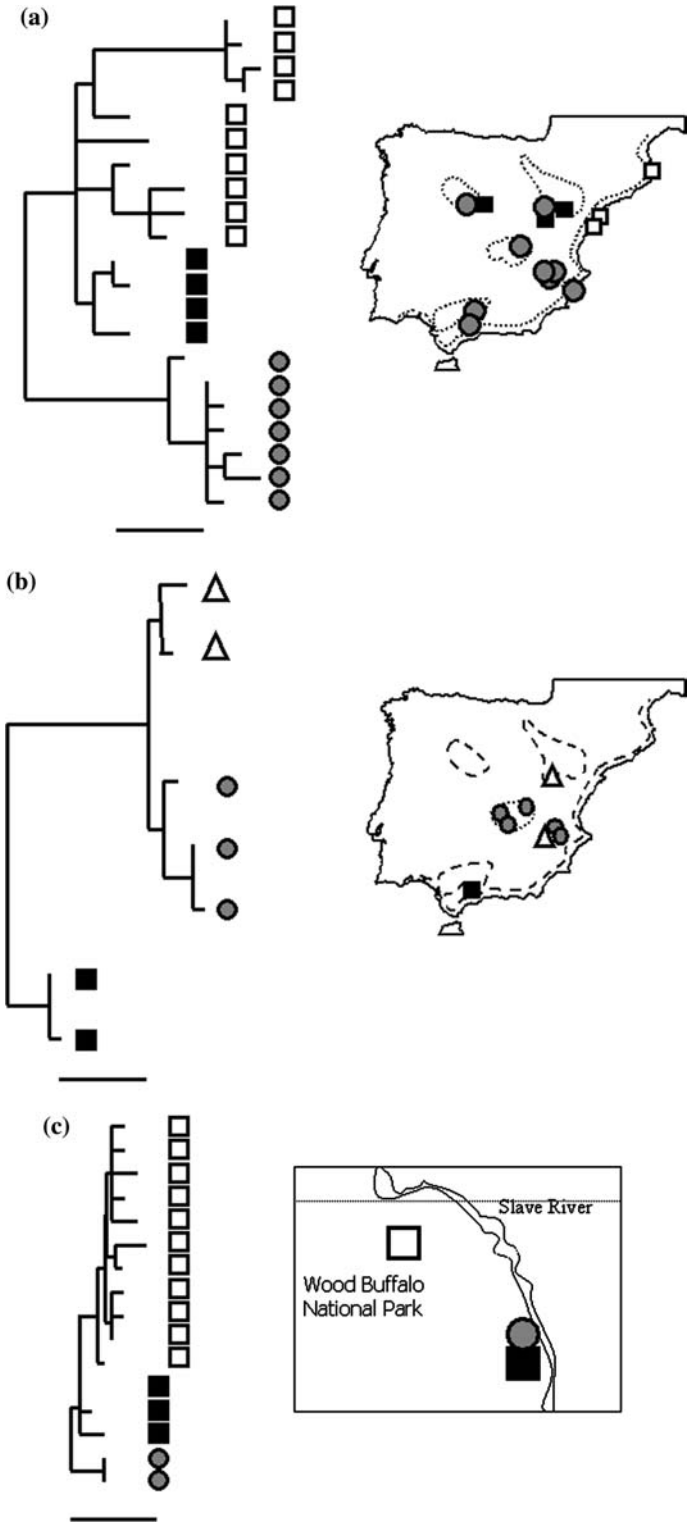
using allozyme analysis in several permanent and temporary Mediterranean ponds in a set of sibling species (Ortells, 2002).

The studies to date emphasize the importance of applying molecular tools to the understanding of rotifer clonal structure and the interplay with the resting egg bank. However, the body of research is still limited and, unfortunately, restricted to the *Brachionus plicatilis* complex. Comparisons with other rotifer species, particularly in large lakes, freshwater ponds, and riverine habitats are badly needed for a better understanding of rotifer clonal structure.

Phylogeography

Phylogeography is the study of the patterns and processes governing the geographic distribution of genetic lineages (Avise, 2000). Such analysis allows distinguishing between recurrent processes such as gene flow, and historical processes such as population subdivisions, long distance migration events or range expansions (Templeton, 1998). Phylogeographic information to date comes overwhelmingly from RFLPs, or from sequence variation of mtDNA (see review in Avise, 2000). At the time of Avise's influential book (Avise, 2000) phylogeographic research of freshwater zooplanktonic organisms was just beginning and the treatment they received was rather scant and biased: "This highly dispersive phase of the life cycle (the ephippium) probably accounts for near ubiquity of (*Daphnia*) mtDNA lineages across vast areas such as Northern Eurasia." Avise was reviewing the first mDNA assessments of Holarctic *Daphnia* (Taylor et al., 1996; Weider et al., 1996; Weider & Hobaek, 1997). These pioneering studies had been performed in areas strongly affected by the Pleistocene glaciations, and the results described the colonisation of very recently formed ponds and lake systems of the Arctic and Subarctic. The high dispersal and colonisation abilities of zooplankton (see review in De Meester et al., 2002) do indeed explain the rapid colonisation of such geographic areas. Since then, additional *Daphnia* studies in more temperate areas have supported strong geographic structure and regional endemism of lineages incompatible with high rates of gene flow (see review in De Meester et al., 2002).

The phylogeographic structure of *Brachionus plicatilis* (sensu Ciros-Pérez et al., 2001), in the Iberian Peninsula was investigated by sequencing 653 bp of the mitochondrial gene cytochrome *c* oxidase subunit 1 (COI) (Gómez et al., 2000). DNA was extracted from individual resting eggs retrieved from sediments of salt lakes in the Iberian Peninsula. Sampling resting eggs reduces biases due to stochastic variation in clonal populations due to selection or drift in a given parthenogenetic growth period. *B. plicatilis* s.s. was found in the resting egg banks of 18 of the 47 lakes sampled. A total of 98 individuals were sequenced for the mtDNA gene, yielding 21 different mtDNA haplotypes. Phylogenetic analysis revealed the occurrence of two mtDNA lineages (Fig. 4a). These lineages were strongly structured geographically, with one being present in the southern ponds, and the other in the northern ones. Both lineages were found to coexist in two ponds which formed a contact zone. The northern lineage was further divided in three subgroups (see Fig. 4a). Individual lakes had relatively low genetic diversity, and most of the haplotypes were restricted to single lakes. Examination of the data using Templeton's (1998) Nested Clade Analysis suggested a low level of gene flow, with isolation by distance and some episodes of long distance colonisation. The main process that structured genetic diversity was historical population fragmentation in allopatry. Given the degree of genetic divergence of the two groups of haplotypes, a hypothesis was proposed to explain the observed phylogeographic structure in Iberian rotifer populations. Such a pattern could have arisen from the climatic changes accompanying Pleistocene glaciations, which probably reduced and fragmented the area occupied by salt marshes and lakes in the Iberian Peninsula. Because rotifer resting eggs seem so well


(a)
(b)
(c)
Slave River
Wood Buffalo
National Park

suited for dispersal (King, 1980), and the Iberian Peninsula is one of the main corridors of European waterfowl migration, the persistence of signatures of population fragmentation after several thousand years is surprising.

Recently, Gómez et al. (unpublished results) have examined the phylogeography of another rotifer from the *B. plicatilis* complex in the Iberian Peninsula, the *B.* 'Manjavacas' (see Gómez et al., 2002b). The mtDNA COI gene was sequenced in resting eggs collected from salt lake sediments. In agreement with the findings in *B. plicatilis* s.s., a strong phylogeographic structure was found in one of the lineages (Fig. 4b), but the two most divergent lineages of this species seem to overlap to a large extent (data not shown).

Derry et al. (2003) have recently investigated the sequence variation of two mtDNA genes of *B. plicatilis* in three Canadian salt lakes (Fig. 4c). Their results support the patterns found in the Iberian Peninsula. The species they worked with seems to be *B. plicatilis* s.s. (a 4.1% sequence divergence was found between the COI gene of Canadian and Iberian rotifers) and they found strong geographic structure, with haplotypes restricted to single lakes, and related haplotype lineages found in the same lake (Fig. 4c).

The pattern of intraspecific geographic differentiation found suggests that speciation could happen in allopatry, and that sympatry is secondary, although more detailed analyses are needed to reach conclusions regarding the tempo and mode of speciation in rotifers.

Population structure

The reasons underlying the high levels of interpopulation differentiation, even at local scales, found in *Daphnia* and other zooplanktonic organisms despite their dispersal abilities have been much debated (see reviews in De Meester, 1996; De Meester et al., 2002). Rapid local adaptation might prevent survival of migrants due to inferior competitive abilities in the new habitat compared with the locals; intragenomic interactions, such as outbreeding depression with hybrid breakdown might arise due to genomic incompatibilities established during the historical colonisation process. In addition, habitats can be colonised by a few propagules which will reproduce rapidly, as they would grow unchecked by competitors giving rise to a 'persistent founding effect' (Boileau et al., 1992), by which the allelic frequencies established by the first colonists will be resistant to change due to migration. In order to investigate these processes in rotifers, Gómez et al. (2002a) typed between 20 and 50 rotifer resting eggs retrieved from sediment samples from the same group of salt lakes and coastal lagoons sampled for the mtDNA phylogeographic study for a set of 7 unlinked microsatellite loci. Of the 63 alleles found in the 440 eggs typed, 23 were private alleles, that is, they were alleles found in a single population. In accordance with the mtDNA findings, results show a strikingly high level of population genetic differentiation (global F_{st} estimate 0.43). Thirteen out of the fourteen populations for which more than 9 resting eggs were typed were in genetic equilibrium suggesting that ongoing inbreeding (due for example to a low number of clones in rotifer populations) is not a cause of population differentiation. A Principal Component Analysis revealed some differences with the phylogeographic structure of the species, as the microsatellite differentiation was not correlated with the mtDNA differentiation. Some populations seemed to be responsible for this discrepancy patterns as they were likely part of the contact zone where both historical mtDNA lineages had come into contact (see Berrieman et al., 2004). A strong pattern of isolation by distance was found, independently of the mtDNA constitution of the involved populations. This pattern indicates that populations harbouring different mtDNA lineages, in spite of their differentiation belonged to the same species and that gene flow might play some role at a local scale. It will be interesting to investigate if such local gene flow reflects local colonisation-extinction dynamics or genetic exchange between established populations.

The accumulation of data in several passively dispersed aquatic organisms has led to the proposal of an integrated hypothesis based on several ecological and evolutionary processes (De Meester et al., 2002). This monopolisation hypothesis explains the paradox of strong population structure (low gene flow) despite good colonisation abilities (high dispersal). Both neutral processes (persistent founder effects) and selective processes

(local adaptation) have been shown to be particularly effective in several aquatic organisms and are hypothesised to act synergistically to diminish the genetic consequences of dispersal. The former is due to a 'dilution' effect: the migrant alleles form a much reduced proportion of the local gene pool. The latter acts by reducing the chances of migrants of surviving or leaving descendants in the population. The third component of the monopolisation hypothesis is the presence of large resting egg banks containing past and presently adapted genotypes (an effective archive of the cumulative time-space adaptive spectrum of the population, see for example Cousyn et al., 2001), against which any migrant has to compete.

Thus, in a similar manner to other zooplanktonic organisms, rotifers display marked population differentiation in neutral markers which can largely be explained by historical colonisation events. The importance of the dilution effect against migration afforfed by the resting egg bank can also be safely assumed.

The proximate causes of cyclomorphosis

Cyclomorphosis defines the temporal cyclic morphological changes that occur within a population (Black & Slobodkin, 1987). This widespread phenomenon (investigated particularly in *Brachionus*, *Keratella* and *Asplanchna*) has complicated rotifer taxonomy (Serra et al., 1997). The possible proximate causes determining cyclomorphosis include phenotypic plasticity, genetic replacement of clones, and, using a wider definition, seasonal succession of sibling species. Laboratory culture and exposure of clones to the same or different growing conditions can help disentangle the causes of morphological variation (see Gilbert, 2001 for a recent example). Molecular techniques might afford a more cost-effective and rapid demonstration of the genetic basis of cyclomorphosis, and the discrimination between species and clonal replacement can be more finely resolved. A recent contribution to the understanding of morphological changes in the genus *Keratella* was made by Derry et al. (2003). Samples from three morphs of *K. cochlearis* (*tecta*, *robusta* and *faluta*), 2 morphs of *K. hiemalis* (single spined and two-spined) and *K. quadrata* were collected in several lakes in Wood Buffalo National Park (Alberta, Canada). Fragments of two mtDNA genes, COI and 16S rRNA were sequenced in a total of 42 and 9 individuals respectively. In agreement with previous biometric analysis of seasonal variation (Hofmann, 1983), large sequence differences in their mtDNA suggest that *K. cochlearis* is a species complex (Fig. 5). In contrast, morphological variation in *K. hiemalis* in these lakes seemed to be due to phenotypic plasticity, as the single-spined and two-spined morphs were very similar genetically. Little genetic variation was found in *K. quadrata* in these Canadian lakes. This study illustrates that molecular techniques can be a powerful tool to investigate the nature of phenotypic variation in rotifers. More studies, and particularly, genetic analysis of well known systems, will be required for a more global assessment of the importance of clonal or species succession in cyclomorphosis.

Perspectives and future directions

The application of molecular techniques has given a new impetus to the fields of ecology and evolution of rotifers. There are two main causes for concern regarding general conclusions, though. First, the number of studies is still small, and second, the body of research is largely restricted to the species complex *B. plicatilis*. *B. plicatilis* s.l. inhabits salt lakes and lagoons – habitats that are not considered typical among rotifers, and therefore, the results obtained with this taxon might not be extendable to other monogononts. On the other hand, *B. plicatilis* has proven to be a good model organism and the conclusions attained so far have been largely in agreement with findings in freshwater zooplankters such as cladocerans and copepods. However, it must be emphasised that is important to test the generality of the patterns discovered in other rotifer models, for example inhabiting different habitat types (interstitial, riverine, lacustrine and other freshwater rotifers), or habitats with less seasonality than the ones investigated (tropical lakes, for example).

The future of rotifer Molecular Ecology looks very promising. Many exciting fields that have remained largely untouched in the past can now be tackled. For example, rotifer resting egg banks have not been investigated for historical temporal variation in ecologically relevant traits. This research has indeed provided surprising results in

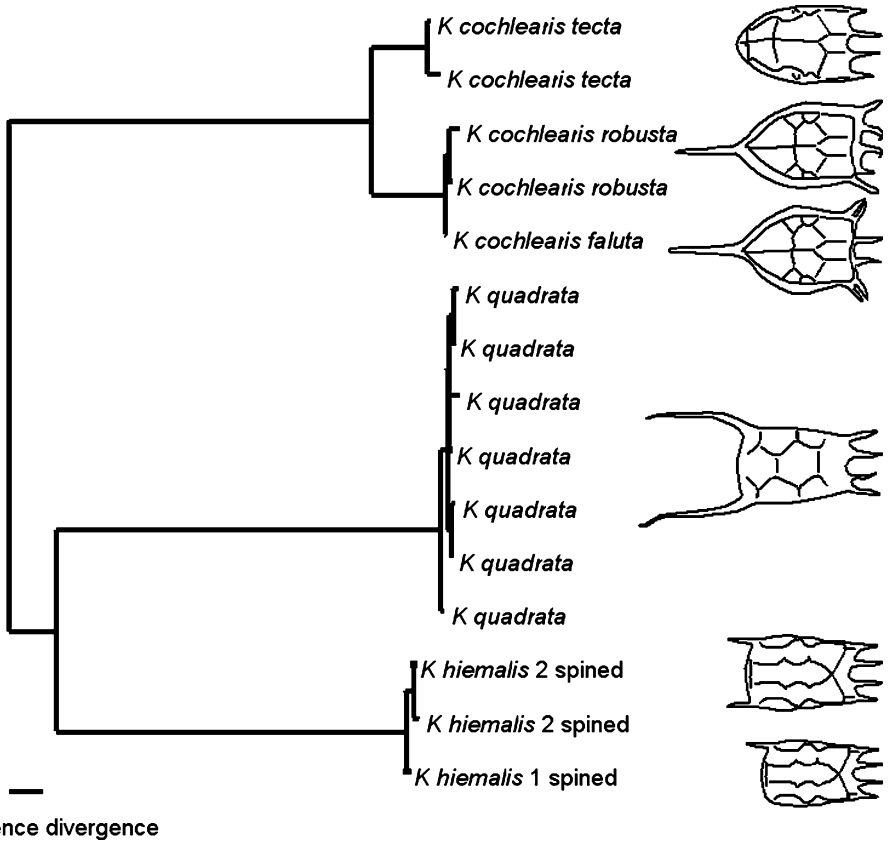


Figure 5. Cyclomorphosis in *Keratella*. Neighbour Joining phylogenetic tree (Log-Det distances) showing the phylogeny of species and morphs of three *Keratella* species collected in Canadian lakes. The tree was built from sequences downloaded from GenBank (AF499073–AF499087) originally published in Derry et al. (2003).

Daphnia (Cousyn et al., 2001). To further understand the structure of rotifer populations, studies must be undertaken on the importance of local adaptation, measuring the level of interpopulation differentiation regarding ecologically relevant traits relative to neutral genetic differentiation.

In addition, the application of metapopulation theory can be quite productive, especially because clusters of lakes or ponds can have very different demographic properties in terms of population extinction and colonisation. Some larger or more stable lakes could act as 'sources' and other smaller lakes – where temporal stochastic variation in resting egg production could lead to population extinctions – could be regarded as 'sinks'. In fact, sets of coastal pools were demonstrated to fit to a metapopulation structure in *Daphnia magna* (Ebert et al., 2002; Haag et al., 2002). The effect that this metapopulation structure, so different in its effects to the traditional island model, could have in explaining the genetic divergence of rotifer populations has not been explored at all.

Intraspecific clonal variation in ecologically relevant traits have been little investigated in rotifers (Zhao & King, 1989). Ecologically relevant traits are often determined by several quantitative trait loci and therefore, populations could harbour large variability for these traits allowing for rapid responses to selection (Lynch et al., 1999; Morgan et al., 2001). Indeed, some rotifer populations, especially those inhabiting temporary environments, have shown few temporal changes in their neutral population structure. However, important temporal population changes in ecologically relevant traits may be accompanied by few or no detectable changes in neutral genetic markers, as it has been shown with antipredator phototactic behaviour in *Daphnia magna* (Cousyn et al., 2001).

There is little information on the occurrence of local adaptation in rotifers and how it is achieved. For example, local adaptation in a population could be a property facilitated by its resting egg bank if adapted genotypes from previously selective environments are present and hatch in the appropriate environment or randomly (resting egg banks would constitute an archive of selections past), or it could be a property of the clonal population due to its rapid response to selection. If the former is important, the consequences of human-induced pollution, climate change or species invasions (or any other new environmental challenge) could have unforeseen effects as rotifer species could well lack the genetic diversity necessary to respond to such changes. On the contrary, if rotifers are capable to respond rapidly to selection, then their populations could be quite resilient to the aforementioned environmental challenges. Indeed microevolution in a rotifer planktonic population without access to a resting egg bank, due to selection against sexual reproduction, has been reported in chemostat cultures of *B. calyciflorus* (Fussmann et al., 2003).

Many interesting questions remain: What is driving speciation in rotifers? Is speciation mostly allopatric? Does reproductive isolation arise as a side-effect of population divergence or is reinforcement important? Is speciation ecological as proposed for *Daphnia* (Pfrender et al., 2000)? Will a global biogeography of the *B. plicatilis* complex reveal true cosmopolitans? What is the importance of inbreeding during population colonisation and its interaction with early dispersal?

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