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Behavioural reproductive isolation in a rotifer hybrid zone

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Abstract

A hybrid zone between two *Brachionus plicatilis* rotifer mitochondrial DNA (mtDNA) lineages was recently described in the Iberian Peninsula between a pond (Santed 2) and a lake (Gallocanta). The patterns of mitochondrial and nuclear genetic variation observed suggested that gene flow is mainly male-mediated from the lake to the pond. Here we test two hypotheses: (a) that male-mediated gene flow occurs through assortative mating between individuals from these ponds, (b) that behavioural isolation occurs between the two mtDNA lineages. We isolated, reared and genotyped rotifer clones from resting eggs collected in the sediments of these and two other distant ponds. We devised a quick, inexpensive RFLP method to discriminate between *B. plicatilis* and its sibling species *B.* 'Manjavaeas' and between both mtDNA *B. plicatilis* lineages. Behavioural no-choice tests using new-born, virgin males and females were performed between five clones. *B.* 'Manjavacas' and *B. plicatilis* were reproductively isolated. *B. plicatilis* clones did not show evidence of reproductive isolation, regardless of their mtDNA lineage, except Santed 2 males, which discriminated strongly against Gallocanta females. These results could help to explain the discrepancies between mitochondrial and nuclear genetic variation reported in the two populations.

Introduction

The application of molecular methods to the analysis of zooplanktonic populations has recently challenged widely held views on the biology of these organisms. First, molecular methods have uncovered a plethora of sibling species complexes (Gómez et al., 2002b) that previously contributed to confounding biodiversity and ecological assessments. Second, important aspects of the population biology of these organisms have been discovered (De Meester et al., 2002), which emphasise the surprisingly low impact of dispersal and gene flow in continental zooplanktonic populations. Third, phylogeographic assessments of continental zooplanktonic organisms, although relatively recent, have revealed an important amount of geographic diversification, leading to important insights into their microevolution and speciation processes (see review in De Meester et al., 2002).

To fully exploit the advantages of using molecular methods in zooplanktonic organisms, a multidisciplinary approach is necessary. Thus, although some of the aforementioned molecular assessments rely on mitochondrial DNA, none investigates reproductive isolation between the taxa involved and, therefore, it is not known if the molecular signatures detected reveal gene patterns or population level phenomena. A recent study of the phylogeography of *Brachionus plicatilis* Müller 1786 in Iberian saline lakes (Gómez et al., 2002a) revealed two distinct monophyletic mtDNA lineages with a geographic structure corresponding to 'northern' and 'southern' lineages. There was also evidence of separate evolutionary histories since the onset of the Pleistocene. A survey of seven unlinked microsatellite loci revealed strong

population structure, but surprisingly little correlation with mtDNA differentiation, population structure showing isolation by distance (Gómez et al., 2002a). Therefore, unexpectedly, populations from two nearby locations – a lake, Laguna de Gallocanta, and a pond, Balsa de Santed 2 – dominated by mtDNA haplotypes from northern and southern lineages, respectively, proved very similar in their nuclear loci allele frequencies. This suggests that gene flow occurs between the two populations, most prominently from the lake to the pond, with some mechanism restricting the amount of female-mediated gene flow. Although hybrid swarms have been described in *Daphnia* (see for example Schwenk et al., 2000), to our knowledge, Gallocanta and Balsa de Santed 2 form the first classic hybrid zone described for a continental zooplanktonic organism.

Claims of reproductive isolation are rarely substantiated with direct evidence from mating tests in zooplanktonic organisms due to the impracticalities of carrying out such experiments. However, mating tests have been used to great success in rotifers to demonstrate assortative mating and behavioural reproductive isolation between different species (e.g., Snell & Hawkinson, 1983; Gómez & Serra, 1995; Rico-Martinez & Snell, 1995a,b). Many characteristics of *B. plicatilis* make it an ideal organism for this type of study: (a) ease of laboratory culture, (b) short generation time, (c) easy handling, (d) well known mating behaviour (Snell & Hawkinson, 1983).

Variation in assortative mating among domesticated and wild strains in *B plicatilis s.l.* has been reported several times (Snell & Hawkinson, 1983; Fu et al., 1993; Rico-Martinez & Snell, 1995a, b). However, few studies dealt with genetically well characterised wild strains (Gómez & Serra, 1996; Gómez & Snell, 1996), and therefore their conclusions have limited value.

In this paper we combine the use of molecular tools to identify rotifer species and genetic lineages from clones isolated from field sediments, and mating behaviour experiments in the laboratory to test two hypotheses: (a) mating behaviour can be responsible for the discrepancies between mitochondrial and nuclear DNA phylogeography in *B. plicatilis* observed in this hybrid zone, and (b) the occurrence of behavioural reproductive isolation between southern and northern mtDNA lineages. Using pair-wise mating tests, we investigate the existence of reproductive isolation between the northern and southern mtDNA lineages of Iberian *B. plicatilis s.s.* and the patterns of mating behaviour from rotifers from Laguna de Gallocanta, and Balsa de Santed 2. The results will help us understand the microevolutionary processes involved in rotifer speciation.

Methods

Experimental design

Individuals of the rotifer *B. plicatilis* sensu stricto (see Ciros-Pérez et al., 2001) from the lakes Balsa de Santed 2 (SA2) and Laguna de Gallocanta (GAL) were used in the mating experiments. Populations from these lakes are dominated by southern and northern mtDNA haplotypes (see Fig. 1), respectively, and are geographically close together (less than 10 km apart). Therefore, both populations are likely to exchange migrants, as suggested by microsatellite results (Gómez et al., 2002a). Individuals from Laguna de las Eras (ERA) (predominantly northern mtDNA) and Laguna de Mojón Blanco (MOJ) (southern) were also included in the experimental design; these lakes are equidistant from the other two. In this way, our experimental design examined reproductive isolation between populations with divergent mitochondrial and nuclear DNA (distant populations) and also between populations with different mitochondrial but similar nuclear DNA (nearby populations in the contact zone between mtDNA lineages). Finally, a strain from the lineage *B.* 'Manjavacas' (Gómez et al., 2002b), isolated from Laguna de Gallocanta, was also included. This lineage is highly divergent from *B. plicatilis* for both nuclear and mitochondrial sequenced genes (Gómez et al., 2002b) and allozyme loci (Ortells et al., 2000). The lack of hybrid genotypes between *B. plicatilis* and *B.* 'Manjavacas' despite co-occurrence in their natural habitat, and the observation that *B. plicatilis* males discriminate against 'Manjavacas' females (Ortells et al., 2000) has led to the suggestion of species status for this lineage (Gómez et al., 2002b).

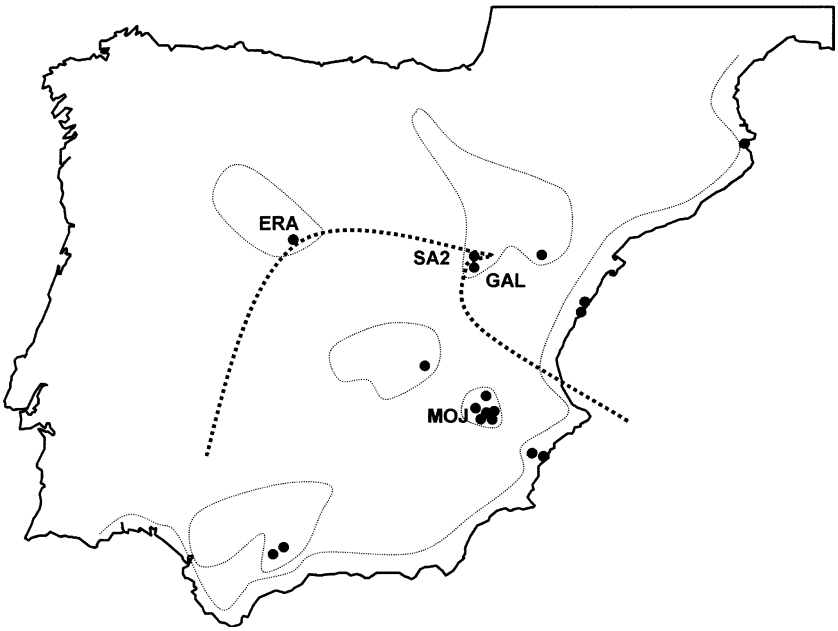

Figure 1. Map of the Iberian Peninsula showing the sampling locations indicated as follows: ERA: Laguna de las Eras; SA2: Balsa de Santed II; GAL: Laguna de Gallocanta; MOJ: Laguna de Mojón Blanco. Unlabelled points are sampling locations used by Gómez et al. (2000). Thin dashed lines indicate drainage basins and the thicker dashed line represents the boundary between northern and southern mitochondrial DNA lineages. Lakes are not drawn to scale.

Resting egg isolation, hatching and DNA extraction

Resting eggs were isolated from sediment samples collected from Laguna de Gallocanta (GAL), Balsa de Santed 2 (SA2), Laguna de Mojón Blanco (MOJ) and Laguna de Las Eras (ERA) (Fig. 1) using a sucrose flotation method (see Gómez et al., 2000 for details of the sampling locations). Approximately 5 ml of sediment was dispersed in 35–40 ml of 1:1 weight:volume sucrose solution and centrifuged at 700 rpm for 5 min. The supernatant was filtered through a 30 μm Nytal mesh and the filtrate rinsed with 6 g l^{-1} saltwater (Instant Ocean®; Aquarium Systems) and then resuspended in fresh saltwater for examination under a stereoscope at ×40 magnification. *B. plicatilis* eggs were identified by their morphology, and kept in a Petri dish at 20 °C under constant illumination (see below) for approximately 48 h to allow hatching. New-born rotifer females were transferred to individual wells in 24-well tissue culture plates (Nalge Nune International) containing 1 ml of growth medium (see below) using a pipette and disposable tips. Rotifers were allowed to reproduce and some of the clones were used for the mating tests. Each isolated clone, offspring of a single hatched female from the sediment sample, was named with the pond initials and a letter.

For DNA extractions, individual live female rotifers from each clone were isolated and washed in fresh 12 g l^{-1} saltwater before being released into a 0.2 ml tube containing 40 μl of Chelex (6% InstaGene™ Matrix; Bio-Rad). Individuals were handled using sterile pipette tips to avoid cross-contamination. The tubes were heated for 20 min at 56 °C followed by 10 min at 99.9 °C and then cooled for 30 min at 4 °C before spinning down the resulting solution. Samples were kept frozen (−20 °C) until needed.

Culture conditions

Growth medium was a monoculture of the unicellular algae *Tetraselmis chuii* Butcher in 12 g l^{-1} saltwater enriched with f/2 medium (Guillard & Ryther, 1962) and aerated using an aquarium pump. Rotifer clones were transferred from culture wells to glass test tubes with fresh growth medium when several individuals could be observed in the growth wells. Algae and rotifer

cultures were kept in a temperature-controlled chamber at 20 °C under constant illumination (ca. 47 Em^{-2} s^{-2}). Rotifers clones were maintained in duplicate and fed once a week by replacing half of the culture with fresh growth medium.

PCR amplification and RFLP analysis of mitochondrial DNA

A 712 bp (base pair) fragment of the mitochondria) gene cytochrome *c* oxidase I (COI) DNA was amplified for each clone using the polymerase chain reaction (PCR). Reactions were carried out in 0.2 ml tubes in a final volume of 10 μl containing 2 μl template DNA, PCR buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl (pH 8.8 at 25 °C), 0.01% Tween-20), 1.5 mM $MgCl_2$, 200 μM of each nucleotide, 2.5 pmol of each primer and 0.125 U *Taq* polymerase. The primers LCO1490 (5′-GGTCAACAAAT-CATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACC AAAAA-ATCA-3′) (Folmer et al., 1994) were used, with the following cycling conditions: 3 min at 93 °C followed by 40 cycles of 15 s at 92 °C, 20 s at 50 °C, 1 min at 70 °C, and a final 3 min at 72 °C.

As detailed sequence information is available for the populations and species used here (Gómez et al., 2000, 2002b), the species and COI haplotype group of each clone was determined by carrying out Restriction Fragment Length Polymorphism (RFLP) analysis on the amplified COI fragment. To find out which restriction enzymes could be used to discriminate between species and haplotype groups, a survey of expected digests for a range of restriction enzymes was carried out using DNASTAR ver. 2.88 (Lasergene Inc.) using consensus sequences of *B.* 'Manjavacas' vs. *B. plicatilis* and between consensus sequences of the northern and southern lineages of *B. plicatilis* (obtained from Gómez et al., 2000, 2002b). Only enzymes cutting non-polymorphic sites within species and lineages were selected. Endonuclease digestions were carried out in 10 μl final volume containing 2 μl DNA, 1 μl buffer ($Kpn1^+$ for *Kpn*I, R^+ for *Mbo*I, O^+ for *Bst*X1; MBI Fermentas) and 2 U restriction endonuclease. Reactions were incubated overnight at 37 °C (55 °C for *Bst*X1). The species or haplotype group was determined by examining the pattern of DNA bands produced on a 2% agarose gel stained with ethidium bromide.

Mating tests

The mating tests carried out here were pair-wise no-choice experiments in which males produced by a given clone were exposed to either homogamic (same clone) or heterogamic (different clone) females. This experimental design is a cost-effective way of performing behavioural reproductive isolation tests in rotifers for two reasons. First, no way of visually discriminating between males or females from different clones has been devised and, second, as mating behaviour in rotifers occurs only after contact between a male and female, there is no detection at a distance and thus choice can only be effected when a male meets a female. Procedures for the mating tests followed Gómez & Serra (1996). Rotifer cultures for mating tests were initiated in 250 ml conical flasks with 200 ml growth medium and fed daily or every other day to ensure high population densities and the presence of many females carrying multiple mictic (male) and amictic (female) eggs. Before each experiment, female rotifers carrying male or female eggs were isolated from the appropriate cultures and placed in tissue culture wells in 12 g l^{-1} saltwater. Male eggs were recognised as being smaller than female eggs. After approximately 2 h, new-born virgin males and females were isolated for use in the experiments. Females were less than 4 h old and males less than 8 h old when the experiments were carried out, because very young females are most attractive to males (Gómez & Serra, 1996) and sexual activity and fertilisation ability in males decreases as they age (Snell & Childress, 1987; Snell & Hoff, 1987).

For each experiment, 25 new-born females of a given clone were isolated in 45 μl saltwater in a small well (96 round-bottom wells per plate; Nalge Nunc International). A male of the same or a different clone was then introduced in a further 5 μl of saltwater and its behaviour monitored for 5 min under a binocular stereoscope at ×20 magnification. Experiments were conducted within the temperature range 21–25 °C. Three types of male behaviour were recognised – contact, circling and copulation. Contact was defined as a head-on collision between a male and female, circling

involved the male completing one or more revolutions around the female's body while maintaining coronal contact, and copulation was recorded when the male stopped circling, attached his penis to the female and coronal contact was lost. Six replicate males from each clone were tested successively on each group of females. The mating tests were performed blind, so that the observer (H.K.B.) was unaware of the identity of the clones until all the experiments had been completed.

Data analyses

The percentages of male-female encounters resulting in circling and copulation per individual male for a given combination of male and female clone were used as raw data. This removed biases due to the fact that males spend different amounts of time copulating and circling females. Normal qantile plots (Sokal & Rohif, 1995) indicated that these data were not normal; therefore, we performed non-parametric analysis to test our hypothesis. Our null hypothesis was that male behaviour was the same to homogamic females as to heterogamic females. To test this hypothesis we used Mann–Whitney tests in which the behaviour (circling or copulation percentages) of the males towards the homogamic females was compared with their behaviour towards the heterogamic females (Zar, 1984) using the six male replicates of each mating experiment. All statistical analyses were carried out using SPSS v. 8.0.2. A p-value of 0.05 was used for all tests.

Results

Genetic characterisation of the clones

Thirty-two hatchlings were obtained from the sediment samples: 8 from ERA, 7 from GAL, 6 from SA2 and 11 from MOJ. Once the genotype of the resulting clones was known, the clones having the desired genotypes from each pond were selected for the experiments. The clones used in the experiments and some representatives of their ponds were kept in culture in the laboratory.

The restriction enzymes *Kpn*I and *Mbo*I were diagnostic to discriminate between amplified COI fragments from *B. plicatilis* and its sibling species 'Manjavacas'. A restriction site for *Kpn*I (5′-GGTAC$^{\downarrow}$C-3′) in *B. plicatilis* DNA generates fragments of 349 and 363 bp when digested, but no restriction site for this enzyme exists in 'Manjavacas' DNA, leaving the 712 bp COI DNA fragment intact. *Mbo*I (5′-$^{\downarrow}$GATC-3′) generates fragments of different size on each species (614, 27 and 71 bp in *B. plicatilis*, and 407, 108, 99, 27 and 71 bp in 'Manjavacas'). *Bst*X1(5′-CCAN$_5^{\downarrow}$NTGG-3′) was used to determine whether mtDNA of the *B. plicatilis* clones belonged to northern or southern haplotype groups (see Gómez et al., 2000 for details). A restriction site for this enzyme in southern haplotype COI DNA generates fragments of 435 and 277 bp, but there is no *Bst*X1 site in northern haplotype DNA. All clones showed the RFLP haplotype expected for the lake of origin, based on the results of Gómez et al. (2000). SA2 and MOJ clones were all southern, ERA clones were all northern and GAL clones were either northern or belonged to the 'Manjavacas' lineage. Examples of the band patterns produced by agarose gel electrophoresis of the RFLP fragments are presented in Figure 2. This RFLP technique was found to be entirely reliable when digestion of previously sequenced clones was performed (data not shown).

Mating experiments

Five clones were selected for the mating experiments: ERA-B and GAL-B, *B. plicatilis* clones with northern haplotypes, SA2-A and MOJ-C, *B. plicatilis* clones with southern haplotypes and MAN, a *B.* 'Manjavacas' clone. All male-female clone combinations were tested, including males and females from the same clone. The mean percentage of encounters ending in circling and copulation of the six replicate experiments (males) for each cross are shown in Figure 3. All male strains displayed a very low percentage of circling with *B.* 'Manjavacas' females (<5%, except for homogamic males, which show a higher value of 5.73%). Significant differences between the circling percentages to homogamic females and 'Manjavacas' females were found for male clones GAL-B, SA2-A and MOJ-C (Fig. 3). With the exception of one male (of strain GAL-B) no copulations were observed with *B.* 'Manjavacas' females but, due to the low number of copulations observed in the

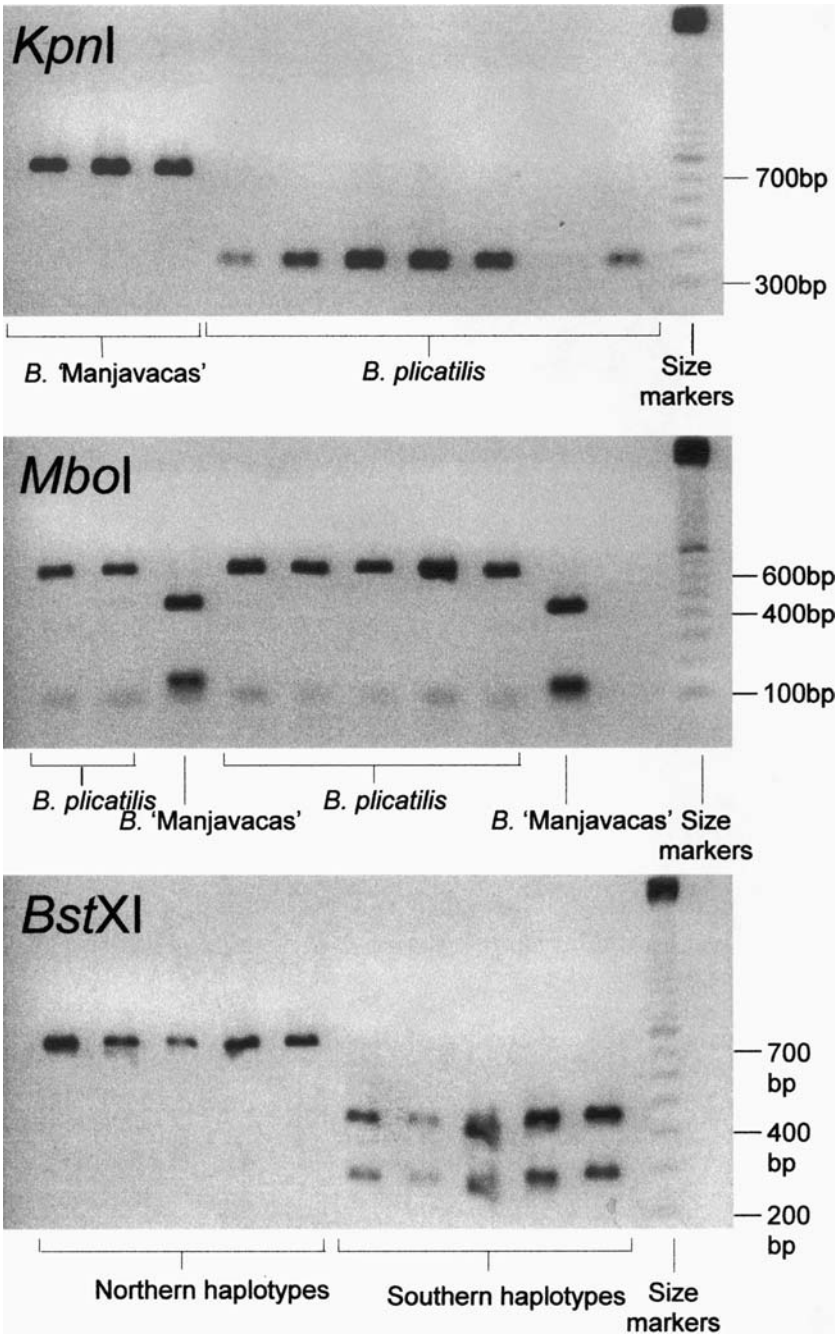

Figure 2. RFLP banding patterns produced when restriction enzyme digests of *B. plicatilis* and *B.* 'Manjavacas' DNA are run on agarose gels. Profiles generated by *Kpn*I (top gel) and *Mbo*I (middle gel) differentiate between *B. plicatilis* and *B.* 'Manjavacas'. *Bst*XI (bottom gel) differentiates northern and southern haplotypes of *B. plicatilis*.

intraspecific cross, these differences were not significant. *B.* 'Manjavacas' males showed significantly increased circling percentages towards females of the clones ERA-B, GAL-B, and SA2-A, and a consistent low percentage of copulations with all female strains (including their own) despite high percentages of circling to other female strains. Within the *B. plicatilis* male strains, the percentage

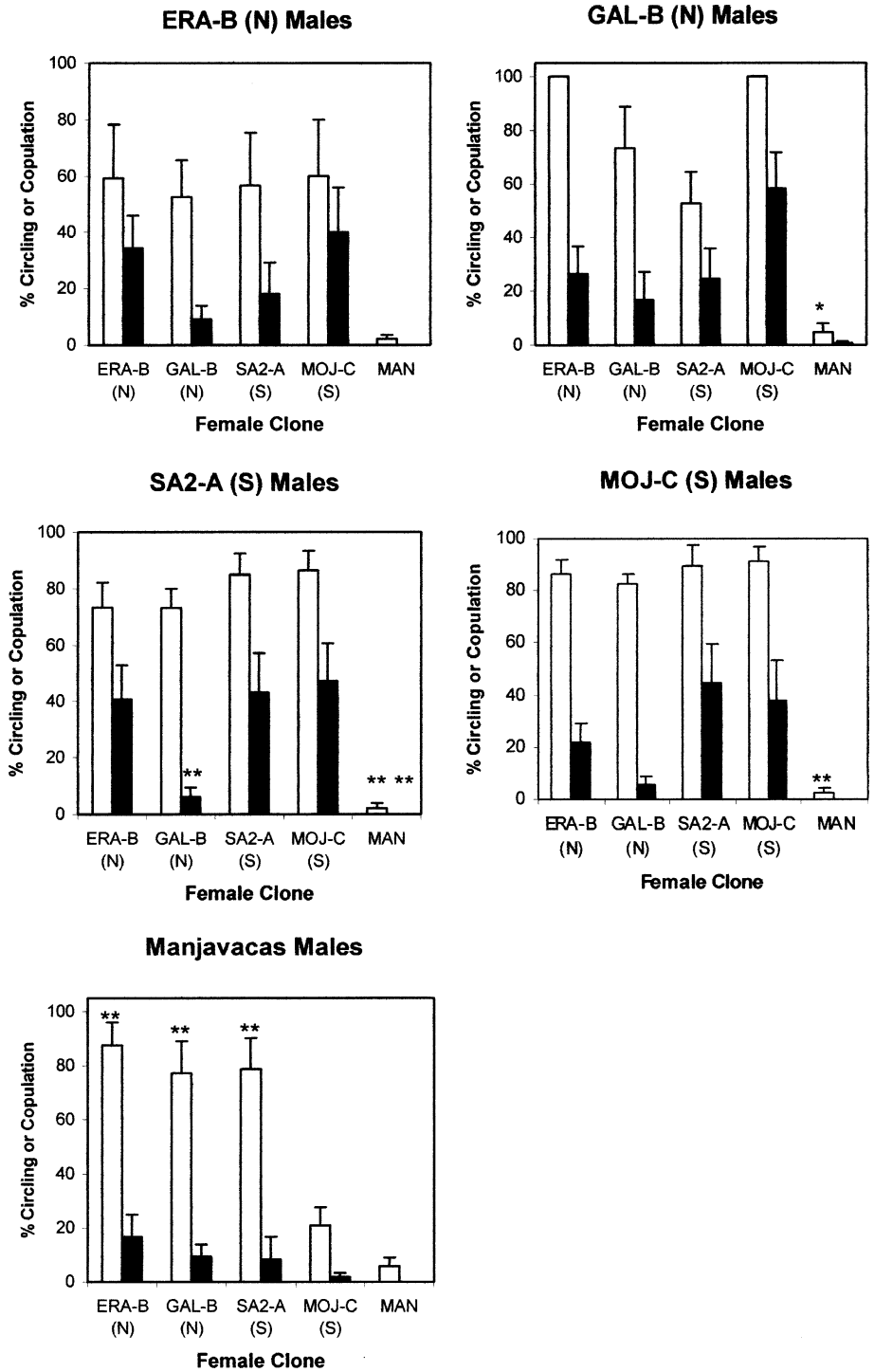

Figure 3. Mating behaviour of males of each clone towards each clone of females. Bars show the mean percentage of encounters ending in circling (clear bars) and copulation (black bars) of six replicate experiments for each cross. Vertical lines are the standard errors. Significant results of the Mann–Whitney tests used to compare the six replicate values of male behaviour (circling or copulation percentages) between homogamic and heterogamic females are also shown (*, $p < 0.05$ level, **, $p < 0.005$). The N and S in parentheses indicate the mtDNA lineage of each clone.

of encounters ending in circling was generally similar for all strains of females. The percentage of encounters ending in copulation was more variable than circling percentages. The least attractive female strain in terms of copulations seems to be GAL-B; this difference is more marked in southern strains than northern strains, but these differences were non-significant.

Discussion

The haplotype results from this study can be combined with those of Gómez et al. (2000), who found that only one out of the 10 individuals tested from ERA was of southern haplotype and only one of the 20 individuals tested from SA2 was of northern haplotype. Combined with the results presented above, a total of 1 southern and 16 northern haplotype individuals have been identified from ERA, and 1 northern and 25 southern from SA2, remaining consistent with the view that ERA is inhabited predominantly by northern haplotypes and SA2 by southern haplotypes. GAL and MOJ were respectively inhabited by northern and southern haplotypes exclusively. Therefore, our results contribute to highlighting the discrepancies between mitochondrial and nuclear DNA in the two nearby locations GAL and SA2. The RFLP technique presented here, given the absence of described diagnostic morphological features between the *B.* 'Manjavacas' lineage and *B. plicatilis*, and between both mtDNA lineages in *B. plicatilis*, is a rapid and cost-effective method of characterising clones isolated from the field.

Pair-wise mating experiments

Interactions between B. plicatilis *and* B. 'Manjavacas'

The mating behaviour data presented here does not lend support to the hypothesis that the northern and southern lineages are reproductively isolated. As for our first hypothesis, the data, although limited, is consistent with the discrepancies found between nuclear and mitochondrial DNA in Gallocanta and Balsa de Santed 2, as SA2-A males showed a significantly lower percentage of copulations to GAL-B females, which, if extended to the population of the pond (Balsa de Santed 2) as a whole, would result in a reduced level of mitochondrial introgression.

Furthermore, the results of the mating tests suggest the existence of behavioural reproductive isolation between *B. plicatilis* and *B.* 'Manjavacas' strains. 'Manjavacas' females consistently elicited fewer mating responses than *B. plicatilis* females with all strains of males, including their own. This result is consistent with that of Ortells et al. (2000), who found that *B. plicatilis* males discriminate against 'Manjavacas' females (the strain GAL-A6 in their paper). *B.* 'Manjavacas' males discriminate against *B. plicatilis* females mostly during the copulation step. The low percentage of homogamic circlings and the absence of homogamic matings between *B.* 'Manjavacas' males and females further supports the view that this lineage differs significantly from *B. plicatilis*, as this result may be due to different optimum culture conditions regarding salinity, temperature or food source. As the experiments involving *B.* 'Manjavacas' rotifers were not all executed at the same time this result cannot be attributed to different conditions on the day of the experiment. However, more work is needed regarding optimal conditions for mating behaviour tests for *B.* (Manjavacas), as previous work has shown that temperature in particular could importantly affect male mating behaviour (Kotani & Hagiwara, 1999, 2003), therefore, our results regarding the behaviour of 'Manjavacas' males must be taken with caution.

Interactions between northern and southern B. plicatilis

The percent of circlings in crosses involving *B. plicatilis* strains are in agreement with published data, with 50–100% of encounters leading to circling in cases where there has not been shown to be discrimination against females (Snell & Hawkinson, 1983; Gómez & Serra, 1995; Rico-Martinez & Snell, 1995a, b). Copulations were observed to occur less frequently than circlings, but often more frequently than has been reported in other studies; many crosses resulted in copulation in around 40% of encounters (maximum 58.33%), compared to published results of up to 12 or 14% (Gómez & Serra, 1995; Rico-Martinez & Snell,1995a, b), although Ortells et al. (2000) have reported copulation percentages up to 94.4% for homogamic matings. These results are most

likely a consequence of performing the crosses with new-born females, as males show a marked preference for them (Gómez & Serra, 1996). In general, there was no clear discrimination for circling or copulations between clones of northern and southern haplotype, with the exception of SA2-A males discriminating against GAL-B females. These results indicate that the northern and southern lineages have not evolved significant behavioural reproductive isolation during the long period of time (since the beginning of the Pleistocene) that they have been in geographic isolation (Gómez et al., 2000), lending support to a hypothesis of slow evolution of mate recognition in allopatry.

The fact that nuclear introgression might explain the discrepancies between the nuclear and mitochondrial genomes in *B. plicatilis* SA2 and GAL populations could seem counter-intuitive given that the dispersing propagules of rotifers (resting eggs) hatch into parthenogenetic females. However, if the clones originating from hatchlings fail to produce resting eggs in their first season, only nuclear genes would be transmitted to the following generation, *via* the males produced by sexual daughters (Gómez et al., 2000), and mitochondrial genes will fail to introgress. According to the results presented here, if SA2-A males discriminate against GAL-B females, but the reverse is not true, GAL females migrating into SA2 will be rare in the population and will most probably not secure matings. They will consequently produce mostly males, not resting eggs, resulting in the biased transmission of nuclear genes. Therefore this pattern of asymmetric mating behaviour could explain the discrepancies between the mtDNA and microsatellite results (Gómez et al., 2000). As GAL-B males do not appear to discriminate against SA2-A females this may, indeed, be the case.

An additional factor affecting the direction of gene flow is the relative size of the two lakes. SA2 is a small temporary pond (0.8 ha), whereas GAL is a large lake (1200 ha). This could affect the net direction of dispersal (from GAL to SA2) and also gene flow, and helps explain why there is no observed nuclear introgression in the direction from SA2 to GAL.

A question still to be answered is: When did this discrimination evolve? An interesting possibility that fits our results is that a process of recent reinforcement of reproductive isolation has taken place in SA2. This requires the existence of decreased hybrid fitness in the offspring generated by crosses between both ponds. As, presumably, many fewer SA2 individuals migrate into GAL this would explain why no similar discrimination has developed between GAL-B males and SA2-A females and therefore there has been less selective pressure for GAL males to discriminate against SA2 females as inter-strain matings in this direction would be relatively rare events. If decreased fitness of hybrid offspring was the selective pressure causing mating discrimination (i.e., reinforcement), it would fit with the observation that behavioural isolation does not happen between allopatric populations, even those that have been isolated for more than 1 million years. The hypothesis of decreased fitness of hybrid offspring will need to be investigated in the future. An alternative to the reinforcement scenario would be that behavioural isolation evolved in allopatry in the SA2 pond, and then favoured male-mediated gene flow when the populations came into contact. The demonstration of reinforcement would imply that pre-mating isolation can evolve in sympatry in rotifers, suggesting that eventually complete reproductive isolation could come to exist via the process of sympatric speciation.

Conclusions

This work has presented a rapid and cost-effective method to discriminate between sympatric rotifer species. Behavioural cross-mating experiments showed that reproductive isolation appears to exist between *B. plicatilis* and *B.* 'Manjavacas' strains. Although there was no conclusive evidence of behavioural reproductive isolation between northern and southern *B. plicatilis* lineages, some reproductive isolation was found between Gallocanta and Santed 2 strains. Our results indicate that the pattern of reproductive isolation may be responsible for the population genetic structure reported for these ponds. These results suggest that further investigating the role reinforcement might have played in the evolution of reproductive isolation in these rotifers is warranted.

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