

Persistent genetic signatures of colonization in *Brachionus manjavacas* rotifers in the Iberian Peninsula

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Abstract

Recent phylogeographical assessments have consistently shown that continental zooplankton display high levels of population subdivision, despite the high dispersal capacity of their diapausing propagules. As such, there is an apparent paradox between observed cosmopolitanism in the zooplankton that is associated with long-distance dispersal, and strong phylogeographical structures at a regional scale. Such population dynamics, far from migration–drift equilibrium, have been shown in the rotifer species complex *Brachionus plicatilis*, a group of over a dozen species inhabiting salt lakes and coastal lagoons worldwide. Here we present the mitochondrial DNA phylogeography of one of these species, *Brachionus manjavacas*, in the Iberian Peninsula, where it often co-occurs with the morphologically similar species *B. plicatilis sensu stricto*. We obtained sequences from 233 individuals from diapausing eggs and clonal cultures from 16 lakes in the Iberian Peninsula, and a Tunisian lake. Two strongly supported deep mitochondrial DNA clades were found (A and B). Phylogenetic and nested clade analysis showed that clade A has a strong phylogeographical structure, with a strong similarity of phylogeographical patterns between *B. manjavacas* clade A and *B. plicatilis s.s.* These include (i) signatures of allopatric fragmentation between central and southern populations, and (ii) range expansions in the Iberian Peninsula, both likely to have occurred during the Pleistocene. We find evidence for a glacial refugium in the Guadiana basin. Clades A and B co-occurred in several of these lakes because of range expansion and secondary contact between both clades. The co-occurrence between *B. plicatilis s.s.* and *B. manjavacas* is not recent, and both species might have experienced similar environmental challenges during the Pleistocene. The strong correlation of genetic and geographical distance found suggests that historical events can lead to such correlation, mirroring the effects of ‘isolation by distance’ in equilibrium populations.

Keywords: COI, diapausing eggs, glacial refugia, passive dispersal, phylogeography, Pleistocene

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Introduction

The importance of regional population subdivision, often triggered by Pleistocene climatic oscillations, has recently been acknowledged for passively dispersed continental zooplankton. These organisms generally show pronounced phylogeographical structure with little or no secondary sympatry between conspecific lineages (Taylor *et al.* 1998;

Gómez *et al.* 2000; Cox & Hebert 2001; Hebert *et al.* 2003; Penton *et al.* 2004). Estimates of genetic differentiation between populations are high, while polymorphism for nuclear loci or mitochondrial DNA (mtDNA) haplotype diversity within populations is typically low (De Meester 1996; Gómez *et al.* 2000; De Meester *et al.* 2002; Gómez *et al.* 2002a). Contrary to previous expectations (Avice 2000), phylogeographical analyses have helped to identify the location of putative Pleistocene refugia in zooplankton, and such refugia are often concordant with those identified in other organisms (e.g. Gómez *et al.* 2000; Cox & Hebert

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2001; Penton *et al.* 2004). Most phylogeographical studies on the zooplankton have been carried out in the cladoceran genus *Daphnia* (Weider *et al.* 1999a; Weider *et al.* 1999b; Hebert *et al.* 2003; Penton *et al.* 2004; De Gelas & De Meester 2005), whereas attention to other groups – other cladocerans, copepods, anostracans, notostracans and rotifers – has been relatively scant (but see Freeland *et al.* 2000; Gómez *et al.* 2000; Cox & Hebert 2001; Freeland *et al.* 2004).

In apparent contradiction to the patterns of strong phylogeographical and population structure described above, high dispersal capacity through diapausing egg stages is a key feature of the life history of continental zooplankton. This includes long-distance dispersal as shown by the rapid colonization of new habitats (Jenkins & Buikema. 1998; De Meester *et al.* 2002; Louette & De Meester 2005). Several habitat features may have led to selection for enhanced colonization abilities in these organisms. First, many aquatic systems behave in an island-like manner, so that the geographical distribution of many taxa is necessarily patchy, and populations are often physically isolated from each other. Second, strong ecological fluctuations mean that appropriate conditions might only be present during a brief period each year or even every few years in the lake or pond. Third, many continental aquatic systems (salt lakes, many shallow freshwater lakes, rainpools) have relatively short geological lives because of silting. In summary, there has to be strong selection for good colonization abilities in species living in temporally variable and unstable aquatic systems. Indeed, several instances of long-distance colonization, seemingly unrelated to anthropogenic effects, have been described in rotifers (Gómez *et al.* 2002b).

To conciliate these contradictions, it has been proposed that once local habitats are colonized, a strong habitat monopolization reduces the importance of gene flow in these organisms, creating long-lasting founder effects in populations (Boileau *et al.* 1992; De Meester *et al.* 2002). This monopolization effect is promoted by fast growth rates, the presence of diapausing eggs forming egg banks in the sediment and the rapid build-up of local adaptation (De Meester *et al.* 2002). The immediate consequence of such an effect for neutral genetic markers is a very slow progress towards migration–drift equilibrium in populations. In support of this, isolation-by-distance patterns are weak or nonexistent in some *Daphnia* or *Artemia* studies (Vanoverbeke & De Meester 1997; Naihong *et al.* 2000; De Meester *et al.* 2002; De Gelas & De Meester 2005). However, an association between current estimates of waterfowl movements and the level of population differentiation of several aquatic organisms has been found (Taylor *et al.* 1998; Freeland *et al.* 2000; for a review see Figuerola *et al.* 2005), which could be interpreted in terms of birds determining ongoing gene flow among populations. This correlation could be attributed, however, to a common

phylogeographical patterns of waterfowl (Johnson & Cicero 2004) and aquatic organisms, reflecting a common history rather than ongoing dispersal. In contrast to these results, however, strong patterns of correlation of genetic and geographical distances have been described in a rotifer species both at regional and global scales (Gómez *et al.* 2002a; Mills 2006). Unexpectedly, in rotifers no correlation of lineage distributions or population differentiation and main bird migratory pathways has been found (Gómez *et al.* 2000), indicating that ongoing gene flow (assumed to be aided by bird movements between suitable habitats) is not responsible for the phylogeographical patterns observed, notwithstanding the role of birds as vectors of dispersal and colonization of aquatic organisms (Figuerola & Green 2002).

Until recently, patterns of association between genetic and geographical distance were commonly attributed to the attainment of equilibrium conditions in a model of gene flow which favoured short-distance dispersal ('stepping-stone models') (Rousset 1997; Hutchison & Templeton 1999). New research, however, has shown that the cumulative effect of founding events during colonization of new habitats, when founders come from adjacent populations, has long-lasting consequences on genetic population structure (Le Corre *et al.* 1997). First, it results in gradual loss of heterozygosity (Le Corre *et al.* 1997; Prugnolle *et al.* 2005) and second it can produce a correlation between genetic estimates of population differentiation and geographical distance (Ramachandran *et al.* 2005). Apparent 'isolation-by-distance patterns' could therefore reflect nonequilibrium conditions as they will be the result of persistent colonization effects (Ramachandran *et al.* 2005), especially when gene flow is restricted. Therefore, this nonequilibrium cause for correlations between genetic and geographical distances could be common in plants and animals with limited dispersal or strong priority effects (e.g. Abbott *et al.* 1995; Tremblay & Schoen 1999; Pruett & Winker 2005; Derycke *et al.* 2007; Gómez *et al.* 2007) where the signatures of colonization will take longer to be erased because of gene flow (Boileau *et al.* 1992).

To better understand the importance of gene flow vs. historical effects, more research is needed into the phylogeographical patterns of continental zooplankton. In addition, the study of phylogeographical concordance between co-occurring species could help weight the relative strength of long-distance vs. local dispersal and gene flow in continental zooplankton populations.

Here we present a phylogeographical assessment of the salt lake rotifer *Brachionus manjavacas* in the Iberian Peninsula. This species belongs to the *Brachionus plicatilis* species complex (Gómez *et al.* 2002b) and is often sympatric to the morphologically very similar *B. plicatilis* s.s. (Campillo *et al.* 2005; Fontaneto *et al.* 2007). Both species are reproductively isolated (Ortells *et al.* 2000; Berrieman *et al.* 2005)

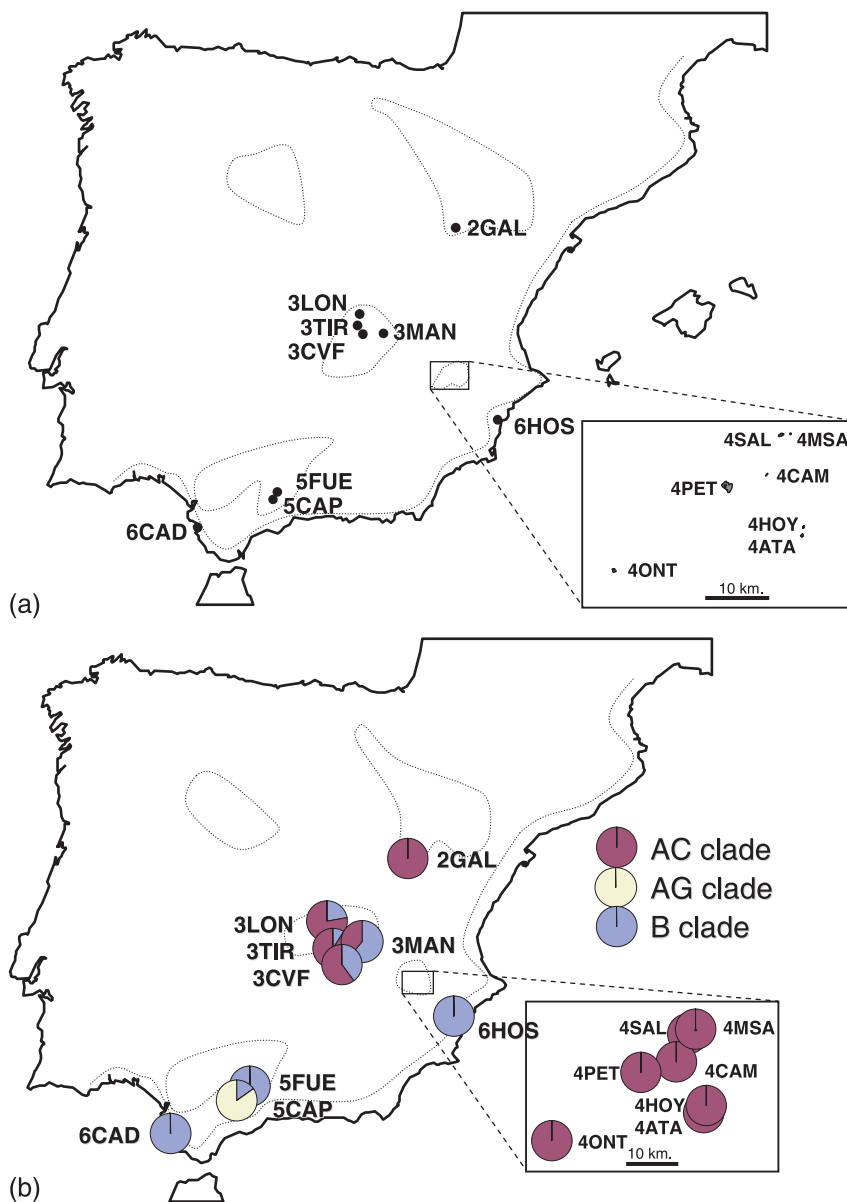


Fig. 1 (a) Distribution of *Brachionus manjavacas* in the Iberian Peninsula. See lake codes in Table 1. (2, Ebro basin; 3, Guadiana basin; 4, Júcar-Segura basin; 5, Guadalquivir basin; 6, coastal lagoons). (b) Relative distribution of each lineage in the different populations (see Fig. 2).

and were estimated to have long, independent evolutionary histories (Gómez *et al.* 2002b). As the phylogeographical patterns of *B. plicatilis* s.s. are already known in this area (Gómez *et al.* 2000) this offers us an opportunity to explore concordance patterns in the phylogeography of Iberian *Brachionus* species.

Materials and methods

Sample collection, diapausing egg isolation and DNA extraction

Sediment samples were available from a total of 56 salt and brackish lakes, ponds and lagoons sampled between 1998

and 2005 as part of several projects (Fig. 1a). These samples covered the five endorheic basins in Spain and the eastern and southern coastlines. Samples were sometimes available from 2 years (Table 1. See further sampling details in Gómez *et al.* 2000). We tried to retrieve large sample sizes of diapausing eggs from lake sediment where *Brachionus manjavacas* was known to occur (Ortells *et al.* 2000; Gómez *et al.* 2002b) and also from new localities discovered while developing other projects. We isolated further *B. manjavacas* eggs from a Tunisian locality (Gómez *et al.* 2002b). We isolated diapausing eggs from lake sediments, and performed DNA extractions as described previously (Onbé 1978; Gómez & Carvalho 2000). Diapausing eggs of *B. manjavacas* were discriminated from other species in the

Table 1 Details of the sampling sites, number of individuals sequenced (*N*), number of haplotypes (*A*) and number of individuals per haplotype found in each lake. Acronyms of sample sites indicate the basin in the Iberian Peninsula samples and a three-letter code for the lake. *Hd* represents the haplotype diversity (standard deviation within parentheses)

Lake	Code	Location	Collecting dates	<i>N</i>	<i>A</i>	<i>Hd</i> (SD)	Haplotypes and number of individuals
Gallocanta	2GAL	40°58'12"N, 1°30'00"W	13/3/99	10	1	0 (0)	H3(10)
Longar de Lillo	3LON	39°42'11"N, 3°19'22"W	20/4/99	9	4	0.83 (0.08)	H2(2), H6(3), H7(2), H11(2)
Tírez	3TIR	39°32'15"N, 3°21'24"W	16/6/01	33	6	0.76 (0.05)	H2(3), H7(8), H11(2), H12(1), H13(6), H14(13)
Camino de Villafranca	3CVF	39°24'43"N, 3°15'13"W	14/6/01, 20/4/99	10	3	0.71 (0.09)	H6(4), H7(2), H11(4)
Manjavacas	3MAN	39°25'03"N, 2°51'43"W	3/4/00	28	2	0.50 (0.05)	H11(17), H7(11)
Salobrejo	4SAL	38°54'50"N, 1°28'06"W	1/8/03, 8/3/99	18	1	0 (0)	H6(18)
Pétrola	4PET	38°50'28"N, 1°33'54"W	1/8/01, 8/3/99	31	2	0.52 (0.02)	H4(16), H6(15)
Campana	4CAM	38°51'27"N, 1°29'35"W	12/11/04	3	1	—	H6(3)
Morras del Salobrejo	4MSA	38°51'27"N, 1°27'03"W	12/11/04	5	2	0.40 (0.24)	H6(4), H4(1)
Hoya Rasa	4HOY	38°47'05"N, 1°25'39"W	03/11/04	3	2	—	H4(2), H6(1)
Atalaya de los Ojicos	4ATA	38°46'22"N, 1°25'50"W	03/11/04	16	2	0.23 (0.13)	H6(14), H4(2)
Fuente de Piedra	5FUE	37°06'38"N, 4°46'07"W	19/6/99	6	1	—	H11(6)
Capacete	5CAP	37°01'21"N, 4°49'35"W	19/6/99	13	3	0.60 (0.12)	H8(8), H9(3), H11(2)
Universidad de Cádiz	6CAD	36°31'58"N, 6°12'40"W	20/8/99	2	2	—	H10(1), H11(1)
El Hondo Sur	6HOS	38°10'04"N, 0°43'34"W	16/3/04	12	2	0.41 (0.13)	H15(9), H12(3)
Ontalafia	4ONT	38°43'15"N, 1°46'07"W	12/1/05	21	1	0 (0)	H6(21)
Korba Sebkhet (Tunisia)	KOR	36°39'N, 10°57'E	18/9/00	12	2	0.17 (0.13)	H5(1), H3(11)
Mangyshlak Peninsula (Caspian Sea, Kazakhstan)	RUS*	43°53'N, 51°0'20"E	?	1	1	—	H1(1)

*This clone was previously assumed to originate from the sea of Azov (Russia) and has been previously named Russian strain. It has been used in several published genetic analyses.

species complex either by direct sequencing of polymerase chain reaction (PCR) products or by restriction fragment length polymorphism (RFLP) analysis (see Berrieman *et al.* 2005 for details; Campillo *et al.* 2005). Two clones of known origin maintained in the Evolutionary Ecology laboratory of the University of Valencia (3TIR-L5 and 4SAL-L5) were also included in the data set.

Amplification and sequencing

We used primers LCO1490 and HCO2198 (Folmer *et al.* 1994) to amplify part of the mitochondrial gene cytochrome *c* oxidase subunit I (COI) following procedures slightly modified from Gómez *et al.* (2000). PCRs typically included 2-μL template DNA, 1.5 mM MgCl₂, 200 μM of each nucleotide, 2.5 pmol of each primer, 16 mM (NH₄)₂SO₄, 67 mM Tris-HCl (pH 8.8 at 25 °C), 0.01% Tween 20 buffer, and 0.125 U of *Taq* DNA polymerase for a final volume of 10 μL. PCR conditions were: 3 min denaturing at 93 °C; (15 s at 92 °C, 20 s at 50 °C, 1 min at 70 °C) × 40, and 3 min at 72 °C. Sequences were obtained through cycle sequencing of PCR-amplified DNA.

The new sequences were deposited in GenBank as a PopSet under accession nos DQ089826–DQ089998 and EF017608–EF017655. Previously published sequences of

the same COI fragment in 12 *B. manjavacas* individuals (Gómez *et al.* 2002b) were also included in our analysis (accession nos AF387250–61).

Data analysis

We computed the number of haplotypes per population (*A*), haplotype diversity (*Hd*), and between-population differentiation (*N_{ST}*) using DNASP version 4.00.6 (Rozas *et al.* 2003). The global population differentiation parameter *F_{ST}* for nucleotide sequence data (Hudson *et al.* 1992) was also calculated with DNASP version 4.00.6 (Rozas *et al.* 2003) in those populations with five or more individuals sequenced. The statistical significance of the correlation between the genetic (*F_{ST}*) and the geographical distance between sites was tested with a one-tailed Mantel test included in POPTOOLS (Hood 2005). The Mantel test was used to calculate the probability of the observed correlation coefficient by computing correlation coefficients between randomized matrices (1000 replications). The obtained coefficients were ordered and the probability was assigned to the observed correlation coefficient as the frequency of equal and higher simulated correlation coefficients. The test was one-tailed as only a positive correlation is expected when matrices involve geographical and genetic distances.

The phylogeny of the mtDNA haplotypes was inferred using maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). We used the program MODELGENERATOR version 0.63 (Keane *et al.* 2006) to find the most appropriate model of evolution for maximum likelihood and PHYML (Guindon & Gasquel 2003) was used to construct an ML tree. MP reconstructions (branch and bound) were performed with PAUP* 4.0b8 (Swofford 1998). In both analyses, topology support was assessed through nonparametric bootstrapping (1000 pseudoreplicates). MRBAYES version 3.1.1 (Ronquist & Huelsenbeck 2003) was used to perform a Bayesian search with data partitioned by codon positions. Two simultaneous Markov chain Monte Carlo analyses were run for 2 000 000 generations, sampling trees and parameters every 100 generations. We discarded the first 250 000 generations (2500 trees) on each run as 'burn-in' after visually confirming chain stationarity from plots of likelihood against generation and calculated a 50% consensus tree.

We used nested clade analysis (NCA) (Templeton 1998) to test for associations between genealogy and geography in the Iberian Peninsula data set, and to discriminate 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 (developed by D. Posada) was used to calculate the probability of the most parsimonious solution between each clade. We followed rules by Templeton *et al.* (1992) and Crandall (1996) to construct a nested design based on the network. The program GEODIS 2.4 (Posada *et al.* 2000) was used to implement the calculations of the distance measures and their statistical significance. The statistical distribution of the distance measures was determined recalculating all distances after 1000 random permutations of clades against sampling locality.

NCA does not provide criteria for detecting secondary contact with population admixture or hybridization of lineages that were previously isolated (Templeton 2001). Therefore, to complete NCA regarding the possibility of secondary sympatry of *B. manjavacas* clades, an analysis suggested by Templeton (2001) was carried out. This consists of computing an index for the haplotypes and n -step clades ($n = 0, 1, 2 \dots$) found in each sampling site. The index is calculated by averaging the pairwise geographical distances between the geographical centres of the haplotypes or clades found in each sampling site, which are provided by the GEODIS output. Thus, a high value of the index is obtained for a sampling site if the haplotypes or clades found in that site have geographical centres away from each other (i.e. they come from sites distant among them). The indexes computed for a sampling site at different hierarchical levels are inspected. The results are interpreted as follows: if a process of 'isolation by distance' in

equilibrium populations is responsible for the observed pattern of haplotype occurrence in a given sampling site, closely related haplotypes are expected, and then the index should decay with the hierarchical level. A hallmark of secondary contact occurs when some localities contain very divergent haplotypes, belonging to higher level clades. Then, the index stays stable or increases with the hierarchical level.

Results

Geographical distribution of Brachionus manjavacas

Brachionus manjavacas was found in 16 salt lakes in the Iberian Peninsula (in the basins of the rivers Ebro, Júcar-Guadiana and Guadalquivir), and in two coastal lagoons, one near the Guadalquivir river basin, and the other close to the Júcar-Guadiana basin (see Fig. 1a). Outside the Iberian Peninsula, this species has been found in Tunisia (coastal lagoon), Russia (Caspian Sea) (Gómez *et al.* 2002b) and in the USA (Pettaquamscutt Estuary, Rhode Island) (Suatoni 2003). At least nine out of the 16 Iberian lakes for which we have information are hypersaline during some part of the year cycle or at least during some years and all of them are temporary or semipermanent, drying out completely either seasonally or during dry years.

Sequence diversity

We sequenced an average of 14 individuals per lake (Table 1). The COI alignment was unequivocal and included a total of 233 individual sequences which were collapsed into 15 haplotypes, 13 of them present in the Iberian Peninsula. The alignment included 661 bp of which 62 were polymorphic and 56 were parsimony informative. No indels or stop codons were found and all changes involved synonymous substitutions. Seven of the 13 Iberian haplotypes were found in single lakes, two haplotypes were found widespread in lakes from the same basin, two were found in two basins and the most widespread haplotype (H11) was found in seven lakes from three basins (average 2.69 lakes per haplotype). Each Iberian lake contained an average of 2.18 haplotypes (range 1–6). There were two haplotype-rich lakes, located in the Guadiana basin (Tírez and Longar, with six and four haplotypes, respectively). The Tunisian locality (KOR) had two haplotypes. Haplotype (gene) diversity (H_d) for lakes with more than five individuals sequenced ranged from 0 to 0.83 (Table 1).

Phylogenetic analysis

Maximum likelihood, maximum parsimony, and Bayesian inference retrieved a very similar tree topology (see Fig. 2). The maximum parsimony unweighed branch and bound

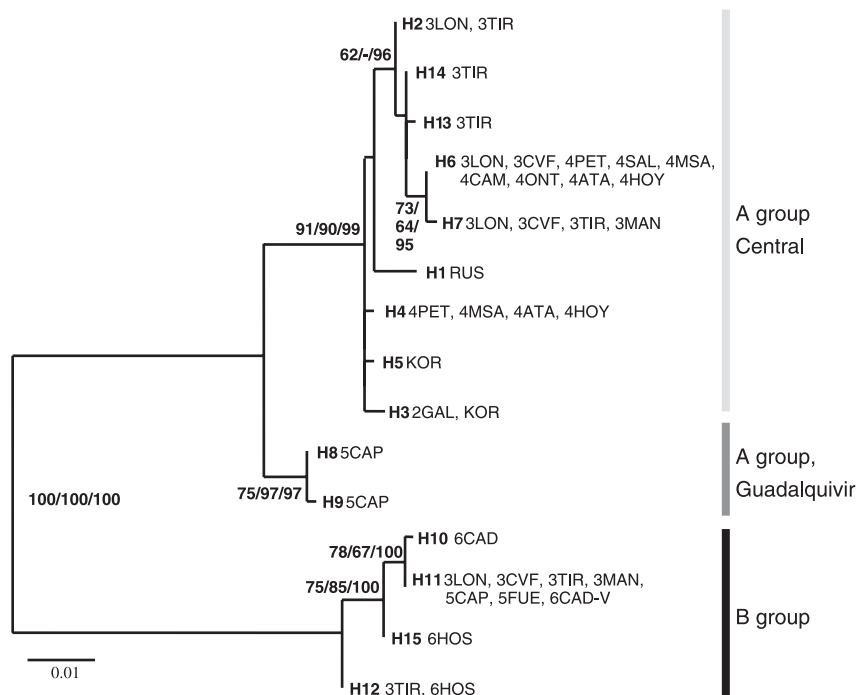


Fig. 2 Midpoint rooted maximum-likelihood phylogram representing the relationships of *Brachionus manjavacas* mtDNA haplotypes. Percent bootstrap support in the maximum likelihood and maximum parsimony and posterior probability in the Bayesian Analysis is shown by major branches (only values > 50% are shown).

search revealed 45 best trees of 74 steps with similar general topology, which differed in the relative position of haplotypes in one of the main branches (AC, see below). The Akaike Information Criterion II and Bayesian Information Criterion in MODELGENERATOR version 0.63 selected the Hasegawa–Kishino–Yano plus invariable sites model (HKY + I) (Hasegawa *et al.* 1985) as the best fit model of evolution for ML, with base frequencies of A = 0.24, C = 0.19, G = 0.18 and T = 0.39, and proportion of invariable sites was 0.79. The midpoint rooted phylogenetic tree showed three strongly supported monophyletic clades in ML, MP and BI. A basal division between a high-diversity clade, with 11 haplotypes (clade A) and a low-diversity clade, with 4 haplotypes (clade B) had 100% bootstrap support in all analyses. In addition, within clade A, a strongly supported division between a central (AC) and a Guadalquivir basin (AG) clades were also strongly supported by all criteria. Within the AC clade there is also some support for a monophyletic group of five haplotypes found only in the Guadiana and Júcar-Segura basins. Average sequence divergence between clades A and B was 7.1% (uncorrected *p*) and between clades AC and AG was 2.3%. Both the Russia strain (RUS) and the Tunisian haplotypes (KOR) belonged to the AC clade and there was no evidence of the Iberian haplotypes being monophyletic. In fact, haplotype H3 was found in both Tunisia and in an Iberian lake.

In the Iberian Peninsula, clades A and B were found in 13 and 8 lakes, respectively, and they co-occurred in five

lakes in the Guadiana and Guadalquivir basins (Fig. 1b) in a frequency of 9–71% for clade B in the diapausing egg bank (average 30.1%).

Mismatch analysis using DNASP version 4.00 (Rozas *et al.* 2003) for clade B to test for population expansion showed that Tajima's *D* was negative (−0.18), but nonsignificant, probably because of the low number of haplotypes involved.

Nested clade analysis

The haplotype network and the associated nested design with four nested levels within the total cladogram are shown in Fig. 3. The maximum number of parsimonious steps ($P > 0.95$) between haplotypes was 11. This meant the occurrence of three disjunct clades at the 3rd haplotype level nesting, which coincided with the highly supported clades in the phylogenetic analyses described above (clade AC, clade AG, and clade B). GEODIS results indicated that eight out of the 10 clades with geographical and genetic variation had significant permutational contingency tests indicating nonrandom geographical distribution of haplotypes (Table 2). The inference of the processes causing this geographical patterning was resolved in six of the clades, with two of them giving inconclusive outcomes because of the unresolved status of tip-interior position of the clades. Three clades showed significant evidence of allopatric fragmentation, including between clades AC (Ebro, Guadiana and Júcar-Segura basins) and AG (Guadalquivir basin) within clade A, but also in two lower level clades

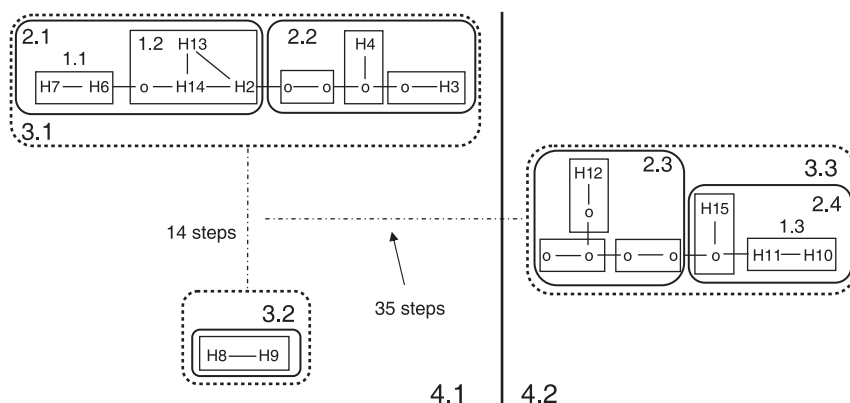


Fig. 3 Unrooted nested minimum-spanning cladogram derived from the 15 haplotypes found in *Brachionus manjavacas*. Haplotypes are indicated by an H followed by their assigned number; zeros represent missing intermediate haplotypes. Informative clades are designated as *x.y*, where *x* represents the nesting level (from the haplotype level to the 4th level) and *y* represents the number assigned to that particular clade. Except when indicated, each line represents one mutation. All connections indicated by solid lines between haplotypes are supported to be parsimonious at the 95% level; the dashed line indicates an uncertain number of steps between haplotypes.

Table 2 Nested clade analysis of the geographical distribution of *Brachionus manjavacas* haplotypes. Only the eight clades with significant GEODIS results are shown. The *P* value of the chi-square test is shown. Haplotype and clade distributions are shown to aid in the interpretation of results. If a clade was found in all lakes in a basin only the basin number is given. The sequence followed in the inference key (version 11 November 2005) is shown. See Fig. 3 for the haplotype network and nested design with haplotype and clade designations

Clade	Distribution	<i>P</i> value	Inference key sequence	Biological inference
1-1: H6 H7	3LON, 3CVF, Basin 4 Basin 3	0.0000	1, 2, 11, 12, 13 yes	Range expansion with long distance colonization
1-2: H2 H14 H13	3LON, 3TIR 3TIR 3TIR	0.038	1, 2, 11, 17, 4 no	Restricted gene flow with isolation by distance
2-1: 1-1 1-2	Basins 3 and 4 3LON, 3TIR	0.0000	1, 2, 11, 12, 13 yes	Range expansion (possibly long-distance colonization with subsequent fragmentation)
2-2: H3 H4	2GAL 4PET, 4MSA, 4HOY, 4ATA	0.0000	1, 19 no	Allopatric fragmentation
3-1 (AC): 2-1 2-2	Basins 3 and 4 2GAL, 4PET, 4MSA, 4HOY, 4ATA	0.0000	1, 2 tip-interior status cannot be determined	Inconclusive outcome
4-1 (A): 3-1 (AC) 3-2 (AG)	Basins 2, 3 and 4 5CAP	0.0000	1, 19 no	Allopatric fragmentation
2-4: 1-3 H15	Basin 3, Basin 5, 6CAD 6HOS	0.0000	1, 19 no	Allopatric fragmentation
Total: 4-1 (A) 4-2 (B)	Basin 2, 3, 4, 5CAP Basin 3, 5, 6CAD, 6HOS	0.0000	1, 2 tip-interior status cannot be determined	Inconclusive outcome (see further analysis related to secondary contact)

indicating fragmentation between the southeastern (HOS lagoon) and southwestern populations (Guadiana and Guadalquivir) in clade B, and between Ebro basin (GAL) and Júcar-Segura (PET, MSA, HOY, ATA) basin in clade A. According to NCA inference key, some support for 'restricted gene flow with isolation by distance' was found in a lower level clade distributed in the Guadiana basin. Finally, strong evidence of range expansion with long-distance colonization was found in two clades nested within each other in the Guadiana and Júcar-Segura basins. In both cases, the clade with a more restricted geographical distribution was limited to the Guadiana basin,

indicating that a range expansion within the Guadiana basin and into the Júcar-Segura basin had taken place.

The NCA results for the total cladogram (containing clades A and B) were inconclusive because of the unresolved tip-interior status of both clades and the partial sympatry of both clades. Therefore, we carried out the analysis suggested by Templeton (2001) to test for secondary sympatry (Fig. 4). As we move towards the bottom graphs (highest level clades) five populations (in the Guadiana and Guadalquivir basins) occur where the highest level clades (4th level, A and B clades) co-occur. This indicates that these lakes have undergone secondary

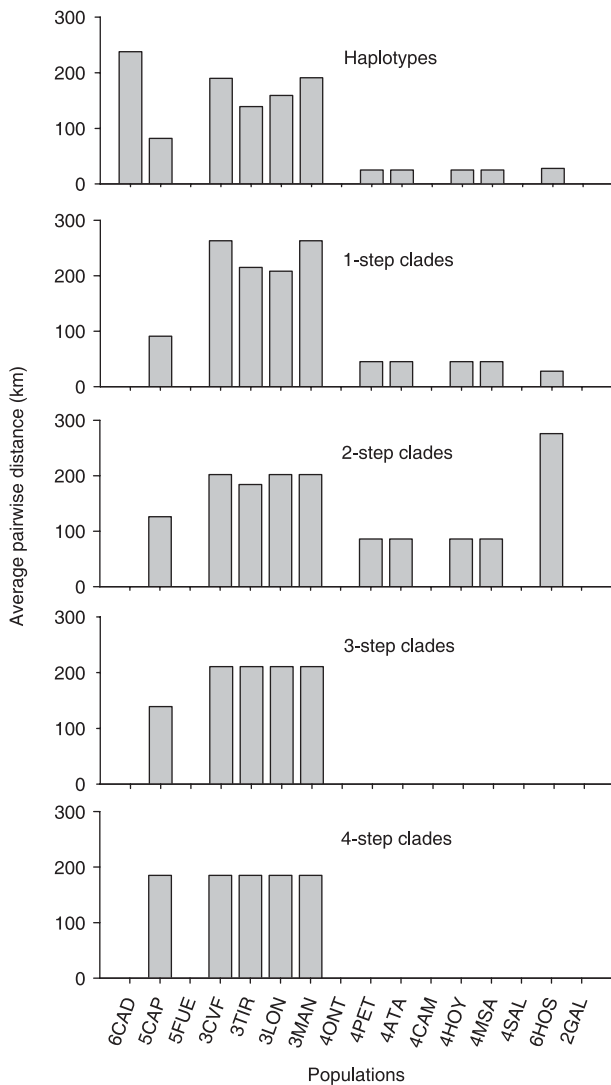


Fig. 4 Average pairwise distances for haplotypes and clades on each sampling site for *Brachionus manjavacas* mtDNA. Lakes where the species was found are ordered on the x axis in increasing distances from the most westerly lake (6CAD, in the left) to the most easterly (2GAL in the right). The bars in each panel show the average pairwise geographical distance between the geographical centres of haplotypes and clades found at each of the lakes calculated from the coordinates for haplotypes and clades given in the GEODIS output.

admixture. These results show that the geographical distribution of the divergent clades A and B is likely to be due to divergence in allopatry followed by range expansion and subsequent secondary contact.

Patterns of population subdivision and correlation of genetic and geographical distance

Considering both clades together, *B. manjavacas* populations were highly subdivided (see Table 3) with $F_{ST} = 0.67$

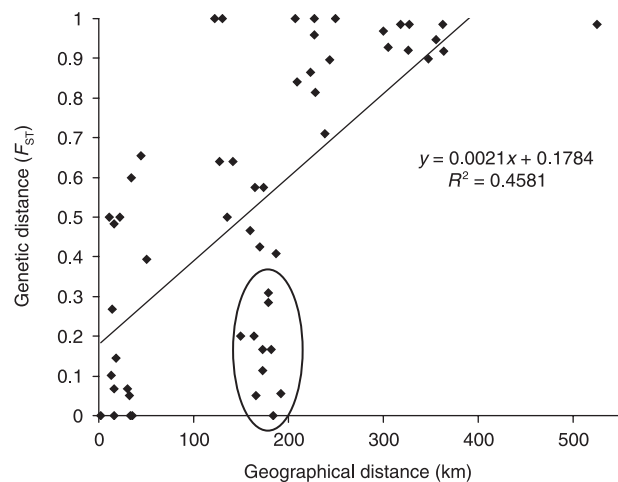


Fig. 5 Plot of the genetic differentiation (F_{ST}) vs. the geographical distance between Iberian lakes containing clade A haplotypes. Only lakes with five or more individuals sampled were included in the data set. Encircled points represent pairs of populations from the Guadiana and Júcar-Segura basins (see text for details).

(assuming an island model Nm would be estimated at 0.12 individuals). Considering only the Iberian clade A, global is $F_{ST} = 0.80$ (estimated Nm assuming an island model would be 0.06 individuals). Before Mantel tests were applied, two negative F_{ST} values were adjusted to 0. Although the correlation between genetic and geographical distance was nonsignificant when the Tunisian population was included ($R^2 = 0.092$, $P = 0.12$), it was significant when only the Iberian populations were used ($R^2 = 0.29$, $P = 0.0039$). This pattern was even more marked when only clade A was used in the Mantel test (11 populations, $R^2 = 0.46$, $P < 0.0015$) (Fig. 5). An inspection of the genetic vs. geographical distance plot revealed that a cluster of points (encircled) represented pairs of populations from the Guadiana and Júcar-Segura basins (including 3CVF and 3LON from the Guadiana basin and ONT, MOR, SAL and ATA from the Júcar-Segura) which contained the haplotypes and clades for which NCA found evidence for long-distance colonization and range expansion.

Discussion

Status of the divergent clades and secondary sympatry

We found two strongly supported deep mtDNA clades (A and B) which were only partially segregated in their geographical distribution in the Iberian Peninsula. Clade A was more diverse and highly structured whereas clade B was less diverse and more restricted in its distribution. Our results indicate that both clades evolved in allopatry and

Table 3 Population subdivision in *Brachionus manjavacas*. F_{ST} values *sensu* Hudson *et al.* (1992) are shown. Only populations with five or more individuals are included

	2GAL	3LON	3TIR	3CVF	3MAN	4SAL	4PET	4MSA	4ONT	4ATA	5FUE	5CAP	6HOS	KOR
2GAL	—													
3LON	0.32	—												
3TIR	0.52	0	—											
3CVF	0.4	-0.04	0.17	—										
3MAN	0.61	0.2	0.43	0.02	—									
4SAL	1	0.16	0.24	0.34	0.61	—								
4PET	0.71	0.15	0.22	0.32	0.57	0.5	—							
4MSA	0.82	0.12	0.16	0.31	0.58	0	0.10	—						
4ONT	1	0.16	0.24	0.34	0.61	0	0.5	0	—					
4ATA	0.90	0.13	0.19	0.32	0.59	0.07	0.27	-0.15	0.07	—				
5FUE	1	0.74	0.89	0.55	0.37	1	0.97	0.98	1	0.99	—			
5CAP	0.67	0.31	0.48	0.29	0.41	0.68	0.6	0.63	0.68	0.65	0.83	—		
6HOS	0.98	0.72	0.87	0.54	0.38	0.98	0.96	0.96	0.98	0.97	0.73	0.81	—	
KOR	0	0.31	0.49	0.39	0.6	0.96	0.65	0.77	0.96	0.85	0.99	0.65	0.98	—

the sympatry is secondary because of the range expansion of one or both of the clades. Our data permit us to speculate on the temporal sequence of events leading to the secondary sympatry. Likely, the genetically more diverse and structured clade A arrived in the area first, which allowed enough time for population divergence, while clade B expanded in range later, explaining its lower haplotype diversity.

The large mitochondrial COI sequence divergence (7.1%) between clades A and B indicates that the separation between them is not recent and could predate the Pleistocene ice ages. Despite the relatively marked differentiation in mtDNA, the nuclear locus ITS1 was found to be identical in sequence in individuals analysed in both clades (Gómez *et al.* 2002b). Although ribosomal sequences typically accumulate differences much slower than mtDNA, the data currently available does not eliminate the possibility of hybridization in sympatry between both clades. If the range expansion (likely of clade B into range previously occupied by clade A) has resulted in the hybridization and introgression between differentiated clades in the lakes where they co-exist, this would be difficult to explain in the context of the Monopolization hypothesis, as massive gene flow (or an adaptive advantage of individuals bearing colonizing haplotypes) would be needed to account for the high proportion of the B clade found in those lakes. In contrast, if both clades had some form of reproductive isolation and their sympatric character indicated some niche segregation, this would fit with the pattern of low intraspecific gene flow, but the apparent lack of barriers to colonization of lakes already occupied by congeneric species even from the same species complex (Gómez *et al.* 2002b). The co-existence of ecologically similar rotifer species could be facilitated by the large environmental

variation at different temporal scales showed by Iberian salt lakes. This variation is partly due to a large interannual variation in rainfall (Rodríguez-Puebla *et al.* 1998; Serrano *et al.* 1999). Co-existence would also be facilitated if they differed in their reaction norms to a relevant ecological parameter (i.e. salinity) and they held diapausing egg banks (J. Montero-Pau, unpublished manuscript), what is known as the 'storage effect' (Cáceres 1997). The fact that both clades are closely related makes the system a potentially useful model to investigate the development of reproductive and ecological barriers and speciation in rotifers. Future studies should address this issue, possibly using polymorphic microsatellite loci.

Phylogeographical and population structure within clades

Brachionus manjavacas COI haplotype diversity was low but comparable to that found in other rotifer species (Gómez *et al.* 2000). The level of population subdivision ($F_{ST} = 0.67$) is very high and supports that drift and historical processes are much more important than gene flow in this taxon. Clade A shows highly subdivided populations, displaying signatures of population fragmentation, strong subdivision between central (AC) and southern (AG) populations and a subsequent range expansion in the central area (Guadiana and Júcar-Segura basins) with a strong pattern of association between genetic and geographical distance. The lower diversity of the clade B, with four haplotypes in 48 individuals sequenced in eight lakes may suggest either its recent expansion from outside the sampled area, or a population bottleneck, with glacial survival in a restricted local area. Subsequent to this, the clade has colonized sites already occupied by the populations from clade A creating the

secondary contact we observe. Clade A seems to have been present in the Iberian Peninsula for longer and the level of mean sequence divergence between the central (AC) and Guadalquivir basin (AG) clades (2.3%) is of a scale usually associated with a Pleistocene split in phylogeographical studies.

Refugia

The results allow us to hypothesize that *B. manjavacas* populations survived in two or three distinct glacial refugia. Survival during the Pleistocene glaciations *in situ* of all clades (B, AG and AC) in the Iberian Peninsula is possible but, given the long-distance dispersal abilities of rotifers, until sampling around the Mediterranean is more exhaustive it must be supported with additional data. Taking into account this caveat, our data strongly suggests that at least one glacial refugium is likely to be located in the centre of Iberian Peninsula (Guadiana basin), and would have held the ancestors of clade AC. In support of this, the Guadiana basin (i) contains lakes with high haplotype diversity, (ii) shows widespread distribution of clade AC, (iii) has a monophyletic cluster of haplotypes, and (iv) is in part responsible for the pattern of association between genetic and geographical distance detected. The other glacial refugium could have been located in the South of the Iberian Peninsula, either in the Guadalquivir basin or in the coast, but the haplotype diversity and number of lakes where clade AG is found is small, therefore it is less well supported. Again, no direct evidence of survival of clade B in the Iberian Peninsula was found, although if a refugium existed it would have been located either in the South or Centre of the Iberian Peninsula.

Phylogeographical concordance in the Iberian Peninsula

We now have two examples of regional phylogeography in the *Brachionus plicatilis* species complex and we can gain valuable insights by comparing these. *Brachionus plicatilis* s.s. was suggested to have survived the Pleistocene ice ages in one or two refugia in the Iberian Peninsula (Gómez *et al.* 2000), the best supported one of them located in the Ebro basin and/or adjacent Mediterranean coast (basin 2 in Fig. 1a) and the other in the Guadalquivir basin (basin 5 in Fig. 1a). In *B. manjavacas*, there is strong indication of glacial survival of the central lineage in the Guadiana basin, which shows high haplotype richness, widespread distribution of lineage A (four lakes) and monophyly of the haplotypes. A weaker support for a refugium in the Guadalquivir basin for the A southern lineage is found. Remarkably, the level of divergence within clade A (2.3%) is similar to that found within *B. plicatilis* s.s. (2.8% uncorrected sequence divergence) (Gómez *et al.* 2000), and this would suggest that both species must have been sympatric

in the Iberian Peninsula for a long time. For both *B. plicatilis* s.s. and *B. manjavacas* there is strong evidence of a central refugium (Ebro for the former and Guadiana for the latter) and a secondary refugium in the South (both suggesting the Guadalquivir basin). In conclusion, the genetic diversity and refugial patterns found are similar, but not identical to those found in a phylogeographical analysis of *B. plicatilis* s.s. and support multiple glacial refugia in the Iberian Peninsula (Gómez *et al.* 2000; Gómez & Lunt 2006). The patterns of genetic diversity of the central and Guadalquivir clades and the level of divergence are similar to that in *B. plicatilis* s.s., with the former clade showing higher genetic diversity than the latter, which suggests that populations survived in a refugium persisting through several ice ages, or a larger, more structured refugium.

The Iberian Peninsula has been recognized as one of the main European glacial refugia during the Pleistocene (Hewitt 1996; Hewitt 2000). However, evidence for a complex pattern of several glacial refugia within the area for a wide range of flora and fauna is accumulating (Gómez & Lunt 2006). In spite of the localized and isolated character of the habitats of these rotifers (salt lakes) both rotifer species investigated so far support the view of at least one, and possibly two, glacial refugia for salt lake fauna in the Iberian Peninsula. Concordance patterns found are only rough, indicating that each species might have survived in different basins, possibly varying in their salinity regime. Although *B. manjavacas* has been found mainly in hypersaline lakes, *B. plicatilis* seems to have a wider tolerance of salinity and has been found in habitats from hyposaline to hypersaline conditions, including coastal lagoons (Gómez *et al.* 1995; Aparici *et al.* 2002; Ortells *et al.* 2003; Lowe *et al.* 2005; E. Ramos, personal communication).

We have no direct estimates on the age of salt lakes in the Iberian Peninsula and little evidence for their persistence during the Pleistocene glaciations. The inland salt lake basins in the Iberian Peninsula are known to date from the Miocene (Comín & Alonso 1988), although it is unlikely that any given lake has persisted for that long. There is evidence for persistence of saline conditions in lake systems at least through late-glacial conditions in the Ebro basin and near the Júcar-Segura basin, although they ranged from permanent saline lakes to hypersaline salt mud pans (Giralt *et al.* 1999; González-Sampériz *et al.* 2004). Data is lacking for the Guadiana basin, but indirect support for a glacial refugium in the Guadiana basin comes from the persistence of tertiary relics such as *Branchinecta orientalis* (syn. *cervantesi*) (Alonso 1990).

The ability for long-distance dispersal of these organisms provides an important caveat to these interpretations however. There is wide evidence of long-distance — even intercontinental — natural dispersal in the zooplankton (Gómez *et al.* 2000; Gómez *et al.* 2002b; De Gelas & De Meester 2005). The lack of monophyly of the Iberian haplotypes of

A lineage central-north, with haplotypes from Russia and Tunisia nested within it, suggests that at some point in the past, episodes of range expansion or long-distance dispersal and colonization involving the Mediterranean basin took place. The low diversity, with a single haplotype found, in the Ebro basin, which is found also in Tunisia, would suggest a pattern of recent colonization of the Ebro basin from an area outside the Iberian Peninsula, accounting for NCA significant evidence of past allopatric fragmentation involving this basin (clade 2-2). In contrast, the level of diversity and patterns of relatedness of the haplotypes suggest that clade AC has survived in the Peninsula for at least several glacial ages.

Colonization effects

Our results aid in the interpretation of the contradictions between the development of an association of genetic and geographical distance in rotifers and the Monopolization hypothesis. Traditionally, patterns of correlation of genetic and geographical distance were interpreted as a direct effect of migration–drift equilibrium conditions when nearby populations exchange migrants at a higher rate than farther populations (Rousset 1997; Hutchison & Templeton 1999), and therefore, they were used to support a role for gene flow in the genetic structure of populations. However, populations of continental invertebrates have been shown to be slow in developing migration–drift equilibrium because of a strong founder effect (Boileau *et al.* 1992), and this is one of the tenets of the Monopolization hypothesis (De Meester *et al.* 2002). The high F_{ST} found indicates that drift plays a much more important role than gene flow in most rotifer populations, allowing allopatric fragmentation and local adaptation to proceed in these organisms. Recently, simulations showed that the pattern of association of genetic and geographical distance in humans worldwide can be explained by serial founder events (Ramachandran *et al.* 2005). Although both equilibrium and nonequilibrium explanations are not mutually exclusive — that is, local migration could reinforce the already existing pattern (Pruett & Winker 2005) — we speculate that in rotifers the strong correlation of genetic and geographical distances could be mostly due to colonization effects from glacial refugia. This might also explain why a significant correlation between genetic and geographical distances was found also in *B. plicatilis* s.s. in the Iberian Peninsula (Gómez *et al.* 2002a), which is thought to contain at least one glacial refugium for this species. Although Gómez *et al.* (2002a) concluded that the correlation between genetic and geographical distance indicates local gene flow, this is incompatible with the nonequilibrium status of rotifer populations and we suggest instead that the pattern found in *B. plicatilis* s.s. is likely to be a consequence of historical colonization. It is

possible that continental zooplankton populations where no correlation between genetic and geographical distances has been found are located in recently colonized areas where the refugial area itself had not been sampled (i.e. High Arctic, Northern Europe). Although the conclusions of our analyses are highly relevant to understand the population structure of zooplankton populations, they should also apply to other organisms with restricted gene flow, rapid population growth and large population sizes, including plants living in patchy habitats and many coastal marine organisms (Gómez *et al.* 2007).

Conclusions

This study has shown that a rotifer taxon has a complex phylogeographical structure in the Iberian Peninsula. *Brachionus manjavacas* consists of two deeply divergent parapatric clades, A and B. Clade A shows higher diversity and seems to have persisted for longer in the Iberian Peninsula. We found strong evidence for at least one refugium for this clade in the Guadiana Basin. Despite strong population subdivision and NCA supporting a strong effect of historical events, a pattern of correlation between genetic and geographical distances was found in this clade in the Iberian Peninsula, indicating that patterns of colonization can result in significant associations between genetic and geographical distances. Clade B has lower diversity and evidence points to a range expansion into previously occupied lakes. We discuss some potential mechanisms allowing for the coexistence of these clades.

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References

- Abbott RJ, Chapman HM, Crawford RMM, Forbes DG (1995) Molecular diversity and derivations of populations of *Silene acaulis* and *Saxifraga oppositifolia* from the high

- Arctic and more southerly latitudes. *Molecular Ecology*, **4**, 199–207.
- Alonso M (1990) Anostraca, cladocera and copepoda of Spanish saline lakes. *Hydrobiologia*, **197**, 221–231.
- Aparici E, Carmona MJ, Serra M (2002) Evidence for an even sex allocation in haplodiploid cyclical parthenogens. *Journal of Evolutionary Biology*, **15**, 65–73.
- Avice JC (2000) *Phylogeography: the History and Formation of Species*. Harvard University Press, Cambridge, Massachusetts.
- Berrieman HK, Lunt DH, Gómez A (2005) Behavioural reproductive isolation in a rotifer hybrid zone. *Hydrobiologia*, **546**, 125–134.
- Boileau MG, Hebert PDN, Schwartz SS (1992) Nonequilibrium gene frequency divergence – persistent founder effects in natural populations. *Journal of Evolutionary Biology*, **5**, 25–39.
- Cáceres CE (1997) Temporal variation, dormancy, and coexistence: a field test of the storage effect. *Proceedings of the National Academy of Sciences, USA*, **94**, 9171–9175.
- Campillo S, García-Roger EM, Martínez-Torres D, Serra M (2005) Morphological stasis of two species belonging to the L-morphotype in the *Brachionus plicatilis* species complex. *Hydrobiologia*, **546**, 181–187.
- Comin F, Alonso M (1988) Spanish salt lakes: their chemistry and biota. *Hydrobiologia*, **158**, 237–245.
- Cox AJ, Hebert PDN (2001) Colonization, extinction, and phylogeographic patterning in a freshwater crustacean. *Molecular Ecology*, **10**, 371–386.
- Crandall KA (1996) Multiple interspecies transmissions of human and simian T-cell leukemia/lymphoma virus type I sequences. *Molecular Biology and Evolution*, **13**, 115–131.
- De Gelas K, De Meester L (2005) Phylogeography of *Daphnia magna* in Europe. *Molecular Ecology*, **14**, 753–764.
- De Meester L (1996) Local genetic differentiation and adaptation in freshwater zooplankton: patterns and processes. *Ecoscience*, **3**, 385–399.
- De Meester L, Gómez A, Okamura B, Schwenk K (2002) The Monopolization Hypothesis and the dispersal-gene flow paradox in aquatic organisms. *Acta Oecologica-International Journal of Ecology*, **23**, 121–135.
- Derycke S, Van Vynck R, Vanoverbeke J, Vincx M, Moens T (2007) Colonisation patterns of Nematoda on decomposing algae in the estuarine environment: community assembly and genetic structure of the dominant species *Pellioditis marina*. *Limnology and Oceanography*, **52**, in press.
- Figuerola J, Green AJ (2002) Dispersal of aquatic organisms by waterbirds: a review of past research and priorities for future studies. *Freshwater Biology*, **47**, 483–494.
- Figuerola J, Green AJ, Michot TC (2005) Invertebrate eggs can fly: Evidence of waterfowl-mediated gene flow in aquatic invertebrates. *American Naturalist*, **165**, 274–280.
- 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. *Molecular Marine Biology and Biotechnology*, **3**, 294–299.
- Fontaneto D, Giordani I, Melone G, Serra M (2007) Disentangling the morphological stasis in two rotifer species of the *Brachionus plicatilis* species complex. *Hydrobiologia*, **583**, 297–307.
- Freeland JR, Noble LR, Okamura B (2000) Genetic diversity of North American populations of *Cristatella mucedo*, inferred from microsatellite and mitochondrial DNA. *Molecular Ecology*, **9**, 1375–1389.
- Freeland JR, Rimmer VK, Okamura B (2004) Evidence for a residual postglacial founder effect in a highly dispersive freshwater invertebrate. *Limnology and Oceanography*, **49**, 879–883.
- Giralt S, Burjachs F, Roca JR, Julia R (1999) Late glacial to early holocene environmental adjustments in the Mediterranean semi-arid zone of Salines playa-lake (Alicante, Spain). *Journal of Paleolimnology*, **21**, 449–460.
- Gómez A, Temprano M, Serra M (1995) Ecological genetics of a cyclical parthenogen in temporary habitats. *Journal of Evolutionary Biology*, **8**, 601–622.
- Gómez A, Carvalho GR (2000) Sex, parthenogenesis and genetic structure of rotifers: microsatellite analysis of contemporary and resting egg bank populations. *Molecular Ecology*, **9**, 203–214.
- Gómez A, Lunt DH (2006) Refugia within refugia: patterns of phylogeographic concordance in the Iberian Peninsula. In: *Phylogeography of Southern European Refugia* (eds Weiss S, Ferrand N), pp. 155–188. Springer, Dordrecht, The Netherlands.
- Gómez A, Carvalho GR, Lunt DH (2000) Phylogeography and regional endemism of a passively dispersing zooplankton: mitochondrial DNA variation in rotifer resting egg banks. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **267**, 2189–2197.
- Gómez A, Adcock GJ, Lunt DH, Carvalho GR (2002a) The interplay between colonization history and gene flow in passively dispersing zooplankton: microsatellite analysis of rotifer resting egg banks. *Journal of Evolutionary Biology*, **15**, 158–171.
- Gómez A, Serra M, Carvalho GR, Lunt DH (2002b) Speciation in ancient cryptic species complexes: evidence from the molecular phylogeny of *Brachionus plicatilis* (Rotifera). *Evolution*, **56**, 1431–1444.
- Gómez A, Hughes RN, Wright PJ, Carvalho GR, Lunt DH (2007) Mitochondrial DNA phylogeography and mating compatibility reveal marked genetic structuring and speciation in the NE Atlantic bryozoan *Celleporella hyalina*. *Molecular Ecology*, **16**, 2173–2188.
- González-Sampériz P, Valero-Garcés B, Carrión J (2004) Was the Ebro valley a glacial refugium for temperate trees? *Anales de Biología*, **26**, 13–20.
- Guindon S, Gasquet O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology*, **52**, 696–704.
- Hasegawa M, Kishino H, Yano T (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution*, **22**, 160–174.
- Hebert PDN, Witt JDS, Adamowicz SJ (2003) Phylogeographical patterning in *Daphnia ambigua*: regional divergence and intercontinental cohesion. *Limnology and Oceanography*, **48**, 261–268.
- Hewitt G (2000) The genetic legacy of the Quaternary ice ages. *Nature*, **405**, 907–913.
- Hewitt GM (1996) Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society*, **58**, 247–276.
- Hood GM (2005) PopTools Version 2.6. CSIRO Canberra, Australia. URL: <http://www.cse.csiro.au/poptools>.
- Hudson RR, Slatkin M, Maddison WP (1992) Estimation of levels of gene flow from DNA-sequence data. *Genetics*, **132**, 583–589.
- Hutchison DW, Templeton AR (1999) Correlation of pairwise genetic and geographic distance measures: inferring the relative influences of gene flow and drift on the distribution of genetic variability. *Evolution*, **53**, 1898–1914.

- Jenkins DG, Buikema AL Jr (1998) Do similar communities develop in similar sites? A test with zooplankton structure and function. *Ecological Monographs*, **68**, 421–443.
- Johnson NK, Cicero C (2004) New mitochondrial DNA data affirm the importance of Pleistocene speciation in North American birds. *Evolution*, **58**, 1122–1130.
- Keane T, Creevey C, Pentony M, Naughton T, McInerney J (2006) Assessment of methods for amino acid matrix selection and their use on empirical data shows that ad hoc assumptions for choice of matrix are not justified. *BMC Evolutionary Biology*, **6**, 29.
- Le Corre V, Machon N, Petit RJ, Kremer A (1997) Colonization with long-distance seed dispersal and genetic structure of maternally inherited genes in forest trees: a simulation study. *Genetical Research*, **69**, 117–125.
- Louette G, De Meester L (2005) High dispersal capacity of cladoceran zooplankton in newly founded communities. *Ecology*, **86**, 353–359.
- Lowe CD, Kemp SJ, Bates AD, Montagnes DJS (2005) Evidence that the rotifer *Brachionus plicatilis* is not an osmoconformer. *Marine Biology*, **146**, 923–929.
- Mills S (2006) *Investigations of the Brachionus Plicatilis Species Complex, with Particular Reference to Southwest Western Australia*. University of Western Australia, Australia.
- Naihong X, Audenaert E, Vanoverbeke J *et al.* (2000) Low among-population genetic differentiation in Chinese bisexual *Artemia* populations. *Heredity*, **84**, 238–243.
- Onbé T (1978) Sugar flotation method for sorting the resting eggs of marine cladocerans and copepods from sea-bottom sediment. *Bulletin of the Japanese Society of Scientific Fisheries*, **44**, 1411.
- Ortells R, Snell TW, Gómez A, Serra M (2000) Patterns of genetic differentiation in resting egg banks of a rotifer species complex in Spain. *Archiv für Hydrobiologie*, **149**, 529–551.
- Ortells R, Gómez A, Serra M (2003) Coexistence of cryptic rotifer species: ecological and genetic characterisation of *Brachionus plicatilis*. *Freshwater Biology*, **48**, 2194–2202.
- Penton EH, Hebert PDN, Crease TJ (2004) Mitochondrial DNA variation in North American populations of *Daphnia obtusa*: continentalism or cryptic endemism? *Molecular Ecology*, **13**, 97–107.
- Posada D, Crandall KA, Templeton AR (2000) GEODIS: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology*, **9**, 487–488.
- Pruett CL, Winker K (2005) Northwestern song sparrow populations show genetic effects of sequential colonization. *Molecular Ecology*, **14**, 1421–1434.
- Prugnolle F, Manica A, Balloux F (2005) Geography predicts neutral genetic diversity of human populations. *Current Biology*, **15**, R159–R160.
- Ramachandran S, Deshpande O, Roseman CC *et al.* (2005) Support from the relationship of genetic and geographic distance in human populations for a serial founder effect originating in Africa. *Proceedings of the National Academy of Sciences, USA*, **102**, 15942–15947.
- Rodríguez-Puebla C, Encinas AH, Nieto S, Garmendia J (1998) Spatial and temporal patterns of annual precipitation variability over the Iberian Peninsula. *International Journal of Climatology*, **18**, 299–316.
- Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572–1574.
- Rousset F (1997) Genetic differentiation and estimation of gene flow from *F*-statistics under isolation by distance. *Genetics*, **145**, 1219–1228.
- Rozas J, Sánchez-Del Barrio JC, Messeguer X, Rozas R (2003) DNASP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, **19**, 2496–2497.
- Serrano A, Mateos VL, Garcia JA (1999) Trend analysis of monthly precipitation over the Iberian Peninsula for the period 1921–95. *Physics and Chemistry of the Earth, Part B: Hydrology, Oceans and Atmosphere*, **24**, 85–90.
- Suatoni EM (2003) *Patterns of Speciation in the Rotifer Species Complex Brachionus Plicatilis*. University of Yale, New Haven, Connecticut.
- Swofford D (1998) *PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods)*, Version 4. Sinauer & Associates, Sunderland, Massachusetts.
- Taylor DJ, Finston TL, Hebert PDN (1998) Biogeography of a widespread freshwater crustacean: pseudocongruence and cryptic endemism in the North American *Daphnia laevis* complex. *Evolution*, **52**, 1648–1670.
- Templeton AR (1998) Nested clad analyses of phylogeographic data: testing hypotheses about gene flow and population history. *Molecular Ecology*, **7**, 381–397.
- Templeton AR (2001) Using phylogeographic analyses of gene trees to test species status and processes. *Molecular Ecology*, **10**, 779–791.
- Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. 3. Cladogram estimation. *Genetics*, **132**, 619–633.
- Tremblay NO, Schoen DJ (1999) Molecular phylogeography of *Dryas integrifolia*: glacial refugia and postglacial recolonization. *Molecular Ecology*, **8**, 1187–1198.
- Vanoverbeke J, De Meester L (1997) Among-population genetic differentiation in the cyclical parthenogen *Daphnia magna* (Crustacea, Anomopoda) and its relation to geographic distance and clonal diversity. *Hydrobiologia*, **360**, 135–142.
- Weider LJ, Hobaek A, Colbourne JK *et al.* (1999a) Holarctic phylogeography of an asexual species complex I. Mitochondrial DNA variation in arctic *Daphnia*. *Evolution*, **53**, 777–792.
- Weider LJ, Hobaek A, Hebert PDN, Crease TJ (1999b) Holarctic phylogeography of an asexual species complex – II. Allozymic variation and clonal structure in Arctic *Daphnia*. *Molecular Ecology*, **8**, 1–13.

This paper is part of an ongoing collaboration between the Molecular Ecology Laboratory in Hull and the Evolutionary Ecology Group in Valencia. Africa Gómez is an advanced NERC fellow in Hull and is interested in the population structure and phylogeography of clonal organisms. Manuel Serra is Professor at the University of Valencia and has a long-term interest in the ecology and evolution of cyclical parthenogenetic organisms, in particular *Brachionus*. Sergi Campillo and Javier Montero-Pau are carrying out their PhDs on ecological and genetic differentiation in *Brachionus*. David Lunt is a lecturer in Molecular Ecology at the University of Hull whose interests include phylogeography and asexual organisms.
