THE ROYAL SOCIETY

doi 10.1098/rspb.2000.1268

Phylogeography and regional endemism of a passively dispersing zooplankter: mitochondrial DNA variation in rotifer resting egg banks

Africa Gómez[1,2*], **Gary R. Carvalho**[1] **and David H. Lunt**[1]

[1]*Department of Biological Sciences, University of Hull, Cottingham Road, Hull HU6 7RX, UK*
[2]*Instituto Cavanilles de Biodiversitat i Biologia Evolutiva, Polígono La Coma s/n, 46980 Paterna, Valencia, Spain*

We investigated the phylogeography of the salt water rotifer *Brachionus plicatilis*, a cyclical parthenogen with passive dispersal mechanisms, using resting eggs recovered from saline lake sediments. Individual resting eggs were obtained from a large selection of lakes which were representative of five endorheic basins and the chain of coastal ponds in the Iberian Peninsula. The novel use of resting eggs allows the integration of seasonal and annual variations as well as the impact of stochastic effects such as drift and local extinction. A 653 bp fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene was sequenced from 98 eggs. Our results revealed a deep phylogeographical structure in this species, with a division into two main lineages with distinct geographical distributions, which probably diverged at the beginning of the Pleistocene period. Most of the mitochondrial DNA haplotypes were restricted to single lakes. Nested clade analysis supported Early Pleistocene fragmentation of populations, low gene flow and some long-distance colonization. These conclusions contrast strongly with previous ideas on rotifer biogeography and this pattern is consistent with a recolonization of the Iberian Peninsula from two glacial refugia. The results provide new insights into the processes responsible for the genetic diversification of passive dispersers, a life-history trait typical of zooplanktonic biotas.

Keywords: *Brachionus plicatilis*; cytochrome oxidase I; passive dispersal; nested clade analysis; resting eggs; Pleistocene

1. INTRODUCTION

Phylogeographical analysis is crucial to understanding processes such as population subdivision, speciation, ecological adaptation and historical climate change (Avise 2000). However, it is apparent from the literature that there is a very biased sampling of existing biotas, with most species representative of vagile taxa occupying relatively continuous habitat for a significant part of their recent history. Far less attention has focused on widely distributed organisms with apparently restricted dispersal in fragmented habitats.

Zooplanktonic organisms are important components of continental aquatic ecosystems and often have dormant stages in their life cycles, i.e. resting eggs, which only allow for passive dispersal through wind or waterfowl. In addition, they display a range of breeding systems and life cycles, including sexuals, obligate parthenogens and cyclical parthenogens. Finally, the patchy, insular nature of their habitats is likely to influence both their colonization patterns and local adaptive divergence (Boileau & Hebert 1991; De Meester 1996). However, despite the large number of taxa involved and their ecological importance, there have been relatively few studies addressing the phylogeography of lake zooplankters and most of this research has largely focused on the freshwater genus *Daphnia* (Chaplin & Ayre 1997; Crease *et al.* 1997; Weider *et al.* 1999; Straughan & Lehman 2000). The results of the rather restricted research effort have nevertheless forced a re-examination of the accepted wisdom that aquatic, passively dispersing organisms are cosmopolitan due to high gene flow rates. On the contrary, *Daphnia* studies to date have revealed high geographical genetic differentiation and the widespread presence of species complexes and 'cryptic' endemics, with a striking decoupling of morphological and genetic diversification (Colbourne *et al.* 1997; Hebert 1998). However, breeding system variation and interspecific hybridization are widespread in *Daphnia* and these processes may have often obscured the interpretation of its intraspecific phylogeographical patterns (Hebert & Wilson 1994; Colbourne *et al.* 1998). Thus, there is a need for comparative studies on aquatic organisms with similar dispersal patterns but lacking the reproductive complexities characteristic of hitherto studied taxa.

In order to assess the relative importance of ecological and evolutionary processes in shaping the phylogeography of aquatic passive dispersers on an intermediate geographical scale, we investigated mitochondrial DNA (mtDNA) sequence variation in resting egg banks of the planktonic rotifer *Brachionus plicatilis* in the Iberian Peninsula. Rotifers are a major group of zooplankters and provide an excellent system for studying these questions because, although sharing the low dispersal abilities and habitat patchiness of *Daphnia*, they do not suffer from the confounding effects of hybridization and obligate asexuality. These organisms are cyclical parthenogens and disperse passively via resistant, sexually produced resting eggs (Gilbert 1974) which accumulate in lake sediments forming resting egg banks (Snell *et al.* 1983; Marcus *et al.* 1994). *Brachionus plicatilis* is a rotifer species complex inhabiting salt lakes and ponds worldwide (Gómez *et al.* 1995; Gómez & Snell 1997), habitats which are of a highly patchy nature. Although only two species have been

*Author for correspondence (a.gomez@biosci.hull.ac.uk).

Received 27 April 2000 *Accepted* 10 August 2000

named taxonomically (Segers 1995), at least three sibling species have been recognized in the Iberian Peninsula (Gómez *et al*. 1995; Ortells *et al*. 2000).

Resting egg banks are archives of genetic variability that integrate the seasonal and annual variations of a species in lake and pond sediments (Hairston 1996). As the sampling success of individuals in the water column is often highly unpredictable, due to the ephemeral or seasonal nature of species and habitats (Gómez *et al*. 1995), sampling from sediments provides a more representative approach of detecting long-term trends. The study of egg banks has recently been facilitated by the development of techniques for extracting DNA from minute rotifer resting eggs (Gómez & Carvalho 2000).

The Iberian Peninsula has an exceptional diversity of salt lakes (Reed 1998). Salt lakes cluster in five isolated endorheic basins and a chain of coastal lagoons (Comín & Alonso 1988; Roselló 1993) (figure 1). Although many salt lakes were formed in the mid- and recent Quaternary period (Ibáñez 1975; see also Roca & Juliá 1996), the endorheic character of these basins has existed since the Early Miocene period (Plans 1969). In contrast, current coastal lagoons formed *ca*. 6000 years before present (Roselló 1993). Iberian salt lakes vary widely in their degree of seasonal permanency and chemical characteristics (Alonso 1990), although most are small and temporary. Salt lakes were probably significantly affected during the Pleistocene glacial periods, resulting in a likely reduction in extent and occurrence.

Here we present, to our knowledge, the first phylogeographical survey using the resting egg stages of a zooplanktonic organism. In order to determine the interaction of past population processes and population structure in these passive dispersers, we discriminated between current patterns of gene flow and historic processes on mtDNA variability using phylogeographical methods, including nested clade analysis (Templeton 1998). The resultant phylogeographical data provide novel insights into the population diversification of these rotifers and have wider implications for the biodiversity of zooplanktonic biotas as a whole.

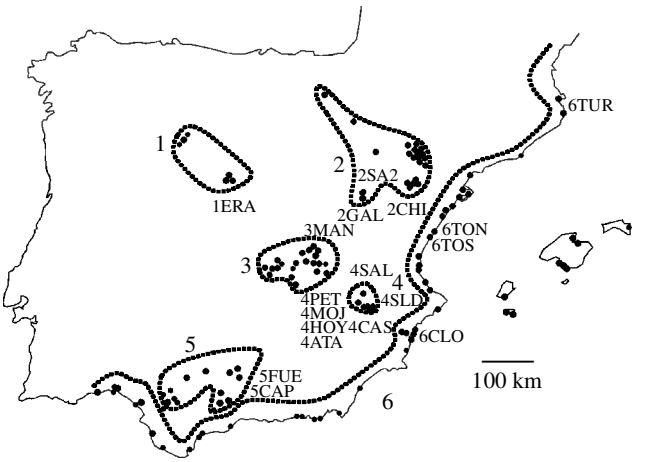

Figure 1. Map of the Iberian Peninsula showing the location of the salt lakes in the endorheic basins (1, Duero Basin; 2, Ebro Basin; 3, Guadiana basin; 4, Júcar-Segura Basin; 5, Guadalquivir Basin) and the chain of coastal lagoons (site 6). Lakes yielding *B.plicatilis* resting eggs are indicated by their acronyms: 1ERA, Laguna de las Eras; 2SA2, Balsa de Santed; 2GAL, Laguna de Gallocanta; 2CHI, Salada de Chiprana; 3MAN, Laguna de Manjavacas; 4PET, Laguna de Pétrola; 4SAL, Laguna del Salobrejo; 4SLD, Laguna del Saladar; 4MOJ, Laguna de Mojón Blanco; 4HOY, Laguna de Hoya Rasa; 4CAS, Laguna de Casa Nueva; 4ATA, Laguna de Atalaya de los Ojicos; 5CAP, Laguna de Capacete; 5FUE, Laguna de Fuente de Piedra; 6TUR, Estany d'en Túries; 6TON, Poza Norte; 6TOS, Poza Sur; 6CLO, Clot de Galvany.

2. MATERIAL AND METHODS

(a) *Sample collection*

Forty-seven salt lakes, ponds and brackish lagoons covering the five endorheic basins in the Iberian Peninsula and the coastal chain were sampled in 1998 and 1999 (the complete list of lakes sampled and further information can be obtained from the authors on request). An effort was made to sample a large number of salt lakes per basin. Superficial mud likely to contain recent resting eggs at high densities (Carvalho & Wolf 1989) was collected using a scoop from the deepest part of each habitat. Sediments were stored in dark and cool conditions until required.

(b) *Resting egg isolation and DNA extraction*

We followed the procedures detailed in Gómez & Carvalho (2000) for isolating resting eggs from pond sediments and for DNA extractions. *Brachionus* resting eggs were recognized by their morphology under a stereoscope and individual resting eggs were rinsed in 6% seawater before DNA extraction. If available, 20 resting eggs were isolated per pond. The taxonomical status of *B.plicatilis* was assessed through hatching of eggs from each site and examining the morphology of the spines and the size of newborn individuals.

(c) *Amplification and sequencing*

Mitochondrial DNA sequences from the cytochrome oxidase subunit I gene (COI) were obtained by cycle sequencing of polymerase chain reaction (PCR)-amplified DNA. PCR reactions were performed in 10 µl final volume containing 2 µl template DNA, 1.5 mM $MgCl_2$, 200 µM of each nucleotide, 2.5 pmol of each primer, 16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl (pH 8.8 at 25 °C), 0.01% Tween-20 buffer and 0.125 U *Taq* polymerase. The reactions were amplified using the following cycling conditions: 3 min denaturing at 93 °C followed by 15 s at 92 °C, 20 s at 50 °C and 1 min at 70 °C (×40) and then 3 min extension at 72 °C. The primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al*. 1994) were used in the PCR reaction and the Cy5 end-labelled versions for cycle sequencing of the double-stranded PCR products using the Thermo Sequenase cycle sequencing kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). Both strands were sequenced in all individuals on an ALFexpress^{TM} (Amersham Pharmacia Biotech) automated sequencer. Forward and reverse sequences were aligned and checked using an ALFwin v. 2.00 sequence analyser (Amersham Pharmacia Biotech). Multiple sequences were aligned by eye and polymorphic sites were manually double checked.

(d) *Data analysis*

The phylogeny of the mtDNA haplotypes was inferred using two optimality criteria: maximum parsimony and maximum likelihood. We used the program MODELTEST v. 3.0 (Posada &

Table 1. *Polymorphic positions of the 21* B. plicatilis *haplotypes and their GenBank accession numbers*

haplotype	accession number	position 0 0 0 0 0 0 0 0 0 0 1 1 1 1 1 1 1 2 2 2 2 2 2 3 3 4 4 4 4 4 4 4 4 4 5 5 5 5 5 5 5 5 6 6 6 / 0 0 2 4 5 6 7 8 8 0 0 1 2 2 3 5 9 0 1 1 2 3 5 1 8 0 0 1 3 3 3 5 6 9 9 0 0 3 4 5 6 7 8 9 2 4 4 / 3 9 4 5 7 3 3 7 8 2 5 4 6 9 2 6 5 1 6 9 8 1 5 5 4 2 5 7 2 8 9 0 2 2 8 1 4 7 3 9 4 0 3 7 1 3 5
H1	AF266853–5	T T C T T T C C T C G T G C A C G C T T G C C A T A C T T C A C T T A C A C T C T T T C G T G
H2	AF266856–7	. . . . . C . . . .
H3	AF266858	. . . . . A . . . . . G . . . . .
H4	AF266859	. . . . . C . . . . A . . . . .
H5	AF266860	. . . . . T . . . . . T . G . . . C . . . T . C G . . . . . T T . .
H6	AF266861	. . . . . T . . A T . . . T T G . . . . . T . C G . . . . . C T . . A
H7	AF266862	. . . . . T . . . T . C A T . G C . . . . . T . C G . . . . . T . . .
H8	AF266863–8	. . T . . . . T . . . . . T . G C . . . . . T . C G . . T . . G . . T . . .
H9	AF266869–70	. . T . C C . . . . . T . . . T . C . T . G C . . . . . T . C G . . T . . . . . T . . .
H10	AF266871	. . T . . . . T . . . . . T . . . T . C . T . G C . . . . . T . C G . . T . . . . . T . . .
H11	AF266872–7	. . . . . T . . . T . . . T . G . . . . . T C C G T . . . . . T . . .
H12	AF266878	. . . . . T G . . T . . . T . G . . . . . T C C G T . . . . . T . . .
H13	AF266879–85	. . . . . T . . . T . . . T . G . . . . . T . C G T . . . . . T . . .
H14	AF266886–94	. . . . . T . . . T . . . T . G . . . . C . . T . C G T . . . . . T . C .
H15	AF266895	C . . C C . T . C . . C . T . . . T . . . T . G . C . . . T . T . C G . G . C . . . . T A . .
H16	AF266896–910	C C . C C . T . . . . C . T . . . T . . . T . G . C . . . T . T . C G . G . C T . . . T A . .
H17	AF266911–2	C C . C C . T . . . . C . T . . . T C . . T . G . C . . . T . T . C G . G . C T . . . T A . .
H18	AF266913	C C . C C . T . . . . C . T . . . T . . . T . G . C . . . T . T . C G . G . C T . . C T A . .
H19	AF266914–22	C C . C C C T . . . . C . T . . . T . . . T . G . C T . . T . T . C G . G . C T . . . T A . .
H20	AF266923–8	C C . C C . T . . T . C A T . . . T . . . T . G . C T . . T . T . C G . G . C T . . . T A . .
H21	AF266929–50	C C . C C . T . . . . C . T . T . T . . . T . G . C . . . T . T . C G . G . C T . . . T A . .

Crandall 1998) in testing the model of evolution for the maximum-likelihood criteria. The Hasegawa–Kishino–Yano model (Hasegawa *et al.* 1985), with a transition–transversion ratio of 17.0069 and base frequencies of A, 0.1988; C, 0.2255; G, 0.2022; and T, 0.3735, proved to be the best fit model. All analyses were performed with PAUP* 4.0b4a (Swofford 1998). Sets of shortest trees were found with and without the outgroup and node support was assessed though bootstrapping (1000 replicates). The tree search algorithms included ten random additions of sequences. Two individuals from another species from the *B. plicatilis* species complex were included as outgroups.

We used nested clade analysis (Templeton (1998) and references therein) for testing the null hypothesis of no associations between haplotypes and geographical locations at different genealogical levels and discriminating between current patterns of gene flow and past events of population subdivision or range expansions. A maximum-parsimony unrooted network of haplotypes was constructed manually and the program PARSPROB 1.1 (available at http://bioag.byu.edu/zoology/crandall_lab/programs.htm) was used for assigning probabilities to the most parsimonious solution between each clade. The nesting design was constructed following the rules described in Templeton *et al.* (1992) and Crandall (1996). The program GEODIS 2.0 (Posada *et al.* 2000) was used for implementing the calculations of the distance measures and their statistical significance. The geographical distances estimated from the data were $D_{c(x)}$, $D_{n(x)}$, $I-T_{c(x)}$ and $I-T_{n(x)}$. $D_{c(x)}$ is the average distance of all individuals of clade x from their geographical centre, indicating how widespread a clade is. $D_{n(x)}$ is the average distance of all members of clade x from the geographical centre of the nesting clade y, estimating how far individuals of clade x haplotypes are from all individuals bearing clade y haplotypes. $I-T_{c(x)}$ and $I-T_{n(x)}$ are the average D_c- or D_n-values of all the interior clades within the nesting clade minus the average D_c- or D_n-values for all interior clades within the nesting clade y. These give an estimation of the distribution of old versus young clades. The statistical distribution of the distance measures were determined by recalculating all distances after 1000 random permutations of clades against sampling locality.

3. RESULTS

(a) *Mitochondrial DNA diversity*

A total of 361 *Brachionus* resting eggs were found in 26 of the 45 lakes sampled. Out of these, 18 lakes from all basins yielded 243 *B. plicatilis* eggs, as confirmed by *Bp1b* typing (a species-specific microsatellite locus) of all *Brachionus* eggs retrieved (Gómez *et al.* 1998). One to ten *B. plicatilis* eggs per lake were sequenced (an average of 5.4 per lake) and 653 bp of the COI gene were obtained in the 98 *B. plicatilis* eggs sequenced (deposited in GenBank as a PopSet with accession numbers AF266853–AF266950). Twenty-one unique haplotypes were identified, with 47 variable sites and 30 parsimony informative sites (table 1). Forty-one substitutions were at third codon positions, with the other six at first codon positions and there were only three transversions. Transitions at position 88 T-C (haplotype H15) and 439 A-G (haplotype H3) were in first codon positions and resulted in an amino-acid replacement. The number of substitutions between haplotypes compared pairwise ranged from one to 25, corresponding to a net proportion of nucleotide substitutions of 0.15–3.83%, respectively. The sequences were moderately A+T rich (mean AT content = 60.0%). No indels were observed within the group, although a triple A insertion at position 519 from the

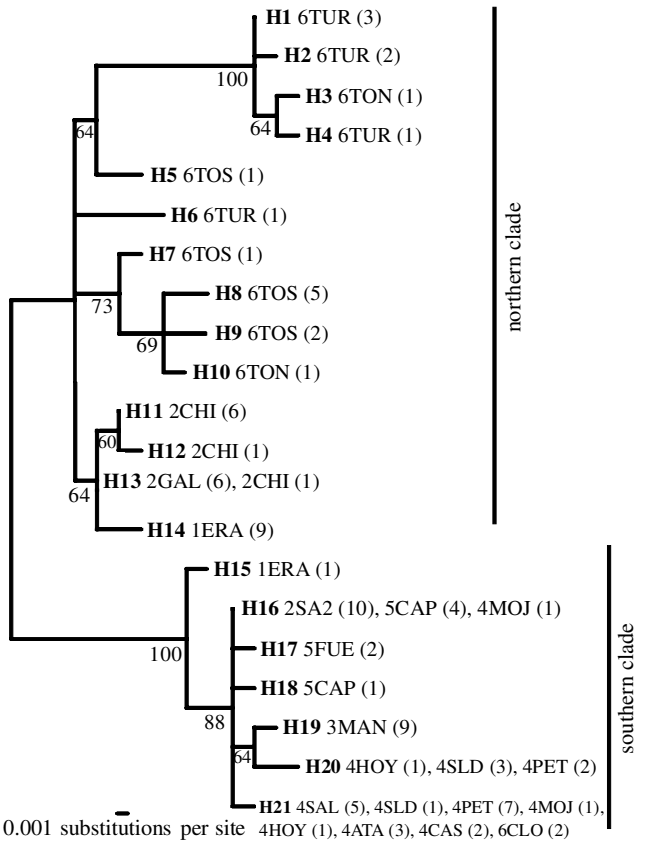

Figure 2. Maximum-likelihood phylogram representing the relationships and geographical distributions of 21 *B. plicatilis* mtDNA haplotypes. Haplotype numbers are accompanied by the lake in which they were isolated; values within parentheses indicate the number of individuals displaying that particular haplotype. Numbers by major branches are the percentages of bootstrap support in the maximum-likelihood analysis (only values $> 50\%$ are shown).

start of the COI gene (corresponding to a lysine in amino-acid position 173 in internal loop 2) was detected in all rotifer COI sequences when compared with other available taxa in GenBank.

(b) *Phylogenetic relationships and geographical structure of the COI haplotypes*

Both optimality criteria produced nearly identical topologies (see figure 2), supporting two monophyletic mtDNA clades (100% bootstrap support with 1000 replicates) which displayed a strong geographical orientation corresponding to 'northern' and 'southern' lineages. This topology was insensitive to the choice of outgroup taxa or midpoint rooting. The two lineages displayed an average uncorrected distance of 2.8% between them. The southern lineage included seven closely related haplotypes that occurred mainly in the three southern basins and in a southern coastal lagoon (6CLO). However, two of these haplotypes were also found in two lakes in the north basins—lake 1ERA, which contained haplotypes from both clades and lake 2SA2, in which only southern haplotypes were found ($n = 10$). Restriction fragment length polymorphism (RFLP) analysis in ten more eggs retrieved from lake 2SA2 confirmed the occurrence of nine additional individuals bearing southern haplotypes and one with a northern haplotype (data not shown). Surprisingly, lake 2SA2 is less than 10 km from lake 2GAL, a large lake dominated by northern haplotypes. The northern lineage included three groups of haplotypes with unresolved relationships between them and was restricted to the two northern inland basins (1 and 2) and the three northern coastal lagoons sampled (6TOS, 6TON and 6TUR). Two branches within this main clade showed strong bootstrap support ($> 70\%$) and contained haplotypes which were only found in coastal lakes. The other, less well-supported branch (64% bootstrap support) included four haplotypes found in the northern inland basins.

The COI haplotypes were strongly localized geographically. Out of the 21 different haplotypes, 17 were restricted to a single lake and two haplotypes were only found within two or more lakes from the same basin. Two haplotypes, both from the southern clade, were present in two and three basins, respectively.

(c) *Population diversity and divergence*

The number of haplotypes per lake was low, ranging from one to four (average 1.8) (see table 2). The haplotype diversity per nucleotide (π) in lakes in which more than five individuals were sequenced ranged from 0 to 0.20. Many lakes contained a single haplotype, with the most diverse lakes occurring on the northern coast (6TUR and 6TOS) (see table 2) and there was a single inland lake (1ERA) which was the only lake containing haplotypes from both the northern and southern clades.

N_{st}-values (Lynch & Crease 1990) were calculated using DnaSP v. 3 (Rozas & Rozas 1999) for estimating the distribution of genetic variation between populations (table 3). The N_{st}-values were very high and ranged between 0.13 (between two nearby lakes) and 1.00 with an average of 0.81 (s.e. = 0.03), which is indicative of low historical levels of gene flow and a strong geographical partition of genetic variance.

(d) *Population history inferred from COI*

Mitochondrial DNA haplotypes separated by up to 11 mutational steps have a $\geqslant 95\%$ probability of being connected in a parsimonious fashion. Using parsimony within these limits, two disjoint networks were obtained (figure 3), each with no internal ambiguities. In agreement with the phylogenetic analysis, these two networks coincided with the northern and southern clades. Figure 3 also shows the nested clade design used in the nested clade analysis. Figure 4 presents the results of the nested clade analysis of the geographical distances for the mtDNA data. The inferences reached emphasize the important role of both population structure and population history in determining the distribution of *B. plicatilis* COI haplotypes (figure 4). There was strong evidence of several episodes of population subdivision, the oldest one being between the southern clade and two northern clades and there was also a more recent one between the inland and coastal clades in one of the northern clades. Long-distance colonization seems to account for the presence of a northern clade haplotype (H14) in pond 1ERA. There has been at least two episodes of past population fragmentation in the southern clade (nested clade 3-1). There was strong evidence here, where a larger

Table 2. *Population locations sampled and haplotype and sequence diversity measures*

(The haplotypes found and their sample sizes (within parentheses) are also included. The nucleotide diversity index is Tajima's π and values per nucleotide are given with variances in parentheses. The values of θ (from the number of segregating sites) are per nucleotide.)

site	location	n	haplotypes	% variable sites	nucleotide diversity index	θ
1ERA	41°10′ N 4°35′ W	10	H14 (9), H15 (1)	2.14	0.005 (0.000 02)	0.0076
2GAL	40°59′ N 1°31′ W	6	H13	0.00	0.000 (0.00)	0.0000
2SA2	41°01′ N 1°30′ W	10	H16	0.00	0.000 (0.00)	0.0000
2CHI	41°14′ N 0°11′ W	8	H11(6), H12 (1), H13(1)	0.31	0.085 (0.00)	0.0012
3MAN	39°25′ N 2°53′ W	9	H19	0.00	0.000 (0.00)	0.0000
4PET	38°50′ N 1°34′ W	9	H21 (7), H20 (2)	0.61	0.071 (0.00)	0.0023
4SAL	38°55′ N 1°27′ W	5	H21	0.00	0.000 (0.00)	0.0000
4SLD	38°48′ N 1°25′ W	4	H20 (3), H21 (1)	—	—	—
4MOJ	38°48′ N 1°26′ W	2	H21 (1), H16 (1)	—	—	—
4HOY	38°47′ N 1°26′ W	2	H20 (1), H21 (1)	—	—	—
4CAS	38°46′ N 1°26′ W	2	H21 (2)	—	—	—
4ATA	38°46′ N 1°25′ W	3	H21 (3)	—	—	—
5FUE	37°06′ N 4°45′ W	2	H17 (2)	—	—	—
5CAP	37°01′ N 4°51′ W	5	H16 (4), H18 (1)	0.15	0.052 (0.00)	0.0007
6TUR	42°15′ N 3°06′ E	7	H1 (3), H2 (2), H4 (1), H6 (1)	3.22	0.202 (0.000 06)	0.0131
6TON	40°10′ N 0°10′ E	2	H3 (1), H10 (1)	—	—	—
6TOS	40°10′ N 0°10′ E	9	H5 (1), H7 (1), H8 (6), H9 (1)	1.83	0.061 (0.000 02)	0.0065
6CLO	38°16′ N 0°31′ W	2	H21 (2)	—	—	—

Table 3. *Population subdivision in* B. plicatilis

(N_{st}-values *sensu* Lynch & Crease (1990) for ponds with sample sizes of $n \geqslant 5$.)

	1ERA1	2GAL	2SA2	2CHI	3MAN	4PET	4SAL	5CAP	6TUR
2GAL	0.53	—	—	—	—	—	—	—	—
2SA2	0.90	1.00	—	—	—	—	—	—	—
2CHI	0.59	0.75	0.98	—	—	—	—	—	—
3MAN	0.91	1.00	1.00	0.98	—	—	—	—	—
4PET	0.86	0.95	0.46	0.93	0.74	—	—	—	—
4SAL	0.91	1.00	1.00	0.98	1.00	0.13	—	—	—
5CAP	0.89	0.98	0.00	0.97	0.91	0.40	0.83	—	—
6TUR	0.69	0.75	0.89	0.75	0.90	0.86	0.89	0.88	—
6TOS	0.61	0.67	0.89	0.68	0.90	0.86	0.90	0.88	0.67

number of ponds and individuals were sampled, of isolation by distance with restricted gene flow.

There was some evidence that the southern basins were colonized later than those in the north, as shown by the shallower branch topology and fewer haplotypes in the south. In addition, three southern haplotypes were shared by different ponds and basins.

4. DISCUSSION

Phylogenetic and cladistic nested analysis of mtDNA and geographical distribution has shown that a passively dispersing rotifer inhabiting patchy, temporary habitats displays significant geographical structuring. Traditionally, the geographical distribution of continental aquatic invertebrates was thought to depend mainly on ecological constraints as they were believed to disperse widely through wind, waterfowl or water currents (Jenkins & Underwood 1998). The current study and several other recent studies on zooplankters are not in accordance with such views (Hebert 1998) as they support strong phylogeographical structures in these organisms. Rotifers, copepods, cladocerans and ostracods display a pattern of persistent founding events, regional endemism and significant geographical structuring with an underlying pattern of reduced gene flow (e.g. Boileau *et al.* 1992; Hebert & Wilson 1994; Weider *et al.* 1999; Schön *et al.* 2000). In addition, they can reflect the effects of glacial refugia and recolonization in a similar way to terrestrial fauna (Boileau & Hebert 1991).

Our analyses established the presence of two main, divergent mtDNA lineages in Iberian *B. plicatilis* with strong geographical segregation forming southern and northern clades, which indicates a deep phylogeographical structure. This division into two mtDNA lineages was probably produced through a past event of population fragmentation (Avise 2000). The pattern found is perhaps surprising as species in this region inhabit one of the most important migratory routes for waterfowl in Europe, i.e. through the Gibraltar Straits. Despite the north–south axis of the migratory route, the geographical localization of the haplotypes suggests that

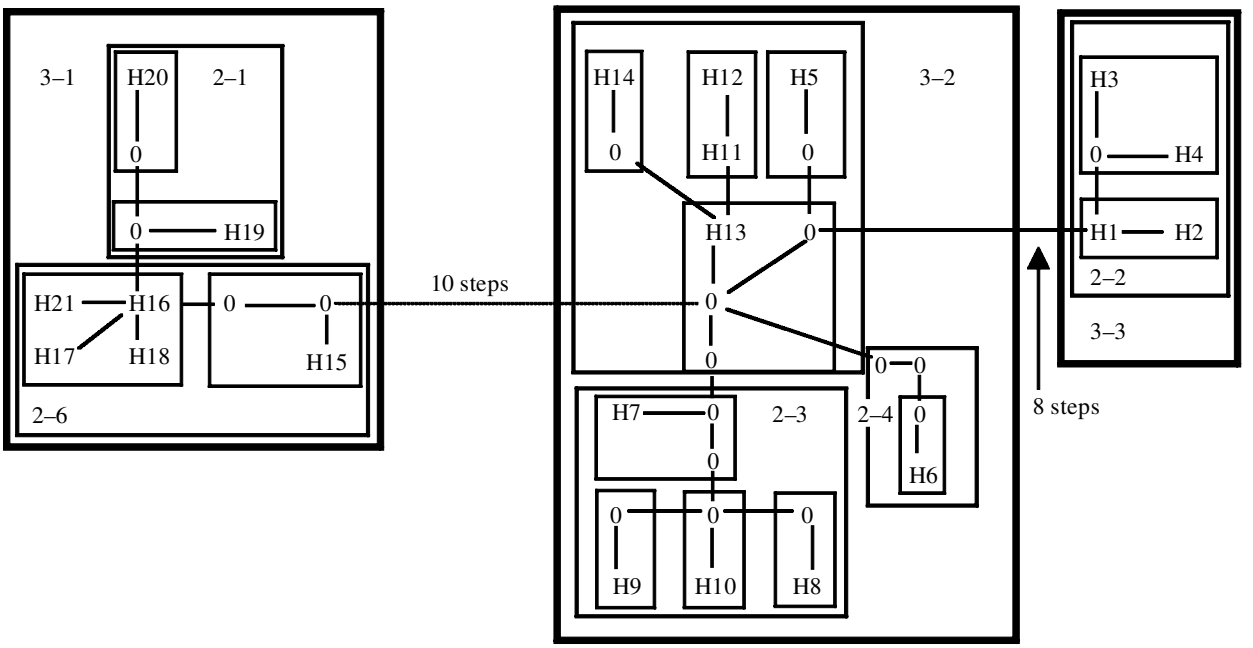

Figure 3. Unrooted, nested, minimum-spanning cladogram derived from the 21 haplotypes found in *B.plicatilis*. Haplotypes are indicated by an H followed by their assigned number and zeros represent missing intermediate haplotypes. Clades are designated by the *x–y* system, where *x* represents the nesting level (from the haplotype level to the fourth level) and *y* represents the number assigned to that particular clade. Clades of level 1 are indicated in figure 4. Each line represents one mutation except where indicated. All connections between haplotypes indicated by solid lines are supported as being parsimonious at the 95% level and the dashed line indicates an uncertain number of steps between haplotypes.

waterfowl transfer of rotifer propagules does not have a major influence on gene flow and population structure over an evolutionary time-scale. This independence of geographical distribution of genetic variation with respect to bird migratory flyways has also been pointed out in the American *Daphnia pulex* complex (Crease *et al.* 1997; Straughan & Lehman 2000).

As no mtDNA molecular clock for rotifers is available, the divergence time of these haplotypes can only be approximated. An arthropod COI clock can be used as a rough approximation when sequence divergence is not very high (1.4–2.3% sequence divergence per million years (Myr)) (Knowlton & Weigt 1998; Schubart *et al.* 1998). According to these calibrations, the main north–south lineage division was established 1.2–2 Myr ago, pointing to historical fragmentation of rotifer populations after the onset of the Pleistocene period (2.4 Myr ago). Such assertions necessitate the survival of populations during glaciation in at least two and possibly three refugia.

Our data can be contrasted with the little information available on Iberian salt lakes during the Quaternary period. Most habitats, including arid steppe regions containing salt lakes, are likely to have been affected during the Pleistocene ice ages (Hewitt 1996). The number, extent and permanency of salt lakes in the Iberian Peninsula during the Pleistocene period would have determined the distribution of glacial refugia for *B.plicatilis*. Salt lake refugia were most likely to occur in regions where, even during cold periods, precipitation was low due to the rain shadow effect of mountain ranges. However, the reduction in the geographical extent of these salt lake regions could have been important, thus isolating populations and favouring genetic diversification. There is strong biogeographical evidence supporting the persistence of Iberian refugia for terrestrial and aquatic steppe fauna (Miracle 1982; Ribera & Blasco-Zumeta 1998) (see Schön *et al.* (2000) for recent phylogeographic evidence) and flora (Thorne 1972) throughout the Pleistocene period. Arid conditions were not interrupted during the Pleistocene period, at least in the Guadalquivir (basin 5) and Ebro Basins (basin 2) (Plans 1969) and these are therefore suitable candidates for glacial refugia for the northern and southern *B.plicatilis* lineages. However, our data do not permit us to exclude the possibility that refugia for these clades were present outside the Iberian peninsula.

The biogeography of salt lake crustaceans in the Iberian Peninsula (reviewed in Alonso 1985) adds further support to our proposal of population fragmentation and survival of populations in northern and southern glacial refugia. The southern basins (3–5) have an aquatic crustacean fauna similar to Morocco; on the contrary, the fauna in the northern basins (1 and 2) are closer to an area encompassing southern France, Cerdenya, Tunisia and Israel. The presence of some endemic species inhabiting steppe salt lakes and the similarities of Spanish fauna with eastern Europe and central Asia (Iberian–Pontocaspian distribution) also support the hypothesis of at least one glacial refugium for these organisms in the Iberian Peninsula. Although recent post-glacial colonization could have contributed to the biogeographical patterns of crustacean fauna, at least part of the aquatic fauna (including ostracods, euphylopoda and diaptomids) of temporary salt lakes in the Iberian Peninsula is older than the Pleistocene period, probably reflecting the broader extension of salt lakes during the Tertiary period.

The nested clade analysis we used allowed us to recognize the important historical processes shaping rotifer

haplotypes			1-step clades			2-step clades			3-step clades			4-step clades		
no.	D_c	D_n	no.	D_c	D_n	no.	D_c	D_n	no.	D_c	D_n	no.	D_c	D_n
H16	230^L	236^L												
H17	0	290												
H18	0	303												
H21	29^S	71^S	1–3	139	139									
I–T	205^L	138^L												
restricted gene flow with isolation by distance (1,2,3,4, no)														
H15	0	0	1–4	0	352^L	2–6	141	140						
			past fragmentation (1,2,11,17,4,9 no)											
H19	0	0	1–2	0^S	84^L	2–1	68	75^S	3–1			4–1	127^S	147^S
H20	0	0	1–1	4^S	57^S	I–T	73	65^L						
			I–T	−4	27^L	isolation by distance with restricted gene flow (1,2,11,17,4, no)								
			past fragmentation (1,2,3,4,9 no)											
H10	0	0	1–18	0	0.1									
H8	0	0	1–19	0	0									
H9	0	0	1–20	0	0									
H7	0	0	1–21	0	0									
			I–T	0	0.1	2–3	0^S	132						
H6	0	0	1–15	0	0	2–4	0	384^L						
									3–2	142^S	141^S			
H11	0	0										4–2	164	256^L
H12	0	0	1–9	0^S	147	2–5	135	134				past fragmentation (1,2,3,4,9, no)		
H5	0	0	1–17	0	205	I–T	134^L	−21						
						past fragmentation (1,2,3,5,15 no)								
H14	0	0	1–22	0^S	176^L									
H13	0	0	1–23	23^S	49^S									
			I–T	22	−140^S									
			long-distance colonization (1,2,11,12,13,14 yes)											
H3	0	75	1–10	117	193	2–2			3–3	157	242			
H4	0	262							I–T	−15	−100^S			
									1,2,11,12 contiguous range expansion					
H1	0	0	1–11	0	123									
H2	0	0	I–T	−117	−170									

Figure 4. Cladistic nested analysis of the geographical distribution of *B. plicatilis* mtDNA haplotypes. The nested design is given in figure 3, as are the haplotype and clade designations. Interior clades are shaded. The clade (D_c) and nested clade (D_n) distances (see the text for a definition of the distances used) are given in kilometres following the name or number of any given clade. In those clades containing both tip and interior nested clades, the average difference between interior versus tip clades for both distance measures is given in the row labelled I − T. S or L superscripts indicate that the distance measure was significantly small or large at the 5% level. The biological inference following Templeton's (1998) key is given at the bottom of each box representing a clade with significant results.

populations in isolated habitats, that is past fragmentation and long-distance colonization events. In addition to the main north–south divide and probably reflecting the strong habitat patchiness of these organisms, evidence was found for incipient smaller scale, regional differentiation in the north between inland and coastal lakes, with evidence of past population fragmentation. The nested clade analysis also allowed us to draw inferences about rotifer population structure, revealing current isolation by distance with restricted gene flow. This was supported by the N_{st}-values, even when considering only northern or only southern ponds and by the strong localization of haplotypes and the relatively low number of haplotypes per pond. These results might seem surprising given the extraordinary colonizing abilities of these rotifers: resting eggs hatch into parthenogenetic females which can show very high reproductive rates. In experiments with artificial ponds (Jenkins 1995; Cáceres & Soluk 1999), rotifer species (often *Brachionus*) were among the first non-insect colonizers, with colonizing times as short as three weeks. This illustrates the often contradictory character of dispersal and gene flow. As a general conclusion, high gene flow is not a feature of rotifer populations and, therefore, despite the dispersal capacity of their resting propagules, mtDNA variation carries the signature of past population fragmentation events, even on an intermediate geographical scale.

This study has revealed that, what was formerly thought to be a cosmopolitan species, actually possesses a marked phylogeographical structure on a medium geographical scale. This reveals much about the microevolutionary patterns and processes (reduced gene flow with isolation by distance, episodes of population fragmentation and long-distance colonization) that may generate significant interpopulational divergence and, eventually, speciation.

The ability to analyse resting egg banks using molecular tools renders the approach valuable for use in numerous other aquatic organisms with diapausing propagules. Such genetic data could provide insights into the role of egg banks as repositories of biodiversity and their influence on population and community responses to environmental change (Reid *et al*. 2000). Although the palaeolimnology of Spanish saline lakes is still very scarce, the high potential of Iberian salt lakes for palaeoclimate reconstruction (Reed 1998) makes phylogeographical analysis of resting egg banks an excellent opportunity for examining the history of genetic lineages in a geographical and climatic context.

In summary, this study has shown that the rotifer *B. plicatilis* is a powerful system for exploring the interplay between population structure and historical, microevolutionary processes in planktonic organisms. The molecular analysis of resting propagules in a cosmopolitan invertebrate species revealed surprisingly high levels of regional endemism and the existence of at least two refugial populations during the Pleistocene period, supporting biogeographical patterns exhibited by other zooplanktonic taxa. In addition, further evidence has shown reduced levels of current and historical gene flow in a species with an apparently high dispersal capacity. The time is right for reassessing our preconceptions about gene flow and phylogeography in passively dispersing aquatic organisms.

We are indebted to the many people who provided us with samples or helped in sampling collection, including Professor Manuel Serra, Raquel Ortells, Miguel Angel Gómez 'Patinegro', Jordi Domingo 'Calamón', Dr Toni Camacho, Dr Javier Armengol, Dr Eduardo Aparici, Cris Martí, Miguel Angel Trenzano, David Sáez, Sara Lapesa, Jordi Romero (Parque Natural dels Aigüamolls del Empordá) and Jesús González (Gallocanta). We are grateful to D. Manuel Rendón (Málaga) and D. Baldomero Moreno (Córdoba) from the Dirección General del Medio Natural de Andalucía for their valuable help and their speed in processing our permit applications. The Universitat de València partly supported Africa Gómez during her stay in Hull. This research was supported by a grant from the National Environmental Research Council (UK grant no. GR9/04482).

REFERENCES

Alonso, M. 1985 Las lagunas de la España Peninsular: taxonomía, ecología y distribución de los cladóceros. PhD thesis, Universidad de Barcelona, Spain.

Alonso, M. 1990 Anostraca, Cladocera and Copepoda of Spanish saline lakes. *Hydrobiologia* **197**, 221–231.

Avise, J. C. 2000 *Phylogeography*. Cambridge, MA: Harvard University Press.

Boileau, M. G. & Hebert, P. D. N. 1991 Genetic consequences of passive dispersal in pond-dwelling copepods. *Genetics* **45**, 721–733.

Boileau, M. G., Hebert, P. D. N. & Schwartz, S. S. 1992 Non-equilibrium gene frequency divergence: persistent founder effects in natural populations. *J. Evol. Biol.* **5**, 25–39.

Cáceres, C. E. & Soluk, D. E. 1999 Duck's feet revisited: considering mechanisms for zooplankton dispersal and colonisation. Abstract from Proceedings of the American Society for Limnology and Oceanography Meeting in Santa Fe, CA, USA, in 1999.

Carvalho, G. R. & Wolf, H. G. 1989 Resting eggs in *Daphnia*. I. Distribution, abundance and hatching of resting eggs collected from various depths in lake sediments. *Freshwat. Biol.* **22**, 459–470.

Chaplin, J. A. & Ayre, D. J. 1997 Genetic evidence of widespread dispersal in a parthenogenetic freshwater ostracod. *Heredity* **78**, 57–67.

Colbourne, J. K., Hebert, P. D. N. & Taylor, D. J. 1997 Evolutionary origins of phenotypic diversity in *Daphnia*. In *Molecular evolution and adaptive radiation* (ed. T. J. Givnish & K. J. Systma), pp. 163–188. Cambridge University Press.

Colbourne, J. K., Crease, T. J., Weider, L. J., Hebert, P. D. N., Dufresne, F. & Hobaek, A. 1998 Phylogenetics and evolution of a circumarctic species complex (Cladocera: *Daphnia pulex*). *Biol. J. Linn. Soc.* **65**, 347–365.

Comín, F. A. & Alonso, M. 1988 Spanish salt lakes: their chemistry and biota. *Hydrobiologia* **158**, 237–245.

Crandall, K. A. 1996 Multiple interspecies transmissions of human and simian T-cell leukemia/lymphoma virus type I sequences. *Mol. Biol. Evol.* **13**, 115–131.

Crease, T. J., Lee, S.-K., Yu, S.-L., Spitze, K., Lehman, N. & Lynch, M. 1997 Allozyme and mtDNA variation in populations of the *Daphnia pulex* complex from both sides of the Rocky Mountains. *Heredity* **79**, 242–251.

De Meester, L. 1996 Local genetic differentiation and adaptation in freshwater zooplankton populations: patterns and processes. *Ecoscience* **3**, 385–399.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. 1994 DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol.* **3**, 294–299.

Gilbert, J. J. 1974 Dormancy in rotifers. *Trans. Am. Microsc. Soc.* **93**, 490–513.

Gómez, A. & Carvalho, G. R. 2000 Sex, parthenogenesis and the genetic structure of rotifers: microsatellite analysis of contemporanean and resting egg bank populations. *Mol. Ecol.* **9**, 203–214.

Gómez, A. & Snell, T. W. 1997 Sibling species and cryptic speciation in the *Brachionus plicatilis* species complex (Rotifera). *J. Evol. Biol.* **9**, 953–964.

Gómez, A., Temprano, M. & Serra, M. 1995 Ecological genetics of a cyclical parthenogen in temporary habitats. *J. Evol. Biol.* **8**, 601–622.

Gómez, A., Clabby, C. & Carvalho, G. R. 1998 Isolation and characterisation of microsatellite loci in a cyclically parthenogenetic rotifer, *Brachionus plicatilis*. *Mol. Ecol.* **7**, 1619–1621.

Hairston, N. G. 1996 Zooplankton egg banks as biotic reservoirs in changing environments. *Limnol. Oceanogr.* **41**, 1087–1092.

Hasegawa, M., Kishino, H. & Yano, T.-A. 1985 Dating of the human–ape splitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol.* **22**, 160–174.

Hebert, P. D. N. 1998 Variable environments and evolutionary diversification in inland waters. In *Advances in molecular ecology* (ed. G. R. Carvalho), pp. 267–290. Amsterdam: NATO Science Series, IOS Press.

Hebert, P. D. N. & Wilson, C. C. 1994 Provincialism in plankton: endemism and allopatric speciation in Australian *Daphnia*. *Evolution* **48**, 1333–1349.

Hewitt, G. M. 1996 Some genetic consequences of ice ages, and their role in divergence and speciation. *Biol. J. Linn. Soc.* **58**, 247–276.

Ibáñez, M. J. 1975 El endorreísmo de la depresión del sector central de la depresión del Ebro. *Cuadernos Inv. Geogr. Hist.* 35–48.

Jenkins, D. G. 1995 Dispersal-limited zooplankton distribution and community composition in new ponds. *Hydrobiologia* **313/314**, 15–20.

Jenkins, D. G. & Underwood, M. O. 1998 Zooplankton may not disperse readily in wind, rain, or waterfowl. *Hydrobiologia* **387**, 15–21.

Knowlton, N. & Weigt, L. A. 1998 New dates and new rates for divergence across the isthmus of Panama. *Proc. R. Soc. Lond.* B **265**, 2257–2263.

Lynch, M. & Crease, T. J. 1990 The analysis of population survey data on DNA-sequence variation. *Mol. Biol. Evol.* **7**, 377–394.

Marcus, N. H., Lutz, R., Burnett, W. & Cable, P. 1994 Age, viability and vertical distribution of zooplankton resting eggs from an anoxic basin: evidence of an egg bank. *Limnol. Oceanogr.* **39**, 154–158.

Miracle, M. R. 1982 Biogeography of the fresh-water zooplanktonic communities of Spain. *J. Biogeogr.* **9**, 455–467.

Ortells, R., Snell, T. W., Gómez, A. & Serra, M. 2000 Patterns of genetic differentiation in resting egg banks of a rotifer species complex in Spain. *Arch. Hydrobiol.* (In the press.)

Plans, P. 1969 Problemas del endorreismo Español. *Rev. Real Acad. Ciencias Ex. Fis. Nat.* **63**, 271–309.

Posada, D. & Crandall, K. A. 1998 Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818.

Posada, D., Crandall, K. A. & Templeton, A. R. 2000 Geodis: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Mol. Ecol.* **9**, 487–488.

Reed, J. M. 1998 Diatom preservation in the recent sediment record of Spanish saline lakes: implications for palaeoclimate study. *J. Paleolimnol.* **19**, 129–137.

Reid, V., Carvalho, G. R. & George, D. G. 2000 Molecular genetic analysis of *Daphnia* in the English Lake District: species identity, hybridisation and resting egg banks. *Freshwat. Biol.* **43**, 247–254.

Ribera, I. & Blasco-Zumeta, J. 1998 Biogeographical links between steppe insects in the Monegros region (Aragón, NE Spain), the eastern Mediterranean, and central Asia. *J. Biogeogr.* **25**, 969–986.

Roca, J. R. & Juliá, R. 1996 Late-glacial and Holocene lacustrine evolution based on ostracode assemblages in Southern Spain. *Geobios* **30**, 823–830.

Roselló, V. 1993 Albuferas Mediterráneas. *Cuad. Geogr.* **53**, 35–64.

Rozas, J. & Rozas, R. 1999 DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics* **15**, 174–175.

Schön, I., Gandolfi, A., Di Masso, E., Rossi, V., Griffiths, H., Martens, K. & Butlin, R. 2000 Persistence of asexuality through mixed reproduction in *Eucypris virens* (Crustacea, Ostracoda). *Heredity* **84**, 161–169.

Schubart, C. D., Diesel, R. & Hedges, S. B. 1998 Rapid evolution to terrestrial life in Jamaican crabs. *Nature* **393**, 363–365.

Segers, H. 1995 Nomenclatural consequences of some recent studies on *Brachionus plicatilis* (Rotifera, Brachionidae). *Hydrobiologia* **313/314**, 121–122.

Snell, T. W., Burke, B. E. & Messur, S. D. 1983 Size and distribution of resting eggs in a natural population of the rotifer *Brachionus plicatilis*. *Gulf Res. Rep.* **7**, 285–287.

Straughan, D. J. & Lehman, N. 2000 Genetic differentiation among Oregon lake populations of the *Daphnia pulex* species complex. *J. Hered.* **9**, 8–17.

Swofford, D. L. 1998 *PAUP*. Phylogenetic analysis using parsimony* (**and other methods*), v. 4. Sunderland, MA: Sinauer Associates.

Templeton, A. R. 1998 Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. *Mol. Ecol.* **7**, 381–397.

Templeton, A. R., Crandall, K. A. & Sing, C. F. 1992 A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* **132**, 619–633.

Thorne, R. F. 1972 Major disjunctions in the geographic ranges of seed plants. *Quart. Rev. Biol.* **47**, 365–411.

Weider, L. J., Hobæk, A., Colbourne, J. K., Crease, T. J., Dufresne, F. & Hebert, P. D. N. 1999 Holarctic phylogeography of an asexual species complex. I. Mitochondrial DNA variation in Arctic *Daphnia*. *Evolution* **53**, 777–792.

As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.