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An analysis of species boundaries and biogeographic patterns in a cryptic species complex: The rotifer—*Brachionus plicatilis*

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Abstract

Since the advent of molecular phylogenetics, there is increasing evidence that many small aquatic and marine invertebrates—once believed to be single, cosmopolitan species—are in fact cryptic species complexes. Although the application of the biological species concept is central to the identification of species boundaries in these cryptic complexes, tests of reproductive isolation do not frequently accompany phylogenetic studies. Because different species concepts generally identify different boundaries in cryptic complexes, studies that apply multiple species concepts are needed to gain a more detailed understanding of patterns of diversification in these taxa. Here we explore different methods of empirically delimiting species boundaries in the salt water rotifer *Brachionus plicatilis* by comparing reproductive data (i.e., the traditional biological species concept) to phylogenetic data (the genealogical species concept). Based on a high degree of molecular sequence divergence and largely concordant genetic patterns in COI and ITS1, the genealogical species hypothesis indicates the existence of at least 14 species—the highest estimate for the group thus far. A test of the genealogical species concept with biological crosses shows a fairly high level of concordance, depending on the degree of reproductive success used to draw boundaries. The convergence of species concepts in this group suggests that many of the species within the group may be old. Although the diversity of the group is higher than previously understood, geographic distributions remain broad. Efficient passive dispersal has resulted in global distributions for many species with some evidence of isolation by distance over large geographic scales. These patterns concur with expectations that micro-meiofauna (0.1–1 mm) have biogeographies intermediate to microbial organisms and large vertebrates. Sympatry of genetically distant strains is common.

Keywords: Rotifera; *Brachionus*; Cryptic species; COI; ITS1; Zooplankton; Reproductive isolation

1. Introduction

Due to a lack of taxonomically useful morphological characters and widespread dispersal capabilities, many freshwater and marine invertebrates were traditionally—and mistakenly—believed to be single, cosmopolitan species (for reviews see Knowlton, 1993; De Meester et al., 2002). With the advent of molecular systematics however, this view changed. Previously undetected genetic diversity has demonstrated the existence of cryptic species complexes in many marine (Knowlton, 1993) and aquatic (De Meester et al., 2002) invertebrates. The prevalence of these cryptic complexes has not only raised questions about the diversity and taxonomy of many marine and aquatic taxa, but has revealed that extensive genetic and ecological variation can be structured in the absence of apparent morphological differentiation. This observation has raised questions about the relative importance of non-morphological traits (e.g., chemical

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recognition systems) in the divergence and speciation of these taxa (as well as non-cryptic taxa) (Knowlton, 1993).

The first step to addressing these questions in any taxon is to develop a clear understanding of how variation is structured and where species boundaries lay. Because different species concepts identify different stages in the 'speciation' trajectory (Harrison, 1998), the application of multiple methods is desirable (Sites and Marshall, 2004). Phylogenetic approaches are probably the most common in cryptic species delineation. One example is the genealogical species concept (GSC) (Baum and Shaw, 1995) which identifies genealogically exclusive groups at the boundary between reticulate and divergent genealogies. Operationally, the approach entails the creation of genealogies for a number of unlinked loci and the construction of a strict consensus tree to identify concordant, resolved nodes (i.e., the most basal, reciprocally monophyletic groups). Whenever sexual reproduction can be evaluated, the biological species concept (BSC) (Mayr, 1963) is generally accepted because it utilizes reproductive incompatibility to identify diverging groups. Reproductive isolation, however, can be difficult to evaluate in nature and laboratory investigations may constitute incomplete tests of the concept. In addition, intermediate degrees of reproductive compatibility require qualitative judgments. Because phylogenetic species can originate shortly after gene flow is interrupted, phylogenetic methods are generally expected to detect greater numbers of species compared to isolation/cohesion concepts (Agapow et al., 2004; Harrison, 1998). A strict application of the genealogical species concept is a likely exception, however, as genealogical species emerge last (Harrison, 1998).

The goal of the present study is to identify patterns of diversification in a recently documented (Gómez et al., 2002) cryptic species complex by investigating both the genealogical and biological species concepts. The monogonont rotifer *Brachionus plicatilis* Müller is a small, cyclically parthenogenetic invertebrate that inhabits salt lakes and coastal ponds worldwide. It is the subject of extensive and varied research since it has commercial importance as a larval fish food in aquaculture (see the work of A. Hagiwara and E. Lubzens) and plays a prominent role in rotifer research. Because it is easily cultured, has a short generation time, and can be induced to be asexual or sexual, it is a favored rotifer for basic physiological and ecological investigations, as well as applied ecotoxicological evaluations (Ricci et al., 2000). In addition, the utility of *B. plicatilis* as a potential model organism in evolutionary biology has recently been recognized and pursued with investigations on its phylogeography (Gómez and Carvalho, 2000; Gómez et al., 2000; Gomez and Serra, 1996) and mate recognition system (Snell et al., 1995). For these reasons, *B. plicatilis* may also serve as a promising subject for investigations into cryptic species and how variation is structured in these complexes.

Aquaculturists were the first to note the existence of a high degree of discontinuous variation within the salt water rotifer complex, *B. plicatilis* (Fu et al., 1991b). On the basis of morphological and allozyme evidence Fu and colleagues (1991a) proposed the existence of two distinct lineages, the 'L-type' (large) and 'S-type'(small). Over the next five years, these were established as true species based on sexual (Hirayama and Rumengan, 1993), karyotypic (Rumengan et al., 1991), and behavioral evidence (Fu et al., 1993). In 1995, Segers (1995) reviewed the history of nomenclature and renamed 'L'- and 'S-types' as *B. plicatilis* and *Brachionus rotundiformis*, respectively. Since then, a series of investigations on a community of *B. plicatilis* 'strains' inhabiting a coastal lagoon in Spain resulted in the accumulation of evidence (ecological, allozymic, reproductive) to designate three species within the complex ('S', 'L', and 'SM') (Gomez and Serra, 1996; Gómez et al., 1995). These three species were re-described as *B. plicatilis sensu stricto*, *B. rotundiformis*, and a new species, *Brachionus ibericus* (Ciros-Pérez et al., 2001). The discovery of nine deeply diverged lineages in the first phylogenetic analysis of the group, however, prompted Gómez et al. (2002) to increase estimated species numbers to nine. With each investigation the number of proposed species increases, and it is clear that the extent of diversity remains unknown.

The present study generates a species boundary hypothesis for *B. plicatilis* by exploring both tree and non-tree based methods. Utilizing the same molecular markers as Gómez et al. (2002) we expanded the phylogenetic hypothesis (in terms of number of taxa and geographic scope) and applied the genealogical species concept (GSC) to the group (Baum and Shaw, 1995). We then tested for congruence between the genealogical species concept and the biological species concept by exploring the location of reproductive boundaries. To better understand the prevalence of sympatry and the pattern of taxon coexistence, sediments containing resting eggs were collected from as many sites as possible. Finally, we conducted a preliminary analysis of gross morphological features (lorica length and width) to relate our findings to the traditional taxonomic groupings of large ('L'), small ('S'), and medium ('SM') type. A synthesis of the analyses can serve as a framework for inquires into the systematics, biogeography, and speciation of the group.

2. Methods

2.1. Sampling, resting egg hatching, and culturing

Sediment samples and laboratory clones were collected from 25 localities from around the world. Because this study was the first to examine large-scale geographic patterns in *B. plicatilis*, an attempt was made to sample broadly. The distribution of samples reflects the distribution of cooperating park officials and rotifer biologists throughout the world. The sites represent at total of 13 countries in North America, Europe, Asia, Africa, Australia, and the Caribbean.

Because rotifer populations are frequently ephemeral, the most effective sampling protocol is to integrate over time and sample the sediment of a site containing the egg bank (Hairston, 1996); monogonont rotifer resting eggs are actually encysted embryos produced by sexual reproduction and have an obligatory diapause period. Resting eggs were isolated following the protocol of Gómez et al. (2002). A small amount of sediment was mixed with 50 mL of 1.75 M sugar solution in a 50 mL centrifuge tube and was centrifuged for 5 min at 100 g. The supernatant was filtered through 15 μm nylon mesh and washed with 15 practical salinity units (psu) Instant Ocean™ sea water. Resting eggs were hatched in 7 psu Instant Ocean™ sea water approximately 6 in. below 40 W cool light bulbs. The number of eggs hatched and individuals sequenced varied from site to site (Appendix 1).

To enable testing for cross-contamination and to guard from loss of clonal lines, two independent replicate clonal cultures from each rotifer hatchling were established. Rotifer clones were cultured in 15 mL disposable glass test tubes filled with 15 psu Instant Ocean™ sea water enriched with Micro Algae Grow™ Guillard f/2 algal medium. Cultures were fed the microalgae *Tetraselmis chui* (strain PLY429) which was supplied by the National Marine Fisheries Service in Milford, Connecticut.

2.2. DNA sequencing

DNA was extracted from approximately 0.5 mL of fresh or frozen rotifer tissue (this amounted to approximately 500–1000 animals clones) using Invitrogen's Easy-DNA™ kit. Rotifers were starved for 24 h prior to each extraction and rinsed thoroughly to reduce algal contamination. A 713-bp region of the mitochondrial gene cytochrome *c* oxidase subunit I (COI hereafter) was amplified and sequenced using the primers LCO1490 and HCO2198 (Folmer et al., 1994). A ca. 530 bp segment containing the complete nuclear ribosomal internal transcribed spacer 1 (ITS1 hereafter) and flanking portions of the nuclear 18S and 5.8S ribosomal RNA genes was amplified using primers III and VIII (Palumbi, 1996). Standard 50 μL amplification reactions were conducted with Applied Biosystem's PCR chemistry on a Hybaid Touchdown PCR machine following the cycling conditions of Gómez et al. (2002). PCR products were cleaned by glass-milk purification (Q. Biogene geneclean® III kit). Amplified regions were sequenced with Applied Biosystem's ABI Prism™ Big Dye™ chemistry and run out on an ABI Prism™ 377 DNA Sequencer. To ensure accuracy of sequence data, complementary strands of DNA were sequenced, two independent replicate clonal cultures were analyzed in parallel from time to time, and inconsistencies and polymorphisms were manually checked against original chromatographs in the computer program Sequencher™ 4.1. Sequences have been deposited in GenBank (COI: AY785174–AY785235; ITS1: AY772094–AY772160).

2.3. Sequence alignment

Due to the conservation of the amino acid sequence and reading frame, the 625 bp region of COI was aligned manually. Alignments of the entire non-coding ITS1 (which ranged in size from 315 to 331 bp) were performed using the program SOAP 1.1a2 (Loytynoja and Milinkovitch, 2001). Regions of instability (i.e., regions where homologous relationships shifted in different parts of the parameter space) were deleted leaving an alignment of 240 positions. Copies of COI and ITS1 from two outgroup species (*B. quadridentatus* and *B. calyciflorus*) were used to root the tree (*B. quadridentatus* COI: AF387294, ITS1: AF387242 and *B. calyciflorus* COI: AF387296, ITS1: AF387243). Each of these species lies within the genus *Brachionus* but differ markedly in morphology and ecology (e.g., *B. calyciflorus* is a freshwater species).

2.4. Phylogenetic analyses

Phylogenetic relationships were estimated by parsimony (MP) and distance in PAUP* 4.0b10 (Swofford, 1999), and by Bayesian analysis in MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001). The gene regions were analyzed separately and as a combined data set. Congruence in highly supported clades was used as a diagnostic to assess the topological compatibility of the two data sets. This assessment was used for the determination of the genealogical species concept and for the compatibility of topological signal between the data sets. A likelihood ratio test between the combined and separate analyses was conducted to check if the two partitions were compatible with the same model of molecular evolution (Sanderson and Doyle, 2001). The molecular clock was tested using likelihood ratio tests (Felsenstein, 1988) using PAUP* 4.0b10.

Inspection of a saturation graph (available from the authors) guided the choice of using various weighting schemes for COI (equal weighting, a 4:1 tv:ts weighting scheme, and excluding transitions) and using equal weighting for ITS1. MP analysis of the combined data set was done using equal weighting in both COI and ITS1 and applying a weighting scheme according to codon position for COI only (1st position = 2, 2nd position = 10, 3rd position = 1). Searches were heuristic (100 random taxon addition replicates) with tree bisection reconnection (TBR) branch swapping. Nodal support was assessed using nonparametric bootstrapping with 1000 bootstrap replicates in parsimony analyses.

Neighbor-joining analyses incorporated the most appropriate model of nucleotide substitution identified by Modeltest 3.06 utilizing a likelihood ratio test (Posada and Crandall, 1998) for each gene (COI: GTR + G + I; I = .57 G = 1.25) (ITS1: TVM + G; G = .45). For the combined analysis a 6-parameter substitution model was applied to the entire data set and required a single estimation of the gamma distribution. Bootstrap values were calculated with 1000 replicates.

Nuclear substitution parameters used in the Bayesian analyses for the separate genes were the same as those used in NJ analyses. For the combined data set, the chosen model utilized two separate GTR models with varying gamma distributions and branch lengths; only the tree topology was held in common (i.e., 2(GTR + G + BrL) + 1T). All analyses consisted of 2 million generations with six incrementally heated chains, and trees sampled every 100 generations. Two replicate analyses were conducted. Burn-in time was determined by time of parameter convergence. Posterior probabilities were calculated in MrBayes on the remaining topologies following application of the appropriate burn-in value.

2.5. Genealogical species concept

Strongly supported nodes (having $\geqslant$95% posterior probabilities) were identified and compared between the two reconstructed Bayesian gene genealogies. Clades were hypothesized to be genealogical species if they were exclusive (reciprocally monophyletic) groups that were also basal (having no other exclusive groups nested within it) (Baum and Shaw, 1995). From now on, the term species, with no further specification, will refer to genealogical species.

2.6. Assessment of reproductive barriers

2.6.1. Crosses conducted

Reproductive success within each hypothesized species was evaluated when two or more populations existed for that species. In addition, a selection of between species crosses was conducted. Crosses are defined as the set of four "matings" (i.e., pairings between a female and male) conducted when evaluating reproductive success between two populations. In all but five cases, two reciprocal heterotypic (i.e., between two different clones, F1 × M2, F2 × M1) matings and two positive control, homotypic (i.e., between members of a single clone F1 × M1, F2 × M2) matings were conducted for each cross. Crosses focused on sister taxa, however, a few were omitted due to experimental difficulties (e.g., mictic ratios were very low in the Austrian clone in clade 4 and rapid and synchronized mixis induction/egg hatching complicated attempts to collect unhatched embryos in clades 11 and 12). Thus a total of 36 crosses (22 within species and 14 between species) were conducted. Five stages of reproduction were evaluated: parental mate recognition, parental fertilization, first generation (F1) development, F1 viability and asexual reproduction—F1(A), and F1 sexual reproduction—F1(S).

In most instances, the *within* species crosses were evaluated as absolute success or failure. The degree of success, however, was evaluated in the between-species and homotypic control crosses. Degree of success was measured as the number of successful events divided by the total number of trials. The reproductive success of heterotypic matings was then normalized by the percent success of the homotypic control matings. Because male rotifers are haploid and were found to account for a large proportion of the variation in reproductive success (Suatoni, 2003), each heterotypic mating was normalized by the homotypic mating of the male clone that was used in the heterotypic mating (e.g., F1 × M2/F2 × M2). The reproductive success of a cross was the arithmetic mean of the two normalized heterotypic matings.

2.6.2. Parental mate recognition

Behavioral bioassays followed the protocol of Snell and Hawkinson (1983), with minor modifications. Because propensity to mate decreases with age (Gómez and Serra, 1995), neonates aged 4 h or under were used in assays. One neonate male and 20 neonate females were put into a well with 250 μL of Instant Ocean™ at 15 psu at room temperature. Rotifer behavior was observed under a compound Leica MZ95 microscope with dark field illumination and videotaped with a Hitachi KP-D 50 color digital camera. The number of encounters and copulations for each replicate was counted. Ten 5-min replicate assays were conducted for each hetero- and homotypic mating. The posture taken by the male during copulation is consistent and reliably scored (male and female form an 'L' shape during which the male frequently carries the female in his characteristic sporadic swimming motion). Mate recognition was measured as the number of copulations/total number of encounters in the five-minute assay. Failed mate recognition was defined as no copulations in any of the replicate assays. Because mate recognition was observed for only a portion of the rotifer's sexually reproductive life-span, recorded measures are an index.

2.6.3. Parental fertilizations

Paired matings were conducted in the small wells of a plastic tissue culture plate. To ensure that no copulations occurred prior to the experiment, hundreds of eggs (male and female) were shaken off of adult females and isolated by placing them in individual wells during hatching. A single neonate male and female (four hours old and under) were paired in 18 μL of 15 psu, room temperature sea water (15 psu) with 1.5–2 × 10^6 *T. chui* cells per liter. Two hundred replicate pairings were conducted for each mating. Each well was fed 10 μL of the same medium on day two and the fertilization outcome was scored on day three. Success was denoted by the existence of a resting egg(s). Because asexual and sexual females are morphologically indistinguishable prior to sexual maturity, trials conducted with asexual females were discounted a posteriori— this caused the number of replicates to vary among crosses. An average of 52 paired matings was conducted for each of the two reciprocal hetero and homotypic matings. Fertilization success was scored as: (number of fertilized mictic females)/(the total number of mictic females). Note, because successful copulations were not observed during this assay, reported values for "fertilization success" are actually combined measures of mate recognition and fertilization success.

However, because mate recognition and "fertilization success" displayed different patterns of association with genetic distance (Suatoni, 2003), this complex parameter was believed to be largely driven by post-copulatory processes.

2.6.4. F1 development

The development of F1 females was scored as successful resting egg hatching. Following six weeks of incubation in 15 psu sea water at 6 °C in total darkness, resting eggs were put in 15 psu sea water at room temperature 6 in. below 40 W cool fluorescent light bulbs. For a period of one week, eggs were checked every 12 h for hatchlings and neonates were removed and cultured. Average number of eggs incubated for homotypic matings was 32. The average number of eggs produced for the distant heterotypic matings was much lower, ranging from 3 to 21, with a mean of 10. F1 development was scored as: (the number of hatched resting eggs)/(total number of resting eggs produced).

2.6.5. F1 viability and asexual reproduction, F1(A)

Rotifers that hatch from resting eggs are always amictic females; thus F1 viability and asexual reproduction were scored as the survivorship and parthenogenetic reproduction of hatchlings. These two factors could not be disentangled due to time delays in scoring. Cultures were started and maintained in the wells of a 12 well tissue culture plate in 2 mL of 15 psu, room temperature, sea water with approximately $0.5–1 \times 10^6$ *T. chui* cells per liter (high concentrations of food in cultures with such low density of rotifers sometimes resulted in death). The average number of hatchlings in homotypic matings was 24, and the average number of hatchlings for the more distant heterotypic matings was nine. F1(A) was scored as: (number of viable and reproductive hatchlings)/(total number of hatchlings).

2.6.6. F1 sexual reproduction, F1(S)

Cultures from hatchlings were amplified in 50 mL of 15 psu seawater with $1.5–2 \times 10^6$ *T. chui* cells per liter and kept under continuous illumination at room temperature. The presence of resting eggs in the bottom of these cultures was evidence that mixis was induced and that F1 males and sexual females were capable of fertilizing and being fertilized. In the interest of time, if more than 15 eggs hatched, only 15 females were scored for F1(S) fertility. Average sample sizes reflected those of the previous stage. F1(S) was scored as: (number of clonal cultures producing resting eggs)/(total number of viable hatchlings).

2.7. Morphotype classification

To determine whether the main phylogenetic clades are consistent with the major morphological divergences in *B. plicatilis*, one representative from 13 of the clades hypothesized to be species (excluding clades 2 and 15) was classified to morphotype. Lorica length and width, the two principal characters traditionally used in classifying *B. plicatilis* to 'type' (Fu et al., 1991c), were measured. Because environmental conditions (salinity, food type, temperature) and maternal effect are known to affect these characters (Ciros-Pérez et al., 2001; Snell and Carrillo, 1984), care was taken to rear each strain in controlled conditions for at least three generations.

Two replicate cultures were started for each strain to account for culture effects. Twenty egg bearing females of equal age derived parthenogenetically from one individual, were measured (10 from each replicate culture). Rotifers were reared in 15 psu Instant Ocean™ sea water at 23 °C, with constant light and were fed *T. chui* cells from axenic cultures at the concentration of 1×10^6 cells/mL. Axenic algae cultures were produced by the National Marine Fisheries Service, Milford, CT research laboratory. To control for maternal effects and eliminate the effects of competition, each culture was maintained in exponential growth phase for at least three asexual generations. This was achieved by inoculating each 100 mL culture with 50 individual clones and replacing half of the culture every third day with fresh medium and food.

After nine days, 120 egg bearing clones were isolated in fresh medium and observed for neonate hatchlings. Forty neonates (age four hours or less) were isolated in individual wells with 1.5 mL of medium containing 1×10^6 algae cells per mL. Forty-eight hours later 20 of the mature, egg bearing clones (10 from each of the two cultures) were preserved in Lugol's solution and measured with an ocular micrometer of a compound Olympus CH microscope. Lorica length and width were measured following the protocol of Ciros-Pérez et al. (2001). Non-parametric tests comparing means of the three major clades were conducted on length, width, and length/width ratios. Because only one clone from each population and only one population from each hypothesized species were measured, within species variation was not captured. Thus statistical comparisons were limited to the major clades.

3. Results

3.1. Phylogenetic analysis

Parsimony analysis of the COI data set using equal weights, a weighting scheme of tv:ts = 4:1, and exclusion of transitions produced the same consensus tree (data available from authors). No differences in topology were observed between MP, NJ, and Bayesian COI trees. The COI marker generally resolved relationships between individuals that displayed pairwise genetic distances below 16%. Nodal support for these clades was consistently high among the three tree building methods (ranging from 62% to 100%) (Fig. 1A).

There was little to no (0–1.0%) nucleotide variation in ITS1 between the individuals that showed less than 16% (GTR corrected) pairwise genetic distances in COI. Thus these individuals were collapsed into 15 terminal taxa in the ITS1 ingroup. With the exception of clade 1, each strongly

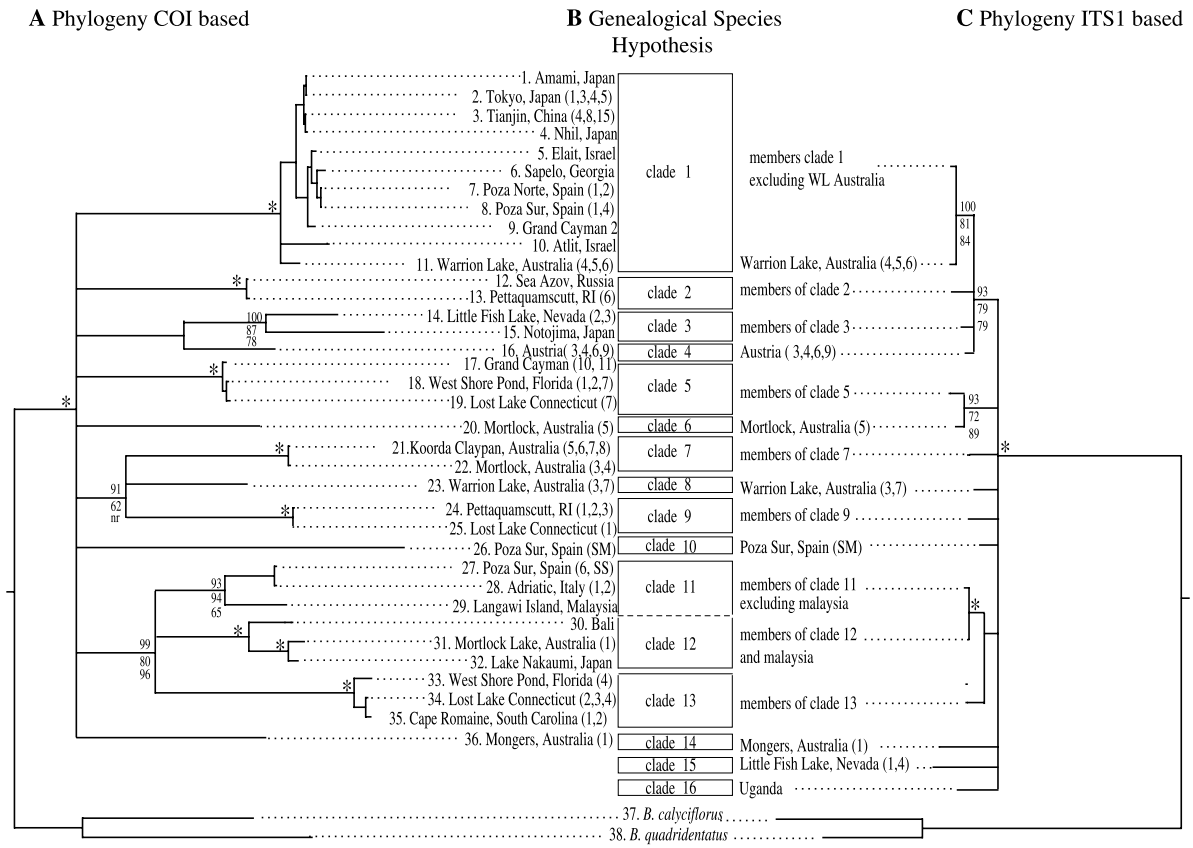


Fig. 1. (A) 90% majority rule consensus Bayesian phylogram of the COI data. Nodal support is given for the Bayesian, NJ, and MP analyses, respectively. * Indicates when posterior probabilities and bootstrap values for Bayesian, NJ, and MP trees are all $\geqslant$95%. Nodes which do not show support values had posterior probabilities or boostrap support values below 90%. (B) Genealogical species hypothesis identifies the reciprocally monophyletic clades between the COI and ITS1 gene trees having $\geqslant$95% posterior probability; such clades are hypothesized to be species. (C) 90% majority rule consensus Bayesian phylogram of the ITS1 data. Nodal support is given for the Bayesian, NJ, and MP analyses, respectively. * Indicates when posterior probabilities and bootstrap values for Bayesian, NJ, and MP trees are all $\geqslant$95%. Nodes which do not show support values had posterior probabilities or boostrap support values below 90%.

supported clade in COI corresponded to one terminal taxon in ITS1. The only discrepancy among these groupings was the placement of the clone from Malaysia which clustered with members of clade 11 in the COI tree and with members of clade 12 in the ITS1 tree (Fig. 1C). Two additional haplotypes, 'Uganda' and 'Little Fish Lake, Nevada 1' and '4', were incorporated into the ITS1 alignment, though there were no corresponding COI data, thus forming a total of 17 terminal taxa in the ITS tree (Fig. 1C). In contrast to COI, ITS1 provided resolution among populations that had higher levels of genetic divergence (i.e., >16% genetic divergence in COI). There was no strongly supported disagreement in topology between the three tree building methods for ITS1 trees.

The likelihood ratio test of the combined analysis was significantly worse ($\delta = 405.45$, $\mathrm{df} = 74$, $p = 3 \times 10^{-47}$) than the sum of the scores of the separated analyses, indicating that the two partitions were not compatible with the same model of molecular evolution. Thus the Bayesian analysis of the combined data set kept the parameters for the two partitions separate and held only tree topology in common. Both gene partitions failed tests of the molecular clock (LRT COI: $p = 1.5 \times 10^{-5}$ ITS1: $p = .016$).

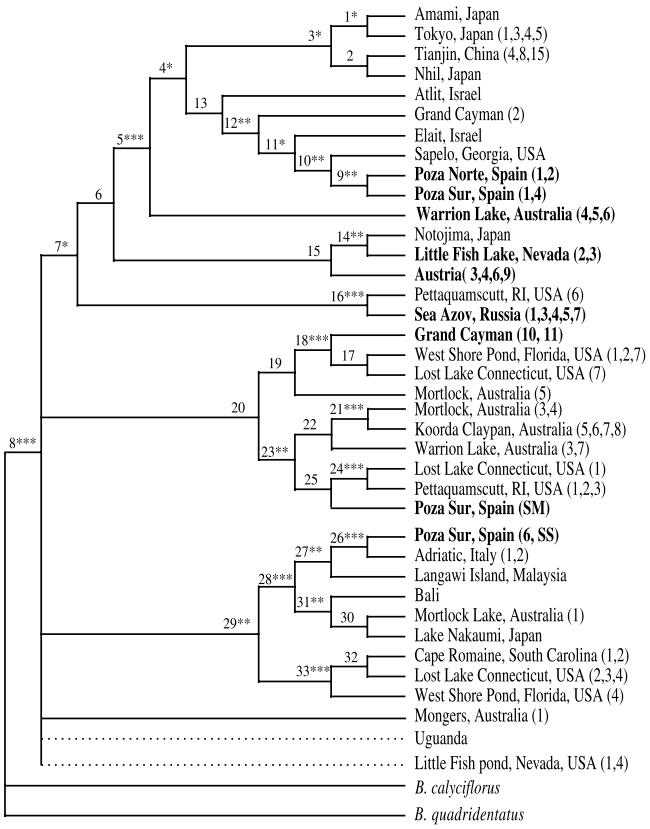
Once the trees from the combined analyses were collapsed into clades with $\geqslant$0.95 nodal support, all three tree building methods (MP, NJ, and Bayesian) agreed in the topology. The combined analysis provided resolution regarding evolutionary relationships at the full range of genetic variation and it is the only analysis to support the clustering of clade B, giving us our most comprehensive understanding of the phylogenetic hypothesis (Fig. 2). Although nodal support was not significant for clade B (0.79 in Bayesian analysis), the grouping is congruent with the presence of unique features like internal resting eggs. The tree showed six distinct lineages (A–F), three of which contained multiple hypothesized species. Rooting by outgroups suggest that the samples comprise a monophyletic clade of what was historically referred to as '*B. plicatilis*'.

Once the phylogenetic hypothesis was formed, we looked for patterns of isolation by distance in the single clade that had a sufficient number of populations (Clade 1), by conducting a Mantel test for genetic and geographic distance. The populations comprising clade 1, demonstrated a

A Support for clade

Clade	Post	NJ	MP
1	0.98	87	86
2	0.59	50	nr
3	0.99	100	100
4	0.98	nr	nr
5	1	100	100
6	0.85	nr	nr
7	0.99	82	65
8	1	100	100
9	1	99	94
10	1	91	78
11	0.98	54	57
12	1	86	91
13	0.65	58	55
14	1	93	78
15	0.84	nr	nr
16	1	100	100
17	0.77	68	63
18	1	100	100
19	0.81	78	nr
20	0.79	61	nr
21	1	100	100
22	0.51	60	nr
23	0.98	62	nr
24	1	100	100
25	0.72	nr	nr
26	1	100	100
27	0.97	94	66
28	1	100	100
29	1	99	96
30	0.93	100	99
31	1	98	98
32	1	100	99
33	1	100	100

B Phylogeny based on combined analysis



C Genealogical species hypothesis

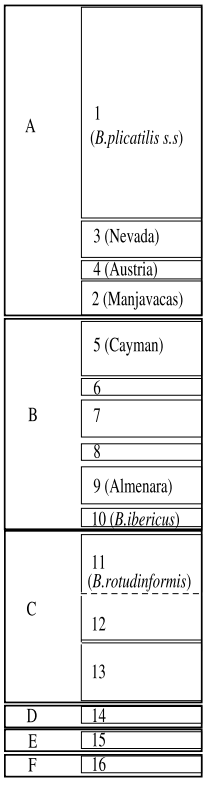


Fig. 2. (A) Nodal support for the phylogenetic hypothesis based on the combined data set of COI and ITS1 sequences. Clade numbers are indicated in the tree (Fig. 2B). 'Post' refers to Bayesian posterior probabilities; 'NJ' refers to bootstrap values for the neighbor joining analysis; 'MP' refers to bootstrap values for the parsimony analysis. (B) Summary of the phylogenetic hypotheses represented by the 50% majority rule consensus Bayesian tree on a combined data set of COI and ITS1 sequences. Taxa are defined by their geographic origin; the individual clones sequenced are indicated by their designated number in parentheses. Populations used in both this study and Gómez et al. (2002) are indicated in bold. * Indicates Bayesian posterior probabilities $\geqslant$95%. ** Indicates Bayesian posterior probabilities and bootstrap values for NJ, and MP $\geqslant$ 85%. *** Indicates when all three levels of support are 100%. Dotted lines attached to tree show taxa for which only ITS1 data existed. Taxa are numbered 1–38 for reference to Fig. 4. (C) The genealogical species hypothesis as derived in Fig. 1. Hypothesized species that coincide with Gómez et al.'s (2002) analysis are indicated with their terminology in parentheses beside the designated clade numbers.

positive correlation between COI genetic distances and degree of geographic separation (Mantel test $\rho = .53$, $p = .0086$).

3.2. The genealogical species concept and biological crosses

Genealogical species were defined as reciprocally monophyletic, basal groups with $\geqslant$95% Bayesian support, shared between the COI and ITS1 gene geneaologies. Fourteen clades fit these criteria suggesting the existence of 14 genealogical species (note: the one exception being the acceptance of clade 11 in the COI gene genealogy with 93% posterior probability) (Fig. 1B). For the ITS1 data set, the most basal monophyletic group that was reciprocal to the groups in the COI data set, were often single haplotypes. Thus, we recognized these collapsed, single haplotype clades as monophyletic groups.

It should be noted that there is a single discrepancy between the trees, confusing the definition of genealogical species. In the COI phylogeny, the clone from Malaysia is grouped with clade 11, yet, in the ITS1 tree, it is collapsed into clade 12. Because of the variable placement of this taxon and the low nodal support between these two clades in COI, the species status of clades 11 and 12 remains in question. Insufficient data in the ITS1 are the likely cause of this ambiguity, as the aligned portions of ITS1 differ by only two substitutions in clades 11 and 12. In addition to the 14 monophyletic groups, there are likely to be additional species as indicated by the unique taxa in clades 15 and 16 in the ITS 1 phylogeny (though there were no COI sequences of these taxa), making a tentative total of 15–16 hypothesized genealogical species.

All reproductive matings within hypothesized species were successful to the point of F1(S) fertility. Of the 14 crosses conducted between hypothesized species, 10 were unsuccessful (eight failed at the mate recognition stage, one failed at the fertilization stage, and one failed at F1

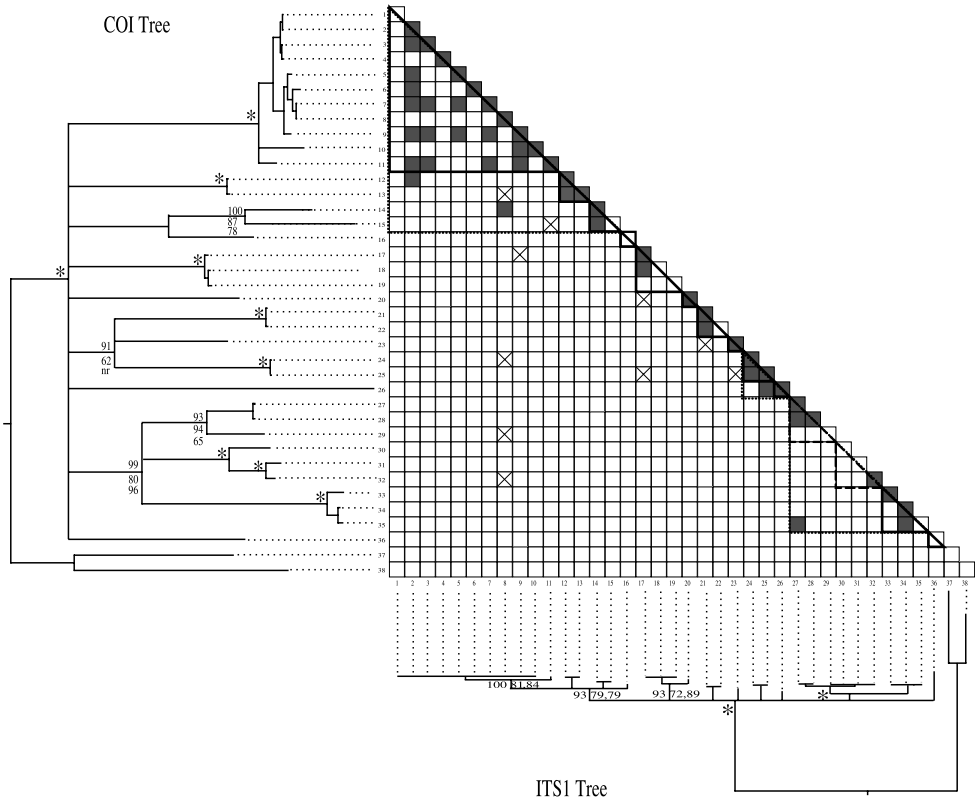


Fig. 3. Biological test of the genealogical species concept. Fig. 1 shows each individual clone enumerated in the two gene trees 1–38. The 38 × 38 matrix shows all possible biological crosses. Darkened squares indicate when reproduction was successful to the F1 generation. X's indicate when reproduction failed. Bold triangles demarcate the GSC. Dashed triangles demarcate discordance between the BSC and GSC. * indicates Bayesian posterior distribution ⩾ 95%.

development) (Fig. 3 and Table 1). Of the four successful crosses, two were successful to the point of F1(S) fertility and two demonstrated very low levels of fertilization success (below 10% of the success of the homotypic controls), and were not able to be scored beyond that stage.

3.3. Morphology

Mean and variance of lorica length (clade A = 281 μm, 824 clade B = 191 μm, 712 clade c = 144 μm, 195), lorica width (clade A = 202 μm, 279 clade B = 154 μm, 628 clade c = 122 μm, 146), and length/width ratios (clade A = 1.39, 0.01 clade B = 1.24,0.01 clade c = 1.18, 0.05) of each of the three major clades were significantly different (Wilcoxon/Kruskal–Wallis Tests all $p < .001$, Fig. 4). Similarly, the *XY* plot of lorica width on length showed that although there is some degree of overlap, the three major clades clustered separately (Fig. 4). Thus the phylogenetic history of the group loosely reflects the morphotype classification that is pervasive in the literature. However, it should be noted that the clone from Mongers, Australia in clade D (Fig. 2C) is a comparatively large clone, and clades E and F have the smallest clones in entire study, by visual estimation. Because the relationships of these clades to the three major clades are not known, there may be more than three basic morphotypes (large, medium, and small), and/or they may not be monophyletic.

4. Discussion

4.1. Taxonomic implications of phylogeny and reproductive barriers

The traditionally recognized taxon, *B. plicatilis* Müller, appears to be a monophyletic group, showing extensive genetic variation, indicating that further revision of the taxonomy is warranted. Consistent with the findings of Gómez et al. (2002), there is evidence of three principal, deeply diverged clades each comprised of lineages likely to have species status (Figs. 2 and 3). Our results, however, increase diversity estimates to a range of 7–14 species (with the potential for more, as some taxa were excluded from the analysis due to lack of COI sequence data (clades 15 and 16) or were not subject to biological crosses).

Based on a high degree of sequence divergence and largely concordant genetic patterns in the COI and ITS1 (Fig. 1), the genealogical species hypothesis posits the existence of 14 species within the *B. plicatilis* complex (with two additional potential species, based on the ITS1 gene tree). Again, there is considerable agreement between our analysis and that of Gómez et al. (2002), as the present study identifies eight of the nine clades previously hypothesized to be species (missing only 'Tiscar') and places them in consistent relationships. Many of the same clones included in Gómez et al. (2002) are contained in the present analysis

Table 1
Reproductive success as indicated by experimental crosses

Cross	Clade # of hypothesized Species	Genetic distance (COI, ITS)	Normalized reproductive success[a]				
			Mate recognition	Fertilization	Resting egg hatching	F1(A) viability and repro.	F1(S) repro.
Within species							
15 crosses in species 1	1	0.00–0.08, 0.0–0.01	0.7–2.33	0.21–1.11	0.67–1.13	0.73–1.06	1.0–1.0
Pettaquamscutt 2 × Lost Lake 1	9	0.002, 0.0	+	+	+	+	+
Poza Sur 6 × Italy 1	11	0.003, 0.0	+	+	+	+	+
Koorda Claypan 7 × Mortlock 3	7	0.003, 0.0	+	+	+	+	+
Russia × Pettaquamscutt 6	2	0.005, 0.0	+	+	+	+	+
Lost Lake 2 × Cape Romaine 1	13	0.006, 0.0	+	+	+	+	+
Grand Cayman 10 × West Shore Pond 1	5	0.010, 0.0	+	+	+	+	+
LFL Nevada 2 × Notojima	3	0.160, 0.0	+	+	+	+	+
Between species							
Koorda Claypan 7 × Warrion Lake 7	7 × 8	0.151, 0.044	0.00	–	–	–	–
Warrion Lake 7 × Lost Lake 1	8 × 9	0.171, 0.045	0.22	0.07	0.00	–	–
Poza Sur 6 × Cape Romaine	11 × 13	0.183, 0.069	0.28	0.09	Not tested	Not tested	Not tested
Grand Cayman 10 × Lost Lake 1	5 × 9	0.183, 0.088	0.00	–	–	–	–
Grand Cayman 10 × Mortlock 5	5 × 6	0.209, 0.134	0.00	–	–	–	–
Poza Sur 1 × LFL Neveda 2	1 × 3	0.213, 0.122	0.16	1.12	+	+	+
Lost Lake 1 × Poza Sur SM	9 × 10	0.228, 0.031	0.23	0.23	+	+	+
Poza Sur 1 × Pettaquamscutt 2	1 × 9	0.254, 0.142	0.00	–	–	–	–
Poza Sur 1 × Malaysia	1 × 11	0.259, 0.140	0.00	–	–	–	–
Grand Cayman 2 × Grand Cayman 10	1 × 5	0.263, 0.165	0.00	–	–	–	–
Tokyo 4 × Russia 5	1 × 2	0.269, 0.085	0.36	0.07	Not tested	Not tested	Not tested
Poza Sur 1 × Lake Nakaumi	1 × 12	0.275, 0.183	0.00	–	–	–	–
Poza Sur 1 × Pettaquamscutt 6	1 × 2	0.292, 0.085	0.00	–	–	–	–
Warrion Lake 5 × Notojima	1 × 3	0.317, 0.031	0.50	0.00	–	–	–

Results are given for five reproductive phases. Reproductive success is the average of the two normalized reciprocal matings.Reciprocal heterotypic matings were normalized by the % success of the homotypic mating.'+' indicates when reproduction was successful (but not measured as % success).'–' indicates when assay was not possible due to failure in a previous stage.

[a] Detailed analysis of reproductive success data in Suatoni et al., submitted crosses in order of increasing genetic distance of COI.

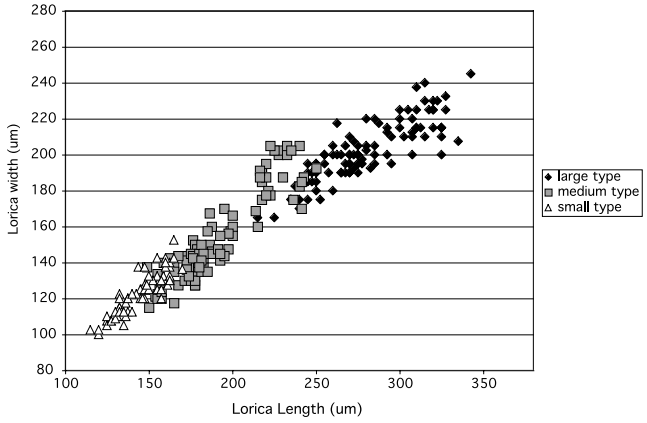


Fig. 4. Comparison of lorica length/width measurements for 'large', 'medium', and 'small' strains of *B. plicatilis*.

making it easy to inter-compare trees (Fig. 2—populations common to both studies are indicated in bold and Gómez et al.'s (2002) species names are indicated in parentheses beside designated clade numbers).

Reproductive crosses yielded evidence for the existence of 7–10 biological species, depending on the criteria adopted to declare reproductive failure. It is important to note, only 11 of the 14 clades identified by the genealogical species hypothesis were tested, thus these numbers exclude three clades. Furthermore, because the number of crosses conducted was a modest subset of the total number of potential crosses, a formal BSC cannot be proposed. Instead, the analysis focuses on the degree of concordance between species concepts.

Four crosses were conducted between taxa from different major clades (i.e., A, B, C) and none resulted in successful reproduction. Ten crosses were conducted between sister taxa within the major clades; six failed and four succeeded (Table 1 and Fig. 3). Of the four cases in which mating was successful, two showed very low levels of fertilization success (<10% of the average success of a homotypic control cross between clones 'Tokyo' × 'Russia' (clades 1 and 2) and 'Poza Sur 6' × 'Cape Romaine' (clades 11 and 12)), and two were relatively successful to the first generation ('Poza Sur 1' × 'LFL Nevada 2' (clades 1 and 3) and 'Lost Lake 1' × 'Poza Sur SM' (clades 9 and 10)). Consequently, if we count any degree of reproductive success as evidence of a single reproductive unit, then the analysis merges seven distinct genealogical species into three (Fig. 3: clades 1, 2, and 3 merge; clades 9 and 10 merge, and clades 11 and 13 merge); the four remaining genealogical species that were tested by crosses remain separate. Whereas, if inconsistent outcomes, or very low levels of reproductive success, between clades (e.g., clades 1 × 2, 1 × 3, 11 × 12) are regarded as evidence of reproductive isolation then only

two genealogical species collapse to form one (clades 9 and 10 merge) and nine remain separate.

Thus the level of concordance between the two species concepts depends largely on the degree of reproductive success used in establishing biological species. In other words, the two methodologies appear to be converging at the fuzzy boundary of reticulate and divergent evolution, necessitating the qualitative judgments of a taxonomist. Whatever approach taxonomists might adopt, it is clear that the lineages within the major clades have become, or are in the process of becoming, reproductively isolated. And, it is on this, and lower genealogical scales that studies of speciation should concentrate.

It is important to emphasize that the present analysis of the biological species concept is a laboratory reduction, testing only intrinsic barriers to reproduction. Consequently, the results are likely to be biased towards grouping clades. Field investigations are necessary to help researchers interpret the significance of low levels of reproductive success in the lab. For example, using ITS1, Ortells et al. (2000) found two clusters of allozyme genotypes, identified as clade 9 (*B.* 'almenara') and clade 10 (*B. ibericus*) (they were identified using ITS1 sequences diagnostically), coexisting in a pond in Spain, with no evidence of hybridization. The present study, however, shows evidence of successful reproduction between members of these clades in the lab (note, however, we crossed *B.* 'almenara' and *B. ibericus* from ponds of two different continents). Divergent local adaptation to environmental cues in factors such as resting egg hatching, or timing of mixis, could help to maintain these potentially reproductive, sympatric species.

High levels of agreement between phylogenetic and biological species concepts are not necessarily expected, as they identify different entities at different stages of the speciation process (Harrison, 1998; Sites and Marshall, 2004). When employing the stringent criterion of reciprocal monophyly at many loci, genealogical species are expected to emerge last and to generally produce lower diversity estimates (Harrison, 1998). Our observation of a higher number of genealogical species than biological species is likely a result of the low number of genes used in generating the hypothesis. Even with only two randomly chosen loci, however, the high degree of congruence between the gene trees, and between the phylogenetic hypothesis and reproductive boundaries, is not necessarily expected due to ancient polymorphism and/or introgression (Ting et al., 2000), and is noteworthy. It may, in fact, be evidence that many of the hypothesized species are old and the time since speciation may have been great enough for coalescence to have occurred.

Although the analysis of lorica size and shape indicates that the three major phylogenetic clades are consistent with the major morphological divergences (Fig. 4), gross morphology does not appear to be a good indicator of species boundaries. Historical reliance on lorica size and shape to distinguish species of *Brachionus* results from the fact that they lack a complex external morphology, having no ornaments or appendages. Biometrical analysis is further complicated by the substantial plasticity of body size and shape, in addition to maternal effects (Ciros-Pérez et al., 2001; Snell and Carrillo, 1984). These factors make properly executed morphological analyses considerably more taxing than molecular studies.

Still, the morphotype classification scheme and its consequent lumping remain dominant in the literature. It is clear that both applied and basic research on *B. plicatilis* will benefit from a taxonomic revision that more closely reflects its evolutionary history, and that continued fruitful interaction between the fields may depend on it. Adherence to the 'L'- and 'S'-type classification is likely to obscure important biological patterns. For example, there is currently a large data base on the diverse physiological responses of *B. plicatilis* strains to environmental factors (e.g., Hagiwara and Lee, 1991; Snell, 1986). The proposed revision could be used to reorganize and clarify the wide diversity of observed responses. The patterns of polymorphisms in the ITS1 offer a promising diagnostic tool for such a re-classification.

4.2. Timing of speciation

Conventional methods to estimate divergence times (absolute or relative) for the *B. plicatilis* complex were not feasible in this study. First, fossil rotifers are rare, with examples from only one genus (*Habrotrocha*) of a Bdelloid rotifer having been found (Waggoner and Poinar, 1993; Warner and Chengalath, 1988). With no known examples of Brachionids, it was impossible to calibrate a molecular clock. Second, a lineage-through time analysis which can reveal general patterns in speciation (Paradis, 1998) was thwarted by non-linear substitution rates throughout the phylogeny and failed attempts to conduct a rate smoothing approach due to these exceptionally heterogeneous substitution rates (Sanderson, 2002). Although a previous analysis estimated hypothesized species within the *B. plicatilis* complex to be 10–20 million years old (Gómez et al., 2002), their conclusions should be regarded as questionable as they relied on calibrations of a molecular clock from distantly related invertebrates. Their use of *Drosophila* and snapping shrimp is especially speculative given evidence that the ingroup itself has experienced changes in substitution rates, and that halophilic invertebrates can display accelerated rates of molecular evolution (Hebert et al., 2002).

Despite these challenges, however, there are patterns in our data to indicate that the species complex is old. First, high concordance between the gene trees of the two loci suggest that sufficient time has passed for coalescence to have occurred. Additionally, the similarities between the genealogical and biological species concepts suggest that they are converging—a pattern expected only after significant time has passed. Lastly, both genetic markers display a noticeable gap between 'within species' distances and 'between species' distances, as defined by the GSC (i.e.,

there are two clusters of branch lengths); this suggests that coalescence time within any given species is almost always much less than the speciation times in the tree (there are a few exceptions). While, in any given monophyletic group coalescence time is always, by definition, less than its speciation time, there is nothing to constrain the coalescence time of every species to be less than the speciation times between all species. This pattern, observed across 13 taxa, supports the idea that all of the species in the group are old ($\gg$4Ne generations of even the largest 4Ne of the 13 species). Attempts to translate $\gg$4Ne generations into absolute times, however, would be speculative. It is commonly believed that the average sexual generation time of *B. plicatilis* is one year, at least for temperate ecosystems (Serra et al., 2004). However, estimation of population sizes (N or Ne) is much more uncertain, as there are few empirical data on the topic; though, they are generally regarded to be large.

Researchers have speculated that the asexual phase of cyclical parthenogens could have population genetic consequences that result in rapid evolution or increased chances of speciation (King, 1977). Because populations can be founded by a single individual that can undergo prolonged periods of parthenogenesis, genotypic frequencies can readily deviate from Hardy–Weinberg equilibrium, and linkage disequilibrium is more likely to arise between traits (Hebert, 1987). In addition, theoretical models have shown that the parthenogenetic phase can accelerate selection and magnify genetic drift (King, 1993). In contrast to these predictions, however, speciation within the *B. plicatilis* group as indicated by the number of hypothesized species over the suggested—long time frame, does not appear particularly rapid.

4.3. Ecological and geographic distribution

Many of the putative species containing more than one population (i.e., 6 out of 9) show a broad, transcontinental geographic distribution indicating that long-range dispersal, though passive, is very effective in this taxon. Zooplankton species, like monogonont rotifers, are frequently widely distributed since long distance dispersal via wind, water, or animals is aided by environmentally resistant resting eggs (De Meester et al., 2002). *B. plicatilis* does not completely lack biogeographic structure, as ubiquitous microbial eukaryotes do (Finlay, 2002), however. In the clade for which there were sufficient numbers of populations (clade 1), a positive correlation between COI genetic distances and degree of geographic separation was demonstrated (Mantel test $\rho = .53$ $p = .0086$), consistent with isolation by distance.

As predicted by Finlay (2002), rotifers with body sizes of approximately 0.1–1 mm (i.e., micro-meiofauna) display a biogeographic pattern that is intermediate to the lack of spatial structure typical of microbial organisms and the high degrees of biogeographical structure usually found in large vertebrates. It is likely that the biogeographic patterns in *B. plicatilis* are formed by sufficiently frequent transcontinental dispersal over long time scales to produce cosmopolitan distributions followed by allopatric divergence over microevolutionary time scales, creating a pattern of isolation by distance. We expect that the speciation events characterized by the present phylogenetic analysis are ancient enough for the dispersal abilities of *B. plicatilis* to have eroded geographic signatures of speciation.

A dominant pattern in this investigation is that *B. plicatilis* species frequently live in sympatry. Of the twelve sites where resting egg hatching was feasible, nine contained two to three species. This frequency of sympatry is expected to be an underestimate since the number of resting eggs hatched from any given site was quite low (for example, two of the sites with only one taxon had only two eggs hatched). Most interesting is the pattern of community composition that is emerging from the study. Seven of the nine sites are comprised of species from two or three of the different major clades (i.e., the deeply diverged clades—either A, B, C, D, or E in Fig. 2C). In the two instances where taxa from the same major clade were seen to coexist (clones 'Mortlock Lake 3' and '5' and 'Lost Lake 1' and '7' in clade B), they were not sister taxa and each exhibited a large degree of genetic divergence. This pattern raises questions about whether more closely related taxa are able to coexist. It is possible that though species within the major clades are reproductively isolated, they have sufficient niche overlap to result in competitive exclusion.

5. Conclusion

This investigation is the first with sufficient breadth to acquire an idea of the extent of species diversity in *B. plicatilis* and to get a glimpse of its large-scale biogeographic and ecological patterns, thus enabling us to formulate a reasonable scenario of the group's radiation and subsequent dispersal. The *B. plicatilis* group is a cryptic species complex containing a minimum of seven—and possibly up to14 or more—species which split greater than 4Ne generations ago. Whatever the exact time, it was sufficiently long ago that passive dispersal has since given many of the species a global distribution. The fact that ponds with more than one species tend to contain representatives of different major clades is probably a result of niche partitioning based on size (or some correlated trait) combined with the fact that different major clades tend to exhibit different body sizes. The current distribution of members of the *B. plicatilis* group is thus a function more of historical colonization and ecology than of phylogeny.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2006.04.025.

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