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SPECIATION IN ANCIENT CRYPTIC SPECIES COMPLEXES: EVIDENCE FROM THE MOLECULAR PHYLOGENY OF *BRACHIONUS PLICATILIS* (ROTIFERA)

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Abstract.—Continental lake-dwelling zooplanktonic organisms have long been considered cosmopolitan species with little geographic variation in spite of the isolation of their habitats. Evidence of morphological cohesiveness and high dispersal capabilities support this interpretation. However, this view has been challenged recently as many such species have been shown either to comprise cryptic species complexes or to exhibit marked population genetic differentiation and strong phylogeographic structuring at a regional scale. Here we investigate the molecular phylogeny of the cosmopolitan passively dispersing rotifer *Brachionus plicatilis* (Rotifera: Monogononta) species complex using nucleotide sequence variation from both nuclear (ribosomal internal transcribed spacer 1, ITS1) and mitochondrial (cytochrome *c* oxidase subunit I, COI) genes. Analysis of rotifer resting eggs from 27 salt lakes in the Iberian Peninsula plus lakes from four continents revealed nine genetically divergent lineages. The high level of sequence divergence, absence of hybridization, and extensive sympatry observed support the specific status of these lineages. Sequence divergence estimates indicate that the *B. plicatilis* complex began diversifying many millions of years ago, yet has showed relatively high levels of morphological stasis. We discuss these results in relation to the ecology and genetics of aquatic invertebrates possessing dispersive resting propagules and address the apparent contradiction between zooplanktonic population structure and their morphological stasis.

Key words.—Cytochrome oxidase I, Internal transcribed spacer 1, mitochondrial DNA, passive dispersal, Rotifera, sibling species, zooplankton.

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Charles Darwin (1859) first pointed out the surprisingly wide geographical distribution of freshwater taxa in spite of the isolation of their habitats. He stressed that the distinctive dispersal of these organisms, that is, passive transport of resting stages through animal vectors such as waterfowl, was likely to permit long-distance dispersal across continents. This view remained almost unchallenged until the late twentieth century, when the introduction of molecular tools revolutionized our views of aquatic invertebrate taxa (see review in De Meester et al. 2002). Many species traditionally seen as cosmopolitan are now recognized as cryptic species assemblages of regionally more restricted taxa, both in the marine and continental realm (Palumbi 1992; Knowlton 1993, 2000; King and Hanner 1998; Taylor et al. 1998; Witt and Hebert 2000). This biodiversity had hitherto remained undetected by traditional taxonomical methods due to a dearth of morphological characters of taxonomic utility and the frequent confounding effects of high phenotypic plasticity or hybridization (Colbourne et al. 1997; Serra et al. 1997; Hebert 1998). However, little attention has been paid to investigating the evolutionary rates and mechanisms of diversification in cryptic species complexes of passively dispersing aquatic invertebrates. To gain a better understanding of the genetic diversification and speciation of these taxa, we need a better knowledge of their phylogenetic patterns, the time frame of their diversification, and data on their biogeography and degree of sympatry.

One taxon in which the presence of cryptic species has been recently documented is the monogonont rotifer, *Brachionus plicatilis* (Gómez and Snell 1996; Serra et al. 1997). Rotifers constitute a relatively small metazoan phylum of about 2000 described species. The class Monogononta, which encompasses most species in the phylum, comprises cyclically parthenogenetic organisms, mainly planktonic suspension feeders, globally widespread in continental aquatic systems. *Brachionus plicatilis* is cosmopolitan and found typically in salt lakes and coastal lagoons. It is the only rotifer with commercial and applied importance through its use as live food for marine fish fry (Lubzens 1987; Lubzens et al. 2001) and in ecotoxicology assessments (Snell and Persoone 1989; Moffat and Snell 1995; Del Valls et al. 1997). In addition, due to the ease with which it can be cultured in the laboratory and its short generation time, *B. plicatilis* has been the subject of much basic research in ecology and physiology, from sex allocation theory to the evolution of aging (e.g., Snell and Hawkinson 1983; Carmona et al. 1989; Aparici et al. 1998). More recently attention has been paid to its population genetic structure and phylogeography (Gómez et al. 1995, 2000; Gómez and Carvalho 2000). Despite its commercial and scientific value, the taxonomic status of *B. plicatilis* remains controversial. Fu et al. (1991a,b) found high morphological and allozyme variation in aquaculture strains and suggested the division of *B. plicatilis* into two groups differing in body size, although the high genetic variation within each group was not addressed. Additional evidence, including karyotype and mating behavior patterns, resulted in the division of *B. plicatilis* into two species (Segers 1995). More recently, however, it has become clear that this division does not fully describe the biological diversity within this group (Gómez and Snell 1996; Serra et al. 1998). A series of studies established the occurrence of three cryptic species (L, SM, and SS) in a single coastal lagoon in Spain. These

three species show morphological (e.g., they rank in size, L being the largest and SS the smallest) and ecological differences and species-specific mating behavior patterns (Gómez et al. 1995; Carmona et al. 1995; Serra et al. 1998), and they have been recently described or redescribed as *B. plicatilis* sensu stricto, *B. rotundiformis*, and the new species *B. ibericus* (Ciros-Pérez et al. 2001a). These three species are more or less widely distributed in coastal lagoons and inland salt lakes in the Iberian Peninsula (Ortells et al. 2000). However, data from mating behavior and allozyme surveys suggested the occurrence of additional species in the complex (Ortells et al. 2000).

Rotifer populations are often seasonal or ephemeral, and sympatric species can be involved in seasonal succession (Gómez et al. 1995). Under adverse environmental conditions, sexual reproduction is induced and sexual resting eggs produced. These so-called resting eggs are actually dormant embryos and are able to withstand desiccation and remain in the habitat sediments until favorable conditions arise. Resting egg banks comprise the total population gene pool when species are absent from the water column and can attain very high densities (Snell et al. 1983; Hairston 1996). In addition, resting eggs are the main dispersal stage in rotifer species. For these reasons, sampling of resting egg banks in wild populations has proven a reliable strategy to maximize survey success and to reduce the impact of seasonal variations in species occurrence (May 1986; Gómez et al. 2000; Ortells et al. 2000).

The current view is therefore that the *B. plicatilis* sensu lato (from here on ''*B. plicatilis* complex'') is a cryptic species complex containing a still-undetermined number of species. Such taxonomic uncertainty constrains the further understanding of rotifer ecological diversification and speciation patterns and processes and hampers applied research in these organisms. To date, no attempt has been made to unravel the phylogenetic relationships and temporal scale of diversification of this species complex or, to our knowledge, of any other in the phylum Rotifera. An initial objective here was to develop a robust phylogenetic framework for the *B. plicatilis* complex. We used an extensive set of samples from saline ponds and lakes in the Iberian Peninsula and approached the collection of specimens using resting egg banks. Sediment samples from non-Iberian locations from four continents were also included in addition to laboratory reference clones representative of presumptive species and for which diagnostic allozyme patterns are known. Two genes, one mitochondrial, the cytochrome *c* oxidase I (COI), and one nuclear, the ribosomal internal transcribed spacer 1 (ITS1), were sequenced. Both genes have proven useful for addressing phylogenetic questions in a wide range of taxa, and their joint use to test phylogenetic hypotheses increases the confidence and power of inferences.

In addition, we attempt to estimate the divergence times to test if the existence of cryptic species is an indication of high speciation rates or suggests high morphological stasis. Finally, we try to integrate the results obtained with salient ecological and genetic features of such passively dispersing invertebrate taxa to understand their genetic diversification and speciation.

Materials and Methods

Sample Collection and DNA Extractions

Sediment samples were collected from 48 salt and brackish ponds, lakes, and lagoons in the Iberian Peninsula and six non-Iberian locations during 1998, 1999, and 2000 (Table 1). Samples were collected from the deepest part of each pond, thus maximizing the inclusion of resting eggs produced under a variety of salinity and depth conditions (lake shores will be enriched in eggs produced at low salinity conditions, when the lake level is higher). The samples were stored in cool, dark conditions until they were processed. In addition, 18 reference clones with known allozyme profiles including presumptive species were obtained from laboratory cultures maintained in several laboratories and aquaculture centers (see Table 2).

Resting eggs were isolated from sediments using a sugar flotation technique (Marcus et al. 1994; Gómez and Carvalho 2000). Representatives of all egg morphologies that could be ascribed to the genus *Brachionus* were isolated from each sample. A fraction of eggs from each sample was hatched in 6 g/L salinity water to confirm their membership of the *B. plicatilis* species complex. A consistent method for total DNA extraction from single eggs and individual rotifers has been described in Gómez and Carvalho (2000). In brief, a single egg/rotifer was transferred to 45 μl of 6% Chelex 100 resin (BioRad, Hemel Hempstead, U.K.), crushed, and the mixture boiled for 10 min to release DNA. From the supernatant, 2 μl was used as DNA template for polymerase chain reactions (PCRs).

COI sequence data for the most common species of this complex in the Iberian Peninsula, *B. plicatilis* sensu stricto, were employed for a phylogeographic survey and are presented elsewhere (Gómez et al. 2000). Four representative COI haplotypes (H5, H11, H15, H16) of this species from Gómez et al. (2000) were included in the COI phylogeny (GenBank accession numbers AF266860, AF266872, AF266895, AF266896). Several individuals from *B. calyciflorus* and *B. quadridentatus* were sequenced and used as outgroups.

DNA Amplification and Sequencing

Sequences were obtained by cycle sequencing of PCR-amplified DNA. Due to the small amount of DNA present in rotifer resting eggs and to avoid contamination, the PCR setup was carried out in a separate containment laboratory where no amplifications had taken place. PCR reactions were performed in 10 μl final volume containing 2 μl template DNA, 1.5 mM $MgCl_2$, 200 μM of each nucleotide, 2.5 pmol of each primer, 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8 at 25°C), 0.01% Tween-20 buffer, and 0.125 U of *Taq* polymerase. Reactions were amplified using the following cycling conditions: one cycle of 3 min denaturing at 93°C; 40 cycles of 15 sec at 92°C, 20 sec at 50°C, 1 min at 70°C; one cycle of 3 min extension at 72°C. A 713-bp region of the COI gene was amplified with primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′; Folmer et al. 1994). The complete ITS1 was amplified using primers

TABLE 1. Details of the sampling sites. Acronyms of sample sites indicate the basin for the Iberian Peninsula samples (1, Duero; 2, Ebro; 3, Guadiana; 4, Júcar-Segura; 5, Guadalquivir; 6, coastal lagoons) and a three-letter code for the pond.

Code	Site	Location (lat. long.)
1ERA	Laguna de las Eras	41°10′N 4°35′W
2GAL	Laguna de Gallocanta	40°59′N 1°31′W
2SA2	Balsa de Santed II	41°01′N 1°30′W
2CHI	Salada de Chiprana	41°14′N 0°11′W
3CVF	Laguna del Camino de Villafranca	39°25′N 3°15′W
3MAN	Laguna del Manjavacas	39°25′N 2°53′W
3LON	Laguna del Longar de Lillo	39°42′N 3°19′W
3RET	Laguna del Retamar	39°26′N 2°58′W
4SLD	Laguna del Saladar	38°48′N 1°25′W
4MOJ	Laguna de Mojón Blanco	38°48′N 1°26′W
4PET	Laguna de Pétrola	38°50′N 1°34′W
4SAL	Laguna del Salobrejo	38°55′N 1°27′W
5TIS	Laguna de Tiscar	37°28′N 4°48′W
5CAP	Laguna de Capacete	37°01′N 4°51′W
5FUE	Laguna de Fuente de Piedra	37°06′N 4°45′W
6TUR	Estany de En Turies	42°15′N 3°06′E
6TOS	Poza Sur (Torreblanca Marsh)	40°10′N 0°10′E
6TON	Poza Norte (Torreblanca Marsh)	40°10′N 0°10′E
6ALM	Laguna de Almenara	39°45′N 0°11′E
6CLO	Clot de Galvany	38°16′N 0°31′W
6CAD	Charca Universidad de Cádiz	36°30′N 6°09′W
6POL	Albufera de Pollensa	39°55′N 3°04′E
Nevada	Little Fish Lake, Nevada (USA)	38°30′N 116°30′W
California	Salton Sea, California (USA)	33°20′N 110°40′W
Australia	Tower Hill, Victoria (Australia)	38°21′S 142°23′E
Cayman	Meagher Pond, Grand Cayman Island (USA)	19°18′N 81°19′W
Tunisia	Korba Sebkhet (Tunisia)	36°39′N 10°57′E
Wales	Kidwelly, Wales (UK)	51°43′N 4°20′W

III (5′-CACACCGCCCGTCGCTACTACCGATTG-3′) and VIII (5′-GTGCGTTCGAAGTGTCGATGATCAA-3′) from Palumbi (1996). The Cy5 end-labeled versions of the primers were used for cycle sequencing of the double-stranded PCR products using the Thermo Sequenase cycle sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Both strands were sequenced in all individuals on an ALFexpress (Amersham Pharmacia Biotech) automated sequencer. For COI, multiple sequences were aligned by eye; for ITS1, a variety of weighting levels and gap extension penalties (from one to 15 for both parameters) for multiple alignment were examined in CLUSTAL X. All polymorphic sites were double-checked manually.

All sequences and alignments (as popsets) were deposited in GenBank (accession numbers AF387189–AF387243 for ITS1 and AF387244–AF387296 for COI).

Phylogenetic Analysis

Phylogenetic analysis were implemented with PAUP* 4.0b4a (Swofford 1998) using neighbor-joining (NJ), maximum-parsimony (MP), and maximum-likelihood (ML) methods. For ML, the hierarchical likelihood-ratio test approach (Huelsenbeck and Crandall 1997) was used to select the model of DNA evolution that best fitted the data, as implemented in the program Modeltest 3.04 (Posada and Crandall 1998). Modeltest was also used to estimate the parameters of the model of evolution for input in PAUP*. Modeltest bases its calculations on an initial NJ tree derived from a Jukes-Cantor distance matrix. This step does not affect which model of evolution is finally selected (Posada and Crandall 2001). Because gaps are likely to contain important phylogenetic information, MP branch-and-bound searches were used both considering gaps as a fifth state and not considering gaps. We used parsimony default options for PAUP* using flat weighting (except for the combined gene analysis, see below). The PAUP* option pairwise deletion of gaps was used to obtain the distance matrix for NJ to try to preserve the phylogenetic information contained in the indels. For COI, due to the high number of taxa involved and the impossibility of obtaining a reasonable number of bootstraps, a NJ tree was obtained on a matrix of ML distances (calculated following the model found to be optimum by Modeltest, and using the parameters estimated by the program). MP with a variety of weighting schemes on codon positions (from 6:9: 1) was also tested. The taxa were added using the option furthest for MP (search and bound) in ITS1, and 100 random-order stepwise addition for MP (heuristic search) in COI. Heuristic searches were performed with TBR branch-swapping. Branches were collapsed if maximum length was zero. In all the trees presented here, confidence in established phylogenetic relationships was determined by 1000 bootstrap pseudoreplicates with the same optimality criterion used to build the tree but with no replicates of taxa addition. Polytomies were forced in the tree if bootstrap support was under 50%.

To examine the congruence between COI and ITS1, a partition homogeneity test (Farris et al. 1995) with 1000 replicates was performed using PAUP*, using the subset of taxa for which both genes had been sequenced. This allowed the inclusion within the dataset, 14 additional COI sequences from Gómez et al. (2000; accession numbers: AF266855, AF266858–266860, AF266863, AF266872, AF266895–

TABLE 2. Reference laboratory strains sequenced for *Brachionus* COI and ITS. For allozyme profiles of most of these strains see Ortells et al. (2000) and Gómez and Snell (1996). Seven microsatellite loci profiles for some of these strains are available from the authors. GenBank accession numbers are shown. Laboratory location: V, University of Valencia, Spain; A, Georgia Institute of Technology, Atlanta, USA; P, Port Erin Marine Laboratory, Isle of Man, UK.

Strain	Species/lineage	Geographic origin	Laboratory	ITS1	COI
6TON-SM6	*B. ibericus*	Poza Norte, Torreblanca Marsh (Spain)	V	AF387223	AF387270
6TOS-SS2	*B. rotudiformiss*	Poza Sur, Torreblanca Marsh (Spain)	V	AF387237	AF387287
6TOS-L4	*B. plicatilis* s.s.	Poza Sur, Torreblanca Marsh (Spain)	V	AF387189	AF266860
6ALM-SM5	*B. ibericus*	Almenara Pond (Spain)	V	AF387224	AF387271
6ALM-SM7	*B.* Almenara	Almenara Pond (Spain)	V	AF387221	AF387268
6ALM-SM32	*B.* Almenara	Almenara Pond (Spain)	V	AF387220	AF387269
6HON-SS	*B. rotudiformis*	Hondo Norte (Spain)	V	AF387238	AF387293
6HOS-L3	*B. plicatilis* s.s.	Hondo Sur (Spain)	V	AF387205	—
6HOS-SM19	*B.* Tiscar	Hondo Sur (Spain)	V	—	AF387282
6HOS-SM7	*B.* Tiscar	Hondo Sur (Spain)	V	AF387234	AF387283
3CVF-4	*B.* Tiscar	Camino de Villafranca (Spain)	V	AF387235	—
3MAN-L5	*B.* Manjavacas	Manjavaca (Spain)	V	AF387213	AF387257
4SAL-L5	*B.* Manjavacas	Salobrejo (Spain)	V	AF387204	—
6CAD-V	*B.* Manjavacas	Charca Universidad de Cádiz (Spain)	V	—	AF387258
Russia	*B.* Manjavacas	Sea of Azov (Russia)	A	AF387218	AF387250
Austria	*B.* Austria	Obere Halbjockchlacke (Austria)	A	AF387208	AF387248
China	*B.* Austria	Tianjin Commercial Salines (China)	A	AF387210	AF387249
Turkey	*B.* sp. Cayman	Unknown location in Turkey	P	AF387230	AF387290

266896, AF266906, AF266914, AF266927, AF266929, AF266942, AF266949), which had ITS1 counterparts in the alignment. A total of 38 different sequences corresponding to 41 individuals formed this dataset.

RESULTS

Of the 29 lakes containing *Brachionus* eggs (of 48 sampled in the Iberian Peninsula), 22 yielded eggs from the *B. plicatilis* complex (Table 1).

ITS Sequence Variation

The entire ITS1 was sequenced in 55 individuals from the *B. plicatilis* complex and two outgroup species. The 19 different sequence types obtained ranged between 314 bp for the reference clone 6TOS-L4 to 330 bp in the Cayman Islands (Caribbean Sea) isolate. ITS1 sequences were very A-T rich (70%), which is not common for ribosomal spacers, but it has also been found in *Drosophila* (Torres et al. 1990; Schlötterer et al. 1994).

After alignment with CLUSTAL X default parameters, pairwise sequence divergences (uncorrected *p*-values) among sequences (with pairwise deletion of gaps) ranged from 0% to 38% overall, with divergence within the *B. plicatilis* complex ranging from 0% to 20%. Alignment length was 354 bp, with 215 variable sites and 185 parsimony informative sites (not counting gaps). Twelve indels within the species complex had to be postulated for the alignment, most of them one or two base pairs long.

ITS1 Phylogeny

Because MP on the ITS1 region data produced a tree topology that did not change significantly across a range of gap weighting schemes in CLUSTAL X, the default options for multiple alignment were employed (gap opening penalty: 15.00, gap extension penalty: 6.66). When gaps were considered as an additional character in PAUP*, two most parsimonious tree were found with a length of 394 steps and a consistency index of 0.84. These trees differed only in the position of the sequence represented by 6TOS-SS2. High (>81%) bootstrap support was found for almost all nodes, but the position of 6TOS-SS2 was unresolved (Fig. 1) and left the relationships of the three main tree branches of the *B. plicatilis* complex as a polytomy. When gaps were excluded from the analysis, two most parsimonious trees were found (not shown), differing from the previous in the position of the sequence represented by 6TON-SM6, which appeared as the sister taxon to the other clades from branch B. The consistency index was 0.81 and the bootstrap supports were also high (>71%) for all nodes except for the position of 6TOS-SS2. Both ML, using the model chosen by Modeltest (TMV + G, transversional model) and the estimated parameters, and NJ using a variety of distance measures yielded trees that did not differ in the main from the topology showed in Figure 1.

Interestingly, the main branches of the ITS1 tree, A, B, C (Fig. 1), showed an association with the three described morphologies in the *B. plicatilis* species complex, L, SM, and SS morphotypes (Fu et al. 1991a; Gómez et al. 1995; Gómez and Snell 1996). Individuals from these three morphotypes differ in size, details of the spination pattern, and the position and number of resting eggs produced, but due to age-related variability and phenotypic plasticity, laboratory culture is often needed to confirm the identity of wild-caught strains. Therefore, and because no morphological analyses are available for the strains sampled as resting eggs, we have termed these main groups simply A, B, and C, and we describe the morphology of the strains or lineages belonging to each group, if known, below.

Group A contained a minimum of four well-supported lineages; with the ones studied displaying an L-like morphology (Gómez and Snell 1996). The clade represented by the reference clone 6TOS-L4 corresponds to *B. plicatilis* s.s. (Ciros-Pérez et al. 2001a). Two very similar ITS1 sequences (one

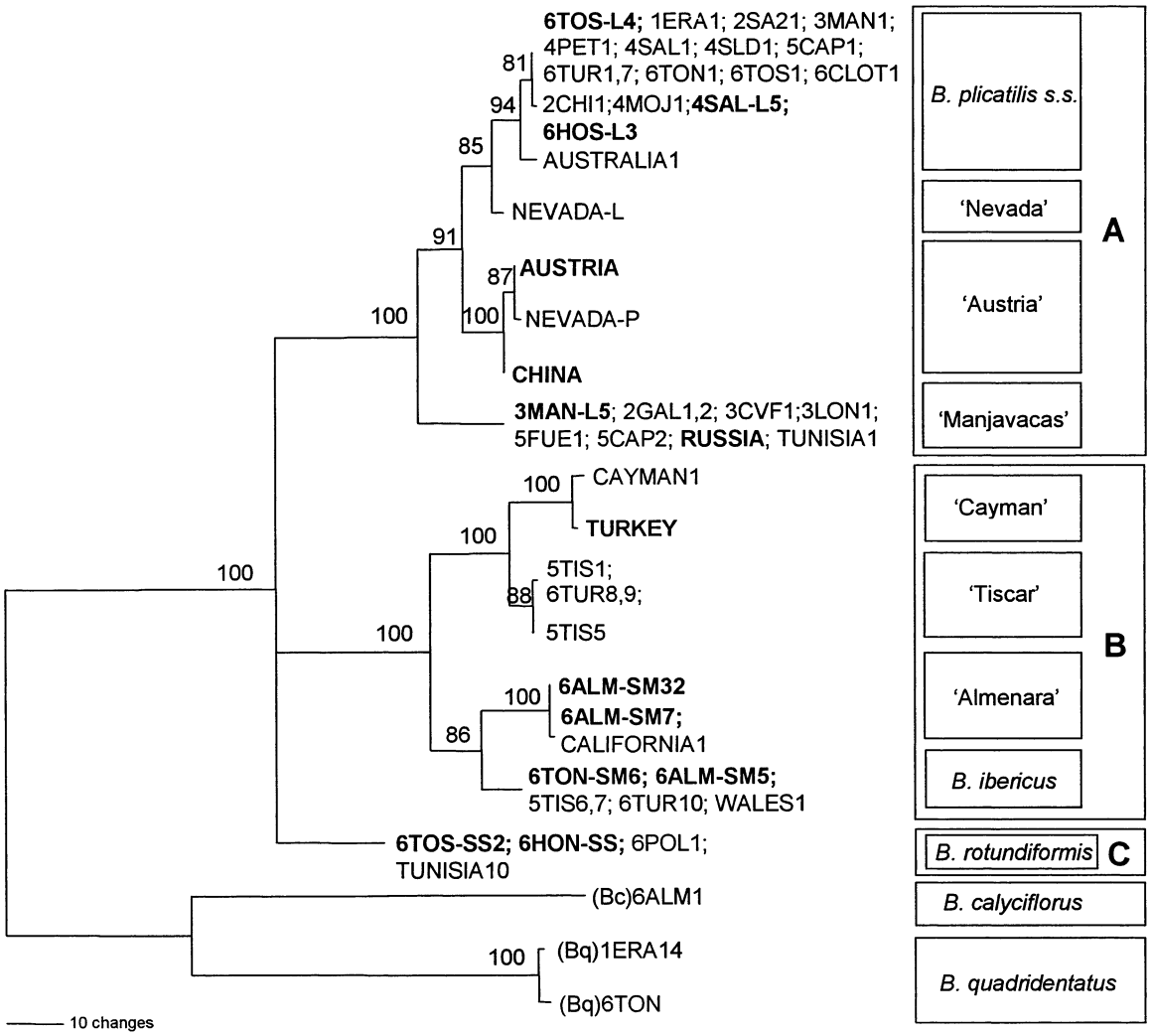

FIG. 1. Branch and bound maximum-parsimony tree of the *Brachionus plicatilis* species complex based on ITS1 sequences. Gaps were treated as fifth base. Identical sequences were collapsed before phylogenetic analysis. Individuals with identical sequences are abbreviated next to each branch. Boldface acronyms indicate reference clones and numbers or letters after sampling site acronym indicate individual sequence. See Tables 1 and 2 for abbreviations and details on the geographic locations and reference clones. Values above branches represent bootstrap support values (1000 replicates; only values higher than 50% are shown). Groups A, B, and C are roughly coincident with the L, SM, and SS morphologies in the species complex, respectively.

substitution apart) were obtained in this species, whose phylogeographic analysis for COI is presented elsewhere (Gómez et al. 2000). In addition, a sequence similar to the Iberian *B. plicatilis* s.s. appeared in Australian samples. A second clade, Nevada, included a sequence from Nevada (USA) sediments (morphology unknown). A third clade, Austria, included sequences from two laboratory strains from Austria and China, displaying an L morphology and an egg sequenced from Nevada (USA). The fourth clade, Manjavacas, named after the first lake in which it was recorded, was represented by the clone 3MAN-L5 (L morphology) and contained a single sequence shared by nine individuals collected in the Ebro, Guadalquivir, and Guadiana Basins, the strain Russia (also with L morphology, originally collected in the Azov Sea and commonly used in aquaculture and ecotoxicology), and an egg isolated in northeastern Tunisia. The relationship between the clades included in group A was well resolved by ITS1. *Brachionus plicatilis* s.s. is a sister taxon to the Nevada lineage. The Austria clade is sister group to them, and the clade Manjavacas is sister to the rest.

Group B contained four well-supported clades. Thorough morphological information is only available for one of them, *B. ibericus*, and this has SM morphology. Clade Cayman included two sequences from the Cayman Islands (resting egg from mud sample) and Turkey (a domesticated strain). Clade Tiscar was found in Tiscar Lake, Can Turies Lagoon, and laboratory clones from El Hondo Lagoon and Camino de Villafranca Lake. Clade Almenara was represented by two laboratory clones, originally collected in Almenara Pond and not observed in other ponds in Spain, although they were also found in the Salton Sea (California, USA). Inspection of some individuals in this clade (A. Gómez, unpubl. data) showed they are more similar to SM morphotype than to any other. A clade formed by the recently described *B. ibericus*

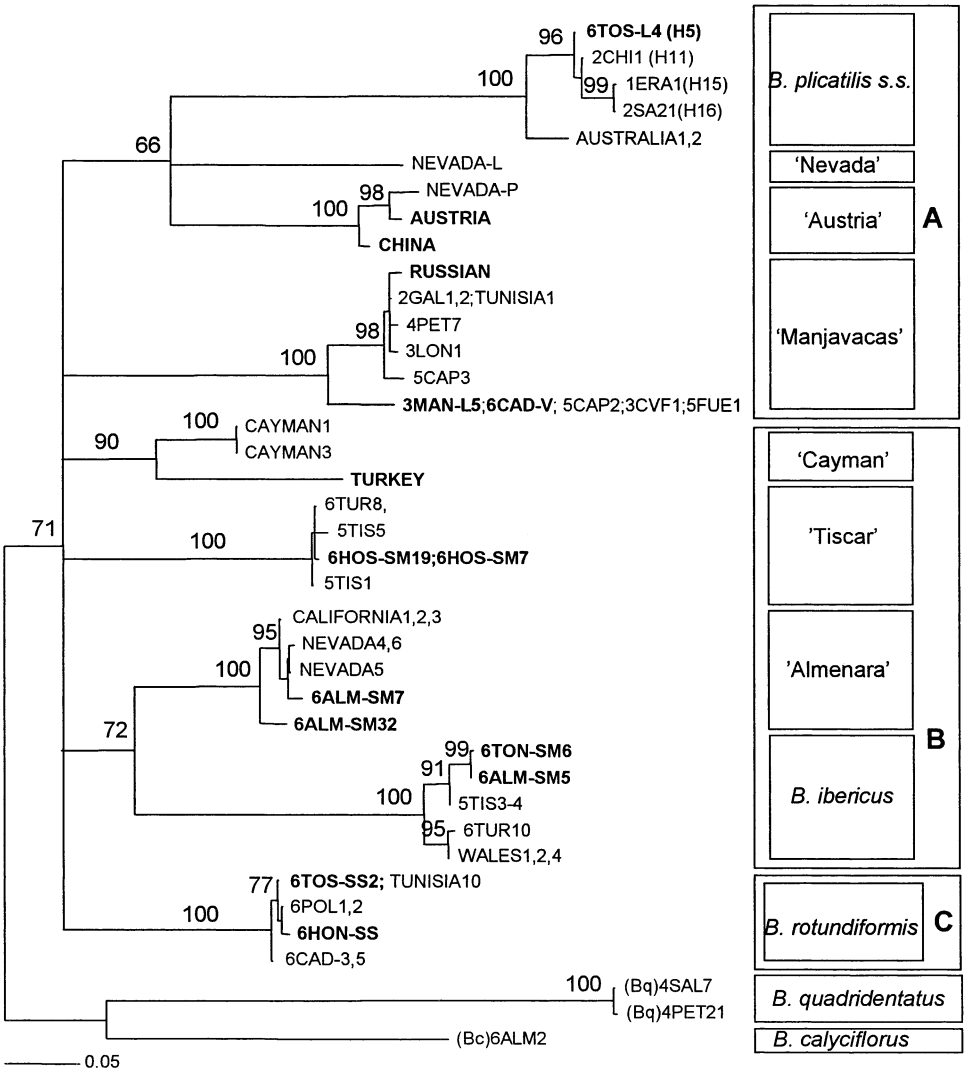

FIG. 2. Neighbor-joining tree based on COI sequences using a matrix of maximum-likelihood distances obtained using the best-fitting model (GTR + I + G). Numbers over branches indicate percent bootstrap support (1000 replicates). Branches with less than 50% bootstrap support were collapsed. The outgroup species are abbreviated: Bc, *Brachionus calyciflorus*; Bq, *Brachionus quadridentatus*.

(Ciros-Pérez et al. 2001a; called *B. rotundiformis* SM in Gómez et al. 1995) was represented by the clone 6TON-SM6 collected in Torreblanca Marsh and was also found in Almenara Pond, Estany d'En Turies, Tiscar, and a Welsh (UK) pond.

The relationships between the clades in group B are well supported by bootstrap, Tiscar and Cayman are sister taxa, as are *B. ibericus* and Almenara, with approximately 10% sequence divergence between the most divergent pairs.

Strains or eggs belonging to group C were represented by a single sequence shared by the reference clones 6TOS-SS2 and 6HON-SS, and eggs from Pollensa Lagoon and northeast Tunisia. The reference clones sequenced here have SS morphology and have recently been redescribed as *B. rotundiformis* (Ciros-Pérez et al. 2001a; called *B. rotundiformis* SS in Gómez et al. 1995).

In summary, ITS1 supports the existence of three main branches in the *B. plicatilis* complex, with a minimum of nine well-defined lineages, six of them present in the Iberian Peninsula.

Mitochondrial DNA Phylogeny

The COI sequence alignment included 603 bp for 57 individuals with a total of 39 unique sequences. Percent sequence divergence (uncorrected *p*) ranged from 0% to 23%.

Likelihood ratio tests performed using Modeltest showed that results fitted best to a GTR + I + G model (general time reversible model). The topology of the NJ COI tree obtained on ML distances and rooted using three sequences from *B. calyciflorus* and *B. quadridentatus* retrieved nine major lineages, the same detected by ITS1 (Fig. 2). Between-lineage divergence (from here on ''lineages'' refer to the nine groups defined as in Fig. 1) was in all cases over 12%. However, COI showed higher sequence diversity than ITS1 within clades (0–12% sequence divergence for COI compared with

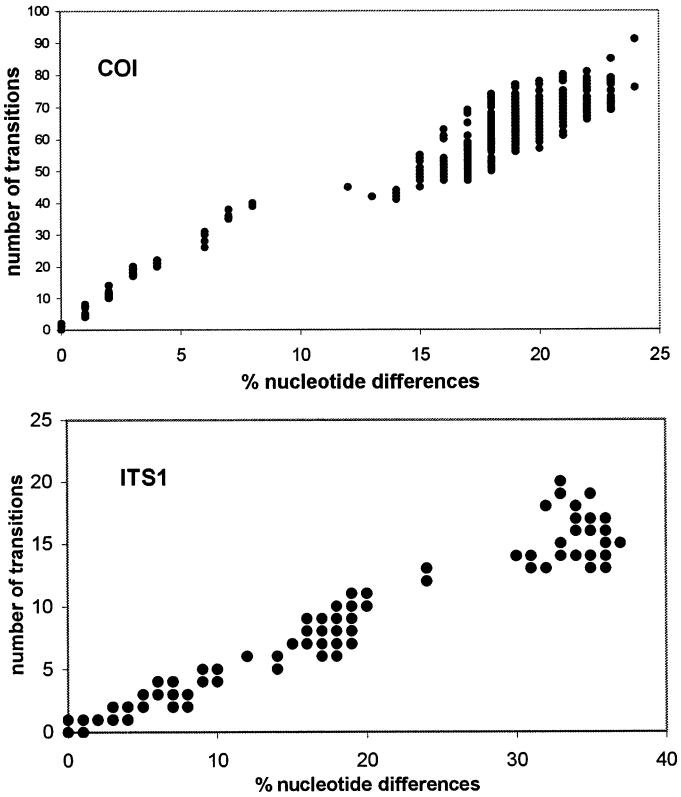

FIG. 3. Plot of number of transitions with the percent sequence divergence in the *Brachionus plicatilis* complex in COI (top) and ITS1 (bottom).

0–2% for ITS1). The diversity of COI for individuals showing identical ITS1 sequences (which are most likely to belong to the same species) was maximum in Cayman and minimum in *B. rotundiformis* and Tiscar. The *B. plicatilis* species complex clustered in six groups in the Iberian Peninsula coincident with those detected by ITS1. *Brachionus ibericus* and Manjavacas presented the highest COI within clade diversity with 4% and 8% sequence divergence, respectively. Outside the Iberian Peninsula the lineage groupings are the same as for ITS1, although COI confirms the separation of the sequence Nevada-L from *B. plicatilis* s.s.: the percent divergence is well in excess of that found within any other single lineage. Transition saturation can be seen in COI over 10% sequence divergences, whereas it does not seem to happen in ITS1 (Fig. 3). Although COI performed very well in detecting intragroup variability, and recovering the different lineages, it performed worse than ITS1 in retrieving the phylogenetic relationships between lineages, as the bootstrap values for the deeper branches were considerably lower. The CO1 phylogeny supported the relationship between *B. ibericus* and Almenara, and *B. plicatilis* s.s. with Austria and Nevada-L (Fig. 2). The rest of the divergences were not well resolved, and this was true as well when MP and NJ using other distances were used. No parsimony weighting scheme, use of outgroups, selection of fewer sequences, use of transversions only, or use of amino acid translations increased the resolution of the phylogeny of the lineages in COI.

Combined Analysis

Partition homogeneity tests using a flat weighting scheme yielded significant noncongruence of both genes. This seemed to be due exclusively to the different mode of evolution of both genes, as weighting COI according to codon position made the partition-homogeneity test nonsignificant. Therefore, MP heuristic searches were performed after applying a weighting scheme according to codon position in COI (first position = 2, second position = 10, third position = 1) and flat weighting in ITS1. A hundred random-order addition replicates were made and 1000 bootstrap pseudoreplicates were performed to assess the confidence of the tree nodes. Four MP trees with length 1269 steps and consistency indexes of 0.52 resulted, which differed only in the topology of the shallow branches. The MP bootstrap consensus topology (Fig. 4) is coincident with that found for ITS1 and COI alone, with strong bootstrap support (>97%) for the lineages discussed above. However, bootstrap support for relationships between lineages was in general under 70% (see Fig. 4). A NJ analysis was carried out using ML distances following the model and parameters found to be optimum by Modeltest for the combined dataset (GTR + I + G). The tree topology was virtually identical to the MP consensus tree, but bootstrap values were generally higher (see Fig. 4) and supported some relationships between lineages unsupported by MP but previously found to be robust in the ITS1 MP analysis. This was confirmed when a ML analysis using representative sequences from each lineage and the outgroup (n = 10) were used. The same topology as above was retrieved (tree not shown), and the bootstrap support for the relationships between lineages was high (>68%), but again, the relationships between the three main clades A, B, and C were unresolved.

Age of the Taxa

No fossils of *B. plicatilis* are available to calibrate a molecular clock for the species complex. Therefore, we attempted to gain a rough estimation of divergence times using calibrations of molecular clocks from other invertebrate taxa. For ITS1, the molecular clock calibrated by Schlötterer et al. (1994) in *Drosophila* was employed. This clock gives 2.4% sequence divergence per million years and was calibrated over the range 30–60 million years. If our taxa are younger, as seems likely, and everything else is equal, their calibration will provide underestimates of the true divergence rates among them. In addition, the rate of accumulation of mitochondrial DNA mutations appears to be approximately linear with time for divergence less than 15–20 million years (Brown 1983) and has been estimated as 1.4% pairwise sequence divergence per million years for COI in snapping shrimps (Knowlton and Weigt 1998). Therefore, the ranges in which both clocks are calibrated are complementary and can yield information on the divergence spectrum of this complex. Such clocks might not exactly extrapolate to rotifers; for instance, rotifers could be evolving at a very different rate than the organisms for which the molecular clocks were calibrated. Therefore, although the estimated divergence times should be taken with caution, they may still distinguish between recent or ancient speciation events.

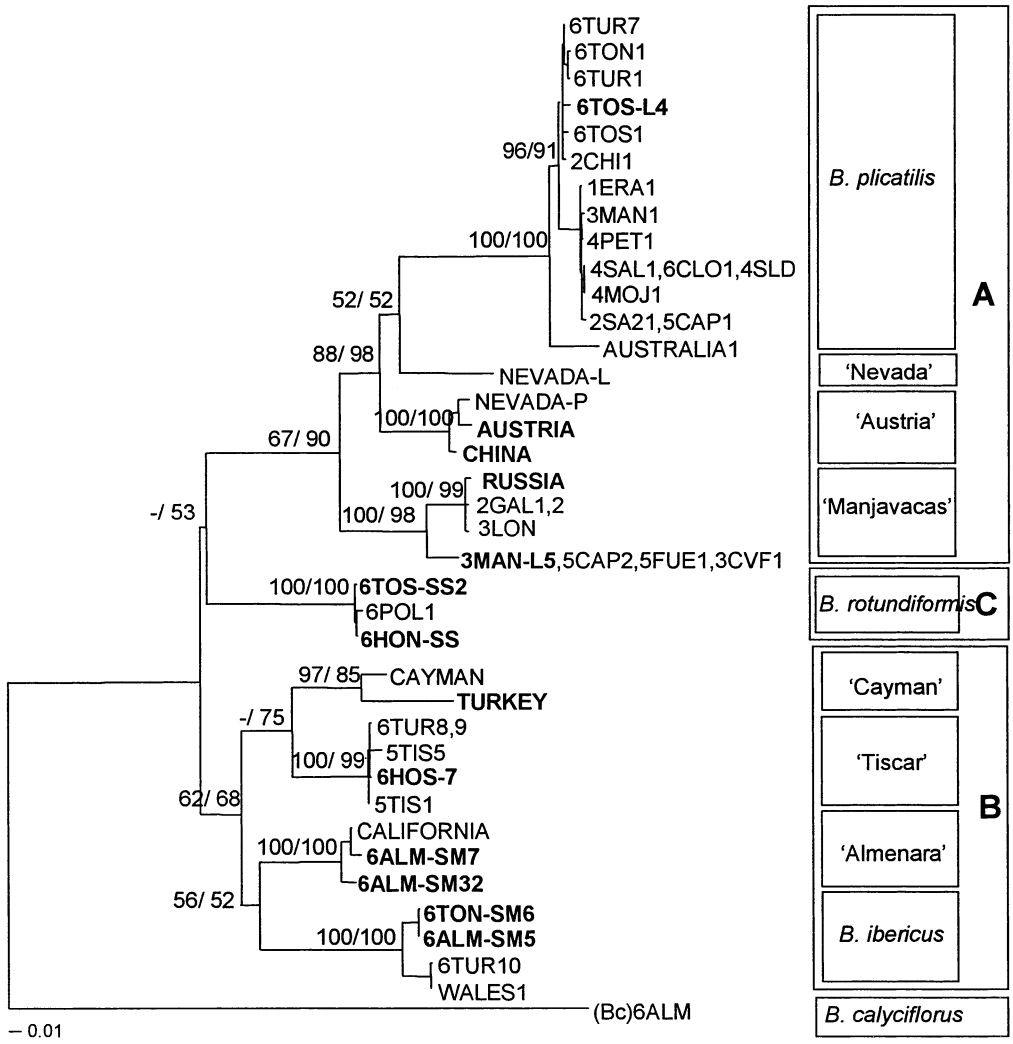

FIG. 4. Combined phylogenetic analysis of ITS1 and COI. The tree shows a neighbor-joining tree based on COI sequences using a matrix of maximum-likelihood distances obtained using the GRT + I + G model (see text for parameters). The consensus tree obtained using maximum parsimony was identical. Values above branches indicate bootstrap support for nodes in both analyses (maximum-parsimony and neighbor-joining); - indicates less than 50% bootstrap value. Bc, *Brachionus calyciflorus*.

We calculated uncorrected (*p*-values) and corrected (ML distances using the optimum model for each gene) distances for both genes for the main splits (see Table 3) and estimated the time to the most recent common ancestor of each pair of lineages using the calibrations above. ITS1 corrected and uncorrected distances were very similar except for the distance between the outgroups and the *B. plicatilis* complex and for the time of diversification of the main branches of the complex. ITS1 tended to yield very low estimates of most recent common ancestors for the shallower branches, and it is possible that this is due to the clock used being calibrated for a time range of 30–60 million years. For these, we will refer to the COI estimates. The corrected and uncorrected distances are quite different for COI, being much higher when corrected. The estimated COI dates are, however, within the range in which it behaves linearly with time for the uncorrected distances and just over these values for the corrected ones.

The average corrected sequence divergences between the ingroup and the two outgroup taxa (*B. quadridentatus* and *B. calyciflorus*) was 80% for ITS1 and 56% for COI suggesting a split that occurred more than 30 million years ago (Oligocene). The major split in the species complex, that is, the separation of group A from groups B and C appears to have occurred more than 20 million years ago (late Miocene or early Oligocene). Radiation within group A is also estimated to have taken place soon after the origin of the group (late Miocene–early Oligocene). The most recent estimated split between lineages is between Cayman and Tiscar and was dated over 19 million years ago (COI).

Distribution of the Taxa

The geographical distribution of the taxa can only be assessed in detail for the Iberian lineages (Fig. 5). Strikingly, 12 ponds of the 26 in which the *B. plicatilis* complex was recorded contained two or more lineages, and coastal lagoons often had three lineages coexisting. In the inland lakes, *B.*

TABLE 3. Average sequence divergences (uncorrected *p*; and maximum-likelihood [ML] distances according to the optimal model and parameters, see text) and estimated times to the most recent common ancestor (MRCA) of selected clades within the *Brachionus plicatilis* species complex.

	ITS1				COI			
Split	Uncorrected *p*	Time to MRCA (million years)	ML distance	Time to MRCA (million years)	Uncorrected *p*	Time to MRCA (million years)	ML distance	Time to MRCA (million years)
Outgroup–*B. p.* species complex	0.35	15	0.80	33	0.21	15	0.56	40
A–B–C	0.16	7	0.23	10	0.18	14	0.38	27
Manjavacas–(*B. p.* ss., Nevada, Austria)	0.09	4	0.11	5	0.20	14	0.44	31
B. p. s.s.–Nevada	0.03	1	0.03	1	0.18	13	0.41	29
B. p. s.s.–Austria	0.06	3	0.07	3	0.18	13	0.39	28
(Cayman + Tiscar)–(Almenara + *B. ibericus*)	0.08	3	0.10	4	0.17	12	0.34	24
Cayman–Tiscar	0.03	1	0.04	2	0.15	11	0.27	19
Almenara–*B. ibericus*	0.06	3	0.06	3	0.17	12	0.30	21

plicatilis s.s. and Manjavacas, the most widespread lineages, often coexisted (five lakes). Of the 15 possible species pairs in the Iberian Peninsula, we found evidence for coexistence of 10 of them. Clade Almenara was restricted to coastal lagoons of low salinity (Ortells et al. 2000), and *B. ibericus* was detected in coastal lagoons of low to medium salinity as well as an inland hypersaline lake. Clade Tiscar has been found in inland and coastal lakes, whereas the Manjavacas lineage is mainly restricted to inland lakes. *Brachionus plicatilis* s.s. is present both in coastal and inland lakes, whereas *B. rotundiformis* is only present in coastal lagoons.

DISCUSSION

The data presented allow the construction of a robust phylogeny entirely concordant between both mitochondrial and nuclear sequences for the *B. plicatilis* complex. Extensive sampling in the Iberian Peninsula and integration of both laboratory strains and isolates from several continents provides estimates of the relative age and extent of divergence within this group.

Results suggest that the divergence within the rotifer species complex *B. plicatilis* is ancient. The deep sequence di-

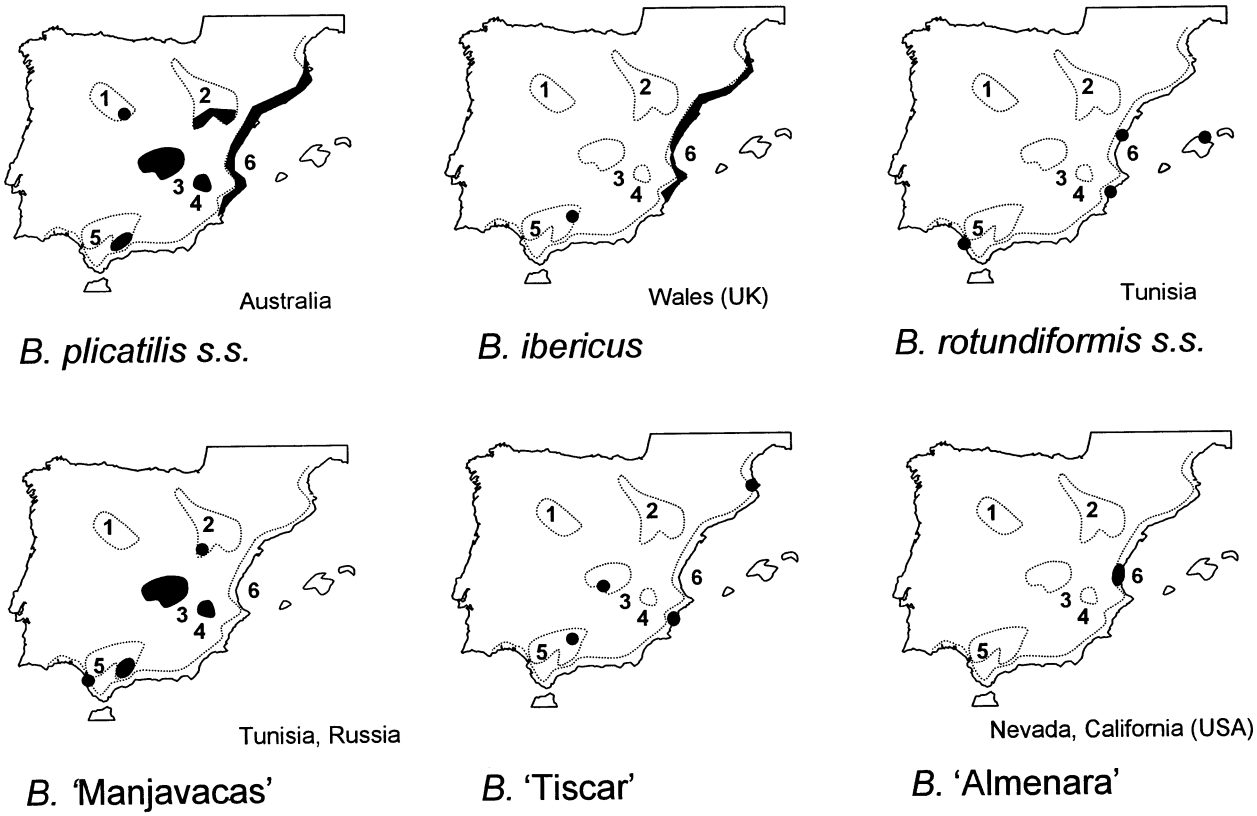

FIG. 5. Distribution of the six *Brachionus* lineages detected in the Iberian Peninsula. Locations in which members of a lineage have been detected outside the Iberian Peninsula are also indicated below each map. Numbers on the maps refer to the inland salt lake basins (1, Duero; 2, Ebro; 3, Guadiana; 4, Júcar-Segura; 5, Guadalquivir) and the chain of coastal lagoons (6).

vergences between these lineages found in both mitochondrial and nuclear genes (15–22% for COI, 3–20% for ITS1), exceed the values usually found between congeneric species (Avise 2000), indicating that each of these lineages has an independent evolutionary history of a scale typical of species or higher taxa. In addition, the magnitudes of genetic divergence among clades do not overlap with those within clades. For ITS1, maximum divergence within lineages was 0.6%, and minimum divergence between lineages was 3%; for COI, maximum within lineage divergence was 12%, and minimum divergence between lineages was 15%. Although many lineages are sympatric, no evidence of hybridization or introgression was found, as both genes produced concordant tree topologies, which suggests a history of reproductive isolation. Phylogenetic concordance between genes has been employed as a tool to recognize species status (Avise and Ball 1990; Baum and Shaw 1995). However, rotifers from these lineages share a very similar morphology, supporting the pattern of morphological stasis described for other zooplanktonic taxa (Colbourne et al. 1997; Hebert 1998).

Taxonomic Assessment of the Species Complex and Consequences for Biodiversity Estimates

Our data, together with other published results, allow a reassessment of the taxonomic status of this species complex. Within the Iberian Peninsula alone, the species complex *B. plicatilis* includes six deep and distinct phylogenetic lineages. The body of data available on the reference clones used (Gómez et al. 1995; Gómez and Serra 1995; Gómez and Snell 1996; Ortells et al. 2000) together with high degree of divergence and concordant patterns of nuclear and mitochondrial DNA sequences and their coexistence in the wild strongly suggest that each of these lineages are distinct biological species or species groups. Three of these species (*B. plicatilis* s.s., *B. rotundiformis*, *B. ibericus*) have recently been described or redescribed (Ciros-Pérez et al. 2001a).

Two of the lineages found in group A, *B. plicatilis*. s.s. and Manjavacas, are highly divergent for both genes analyzed, and allozyme data also support their specific status (Hardy-Weinberg disequilibrium with heterozygote absence when in sympatry; Ortells et al. 2000). These taxa display strong behavioral reproductive isolation (Gómez and Snell 1996; Ortells et al. 2000; H. K. Berrieman, D. H. Lunt, and A. Gómez, unpubl. ms.), and no evidence for hybrids has been found in resting egg banks of ponds where both lineages coexist (Gómez et al. 2002; Ortells et al. 2000; the present study). We suggest that the Manjavacas lineage is a new, hitherto undescribed species.

ITS1 and COI data support the inclusion of at least two other distinct lineages in group A, Nevada and Austria. Gómez and Snell (1996) found that males from a *B. plicatilis* s.s. strain discriminated strongly against females from the strains Austria and China (clade Austria), the same strains that were sequenced in the present study.

Group B was found to include three well-supported lineages in the Iberian Peninsula. Two of these lineages are frequently found sympatrically in coastal and inland ponds. Allozyme surveys (Ortells et al. 2000) have shown that groups represented here by reference strains seemed to be fixed for distinct (private) alleles at some allozyme loci. Although the lineages shared alleles for several other polymorphic allozyme systems, linkage disequilibrium and heterozygote deficits were common when different lineages coexisted, suggesting reproductive isolation. No data are yet available for mating behavior within this group, and the taxonomic description of these species remains incomplete, with only one of them having been described (*B. ibericus*; Ciros-Pérez et al. 2001a). The taxonomic status of the Cayman group is uncertain, with possibly two species, one represented by isolates in the Cayman Islands and the other by the strain from Turkey.

Group C includes a single lineage in the Iberian Peninsula, *B. rotundiformis*, which is quite homogeneous genetically. Our data, along with studies of mating behavior and morphology (Gómez et al. 1995; Gómez and Serra 1995; Ciros-Pérez et al. 2001a), supports its specific status.

The current state of the taxonomy in the species complex *B. plicatilis*, and most probably the genus as a whole, is inadequate as it underestimates the number of evolutionarily independent lineages, thus impeding our understanding of the evolutionary processes and patterns of diversification therein. So far, due to the morphological similarity of these lineages, distinct sympatric cryptic species may have often been mistaken for conspecific clonal groups (e.g., King 1980), which is misleading for the understanding of the ecological and evolutionary phenomena involved in population genetic structure. In addition, according to the current taxonomy of the group, researchers or aquaculturalists working on the same apparent species (i.e., morphospecies) may actually be dealing with completely different, highly divergent species or mixed cultures. The advantages of clearly distinguishing cryptic biological species using molecular tools has proved and promises to be important in revealing the processes involved in the population structure and differentiation of these cyclical parthenogens (Gómez and Carvalho 2000; Gómez et al. 2000). No parallel effort in addressing the taxonomic status using behavioral, population genetic, and phylogenetic approaches has been performed on other rotifer taxa. However, multiple forms and subspecies have been recognized in widely distributed species through morphological inspection of field samples (e.g., Kutikova and Fernando 1995). Knowing whether this variation is due to phenotypic plasticity, within-species variation, or cryptic speciation remains for future work. We anticipate, however, that the latter option will be a frequent one in rotifers (Serra et al. 1997) and, if so, species diversity and coexistence of similar species of this important component of continental zooplankton will have been very largely underestimated.

Distribution of the Taxa in the Iberian Peninsula: Sympatry Supports Specific Status

A significant result of this work is the identification of genetically distinct coexisting lineages in many habitats, which is a strong argument for the species status of those taxa. To what degree the taxon distribution reported herein reflects ecological constraints or a failure to disperse remains unknown. The latter seems unlikely, given the high colonization ability of rotifers in general and the fact that zoo-

planktonic communities seem not to be constrained by dispersal (Jenkins and Buikema 1998; Jenkins and Underwood 1998; Shurin 2000). The high level of sympatry between species and the inferred high colonization potential are in sharp contrast with the low levels of gene flow and high geographic structuring found in studies within rotifer species (Gómez et al. 2000, 2002) as in other zooplankton species (De Meester et al. 2002). The decoupling of high dispersal and colonization abilities, on one hand (which would explain species range and degree of sympatry), and low levels of gene flow in passively dispersing aquatic invertebrates, on the other, has been attributed to the rapid monopolization of resources by the first colonizing migrants aided by their fast growth rates, local adaptation, and the presence of a resting egg bank (De Meester et al. 2002). The high degree of sympatry can be mediated by niche partitioning and different susceptibilities to predators or parasites. In fact, laboratory experiments have shown that sympatric *Brachionus* species are often adapted to different temperature and/or salinity optima (Gómez et al. 1997) or have different food preferences (Ciros-Pérez et al. 2001b) or predation vulnerability (Ciros-Pérez 2001; Lapesa et al. 2002), factors that can mediate coexistence. This ecological segregation is reflected by seasonal succession in the field in a given site (Gómez et al. 1995, 1997). The common coexistence of different taxa may indicate that the range of seasonal or annual ecological variation in single ponds offers several niches, therefore providing ample opportunities for coexistence.

Global Distribution and Long-Distance Dispersal

Despite our restricted sampling outside the Iberian Peninsula for this cosmopolitan species complex, evidence was obtained of several widely distributed lineages, strongly suggesting capabilities for transcontinental long-distance dispersal and colonization. The isolated nature of salt lakes and the absence of commercial traffic among them, suggest that human transportation is an unlikely cause of such transcontinental dispersal. For example, mitochondrial DNA haplotypes from the Almenara group were found in Spain as well as the Salton Sea and Little Fish Pond (California and Nevada, USA), and *B. ibericus* was detected in Spain and Wales. Isolates outside the Iberian Peninsula contained sequences either identical to or falling within the variation of the Iberian species, suggesting recent dispersal and colonization. For non-Iberian lineages, Austria was found in isolates from three continents (from the Nearctic and Palearctic), with similar sequence divergence levels to those observed in Iberian *B. plicatilis* s.s. (Gómez et al. 2000). For other isolates the situation is more ambiguous, as illustrated by Australian *B. plicatilis* s.s., which groups as a sister taxon to the Spanish *B. plicatilis* s.s. Here, the degree of divergence (6% for COI, 2% for ITS1) cannot indicate directly whether this is a closely related species or it represents a highly differentiated geographical isolate.

These results provide evidence for the high capabilities for long-distance dispersal in taxa with passively dispersing resting eggs. Long-distance dispersal and colonization of distant habitats, often aided by waterfowl migrations, has been detected in many zooplanktonic species, although evidence for transcontinental dispersal is rarer (see review in De Meester et al. 2002). Due to the genetic similarity of strains of the *B. plicatilis* complex found in different continents, these colonization events must have happened relatively recently in evolutionary time. This indicates that transoceanic flights are frequent in these organisms and may have had an important impact on their biogeography. More extensive global sampling will be needed for a complete biogeographical description of this cosmopolitan species complex, as the thermophilic character of the genus as a whole suggests a higher speciosity in tropical and subtropical regions, which would have remained undetected by the sampling regime here.

Age of the Species Complex

To discriminate between long-term stasis versus ongoing or recent speciation, the use of molecular clocks, even if only rough approximations, yields critical information. For example, if the major diversity within this group was represented by a pattern of closely related species with genetic divergences in agreement with splits coinciding with the Pleistocene epoch (less than about 2.5 million years ago), it would support ongoing differentiation spurred by recent global climatic changes. Examples of this type of Pleistocene speciation abound in the literature (see Avise 2000; Hewitt 2000). By contrast, our findings strongly suggest that the *B. plicatilis* complex radiation did not happen this recently. Using molecular clocks as rough approximations, both ITS1 and COI sequence divergences indicate that this is an ancient species complex, which probably radiated during the late Oligocene or early Miocene (well over 10 million years ago). Even the genetically closest lineages are likely to have diverged more than 7 million years ago, and only the intraclade genetic diversification seems compatible with Pleistocene glacial-cycle driven vicariant events. We therefore conclude that the *B. plicatilis* species complex radiation does not represent recent speciation, but ancient speciation followed by morphological stasis.

The observed pattern is consistent with that yielded by an increasing number of aquatic invertebrate cryptic species complexes that have been found to be of ancient origin and with relatively constrained rates of speciation. For example, several studies on the phylogenetics and evolution of the speciose genus *Daphnia* (more than 200 described species) have shown that it comprises a minimum of 15 species complexes (Colbourne and Hebert 1996). Most complexes are clusters of ancient cryptic species that diverged over 50 million years ago, and only four, in particular those restricted to arctic regions, show evidence of active speciation in the last 3 million years (Colbourne and Hebert 1996; Colbourne et al. 1997, 1998; Schwenk et al. 2000). More continental planktonic crustacean taxa have also been shown to be composed of ancient cryptic species complexes. The amphipod species complex *Hyalella azteca* comprises a minimum of seven species, often sympatric and thought to have diverged during the mid-Miocene (Witt and Hebert 2000). The North American cryptic species complex *Mysis relicta* (Crustacea: Mysidae) comprises four species of mid Tertiary origin (Vaïnöla et al. 1994). Finally, the anostracan *Artemia salina* (Pérez et al. 1994) and the notostracan complex *Lepidurus*

apus, a living fossil with a paleontological record going back to 200 million years ago (King and Hanner 1998), are also formed by clusters of ancient species. Given how few zooplankters have been studied in detail, it would appear that ancient species complexes are far from uncommon and calls for a common explanation for the observed morphological conservatism.

Morphological Stasis

In spite of having evolved independently for a significant amount of time, these rotifer taxa display remarkably little morphological diversity, supporting the hypothesis that morphological stasis can be a common feature of passively dispersing continental zooplanktonic taxa. The often subtle morphological differences between the species currently recognized (*B. plicatilis* s.s., *B. rotundiformis* and *B. ibericus*; see Ciros-Pérez 2001a) were only reliably detected when individuals were cultured in identical conditions in the laboratory and cohorts of the same age were analyzed using scanning electron microscopy and biometrical statistical tools. In wild-caught samples, individuals of these species are often impossible to discriminate. The fact that sexual signals are of chemical nature, at least in rotifers, copepods, and possibly *Daphnia* (Snell and Morris 1993; Carmona and Snell 1995; Snell et al. 1995; Kelly and Snell 1998; Kelly et al. 1998), means that evolutionary divergence in such signals need not involve morphological change. In addition, divergence in ecological traits need not involve significant morphological divergence if it is based on physiological adaptation to salinity and temperature conditions. Altered food particle size preference or behavioral changes in response to predation pressure may only involve changes in body size. None of these adaptive mechanisms rely on significant morphological change and seem to be widespread in zooplanktonic organisms often underlying differences between related clones or cryptic species (Rothhaupt 1990; De Meester et al. 1995; Gómez et al. 1997; Boersma et al. 1999; Cousyn et al., 2001). In addition, divergence in ecological traits often affects the timing of sexual reproduction; therefore, patterns of seasonal reproductive isolation might also develop concurrently (Lynch 1985). The apparently widespread physiological and behavioral adaptation in the evolution of these organisms and sexual communication through chemical signals may thus be critical in explaining the lack of morphological change associated with local adaptation, population diversification, and cryptic speciation.

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