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Morphological stasis of two species belonging to the L-morphotype in the *Brachionus plicatilis* species complex

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Abstract

Detection and characterization of sibling species complexes in zooplankton are critical to understanding their ecological responses and patterns of evolution. The taxon *Brachionus plicatilis* is a complex of at least 14 species with three major, deeply diverged clades, which are morphologically distinct. We studied morphometric differences between two species – *B. plicatilis* sensu stricto and *B.* 'Manjavacas'– which belong to the L-(large) morphotype and often co-occur in ponds or lakes. *B. plicatilis* s.s. was on average 6% longer than *B.* 'Manjavacas'. They differed significantly in the measurements related to lorica spines. A significant discriminant function relating spine measurements was found, however, individuals from each species showed extensive overlap. Our morphometric data provide additional evidence for the species status of *B. plicatilis* s.s. and *B.* 'Manjavacas'. Since these are ancient species, our results support that a morphological stasis occurs in these taxa. We identified COI restriction sites for *Pvu*II and *Kpn*I which are diagnostic for *B.* 'Manjavacas' and *B. plicatilis* s.s., respectively. We conclude that morphometry is not useful in classifying the two species. At present, this can only be done reliably using molecular methods.

Introduction

Application of molecular markers to the study of aquatic invertebrates has shown that sibling species complexes are more common than previously thought (Knowlton, 1993; Hebert, 1998; Ortells et al., 2000; Gómez et al., 2002; Derry et al., 2003; Suatoni, 2003). In rotifers, genetic divergence resulting in speciation can occur independent of morphological divergence as mate recognition is not visual, and a remarkable morphological stasis can occur (Gómez et al., 2002). Detection and characterization of these species complexes is an important challenge in evolutionary ecology. Failure to do so means that species richness in a habitat may be underestimated and a species' ecological tolerance and habitat range could be overestimated.

The *Brachionus plicatilis* taxon is a complex of at least 14 sibling species (Suatoni, 2003). Most of them cluster into three major phylogenetic clades, which can be categorized as large, medium and small. Evidence for a large number of species comes mainly from molecular (allozyme and DNA) studies (Ortells et al., 2000; Gómez et al., 2002; Suatoni, 2003) and from mating behaviour analysis (Ortells et al., 2000; Suatoni, 2003). Detailed morphological studies generally confirm these species boundaries. An example is Ciros-Pérez et al. (2001) who used morphometry to describe the three formally named species in the complex. However, morphological differences between species in the same body size clade have not been studied yet, so it is not known whether subtle morphological differences can also be used to discriminate these species.

B. plicatilis sensu stricto and *B.* 'Manjavacas' both belong to the large (L-) morphotype (Gómez et al., 2002). Their species status is supported by

the large genetic distance between them and by reproductive isolation in mating tests between sympatric population (Gómez & Snell, 1996; Ortells et al., 2000). The former has been reported in Europe, America, Asia and Oceania and the latter in Europe, America and Africa (Ortells et al., 2000; Gómez et al., 2002; Suatoni, 2003). They have been found in sympatry in 8 ponds of the Iberian Peninsula, while *B. plicatilis* s.s. alone has been reported in 14 ponds and *B.* 'Manjavacas' in 3 ponds (Ortells et al., 2000; Gómez et al., 2002).

We studied morphometry of these two species in order to test if differentiation in genetic markers is associated with morphological divergence, and to know whether we could identify characters that enable us to discriminate these species morphologically.

Methods

Clone collection

For species identification, a collection of 15 L-morphotype clones belonging to the *Brachionus plicatilis* complex was made from isolates collected in the Iberian Peninsula. These included five clones from the collection of the Institute Cavanilles of Biodiversity and Evolutionary Biology (University of Valencia, Spain): GALL1 from Laguna de Gallocanta, L1, L2 and L3 from Torreblanca Marsh (Poza Sur) and L5MAN from Laguna de Manjavacas. The other 10 clones originated from resting eggs that were hatched for this study, and were isolated from sediment samples collected in the following ponds: Salada de Chiprana, Laguna del Camino de Villafranca, Laguna de Pétrola and Laguna de Tírez. We followed the procedure in Gómez & Carvalho (2000) for the isolation of eggs, which were transferred individually to wells (Nunc™ polystyrene 96-well plates) containing 200 μl of 6 g l^{-1} artificial sea water (Instant Ocean™, Aquarium Systems). Hatching conditions were: temperature, 23 °C and light, approximately 150–170 μE m^{-2} s^{-1}. Once hatchlings reproduced parthenogenetically, the morphotypes were identified visually and only L-morphotype clones (Fu et al., 1991a) were selected. These were: ACHI1, ACHI2, ACHI3 and ACHI4 from Salada de Chiprana, ACVF1 and ACVF2 from Laguna del Camino de Villafranca, APET1 from Laguna de Pétrola and ATIR1, ATIR2 and ATIR3 from Tírez (for pond location see Ortells et al., 2000; Gómez et al., 2002). Clones were kept at 19 °C, 12 g l^{-1} of artificial sea water and fed the alga *Tetraselmis suecica*.

Clone identification and selection

The clones were tentatively identified as *B. plicatilis* s.s. or *B.* 'Manjavacas' by allozyme analysis using the loci *pgi*, *pgm*, *mdh-1* and *mdh-2* according to Ortells et al. (2000). For definitive identification, a restriction analysis on the mitochondrial gene COI was carried out on a subset of clones. Analysis of 112 COI sequences of *B. plicatilis* s.s. and *B.* 'Manjavacas' available at GenBank, after the extensive sequencing work by Dr. África Gómez and coworkers, revealed the presence of a *Kpn*I restriction site exclusive to *B. plicatilis* s.s. sequences and a *Pvu*II site only present in *B.* 'Manjavacas'. These two restriction enzymes were, therefore, chosen for species discrimination. For restriction analysis, rotifer DNA extraction was made using Chelex (6% Instagene™ Matrix, BioRad Laboratories; see Gómez et al., 1998). COI was amplified through Polymerase Chain Reaction using the following primers: COIdgF (5′-ggWATYTgAgCWggKCTYATTgg-3′) and COIdgR (5′-ggRTTACCTCCRCCKgCYggRTC-3′). These primers were designed based on the sequences available at GenBank for *B. plicatilis* s.s. and *B.* 'Manjavacas'. PCR was performed in vials containing 3 μl of template DNA, 0.2 mM of each nucleotide, 0.6 μM of each primer and 1.5 U of Taq-polymerase (Amersham Pharmacia Biotech). A Mastercycler™ (Eppendorf) was used for PCR using the following cycling profile: 3 min at 94 °C; 40 cycles of 30 s at 94 °C, 1 min at 48 °C and 1 min at 72 °C; a final step of 7 min at 72 °C was included after cycling. Restriction analysis for *Kpn*I and *Pvu*II was then performed using 15–20 ng of amplified DNA and 5–10 units of the corresponding enzyme in a final volume of 20 μl, and incubated at 37 °C for at least 1 h. Products were separated by standard agarose gel electrophoresis using 1.4% w/v agarose in 0.5 × TBE buffer. Gels were stained with ethidium bromide (0.1 μg ml^{-1}) and exposed to a UV transilluminator for direct DNA fragment visualization.

Morphometric analysis

Eight selected clones based on allozyme and DNA analysis were studied morphometrically. Two replicates per clone were established and grown independently in 250 ml of 12 g l^{-1} artificial sea water at 23 °C and constant illumination (ca. 100 μE m^{-2} s^{-1}) and fed 10^6 cells ml^{-1} of *Tetraselmis suecica*. Twice a week, a fraction of the rotifer culture was replaced with fresh medium (dilution rate: 0.77 $week^{-1}$). *Tetraselmis suecica* provided as food was grown in 22 g l^{-1} artificial sea water at 19 °C and constant illumination (ca. 100 μE m^{-2} s^{-1}) in a semicontinuous culture system (dilution rate: 0.65 day^{-1}). Before using them for rotifer feeding, the algal cells were pelleted by centrifugation (5 min at 3000 rpm), the supernatant was removed and the algal pellet was resuspended in 12 g l^{-1} artificial sea water.

From each replicate rotifer culture, 120 egg-bearing females were transferred to 1.5-ml wells (10 females per well) with 12 g l^{-1} artificial sea water and standard food conditions, and kept at 23 °C. After 5 h, at least 20 newborns per replicate were individually transferred to wells containing fresh medium and cultures as before. After 48 h, individuals were fixed with 4% formaldehyde and 10 females (age: 48–53 h) per replicate were randomly selected for morphometric measurements.

Nine morphometric characters were measured (Fig. 1). Morphometric characters (a–c) and (h) were measured at 400× magnification and (d–g) and (i) at 1000× magnification using a Nikon YS2 microscope. Morphometric characters (a–g) were selected based on Fu et al. (1991a) and (h–i) on Ciros-Pérez et al. (2001). For the statistical analysis of the differences ANOVA, MANOVA and a discriminant analysis were performed using SPSS (release 11.5. SPSS Inc., Chicago, IL).

Results

Species identification based on molecular markers

A restriction analysis of the COI gene was performed for definitive identification of 8 clones isolated from different sites. These clones had been tentatively identified by applying allozyme analysis to 15 L-morphotype clones (data not shown). Results of the digestion with *Kpn*I and *Pvu*II are shown in Figure 2. This restriction analysis allowed us to identify clones ATIR1, ACVF2 and L5MAN as *B.* 'Manjavacas' and GALL1, APET1, ACHI1, L3 and ACHI2 as *B. plicatilis* s.s. These clones were used for morphometric study.

Morphometric analysis

The average morphometric values for 5 *B. plicatilis* s.s. and 3 *B.* 'Manjavacas' clones are shown in

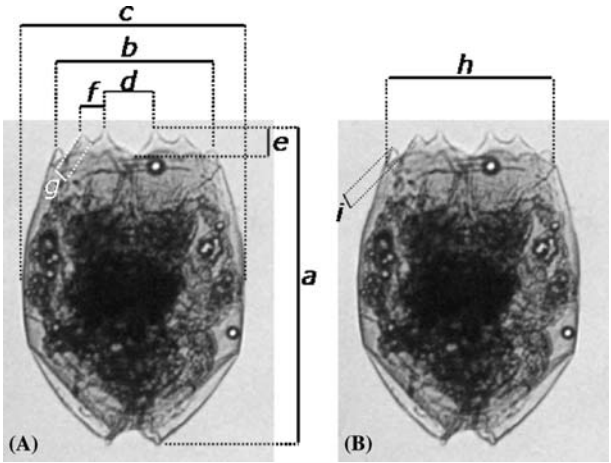

Figure 1. Morphometric characters of the *Brachionus* lorica measured. (A) Characters selected based on Fu et al. (1991a). (B) Characters selected based on Ciros-Pérez et al. (2001). (a) lorica length; (b) distance between lateral spines; (c) lorica width; (d) distance between central spines; (e) dorsal sinus depth; (f) distance between central and medial spines; (g) medial spine length; (h) head aperture; (i) lateral spine length.

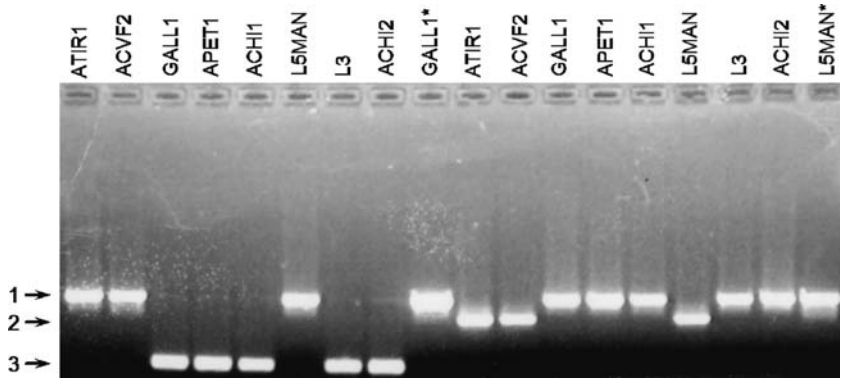

Figure 2. Restriction analysis of COI for eight clones belonging to *B. plicatilis* s.s. or *B.* 'Manjavacas'. A band located at (1) means that the enzyme had not a target; a band at (2) was found when *Pvu*II had a target (*B.* 'Manjavacas'); a band at (3) was found when *Kpn*I had a target (*B. plicatilis* s.s.). *Non-digested DNA.

Table 1. *B. plicatilis* s.s. is larger than *B.* 'Manjavacas' for all the measured lorica traits. The former species is about 6% larger based on major body measurements. However, only three of the nine measured characters (e), (f) and (i) showed significant statistical differences in characters relating to spine morphology, especially the lateral spine length [(i); Fig. 3 and Table 1].

A step-wise discriminant analysis with an associated one-way MANOVA ($F3,139 = 49.67$; $p < 0.01$) selected morphometric characters (e), (f) and (i) as being informative for species morphology discriminations. Thus, each one of these three measurements significantly improves the morphometric discrimination. The step-wise discriminant analysis combined the three significant measurements in a single discriminant axis, which is a new combined morphometric variable allowing for the best discrimination. When the distribution of individuals for this combined variable is plotted (Fig. 4), it is possible to observe a clear differential distribution of the species *B. plicatilis* s.s. and *B.* 'Manjavacas'. However, they extensively overlap in their distributions.

Discussion

The first evidence suggesting that *B. plicatilis* is actually a complex of sibling species comes from morphological data (Fu et al., 1991a) that distinguishes two morphotypes (L and S). According to

Table 1. Means and standard errors (S.E.) (in μm) of the morphometric characters for *B. plicatilis* s.s. and *B.* 'Manjavacas'

Morphometric character	*B. plicatilis* s.s.			*B.* 'Manjavacas'			ANOVA
	Mean	S.E.	*n*	Mean	S.E.	*n*	*p*
(a) Lorica length	385.7	2.8	100	360.7	3.6	60	0.118
(b) Distance between lateral spines	136.0	1.3	99	126.5	1.7	60	0.219
(c) Lorica width	298.2	1.9	100	280.8	2.8	59	0.080
(d) Distance between central spines	30.7	1.0	97	26.5	1.1	55	0.255
(e) Dorsal sinus depth	38.5	0.5	97	31.8	0.4	58	0.011*
(f) Distance between central and medial spines	35.3	0.4	100	31.1	0.5	60	0.021*
(g) Medial spine length	17.6	0.3	97	15.9	0.4	60	0.051
(h) Head aperture	161.3	1.2	100	152.8	1.1	60	0.171
(i) Lateral spine length	18.9	0.4	97	15.2	0.4	59	0.001**

p-values for between-species differences were computed from a four-level (species, clones, replicate, individual or error) nested ANOVA on log-transformed morphometric character (letters in parenthesis refer to Fig. 1). *n*: sample size. *$p < 0.05$ without Dunn–Sidak correction for multiple comparisons. **$p < 0.05$ with Dunn–Sidak correction.

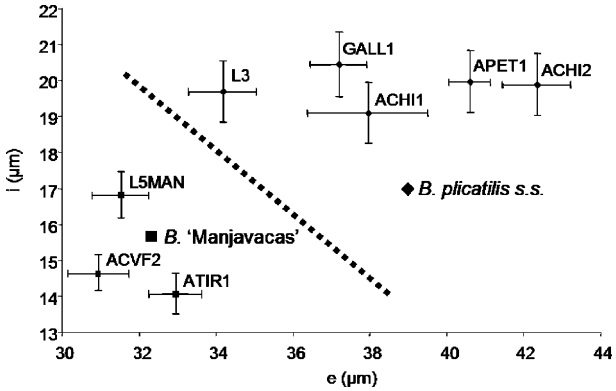

Figure 3. Mean and standard error of the morphometric characters (i) (lateral spine length) and (e) (dorsal sinus depth) for the eight clones. Characters were selected based on ANOVA results (Table 1).

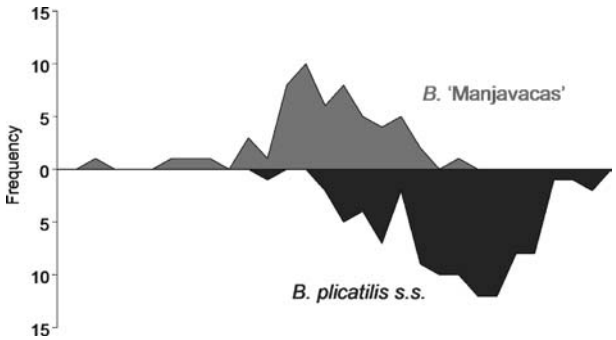

Figure 4. Distribution of individuals in the discriminant axis selected by a step-wise discriminant analysis on the morphometric characters. Characters (i), (e) and (f) were selected by the step-wise procedure.

phylogenetic analysis on DNA markers, the *B. plicatilis* complex is now recognized as a taxon composed of at least 14 species with deep genetic divergence between them (Gómez et al., 2002; Suatoni, 2003). The species are distinguished as three morphotypes: large (L), medium (SM), and small (SS) (Gomez & Serra, 1995; Ciros-Pérez et al., 2001; Suatoni, 2003), and each of these morphotypes corresponds to a major phylogenetic clade in the complex.

B. plicatilis s.s. and *B.* 'Manjavacas' both belong to L-morphotype. Maximum likelihood genetic distances between these two species, estimated on combined COI and ITS1 sequences available at GenBank using the GTR + I + G model, (Gómez et al., 2002), range from approximately 0.3 (strains 3MAN-L5 and AUSTRALIA1) to 0.4 (strains RUSSIA and 6TUR7). Mating behaviour suggests pre-zygotic reproductive isolation between the two species (Gómez & Snell, 1996; Ortells et al., 2000), and no evidence for hybridization in field studies exists, despite the fact that the species co-occur in some ponds in the Iberian Peninsula (Ortells et al., 2000). Our results show that *B. plicatilis* s.s. and *B.* 'Manjavacas' have different morphology, and that the former is on average larger. Traits related to spine morphology significantly differ. However, we do not rule out that there are minor size differences in other body characters that we did not study.

Our data provide additional support for considering *B. plicatilis* s.s. and *B.* 'Manjavacas' as different species, as well as show that if sibling species are first detected by either the molecular, morphological or mating behavioural methods, the divergence among species can be confirmed using the other approaches (Fu et al., 1991a, b; Gómez & Snell, 1996; Ortells et al., 2000; Gómez et al., 2002; Suatoni, 2003).

Phylogenetic analysis has recognized three major species clades in the *Brachionus plicatilis* species complex (Gómez et al., 2002). Each one of these clades corresponds to a clearly-defined different morphology related mainly to body size but also to spine shape (Fu et al., 1991a; Ciros-Pérez et al., 2001; Suatoni, 2003). We found that morphometric differences between these two relatively and closely related species in the complex, *B. plicatilis* s.s. and *B.* 'Manjavacas', although significant, are much smaller than those among the L-, SM- and SS-morphotypes. Therefore, phylogenetic divergence as inferred from molecular markers is correlated to morphological divergence in rotifer sibling species complexes when morphometry is studied in detail. However, the morphometric divergence found by us, though significant, is very small, which contrasts with the reported high genetic divergence based on molecular markers (0.3–0.4; Gómez et al., 2002). This genetic divergence suggests that the species are ancient (Gómez et al., 2002). Therefore, morphological stasis seems to be a characteristic in the evolution of these species. The causes of this stasis should form subject of future research.

The strains studied here were isolated in the Iberian Peninsula. So, they likely represent a small fraction of the diversity within *B. plicatilis* s.s. and *B.* 'Manjavacas'. On the other hand, as revealed by a discriminant analysis, these species show an extensive overlap in the measurements of lorica size and shape. Some individuals are less associated with the distribution of their own species than to the other species, and could be, therefore, easily misidentified based in their morphometric characters. This was observed despite our experimental design, which tended to minimize age and environmental effects. Therefore, we conclude that body measurements are unlikely to be reliable criteria for *B. plicatilis* species identification. As both species can co-occur, correct taxonomic classification is not possible, unless molecular analysis is performed.

Our study shows that deep genetic divergence is possible with high morphological stasis. Morphology is thought to be related to factors like predator vulnerability and feeding preferences. If morphology is similar, it is likely that these factors are also similar for both species. The striking morphological similarity between *B. plicatilis* s.s. and *B.* 'Manjavacas' prompts the question about how so similar species can coexist if differential predation or diet are unlikely to be mediating factors. We also do not know if these species respond differently to abiotic factors like temperature, salinity, pH and ionic composition. Moreover, it is not known whether co-occurrence of these two species in nature is transient and due to the length of the putative exclusion dynamics. Ecological studies and phylogeographic analysis on *B.* 'Manjavacas', similar to those performed on *B. plicatilis* s.s. (Gómez et al., 2000; Ortells et al., 2000; Suatoni, 2003) are needed to address these questions.

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