

2 **Molecular ecology of rotifers: from population differentiation to speciation**3
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8 *Key words:* rotifera, cryptic species, *Brachionus plicatilis*, clonal structure, monopolisation, resting eggs9 **Abstract**10 The development of cost-effective molecular tools allowing the amplification of minute amounts of DNA
11 effectively opened the field of molecular ecology for rotifers. Here I review these techniques and the
12 advances they have provided in the understanding of sibling species complexes, clonal structure, resting
13 egg banks, population structure, phylogeographic patterns and phylogenetic relationships in rotifers.
14 Most of the research to date has focused on the rotifer species complex *Brachionus plicatilis*. The use of
15 DNA sequence and microsatellite variation, in the context of the background knowledge of life history,
16 mating behaviour, and temporal population dynamics in these organisms have revolutionised our views
17 into the processes shaping the genetic diversity in aquatic invertebrates. Rotifers have populations with a
18 very high number of clones in genetic equilibrium. In temporary populations clonal selection is effective
19 in eroding the number of clones. Rotifer populations are strongly differentiated genetically for neutral
20 markers, even at small geographical scales, and exhibit deep phylogeographic structure which might
21 reflect the impact of Pleistocene glaciations. Despite the high potential for dispersal afforded by resting
22 eggs, rotifers display persistent historical colonisation effects, with gene flow effective only at a local scale
23 and with marked isolation by distance. Instances of long-distance transcontinental migration resulting in
24 successful colonisation have also been revealed. *B. plicatilis* is composed of a group of several ancient
25 species and sympatry is common. Despite this, the presence of cosmopolitan species in this species
26 complex cannot be discounted. I discuss future priorities and point out the main areas where our
27 knowledge is still insufficient.

28

29 **Introduction**30
31 Molecular ecology concerns the application of
32 molecular techniques to questions in ecology,
33 evolution, behaviour and conservation biology
34 (Carvalho, 1998). Several technical and methodo-
35 logical developments in molecular biology in the
36 1980s facilitated such application, among these,
37 refinement in DNA extraction protocols, poly-
38 merase chain reaction (PCR), design of conserved
39 primers which allowed amplification and sequencing
40 of genes of virtually any organism, and, the publi-
41 cation of protocols for the development of species-
42 specific microsatellite primers. The use of molecular43 tools has been revolutionary in diverse fields of
44 ecology, evolution and behaviour and has yielded
45 numerous insights into population structure, mating
46 strategies, among many others.47 The field of rotifer biology has much to gain
48 from molecular ecology, as several themes have
49 aroused much theoretical debate with little support
50 from empirical data. Some of these long formu-
51 lated questions include: how many clones are there
52 in rotifer populations? Is clonal selection impor-
53 tant in eroding genetic diversity? Are resting egg
54 banks repositories of past and present genetic
55 variation? And in a related question, is sex serving
56 a function other than the way rotifers make resting

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

68 The small body size of rotifers, and the diffi-
69 culty of laboratory culture for many species, has
70 hindered the application of allozyme electropho-
71 resis to their study (Gómez, 1998). In spite of this,
72 labour-intensive studies using allozyme markers in
73 rotifers have yielded valuable data (King, 1977;
74 King, 1980; Gómez et al., 1995; Ortells et al.,
75 2000). The results based on allozyme markers were
76 often limited due to the reduced amount of poly-
77 morphism detected. Therefore, and unlike in cla-
78 docerans, molecular ecology did not become a
79 strong field in rotifers immediately. Although
80 rotifers have long been a well-established model
81 system in limnology, ecology and life history, the
82 lack of basic genetic knowledge substantially
83 hampered advances in some of these fields. For
84 example, studies involving the rearing and the
85 comparison of the ecological characteristics (e.g.,
86 optimal growth rates, mixis inducing cues) of wild-
87 caught or domesticated clones (for some examples
88 see King, 1980; Snell & Carrillo, 1984; Serra et al.,
89 1994) revealed a strong genetic component in life
90 history trait variation. However, we now know
91 that these studies almost certainly involved com-
92 parison of strains belonging to sympatric cryptic
93 species.

94 Here I will review and discuss (i) methodolog-
95 ical advances that have facilitated the application
96 of molecular techniques to rotifers; (ii) recent
97 advances in the field of rotifer molecular ecology;
98 and (iii) future developments and areas which are
99 likely to benefit further from the molecular eco-
100 logical approach. It must be emphasised that most
101 of the research to date has been performed on the
102 species complex *Brachionus plicatilis*, therefore the
103 results obtained with this taxon will not necessarily
104 apply to other rotifers. For the sake of simplicity
105 however, the name 'rotifers' will be employed
106 throughout the review mostly to refer to this taxon.

Technical and methodological advances in molecular ecology

The number of techniques being applied to molec- 109
ular ecology is quite substantial and new methods 110
are being added to the molecular ecologist's toolkit 111
virtually every year. For the present purposes I will 112
focus on those techniques crucial for the investiga- 113
tion of basic questions in rotifer ecology and evo- 114
lution, and which have already provided some 115
progress. In doing this, I have favoured reviews and 116
monographs instead of the primary literature. The 117
interested reader can trace back the relevant liter- 118
ature cited therein. Although allozyme electropho- 119
resis can be considered a molecular technique, it 120
will not be treated here as the methods and its 121
applications have been reviewed elsewhere (Gómez, 122
1998). 123

DNA extraction procedures 124

The small body size of rotifers means that only 125
procedures that involve PCR (polymerase chain 126
reaction) amplification of extracted DNA can be 127
applied to population problems cost-effectively. 128
Techniques originally developed to recover DNA 129
from forensic material, usually small dried blood 130
or semen samples containing little or degraded 131
DNA (Walsh et al., 1991) were adapted to extract 132
DNA from single rotifer females, males and rest- 133
ing eggs (Gómez et al., 1998; Leutbecher, 2000). 134
The advantages of such techniques were immense, 135
as rotifer genetic variation could be examined 136
without the need to culture them in the laboratory. 137
Moreover, the genetic variation in sexual females, 138
males and sediment borne resting eggs could be 139
analysed, allowing wide-scale analysis of popula- 140
tion structure, as well as long-term genetic 141
temporal variation. 142

Polymerase Chain Reaction (PCR) amplification using conserved primers 143 144

The minute amounts of DNA recovered from 145
single rotifers must be amplified prior to analysis 146
PCR (Palumbi, 1996; Birt & Baker, 2000), and this 147
amplification necessitates a pair of oligonucleotides 148
(the so called 'primers') of sufficient sequence 149

150 similarity to regions flanking the target organism
 151 DNA sequence. Several sets of 'universal' primers,
 152 have been designed from conserved gene regions in
 153 mitochondrial DNA (mtDNA) and nuclear DNA
 154 (nDNA), and used to amplify rotifer DNA suc-
 155 cessfully. To amplify mtDNA regions, these
 156 include cytochrome *c* oxidase I primers developed
 157 by Folmer et al. (1994) used by Gómez et al.
 158 (2000; 2002b) and Derry et al. (2003), and 16S
 159 ribosomal genes developed by Palumbi (1996) used
 160 by Derry et al. (2003). Among nuclear genes
 161 Gómez et al. (Gómez et al., 2002b) amplified and
 162 sequenced the ITS1 ribosomal DNA using primers
 163 developed by Palumbi (1996), and Mark Welch &
 164 Meselson (2000) described and used several coding
 165 gene primers in bdelloids. Sufficient variation at a
 166 local or regional scale can often be found in a
 167 species when relatively fast-evolving genes are
 168 examined, and this is one reason why mtDNA is
 169 favoured for phylogeographic investigations and
 170 phylogenetics at low taxonomic ranks. Other rea-
 171 sons for the choice of mtDNA are (1) its haploidy,
 172 clonality and uniparental mode of inheritance
 173 (usually mother to offspring in animals), which
 174 reduces to $\frac{1}{4}$ its effective population size relative
 175 to nuclear markers making it more sensitive to
 176 demographic and evolutionary relevant events
 177 such as bottlenecks and population subdivision
 178 (Birky et al., 1989; Birky, 2001), (2) the fact that it
 179 occurs in multiple copies per cell, which favours its
 180 preservation and retrieval from ancient, poorly
 181 preserved or small tissue samples (Wayne et al.,
 182 1999). A cautionary note on using mtDNA used
 183 on its own is that, being a maternally inherited
 184 molecule, introgression and hybridisation may not
 185 be detected, therefore calling for the use of nuclear
 186 markers to support it. Although hybridisation not
 187 been detected in *B. plicatilis*, it might well be
 188 present in other monogononts.

189 *Microsatellite loci*

190 Population genetic studies in rotifers have been
 191 hampered by the scarcity of known polymorphic
 192 genetic markers. Although allozyme loci proved to
 193 be useful tools to detect sibling species complexes,
 194 little or no genetic variation has been reported
 195 within populations (Gómez et al., 1995; Ortells
 196 et al., 2000; but see Ortells, 2002). Furthermore,
 197 allozyme loci do not allow for the exploration of

genetic diversity stored in rotifer dormant egg
 banks in lake sediments, or in the sexual individ-
 uals 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, par-
 entage, 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 fol-
 lowing 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 devel-
 oped 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, micro-
 satellites 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 poly-
 morphism) (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

250 *Cryptic species complexes and biogeography*

251 Rotifera is considered a relatively minor metazoan
252 phylum with less than 2000 described species
253 (Segers, 2002). Due to their assumed considerable
254 abilities for passive dispersal, rotifers were long
255 considered to comprise mostly cosmopolitan spe-
256 cies. However, in his review on the biogeography
257 of rotifers, Dumont (1983) argued that rotifers
258 show some evidence for vicariance and illustrated
259 the levels of endemism of the group in several
260 continents. However, Segers (1996) noticed that in
261 comparison with other animal groups, rotifer
262 morphospecies display large distribution ranges,
263 which could at least partly be a consequence to
264 widespread dispersal, with vicariance playing a
265 subordinate role. Both Dumont (1983) and Segers
266 (1996) concur in attributing the apparently high
267 proportion of widely distributed taxa to insuffi-
268 cient taxonomic resolution, that is, to the presence
269 of cryptic taxa within the described morphospe-
270 cies. In addition to increased sampling of poorly
271 known habitats or regions of the world, the des-
272 cription of cryptic or sibling species could contribute
273 substantially to increase our knowledge of rotifer
274 biodiversity. King (cited in Dumont, 1983) sug-
275 gested that, in addition to plankton nets, rotifer
276 researchers should carry electrophoretic equipment
277 with them. Although allozyme electrophoresis has
278 indeed been used to identify cryptic species (Gómez
279 & Snell, 1996; Ortells et al., 2000; Ortells, 2002),
280 King's advice has not been followed widely and the
281 confusion between cryptic species (compounded by
282 cyclomorphosis, see section *The Proximate Causes*
283 *of Cyclomorphosis* below) has crippled much of
284 rotifer basic research.

285 The wealth of ecological information on many
286 rotifer taxa has very little value unless the species
287 used are 'real' biological entities. For example,
288 variation among isolates of the same taxonomic
289 species attributed to cyclomorphosis *sensu stricto*,
290 or to intraspecific ecological variation, led to
291 proposals of models of temporal adaptation of
292 populations which had little connection with the
293 reality of rotifer populations. As King (1980) put it
294 the "population" investigated in many limnologi-
295 cal 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 com-
patibility, many species have been lumped together
and labelled 'generalists' or euryoic, polymorphic
and cosmopolitan. In addition to seriously under-
estimate rotifer biodiversity, the lack of knowledge
of cryptic species is preventing rotifer researchers
from studying niche partitioning, population
dynamics and many other ecological and evolu-
tionary 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 morphologi-
cally 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 con-
clude that two taxa are good species (see for
example Baum & Shaw, 1995). Since the advent of
PCR based techniques the number of cryptic spe-
cies described in a variety of taxa is increasing
steadily (Fig. 1) reflecting a tradition of lumping
by taxonomists, but also morphological conser-
vatism (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 subse-
quently 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

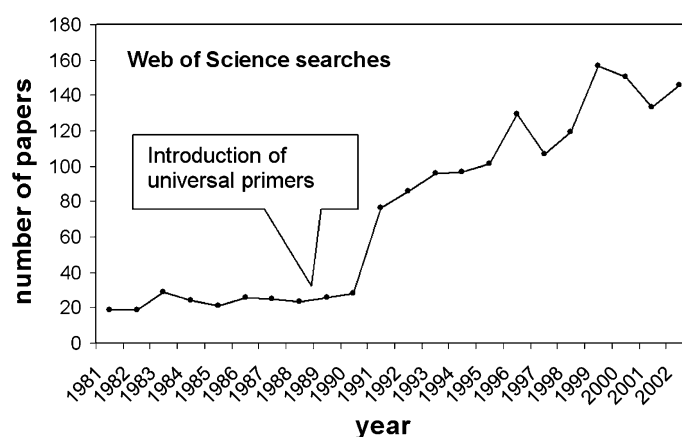


Figure 1. Effect of molecular tools on the recognition of cryptic species. Plot showing *Web of Science* searches on the keywords 'sibling species' or 'cryptic species'. The introduction of universal PCR primers is shown.

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

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

Tentative dating of the radiation of the com- 379
 plex using molecular clocks for each gene goes 380
 back to 10–27 mya (Miocene) (Gómez et al., 381
 2002b). Several additional lines of evidence sup- 382
 port that these genetic lineages are different species 383
 or groups of species. First, the previously men- 384
 tioned lack of hybridisation of these lineages when 385
 in sympatry (Gómez et al., 1995; Ortells et al., 386
 2000; Ortells, 2002); second, cross-mating experi- 387
 ments performed between strains belonging to 388
 different lineages indicate behavioural reproduc- 389
 tive isolation between them (Gómez & Serra, 1995; 390
 Gómez & Snell, 1996; Ortells et al., 2000; Berri- 391
 eman et al., 2004); and third, ecological differences 392
 have been found when clones belonging to differ- 393
 ent lineages have been tested in the laboratory for 394
 optimal growth rates and mixis patterns (Gómez 395
 et al., 1997). The molecular phylogenetic assess- 396
 ment of Gómez et al. (2002b) was performed on a 397
 very patchy sampling of the geographical distri- 398
 bution of the *B. plicatilis* complex – Sub-Saharan 399
 Africa, South and Central America and most of 400
 Asia were not sampled – therefore it is very likely 401
 that several other species are present in this taxon, 402
 especially considering its thermophilic character. 403
 In addition, information on the geographic, genetic 404
 and ecological diversity and mating behaviour of 405
 some of these lineages is scarce or absent and 406
 therefore the detection of additional species in the 407
 already sampled lineages is likely. 408

A further consequence of this study concerns 409
 the issue of cosmopolitanism. Rotifer species show 410
 a high propensity towards local and regional 411

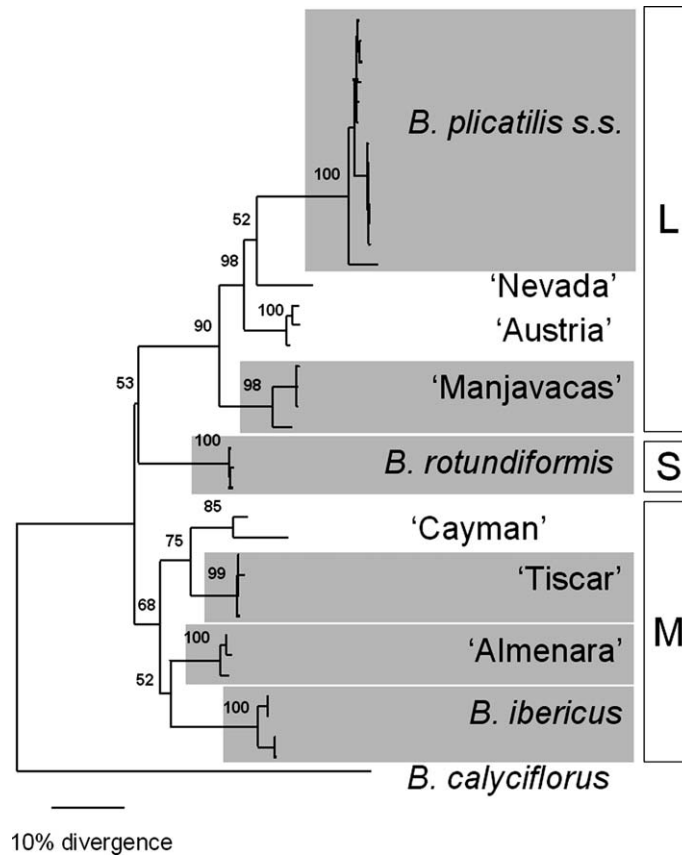


Figure 2. Phylogeny of the *Brachionus plicatilis* complex using *B. calyciflorus* as outgroup. Neighbour Joining tree based on a combined dataset using ITS1 and COI using a matrix of ML distances (see Gómez et al., 2002b for details). Values above branches indicate bootstrap support for nodes. The boxes 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.

412 genetic differentiation in spite of their capabilities
 413 for dispersal. Does the resolution of sibling species
 414 complexes in rotifers mean that good species will
 415 be regional endemics instead of cosmopolitan
 416 species? The answer seems to be: not completely.
 417 Undoubtedly, better geographic sampling is needed
 418 before strong conclusions can be drawn, but
 419 even with the relatively small effort undertaken so
 420 far, several of the lineages found are distributed in
 421 several continents (see Table 1). Whereas some of
 422 these lineages display a pattern of regional differ-
 423 entiation, others show very little genetic differences
 424 when clones retrieved from very distant geo-
 425 graphical locations are compared, indicating that
 426 long-distance (even transcontinental) migration
 427 and colonisation was relatively recent. Human
 428 induced transportation is an issue to be considered
 429 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
<i>B. plicatilis s.s.</i>	x		x		x
<i>B. 'Manjavacas'</i>	x	x		x	
<i>B. 'Austria'</i>	x	x	x		
<i>B. 'Nevada'</i>			x		
<i>B. 'Cayman'</i>		x	x		
<i>B. 'Tiscar'</i>	x				
<i>B. 'Almenara'</i>	x		x		
<i>B. ibericus</i>	x				
<i>B. rotundiformis</i>	x			x	

448 migration, rotifer species are always in the pro-
 449 cess of becoming cosmopolitan. A role for some
 450 dispersal limitation cannot be discarded, specially
 451 for very small and isolated lakes or for very large
 452 distances, although evidence for dispersal limita-
 453 tion in zooplankton is still controversial (Jenkins
 454 & Buikema Jr., 1998; Shurin, 2000).

455 A surprising finding of this study which sup-
 456 ported previous data was that many species in the
 457 complex were often found in sympatry (Gómez
 458 et al., 2002b) (Fig. 3). Lakes containing two and
 459 three rotifer species were not rare and a lake has
 460 been found where four of the species coexist
 461 (Ortells, 2002). Due to the strong seasonality of
 462 salt lakes and coastal lagoons, the high level of
 463 sympatry in rotifers could be due to seasonal
 464 succession and temporal niche partitioning and/or
 465 different susceptibilities to predators and parasites.
 466 In fact, species in the *B. plicatilis* complex can be
 467 involved in seasonal succession (Gómez et al.,
 468 1995), and this has been attributed to their eco-
 469 logical specialisation to different salinities or tem-
 470 peratures (Serra et al., 1998) and also to their
 471 different food preferences (Ciros-Perez et al.,
 472 2001). If this is a common pattern, then it should
 473 be possible to predict the number of species from
 474 the complex likely to be found in a lake, based on
 475 the degree of temporal variability of that lake
 476 (ideally estimated across several years to account
 477 for the interannual variation of the habitats).
 478 However, Ortells et al. (2003) found that in some
 479 cases two species from the *B. plicatilis* complex
 480 coexist throughout most of their presence in the
 481 pond, which seems to suggest that factors medi-
 482 ating coexistence (disturbance, predators, etc.) must

play a role in facilitating sympatry (Ciros-Perez
 et al., 2001).

483
 484
 485 A pattern of common sympatry reflects several
 486 processes: each species should (i) reach the lake or
 487 pond, (ii) establish populations with positive
 488 growth rates, and (iii) persist through time in the
 489 face of environmental variation. I have already
 490 mentioned that long-distance dispersal seems to be
 491 a common phenomenon in this species complex. A
 492 better understanding of ecological preferences
 493 (including pre-competitive and post-competitive
 494 niches) in these species would allow us to further
 495 understand the factors contributing to the estab-
 496 lishment success of rotifer species when they reach
 497 new habitats. It is clear that the fact that these
 498 organisms maintain resting egg banks will con-
 499 tribute to the long-term persistence of species in a
 500 lake, even if its conditions are unsuitable for a few
 501 years (what has been termed a 'storage effect'
 502 (Cáceres, 1997). Thus, a combination of effective
 503 dispersal and colonisation, successful niche parti-
 504 tioning and occurrence of factors mediating
 505 coexistence, and storage-effect allowed by the
 506 resting egg bank, seems to be responsible for the
 507 high degree of sympatry observed in rotifer species
 508 from this complex.

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

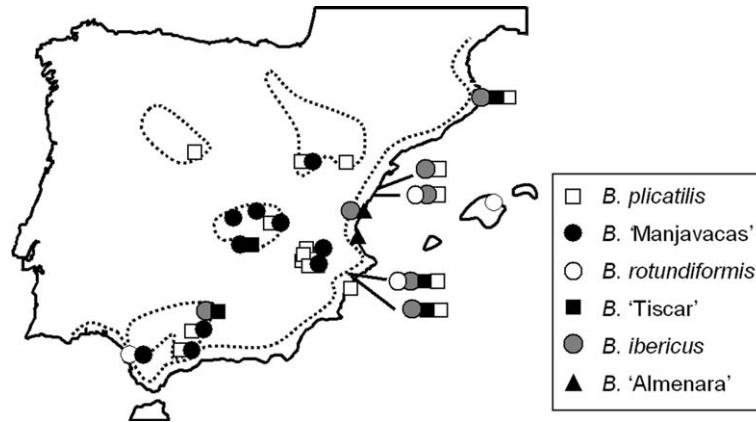


Figure 3. Sympatry in the *B. plicatilis* complex. Map of the Iberian Peninsula showing the distribution of the six species detected so far.

518 reflecting the colonisation abilities of the different
519 species.

520 Finally, phenotypic plasticity, but also mor-
521 phological conservatism, and a pattern of exten-
522 sive sympatry among cryptic species are factors
523 that might have contributed to the difficulties of
524 recognising good rotifer species and led the aver-
525 age rotifer taxonomist to become a 'lumper'. If
526 the patterns found in *B. plicatilis* are found to be
527 common in other rotifer morphospecies the
528 amount of hidden biodiversity in rotifers could be
529 substantial. Indeed, the presence of cryptic species
530 has been reported in *B. calyciflorus* using mating
531 behaviour and sequence variation data (Gilbert &
532 Walsh, this volume, Part V) and *Keratella* (Derry
533 et al., 2003). The day might come when rotifers are
534 not considered a minor phylum.

535 *Routine species identification*

536 Once the members of a species complex have been
537 described using molecular techniques, a simple
538 method available to a large number of rotifer
539 researchers must be developed so that species can
540 be identified from field samples routinely, or con-
541 tamination detected in aquaculture facilities. In the
542 case of *B. plicatilis*, several techniques are avail-
543 able, and they could be applied to other rotifer
544 species. First, allozyme electrophoresis, which can
545 be set up relatively cheaply (Gómez, 1998), could
546 provide 'diagnostic loci' (Ortells et al., 2000;
547 Ortells et al., 2003) which could be genotyped in
548 laboratory grown clones. As mentioned before, the
549 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 restric-
tion profile (restriction fragment length polymor-
phism, RLFP) when samples are run on a gel.
Although this procedure requires the use of a
thermocycler and a basic electrophoresis tools,
such equipment is basic to genetic and evolution-
ary 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., 2004) to discriminate between wild caught
clones of the sympatric and morphologically very
similar *Brachionus plicatilis* s.s. and *B. 'Manjava-*
cas', and between 'northern' and 'southern' *B. pli-*
catilis 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 partheno-
genetic females that hatched from sexually pro-
duced resting eggs in the resting egg bank. In lakes
and ponds that undergo periodic drying or freez-
ing over, these planktonic populations are neces-
sarily reconstituted every year from the resting egg
bank. In mild years, parthenogenetic populations

582 might survive several growing seasons with a varia-
 583 ble input from the resting egg bank. In at least
 584 some cases, species are present in the water column
 585 during part of the year, partly reflecting their tol-
 586 erance ranges or competitive abilities. The clonal
 587 composition and dynamics of planktonic rotifer
 588 populations and the interplay with the resting egg
 589 bank was virtually unknown until recently.
 590 Meanwhile, our understanding of clonal structure
 591 had advanced to a mature state in the other group
 592 of aquatic cyclical parthenogens, cladocerans.
 593 Cladoceran researchers working with *Daphnia* and
 594 mainly using allozyme markers had produced two
 595 models that seemingly accounted for the clonal
 596 and genetic structure of populations. In 'intermit-
 597 tent' ponds, where populations were founded every
 598 year from sediment banks, investment in sexual
 599 reproduction was important and genetic analysis
 600 revealed a large number of clones in genetic equi-
 601 librium (both Hardy-Weinberg and linkage) (see
 602 review in De Meester, 1996). No evidence for
 603 clonal selection was found in these ponds. In
 604 contrast, in permanent ponds some *Daphnia* pop-
 605 ulations persisted among years, sexual investment
 606 was reduced and there were often a low number of
 607 clones which underwent rapid changes in fre-
 608 quencies, indicating clonal selection. Although
 609 these models are simplifying and exceptions have
 610 been found, they can be used as a framework to
 611 help us understand what takes place in rotifer
 612 populations.

613 Gómez & Carvalho (2000) used a set of seven
 614 polymorphic microsatellite markers to screen an
 615 intermittent population of *Brachionus plicatilis* (Poza
 616 Sur, in Prat de Cabanes-Torreblanca Marsh). There
 617 were three consecutive planktonic samples along a
 618 parthenogenetic phase, a sample from the resting
 619 egg bank, and a sample after the re-establishment
 620 of populations after the summer drought. The set
 621 of seven polymorphic microsatellite loci pre-
 622 viously developed for *Brachionus plicatilis* (Gómez
 623 et al., 1998) proved to be a useful tool for clonal
 624 identification because the probability that two
 625 clones produced by separate sexual recombination
 626 events (hatching from two different resting eggs)
 627 have the same multilocus genotype is very low.
 628 Overall, 349 different genotypes were found in the
 629 390 individuals screened. A graph of the number of
 630 genotypes found versus sample size did not plateau,
 631 indicating that rotifer populations are made of a

632 very large number of clones. Unexpectedly, most
 633 samples, including the resting egg bank, were in
 634 genetic equilibrium. However, evidence for linkage
 635 disequilibrium due to replicate genotypes was found
 636 in the planktonic sample at the end of the growth
 637 cycle (March), when the effects of clonal selection
 638 are expected to be noticeable. Indeed, in this sample
 639 11 genotypes were found more than once, and a
 640 simulation revealed evidence of significantly small
 641 expected genotypic diversity, probably due to clonal
 642 selection. A different analysis on the same dataset
 643 (Stenberg et al., 2003) showed that at least four of
 644 the repeated multilocus genotypes are likely to be
 645 members of the same clone.

646 This study revealed that, although clonal
 647 selection is significant, and actually reduced the
 648 genotypic diversity along the parthenogenetic
 649 sample, its effects are weaker than might be pre-
 650 dicted (King, 1980), as populations at the end of
 651 the parthenogenetic phase are still made of a very
 652 high number of clones, partly due to the very large
 653 number of initial clones. If the sample sizes in the
 654 study had been halved, the effects of clonal selec-
 655 tion would not have been detected at all. There-
 656 fore, clonal selection might be effective in reducing
 657 genotypic diversity, but allelic diversity (at least in
 658 neutral alleles at significant frequencies) remains
 659 virtually the same. At least in the set of loci
 660 investigated, the observed genetic diversity is gen-
 661 erated every year by recombination (input from
 662 the resting egg bank), rather than by mutational
 663 input, even for loci with relatively high mutation
 664 rates.

665 The clonal structure of this population resem-
 666 bles the 'incomplete genetic discontinuity model'
 667 of King (1977), in which clones might coexist
 668 during long periods, their frequencies fluctuating
 669 depending on the seasonal conditions of the lake.
 670 To further understand to what extent clones are
 671 ecological generalists or specialists, a joint inves-
 672 tigation of genetic diversity and ecological char-
 673 acteristics should be undertaken. In spite of being
 674 a temporary population, the short generation
 675 times of rotifers facilitate the detection of clonal
 676 selection. In contrast, clonal selection in *Daphnia*
 677 has only been detected in permanent populations,
 678 in which selection has much longer time to take
 679 effect (Gómez & Carvalho, 2000). Recently, fur-
 680 ther evidence for the importance of directional
 681 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. collected 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.

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

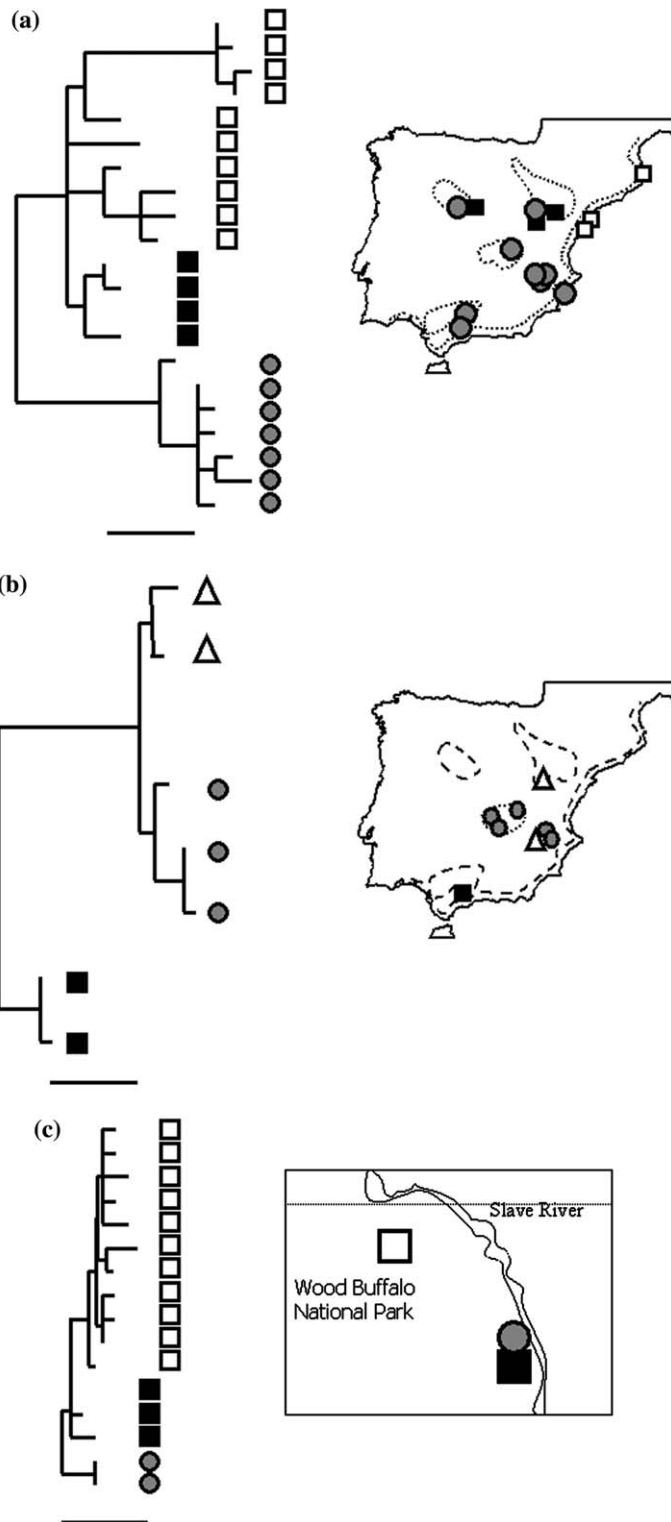
685 The studies to date emphasize the importance
686 of applying molecular tools to the understanding
687 of rotifer clonal structure and the interplay with
688 the resting egg bank. However, the body of research
689 is still limited and, unfortunately, restricted to the
690 *Brachionus plicatilis* complex.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

695 *Phylogeography*

696 Phylogeography is the study of the patterns and
697 processes governing the geographic distribution of
698 genetic lineages (Avice, 2000). Such analysis allows
699 distinguishing between recurrent processes such as
700 gene flow, and historical processes such as popu-
701 lation subdivisions, long distance migration events
702 or range expansions (Templeton, 1998). Phyloge-
703 ographic information to date comes overwhelm-
704 ingly from RFLPs, or from sequence variation of
705 mtDNA (see review in Avice, 2000). At the time of
706 Avice's influential book (Avice, 2000) phylogeog-
707 raphic research of freshwater zooplanktonic
708 organisms was just beginning and the treatment
709 they received was rather scant and biased: "This
710 highly dispersive phase of the life cycle (the
711 ephippium) probably accounts for near ubiquity of
712 (*Daphnia*) mtDNA lineages across vast areas such
713 as Northern Eurasia." Avice was reviewing the
714 first mDNA assessments of Holarctic *Daphnia*
715 (Taylor et al., 1996; Weider et al., 1996; Weider &
716 Hobaek, 1997). These pioneering studies had been
717 performed in areas strongly affected by the Pleis-
718 tocene glaciations, and the results described the
719 colonisation of very recently formed ponds and
720 lake systems of the Arctic and Subarctic. The high
721 dispersal and colonisation abilities of zooplankton
722 (see review in De Meester et al., 2002) do indeed
723 explain the rapid colonisation of such geographic

724 areas. Since then, additional *Daphnia* studies in
725 more temperate areas have supported strong geo-
726 graphic structure and regional endemism of line-
727 ages incompatible with high rates of gene flow
728 (see review in De Meester et al., 2002).

729 The phylogeographic structure of *Brachionus*
730 *plicatilis* (sensu Ciroso-Pérez et al., 2001), in the
731 Iberian Peninsula was investigated by sequencing
732 653 bp of the mitochondrial gene cytochrome *c*
733 oxidase subunit 1 (COI) (Gómez et al., 2000).
734 DNA was extracted from individual resting eggs
735 retrieved from sediments of salt lakes in the
736 Iberian Peninsula. Sampling resting eggs reduces
737 biases due to stochastic variation in clonal popu-
738 lations due to selection or drift in a given par-
739 thenogenetic growth period. *B. plicatilis* s.s. was
740 found in the resting egg banks of 18 of the 47 lakes
741 sampled. A total of 98 individuals were sequenced
742 for the mtDNA gene, yielding 21 different mtDNA
743 haplotypes. Phylogenetic analysis revealed the
744 occurrence of two mtDNA lineages (Fig. 4a).
745 These lineages were strongly structured geo-
746 graphically, with one being present in the southern
747 ponds, and the other in the northern ones. Both
748 lineages were found to coexist in two ponds which
749 formed a contact zone. The northern lineage was
750 further divided in three subgroups (see Fig. 4a).
751 Individual lakes had relatively low genetic diver-
752 sity, and most of the haplotypes were restricted
753 to single lakes. Examination of the data using
754 Templeton's (1998) Nested Clade Analysis sug-
755 gested a low level of gene flow, with isolation by
756 distance and some episodes of long distance colo-
757 nisation. The main process that structured genetic
758 diversity was historical population fragmentation
759 in allopatry. Given the degree of genetic divergence
760 of the two groups of haplotypes, a hypothesis was
761 proposed to explain the observed phylogeographic
762 structure in Iberian rotifer populations. Such a
763 pattern could have arisen from the climatic chan-
764 ges accompanying Pleistocene glaciations, which
765 probably reduced and fragmented the area occu-
766 pied by salt marshes and lakes in the Iberian
767 Peninsula. Because rotifer resting eggs seem so well



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

773 Recently, Gómez et al. (unpublished results)
774 have examined the phylogeography of another
775 rotifer from the *B. plicatilis* complex in the Iberian
776 Peninsula, the *B. 'Manjavacas'* (see Gómez et al.,
777 2002b). The mtDNA COI gene was sequenced in
778 resting eggs collected from salt lake sediments. In
779 agreement with the findings in *B. plicatilis* s.s., a
780 strong phylogeographic structure was found in one
781 of the lineages (Fig. 4b), but the two most diver-
782 gent lineages of this species seem to overlap to a
783 large extent (data not shown).

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

795 The pattern of intraspecific geographic differ-
796 entiation found suggests that speciation could
797 happen in allopatry, and that sympatry is sec-
798 ondary, although more detailed analyses are need-
799 ed to reach conclusions regarding the tempo and
800 mode of speciation in rotifers.

801 *Population structure*

802 The reasons underlying the high levels of inter-
803 population differentiation, even at local scales,
804 found in *Daphnia* and other zooplanktonic
805 organisms despite their dispersal abilities have
806 been much debated (see reviews in De Meester,
807 1996; De Meester et al., 2002). Rapid local adap-
808 tation might prevent survival of migrants due to
809 inferior competitive abilities in the new habitat
810 compared with the locals; intragenomic interac-
811 tions, such as outbreeding depression with hybrid
812 breakdown might arise due to genomic incom-
813 patibilities established during the historical colo-
814 nisation process. In addition, habitats can be

colonised by a few propagules which will repro- 815
duce rapidly, as they would grow unchecked by 816
competitors giving rise to a 'persistent founding 817
effect' (Boileau et al., 1992), by which the allelic 818
frequencies established by the first colonists will be 819
resistant to change due to migration. In order to 820
investigate these processes in rotifers, Gómez et al. 821
(2002a) typed between 20 and 50 rotifer resting 822
eggs retrieved from sediment samples from the 823
same group of salt lakes and coastal lagoons 824
sampled for the mtDNA phylogeographic study 825
for a set of 7 unlinked microsatellite loci. Of the 63 826
alleles found in the 440 eggs typed, 23 were private 827
alleles, that is, they were alleles found in a single 828
population. In accordance with the mtDNA find- 829
ings, results show a strikingly high level of popu- 830
lation genetic differentiation (global F_{st} estimate 831
0.43). Thirteen out of the fourteen populations for 832
which more than 9 resting eggs were typed were in 833
genetic equilibrium suggesting that ongoing 834
inbreeding (due for example to a low number of 835
clones in rotifer populations) is not a cause of 836
population differentiation. A Principal Compon- 837
ent Analysis revealed some differences with the 838
phylogeographic structure of the species, as the 839
microsatellite differentiation was not correlated 840
with the mtDNA differentiation. Some popula- 841
tions seemed to be responsible for this discrepancy 842
patterns as they were likely part of the contact 843
zone where both historical mtDNA lineages had 844
come into contact (see Berrieman et al., 2004). 845
A strong pattern of isolation by distance was found, 846
independently of the mtDNA constitution of the 847
involved populations. This pattern indicates that 848
populations harbouring different mtDNA lineages, 849
in spite of their differentiation belonged to the same 850
species and that gene flow might play some role at 851
a local scale. It will be interesting to investigate 852
if such local gene flow reflects local colonisation- 853
extinction dynamics or genetic exchange between 854
established populations. 855

The accumulation of data in several passively 856
dispersed aquatic organisms has led to the propos- 857
al of an integrated hypothesis based on several 858
ecological and evolutionary processes (De Meester 859
et al., 2002). This monopolisation hypothesis 860
explains the paradox of strong population struc- 861
ture (low gene flow) despite good colonisation 862
abilities (high dispersal). Both neutral processes 863
(persistent founder effects) and selective processes 864

865 (local adaptation) have been shown to be partic- 913
 866 ularly effective in several aquatic organisms and 914
 867 are hypothesised to act synergistically to diminish 915
 868 the genetic consequences of dispersal. The former 916
 869 is due to a 'dilution' effect: the migrant alleles form 917
 870 a much reduced proportion of the local gene pool. 918
 871 The latter acts by reducing the chances of migrants 919
 872 of surviving or leaving descendants in the popu- 920
 873 lation. The third component of the monopolisa- 921
 874 tion hypothesis is the presence of large resting egg 922
 875 banks containing past and presently adapted 923
 876 genotypes (an effective archive of the cumulative 924
 877 time-space adaptive spectrum of the population, 925
 878 see for example Cousyn et al., 2001), against which 926
 879 any migrant has to compete. 927

880 Thus, in a similar manner to other zooplank- 928
 881 tonic organisms, rotifers display marked popula- 929
 882 tion differentiation in neutral markers which can
 883 largely be explained by historical colonisation
 884 events. The importance of the dilution effect
 885 against migration afforded by the resting egg bank
 886 can also be safely assumed.

887 *The proximate causes of cyclomorphosis*

888 Cyclomorphosis defines the temporal cyclic mor- 931
 889 phological changes that occur within a population 932
 890 (Black & Slobodkin, 1987). This widespread phe- 933
 891 nomenon (investigated particularly in *Brachionus*, 934
 892 *Keratella* and *Asplanchna*) has complicated rotifer 935
 893 taxonomy (Serra et al., 1997). The possible prox- 936
 894 imate causes determining cyclomorphosis include 937
 895 phenotypic plasticity, genetic replacement of clones, 938
 896 and, using a wider definition, seasonal succession of 939
 897 sibling species. Laboratory culture and exposure of 940
 898 clones to the same or different growing conditions 941
 899 can help disentangle the causes of morphological 942
 900 variation (see Gilbert, 2001 for a recent example). 943
 901 Molecular techniques might afford a more cost- 944
 902 effective and rapid demonstration of the genetic basis 945
 903 of cyclomorphosis, and the discrimination between 946
 904 species and clonal replacement can be more finely 947
 905 resolved. A recent contribution to the understanding 948
 906 of morphological changes in the genus *Keratella* was 949
 907 made by Derry et al. (2003). Samples from three 950
 908 morphs of *K. cochlearis* (*tecta*, *robusta* and *faluta*), 951
 909 2 morphs of *K. hiemalis* (single spined and two- 952
 910 spined) and *K. quadrata* were collected in several 953
 911 lakes in Wood Buffalo National Park (Alberta, 954
 912 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 pheno-
 typic 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 sys-
 tems, 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 evolu-
 tion 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 there-
 fore, 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 fresh-
 water 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 re-
 search has indeed provided surprising results in

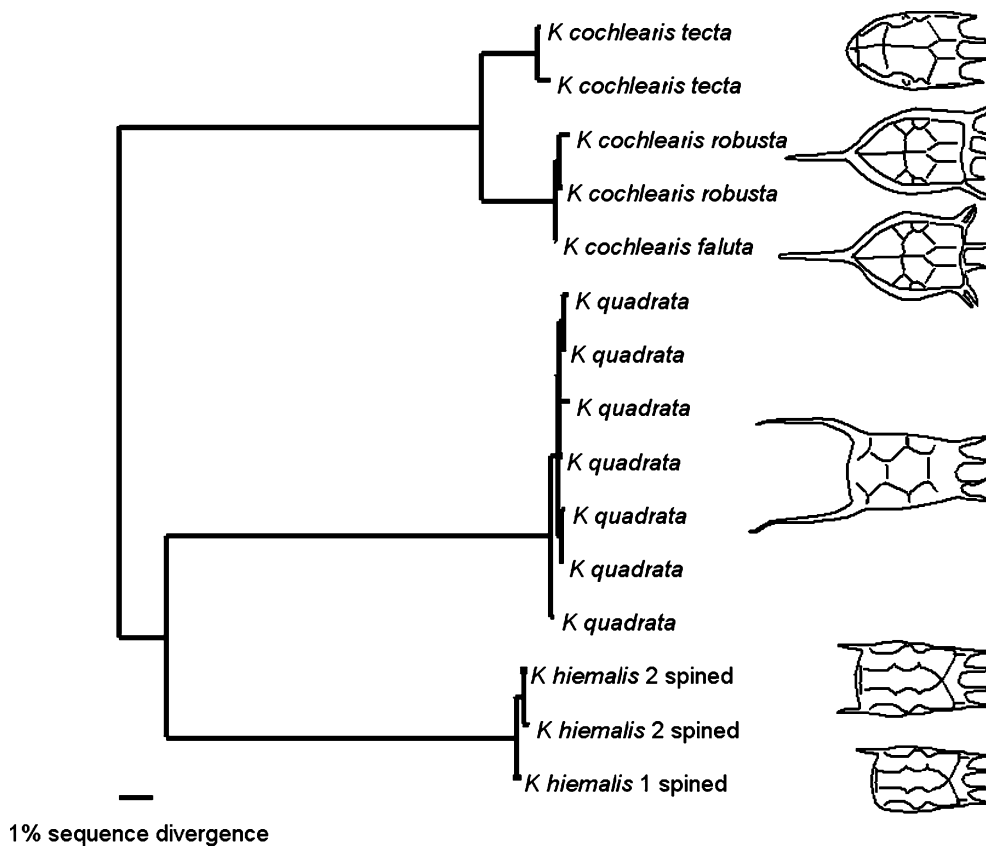


Figure 5. Cyclomorphosis in *Keratella*. Neighbour Joining phylogenetic tree (Log-Det distances) showing the phylogeny of species and morphs of three *Keratella* species collected in Canadian lakes. The tree was built from sequences downloaded from GenBank (AF499073–AF499087) originally published in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

960 *Daphnia* (Cousyn et al., 2001). To further under-
 961 stand the structure of rotifer populations, studies
 962 must be undertaken on the importance of local
 963 adaptation, measuring the level of interpopulation
 964 differentiation regarding ecologically relevant
 965 traits relative to neutral genetic differentiation.

966 In addition, the application of metapopulation
 967 theory can be quite productive, especially because
 968 clusters of lakes or ponds can have very different
 969 demographic properties in terms of population
 970 extinction and colonisation. Some larger or more
 971 stable lakes could act as ‘sources’ and other smaller
 972 lakes – where temporal stochastic variation in
 973 resting egg production could lead to population
 974 extinctions – could be regarded as ‘sinks’. In fact,
 975 sets of coastal pools were demonstrated to fit to a
 976 metapopulation structure in *Daphnia magna*
 977 (Ebert et al., 2002; Haag et al., 2002). The effect
 978 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 rotif-
 ers (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 environ-
 ments, have shown few temporal changes in their
 neutral population structure. However, important
 temporal population changes in ecologically rele-
 vant 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).

998 There is little information on the occurrence of
 999 local adaptation in rotifers and how it is achieved.
 1000 For example, local adaptation in a population
 1001 could be a property facilitated by its resting egg
 1002 bank if adapted genotypes from previously selec-
 1003 tive environments are present and hatch in the
 1004 appropriate environment or randomly (resting egg
 1005 banks would constitute an archive of selections
 1006 past), or it could be a property of the clonal
 1007 population due to its rapid response to selection.
 1008 If the former is important, the consequences of
 1009 human-induced pollution, climate change or spe-
 1010 cies invasions (or any other new environmental
 1011 challenge) could have unforeseen effects as rotifer
 1012 species could well lack the genetic diversity nec-
 1013 essary to respond to such changes. On the con-
 1014 trary, if rotifers are capable to respond rapidly to
 1015 selection, then their populations could be quite
 1016 resilient to the aforementioned environmental
 1017 challenges. Indeed microevolution in a rotifer
 1018 planktonic population without access to a resting
 1019 egg bank, due to selection against sexual repro-
 1020 duction, has been reported in chemostat cultures
 1021 of *B. calyciflorus* (Fussmann et al., 2003).
 1022 Many interesting questions remain: What is
 1023 driving speciation in rotifers? Is speciation mostly
 1024 allopatric? Does reproductive isolation arise as a
 1025 side-effect of population divergence or is rein-
 1026 forcement important? Is speciation ecological as
 1027 proposed for *Daphnia* (Pfrender et al., 2000)? Will
 1028 a global biogeography of the *B. plicatilis* complex
 1029 reveal true cosmopolitans? What is the importance
 1030 of inbreeding during population colonisation and
 1031 its interaction with early dispersal?

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