

Euryhaline *Brachionus* strains (Rotifera) from tropical habitats: morphology and allozyme patterns

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Abstract

The euryhaline rotifer *Brachionus* is a complex of sibling species. Although many investigations have been carried out in the past, the relationships among the Spanish species, the tropical SS strains and the clusters previously described, remained unknown. In this study, allozyme data for five populations from the tropics and two from Spanish lagoons – one of them *B. ibericus* and the other *B. rotundiformis* – were combined with data from the previous studies. Cluster analysis based on genetic distance allowed the 74 strains to be divided into two major groups. One group was associated with *B. plicatilis*-like strains, and the other group was associated with *B. rotundiformis* and *B. ibericus*. This latter group was divided into two clades. One of these clustered most with the S-morphotype strains and the *B. ibericus* species. The other clustered most closely with the tropical (SS) strains and the *B. rotundiformis* Spanish species. These results show a correspondence between the species description based on Spanish strains and the allozyme groups identified in a larger collection of strains.

Introduction

The concept of morphospecies dominated animal taxonomy during the 19th and early 20th centuries. However, as some species were morphologically difficult to distinguish, Mayr (1942) introduced the idea of sibling species. Sibling species are common in aquatic invertebrates, where species recognition is often chemical (Knowlton, 1993; Serra et al., 1998). The failure of investigators to recognize sibling species has led to confusion in the interpretation of ecological processes (Paterson, 1991; Knowlton, 1993; Knowlton & Jackson, 1994).

Since euryhaline rotifers of the genus *Brachionus* are an essential live food in finfish larviculture,

the correct determination of their taxonomy is important. Otherwise, their sibling species may confuse the determination of ecological tolerance limits and reproductive patterns. Initially, they were all classified into a single species, *B. plicatilis*. However, it was later discovered that *B. plicatilis* comprised two morphologically and ecologically distinct groups, one of which was called 'L-type' (large) and the other 'S- (small) type' (Oogami, 1976; Segers, 1995; Campillo et al., 2005). Oogami (1976) distinguished them by the shape of their lorica. Fu et al. (1991a, b) analyzed 67 *Brachionus* strains from a wide geographical area and found that these strains were morphologically and genetically divisible into two groups. In addition,

Rumengan et al. (1991) reported that the number of chromosomes differed between the S- and L-types. Moreover, it was shown that S- and L-types were reproductively isolated from each other (Fu et al., 1993; Gómez & Serra, 1995; Rico-Martínez & Snell, 1995; Gomez, 2005). As a result, Segers (1995) re-classified the S- and L-types as different species, and named the S-type *B. rotundiformis* (Tschugunoff, 1921) and the L-type *B. plicatilis* (O.F. Müller, 1786).

Some studies reported that some strains could not be easily classified as either *B. plicatilis* or *B. rotundiformis* (Carmona et al., 1995; Gómez et al., 1995; Gómez & Serra, 1995; Hagiwara et al., 1995). Rotifer strains in the lower size range of *B. rotundiformis* were discovered in tropical regions and were designated SS-type by aquaculturists because of their small size (Hagiwara et al., 1995). Hagiwara et al. (1995) suggested that SS-type rotifers belonged to *B. rotundiformis* because they were morphologically, ecologically and genetically similar and did not have any pre-mating reproductive isolation.

Three morphologically and genetically distinct types of euryhaline rotifers of the species *Brachionus* were also reported from a pond in Torreblanca Marsh in Castellon, Spain (Carmona et al., 1995; Gómez et al., 1995; Gómez & Serra, 1995). The two smaller strains (SM and SS) fitted within the morphospecies *B. rotundiformis*, but, in further studies, they differed morphologically and genetically, and were reproductively isolated from each other (Carmona et al., 1995; Gómez et al., 1995; Gómez & Serra, 1995; Ortells et al., 2000). It was proposed that these two strains were different species (Gómez et al., 1995; Serra et al., 1998). Ciro-Pérez et al. (2001) created a new species, *B. ibericus*, from the SM strain and retained the name *B. rotundiformis* for the SS strains.

The Spanish and newly discovered tropical strains were investigated for morphological and genetic characteristics (Hagiwara et al., 1995), and their genetic similarity was explored using allozyme markers (Ortells et al., 2000). However, the relationships among the Spanish- and tropical-strains and the 67 strains of Fu et al. (1991a, b) remained unclear. This study examines the relatedness of the small tropical rotifers and the Spanish strains by comparing their allozyme patterns at six loci.

Materials and methods

Isozyme analysis was performed on three strains of S morphotype *Brachionus*. These were collected from brackish ponds in Torreblanca Marsh, Spain (*B. rotundiformis* SS2, Gómez et al., 1995; Gómez & Serra, 1995), on the island of Langkawi, Malaysia, and on the island of Bali, Indonesia. The results were analyzed by cluster analysis, together with the allozyme patterns of 67 strains of Fu et al. (1991b), strains from Fiji and Thailand (Hagiwara et al., 1995; Kotani et al., 1997), a strain from Okinawa, Japan (Hagiwara et al., 1995), and *B. ibericus* strain SM1 (Kotani et al., 1997).

Allozyme analysis

Methods for analyzing *Brachionus* allozymes were described by Fu et al. (1991b). Rotifers were cultured under controlled conditions at 25 °C, in 22‰ diluted seawater in 5-l plastic beakers and fed with *Nannochloropsis oculata*. Each culture was started with an initial density of 1 ind ml⁻¹. Rotifer populations were harvested when they reached high densities (~200–300 ind ml⁻¹). To avoid contamination of rotifer enzymes with those of the food organisms, the rotifers were starved for 1 day before harvesting. The animals were filtered with a 43-µm mesh plankton net, and washed several times with clean diluted seawater. Then the rotifer samples were frozen immediately, and stored at –80 °C until they were used in electrophoresis.

The rotifer samples were thawed to provide a crude extract of enzymes just prior to electrophoresis. Approximately 5-µl of rotifer extract was loaded into each lane of a 12% horizontal starch gel using a filter paper (4 × 4 mm). Electrophoresis was carried out for 5 h at a constant current of 3.3 mA cm⁻² of cross-section in a refrigerator at 5 °C. Six enzymes including LDH, MDH, 6PGD, SOD, PGM and GPI were characterized using the staining procedure described by Fu et al. (1991b). The buffer system for electrophoresis was 0.04 M citric acid, pH 6.9 (adjusted with *N*-(3-aminopropyl)morpholine and NaOH) for analyzing LDH, MDH, 6PGD, SOD and PGM, and 0.04 M citric acid, pH 8 (adjusted with Tris-(hydroxymethyl)methylamine) for GPI. As in the study of Fu et al. (1991b), genetic distance was calculated for six loci based on Rogers (1972). A dendrogram was

constructed using the UPGMA procedure. The cluster analysis was conducted using MEGA version 2.1 (Kumar et al., 2001).

Morphological analysis

We compared the morphology of the groups of strains using data from Fu et al. (1991a) and Hagiwara et al. (1995). Seven characters were determined to obtain lorica length (A), lorica shape (C/A and B/C), and shape of anterior spines (E/D and G/F) (Fig. 1). For each strain, the values of seven characters were determined for 20 individuals. A Mann–Whitney's *U* test was performed to compare each index among groups and a scatter graph of A against B was plotted for each strain. Since there were no morphological data for the Indonesia and Malaysian strains, these were not included in the analysis. The two Spanish strains of Ciro-Pérez et al. (2001) were also excluded from the analysis because, although there are some biometrical data in the paper, no raw data are given.

Results

Genotypes for each locus of the strains not described in Fu et al. (1991b) are indicated in Table 1. The dendrogram produced from the data of Fu et al. (1991b) and the data in Table 1 is indicated in Fig. 2. This dendrogram divides the strains into two groups. Group A is consistent with the L morphotype of *B. plicatilis*, and group B is consistent with the S morphotype of the *Brachionus* strains. The genetic distance between group A and group B was 0.349. Group B was subdivided further into two groups (C and D). Group C included the Japanese and *B. ibericus* SM1 strains, whereas group D included the Fiji, Thai, Malaysian, Indonesian and *B. rotundiformis* SS2 strains. The genetic distance between group C and D was 0.330.

The result of the morphological comparison between group C and group D is indicated in Table 2. There were significant differences in each index. This suggests that the lorica of group D is smaller (A) and rounder (C/A) than that of group C, the top opening of the lorica of group D is much narrower than that of group C (B/D),

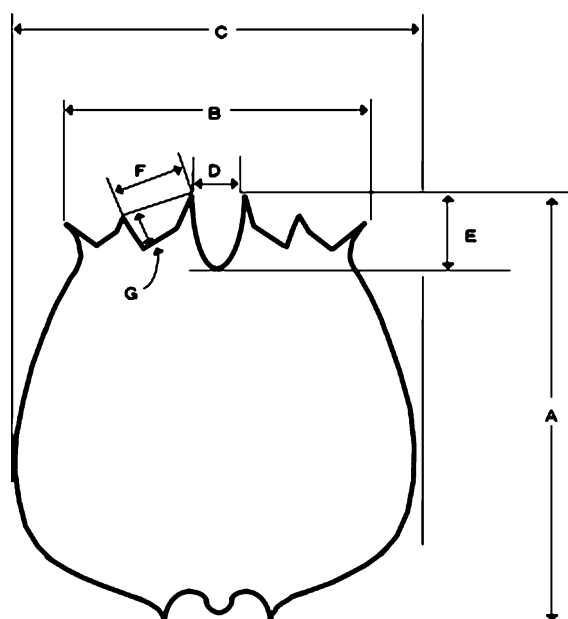


Figure 1. Seven characteristics (A–G) of the lorica of the euryhaline *Brachionus*, as measured by Fu et al. (1991a).

Table 1. Genetic constitution of seven strains detected from electrophoretical analysis of the following six enzymes: lactate dehydrogenase (LDH, EC 1.1.1.27), malate dehydrogenase (MDH, EC 1.1.1.37), 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44), superoxide dismutase (SOD, EC 1.15.1.1), phosphoglucomutase (PGM, EC 2.7.5.1) and glucose phosphate isomerase (GPI, EC 2.6.1.1). Symbols of alleles were based on Fu et al. (1991b). Alleles estimated at each locus were named alphabetically according to their mobility. The alphabetical order followed Fu et al. (1991b)

Strain	Locus and genotype					
	LDH	MDH-1	6PGD	SOD	PGM	GPI
SS2	HH	BB	BB	CC	DD	HH
Indonesia	HH	BB	FF	CC	DD	HH
Malaysia	HH	BB	FF	CC	DD	HH
Fiji ^{a, b}	HH	BB	DD	CC	GG	FF
Thailand ^{a, b}	HH	BB	DD	CC	GG	FF
Japan ^a	FF	AA	IL	CC	FF	BF
SM1 ^b	CC	AA	BB	BB	AA	CC

^a Hagiwara et al. (1995), ^b Kotani et al. (1997).

and that the anterior spine of group D is sharper than that of group C (E/D and G/F). Also, in the scatter graph, group D was more likely to be found in the region of smaller size (Fig. 3).

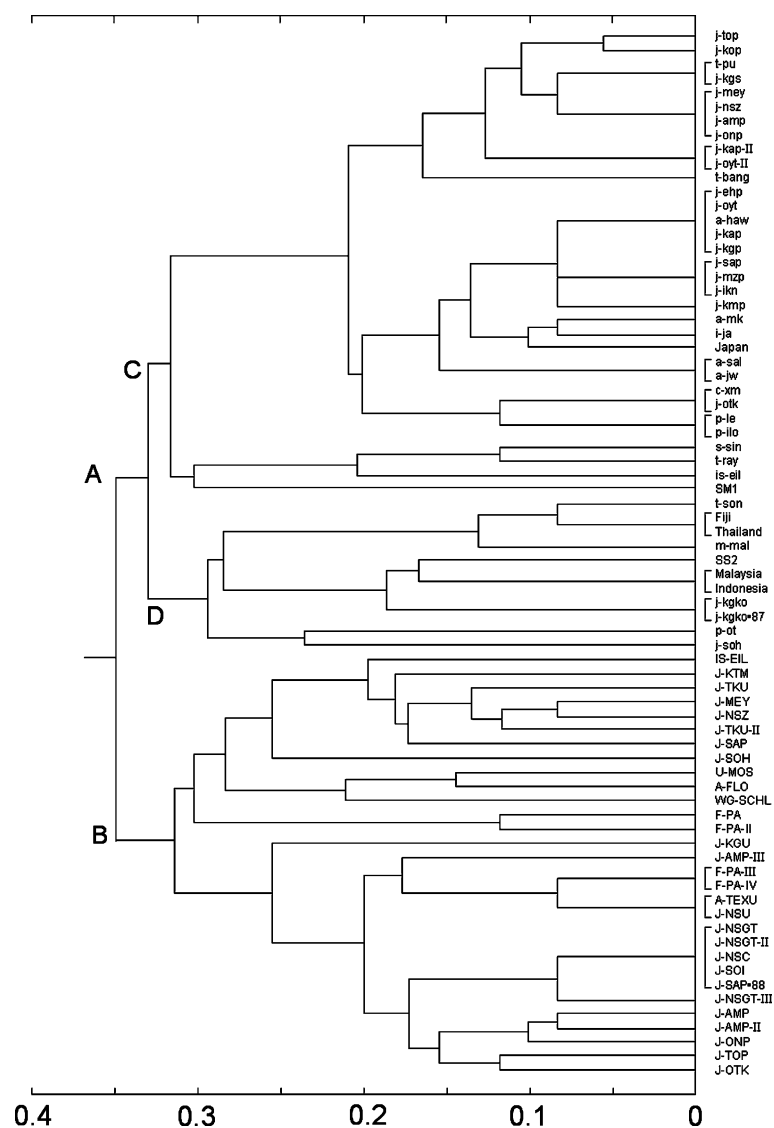


Figure 2. Dendrogram for 74 strains according to a genetic distance index. The dendrogram is the result of the UPGMA analysis of Rogers' genetic distance (1972). Lower case letters indicate the S morphotype; capital letters indicate the L morphotype.

Table 2. Mean and SD values of five variables for group C and D

Variable	Group C ($n = 32 \times 20$)		Group D ($n = 8 \times 20$)		Mann-Whitney's U test		< 0.01
	Mean	SD	Mean	SD	U value	p value	
A (μm)		214.9	19.1	189.3	13.2	14075.0	
C/A	0.805	0.035	0.841	0.035	23863.0	< 0.01	
B/C	0.627	0.038	0.578	0.028	14468.5	< 0.01	
E/D	1.063	0.226	1.000	0.213	44584.0	< 0.05	
G/F	0.552	0.120	0.675	0.096	18288.5	< 0.01	

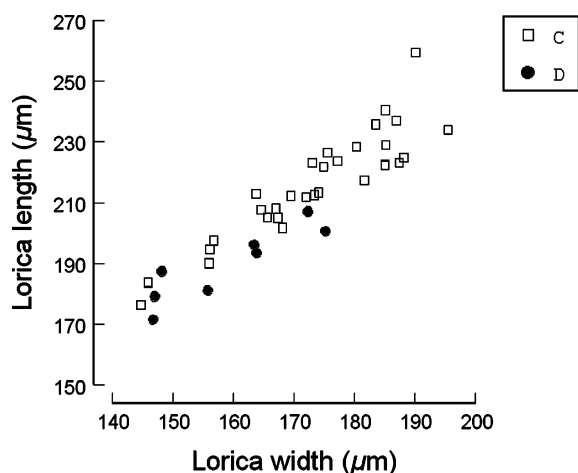


Figure 3. Scatter diagram of lorica length and width in relation to the groups identified by the cluster analysis. The open squares indicate the strains in group C and the solid circles indicate the strains in group D.

Discussion

Our study incorporated new strains into the previous set studied genetically and morphologically by Fu et al. (1991a, b), where euryhaline *Brachionus* was classified into two clades. In the genetic classification of Fu et al. (1991b), S morphotype strains formed two groups, one of them including six strains from tropical regions (t-son, Thailand; m-mal, Malaysia; j-kgko, j-kgko-87, Koshiki islands, Japan; p-ot, Philippines; and j-soh, Hamanako-lake, Japan). In our analysis, these tropical strains clustered into group D (Fig. 2), together with the four small rotifer strains discovered in the tropical regions (Fiji, Thailand, Indonesia and Malaysia) and the Spanish strain *B. rotundiformis* SS2. The latter five strains were newly added from our analysis. The other newly added strains (Japanese strain and *B. ibericus* SM1 strain) also clustered into group C (Fig. 2). The morphological classification of Fu et al. (1991a) divided S morphotype strains into two subgroups, but the morphological division was not consistent with the genetic grouping (See Fu et al., 1991a, b).

Since the results of Fu et al. (1991a, b), a number of analyses using a variety of approaches have been performed on the same and related strains of *Brachionus* for understanding their phylogeny. These studies provide support for both the similarity among strains belonging to the

morphotype S (i.e. our groups C and D) and for the differentiation of clades within the morphotype S. An example is Rumengan's (1990) karyotype analysis on S morphotype strains that did not find differentiation in chromosome number among j-kgko, a-haw (Hawaii, USA) and j-onp (Okinawa, Japan), despite the former belonging to group D and the two latter to group C (Fig. 2). Some mating and copulation have been observed between strains belonging or related to groups C and D. Males of Koshiki (group D, same as j-kgko) strain copulated with the females of the Hawaii strain (group C, Rico-Martínez & Snell, 1995). The *B. ibericus* SM1 (group C) copulated with Fiji, Koshiki and *B. rotundiformis* SS strains (group D) (Gómez & Snell, 1996; Kotani et al., 1997, 2001). Moreover, Kotani et al. (1997, 2001) investigated the differences in the mate recognition pheromone (MRP) among various strains using the antibody of MRP of L and S morphotype *Brachionus*. They did not find differences among S morphotype *Brachionus*, although they studied strains related to both our groups C and D (tropical strains).

Evidence for the differences between groups C and D comes mainly from studies on the Spanish strains. Spanish sympatric *B. rotundiformis* (group D) and *B. ibericus* (group C) were reported to be reproductively isolated in the field, ecologically specialized, biometrically different, possessing strong homotypic mating preferences, and showing no hybridization in the laboratory (Gómez et al., 1995; Gómez & Serra, 1995; Serra et al., 1998; Ciro-Pérez et al., 2001). Molecular phylogenies of COI and ITS genes have shown deep genetic divergence between Spanish strains of *B. rotundiformis* and *B. ibericus* (Gómez et al., 2000). In addition, working with non-Spanish strains, Boehm et al. (2000) found genetic differences between S morphotype *Brachionus* strains and the tropical strains from the analysis of microsatellite DNA sequences. Genetic cohesion of group D is supported by (1) the genetic similarity between tropical small strains (Thailand and Fiji) and the Koshiki strain (Boehm et al., 2000), and (2) the lack of pre- or post-zygotic reproductive isolation (strains j-kgko-89, j-kgko-90, j-soh and m-mal; Fu et al., 1993).

After Segers (1995) re-classified euryhaline *Brachionus*, classification of the tropical strains was attempted by Hagiwara et al. (1995). They

concluded that the tropical strains belonged to S morphotype *Brachionus* because their morphological, ecological, and genetic characters were more similar to S morphotype *Brachionus* than L morphotype *Brachionus*. They were also reproductively isolated from L morphotype *Brachionus* strains, but pre-zygotic isolation with other S morphotype *Brachionus* was incomplete. In this study, we were able to discriminate group C from D in the S morphotypes using cluster analysis of allozyme variation (Fig. 2). When combined with previous findings, our results show that S-morphotype strains tend to group together, and separately from L-morphotype strains. However, there is strong differentiation among S-morphotype strains, with the small strains, usually the tropical ones, being genetically differentiated into group D (*B. rotundiformis*). Despite their size differences, we found an extensive overlap in the lorica size of both groups C and D (Figure 3). Consequently, it is difficult to classify groups C and D with only biometrical methods. Our results emphasize the importance of using morphological, behavioral, and molecular approaches for resolving boundaries species (Knowlton, 2000).

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