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PHYLOGENETIC SYSTEMATICS OF THE AUSTRALIAN FAIRY SHRIMP GENUS *BRANCHINELLA* BASED ON MITOCHONDRIAL DNA SEQUENCES

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A B S T R A C T

Phylogenetic reconstructions derived from analyses of DNA sequence variation in a segment of the mitochondrial ribosomal RNA (16S) gene revealed an extensive radiation of species within the genus *Branchinella* from Australia. Genetic differentiation was evident among all of the species analyzed, including two recently discovered species. There was limited agreement, however, concerning taxon affinities deduced from the 16S data and a previous morphological study. Members of the genus appear to have diversified rapidly, but analyses of other genes and broader taxon sampling are crucial to confirm this hypothesis. Disparities between levels of morphological and molecular change were noted between certain taxa. Whether the radiation of Australian *Branchinella* happened *in situ* or was the product of independent colonization events will require the analysis of branchinellids that occur outside Australia.

Species comprising the fairy shrimp genus *Branchinella* occur in freshwater habitats in Europe, Asia, North and South America, southern Africa, and Australia. They are exceptionally diverse, however, in Australia where 27 species are currently recognized, all of which are endemic. Eighteen of these species have been described in detail by Geddes (1981) based on his critical review of earlier morphological works (e.g., Sayce, 1903; Linder, 1941) along with his own analysis of additional collections. Recent surveys and morphological work have led to the discovery of nine more species of *Branchinella* from this continent (Timms, 2001, 2002; Timms and Geddes, in press). Two of these new species, e.g., *B. campbelli* and *B. budjiti* (Timms, 2001), have been analyzed in the present study; the remaining seven species are formally described elsewhere (Timms, 2002; Timms and Geddes, in press). It should be noted that in Belk and Brtek's (1995) checklist, two of the designated subspecies of *B. nichollsi*, i.e., *B. n. hattahensis* and *B. n. buchananensis* (Geddes, 1981), were elevated to species rank, albeit without justification. Because the present results (see below) support Geddes' (1981) recognition of these two subspecies, we have adopted his taxonomic scheme.

Characters that have been commonly used to differentiate species of Australian *Branchinella* are the presence or absence of frontal appendages (organs) on the second antennae of males, structural details of the phyllopods, and penes. Geddes (1981) proposed a hypothesis of species relationships based primarily on these characters, partitioning branchinellids into three species groups (Table 1). It has been recognized, however, that these characters not only vary among closely related species but also within species, and they may overlap between species (Geddes, 1981; Timms, 2001). Affinities among species based on one set of morphological features and those based on a different set are not always mutually inclusive, suggesting that these characters may have been influenced by convergent evolution. For these reasons, a purely morphological approach is unlikely to produce a robust indication of species relationships.

The high level of species diversity and endemism in Australian *Branchinella*, along with their ability to occupy inland waters with a broad range of salinities (i.e., 0 up to 43 g/L) (Geddes, 1981; Geddes, 1983; Timms and Sanders, 2002), makes this genus an ideal group for evolutionary and ecological studies. Here we used DNA sequences coding for a region of the mitochondrial (mt) ribosomal RNA gene (16S) as an independent source of phylogenetic information to provide an estimate of the evolutionary relationships within Australian *Branchinella*, test the validity of the species groups proposed by Geddes (1981), and distinguish between phylogenetically informative and homoplasious morphological traits within the genus.

Table 1. Species of Australian *Branchinella* arranged according to the scheme of Geddes (1981). Asterisks (*) indicate species (or subspecies) and populations analyzed in this study. Species not included by Geddes in his scheme, as well as the recently discovered species and undetermined populations, are listed as uncategorized. Figures in parentheses after taxon names are the number of individuals examined. NSW, New South Wales; QLD, Queensland; SA, South Australia; WA, Western Australia.

Taxa	State	Localities
Group I		
B. australiensis (Richters, 1876) (3)*	NSW, QLD	Paroo, Claremont 1
B. occidentalis Dakin, 1914 (2)*	NSW, SA	Paroo, Nilpinna
B. nichollsi Linder, 1941		
B. n. hattahensis Geddes, 1981(2)*†	QLD	Paroo-Kaponyee Lake
B. n. buchananensis Geddes, 1981 (2)*†	NSW	Paroo-Gidgee Lake
B. compacta Linder, 1941		
Group II		
B. longirostris (Wolf, 1911) (1)*	WA	King Rocks, via Hyden
B. probiscida Henry, 1924 (2)*	NSW and QLD	Paroo
B. affinis Linder, 1941 (2)*	NSW, SA	Paroo, Pandie Pandie-Appamurra
B. latzi Geddes, 1981		
B. denticulata Linder, 1941		
B. apophysata Linder, 1941		
Group III		
B. pinnata Geddes, 1981(3)*	NSW, QLD	Paroo, Rockwell
B. wellardi Milner, 1929(1)*	NSW	Paroo
B. frondosa Henry, 1924(2)*	QLD	Rockwell
B. arborea Geddes, 1981(1)*	NSW	Paroo
B. basispina		
B. dubia		
Uncategorized		
B. lyrifera Linder, 1941 (4)*	QLD, SA	Paroo-Kaponyee Lake, Bobbiemongie
B. simplex Linder, 1941		
B. budjiti Timms, 2001(5)*	NSW, SA	Paroo, Oodnadatta and Warburton Crossing
B. campbelli Timms, 2001 (1)*	NSW	Paroo
Undetermined (1)*	WA	Kadji Kadji
Undetermined (1)*	QLD	Claremont 3
7 other new species‡		

† Considered as subspecies of *B. nichollsi* by Geddes (1981), but elevated to species rank by Belk and Brtek (1995).
‡ Timms, 2002; Timms and Geddes, in press.

Materials and Methods

Taxa Studied

We examined ethanol-preserved samples for 12 of the 27 known species of Australian *Branchinella*. These samples included two subspecies of *B. nichollsi* as well as specimens from populations of uncertain taxonomic status from two localities (Table 1). We used representatives from two genera, *Thamnocephalus platyurus* and *Streptocephalus dorothae*, as outgroups because on morphological and molecular grounds they appear to be the closest relatives of *Branchinella* (Linder, 1941; Remigio and Hebert, 2000).

DNA Preparation, PCR Amplification, and Sequencing

Procedures for extracting fairy shrimp genomic DNA, PCR amplification of approximately 500 base pair (bp) of the target mt 16S gene region, and generation of 16S sequences from purified PCR products are given in Remigio *et al.* (2001). The PCR products were sequenced in both directions when ambiguous nucleotide positions were detected in the initial analysis.

Sequence and Phylogenetic Analyses

All sequences obtained in this study have been deposited in GenBank (accession codes AF308940–AF308947; AF527556–AF527576). They were aligned "by eye" in ESEE (Eyeball Sequence Editor, version 3.2; Cabot and Beckenbach, 1989). The data matrix used in all analyses is posted at the cladoceran website (www.cladocera.uoguelph.ca). Phylogenetic analyses were performed in PAUP* 4 (beta version 8, Swofford, 1998). A molecular phylogeny was inferred using maximum parsimony (MP) and maximum likelihood (ML) analyses. The MP analysis was performed via heuristic searches using 100 replicates of random sequence addition, tree-bisection-reconnection (TBR) branch swapping, and assuming equal weights for and unordered treatment of all characters. Character states were optimized using accelerated transformation (ACCTRAN). To generate the maximum likelihood (ML) tree

that best fits the 16S data, likelihood-ratio tests (LRTs) (Felsenstein, 1981; Goldman, 1993) were performed. This method involves comparing log-likelihood (−lnL) scores between trees resulting from differing assumptions about the nature of DNA change (e.g., whether nucleotides occur in equal frequencies, whether the occurrence and rates of transitions and transversions are equal, whether there is rate homogeneity, whether sequence changes at certain sites are constrained) (see Huelsenbeck and Crandall (1997), Huelsenbeck and Rannala (1997) for a detailed description of this approach), and testing for significant statistical differences between them via the LRT. Four hierarchically nested models of sequence evolution were used (Table 2), and LRTs were performed following the procedure outlined in Posada and Crandall (1998). Depending on the model of DNA evolution employed, parameters such as transition/transversion ratio, substitution rate matrices, as well as those associated with the assumption of rate heterogeneity (e.g., a proportion of sites is invariable, gamma-distributed rates at all sites) were estimated from actual data. Empirical base frequencies, where applicable, were used. Trees were constructed through heuristic searches, utilizing the neighbor-joining algorithm to generate initial trees and nearest-neighbor-interchange as the branch swapping algorithm. Bootstrap (Efron and Tibshirani, 1993) and decay (Bremer, 1994) analyses were performed to measure support for internal branches. For the former procedure, the data set was resampled 1000 times, whereas the latter method involved analyses of 100 replicates using a full heuristic algorithm. MacClade (version 3.04; Maddison and Maddison, 1992) was used to examine alternative tree topologies, including that constrained to match Geddes' (1981) species groupings. The results were subsequently subjected to the likelihood-based analysis of Shimodaira and Hasegawa (1999; see also Goldman *et al*., 2000), employing 1000 replicates assessed by the resampling estimated log-likelihood method, to determine which topology provides the best estimate of relationships.

Results

The 16S sequences had a high A + T content (mean = 63%). A chi-square goodness-of-fit test indicated, however, that there were no significant changes in nucleotide frequencies across taxa, including those of the outgroups ($\chi^2 = 6.25$, $d.f. = 102$, $P > 0.99$). The aligned data set was 475 bp long. Assessment of positional homology was straightforward as only four alignment gaps (i.e., insertion/deletion events, which were never more than two sites long) were introduced into the alignment. Because these gaps are autapomorphies confined to either or both outgroups, they were excluded from subsequent analyses. The final data set used in all analyses, which included sequences from the two outgroup taxa, contained 145 variable positions, of which 95 were parsimony informative. There was little or no variation between conspecific individuals within a population, but divergences ranging from 2% to 2.5% were detected between populations of *B. lyrifera* from Queensland and South Australia, *B. affinis* from New South Wales and South Australia, and *B. australiensis* from Queensland and New South Wales. Sequence differences among species were highest in comparisons involving *B. longirostris* and the remaining taxa (8–10%). Levels of divergence between the ingroup taxa and each of the designated outgroup genera were comparable (*Thamnocephalus:* 14.7–17.6%; *Streptocephalus*: 15.3–17.6%).

The single most-parsimonious tree resulting from heuristic searches is shown in Fig. 1 (length = 362 steps, consistency index (CI) excluding uninformative sites = 0.48, retention index (RI) = 0.73). This figure also shows variation in the shape of the frontal appendage as well as in mean body length of males. The tree identified a clade (A) composed of *B. n. hattahensis* + *B. n. buchananensis* that is the sister group to a large assemblage (B) consisting of two subclades (b1 and b2). There was generally high bootstrap (bs) and Bremer (di) support for the terminal branches. However, those at the intermediate and deeper portions of the phylogeny (represented by dashed lines in the tree) were resolved with low confidence (bs $< 50\%$, di $\leqslant 2$). The results of MP bootstrap analyses showed that when branches receiving less than 50% bootstrap scores were collapsed (indicated by the dashed lines in Fig. 1), the base of the tree was depicted as an unresolved polytomy. Hierarchical LRTs revealed that a parameter-rich model, GTR + G + I, best fitted the 16S data of Australian branchinellids (Table 2). The single most likely tree (–ln L = 2330.97) based on this model is identical to Fig. 1, except in the differing placements of *B. campbelli* and *B. wellardi* within clade b2. As in Fig. 1, branches at the deeper parts of the ML tree were poorly supported. Regardless of the phylogenetic reconstruction method used, all trees consistently resolved clade b1, but with poor branch support.

Discussion

The present study has shown that an extensive radiation of the genus *Branchinella* has occurred in Australia. There was strong molecular divergence among all of the species analyzed, including two recently discovered species (*B. campbelli* and *B. budjiti*), a finding that was reflected in well-resolved terminal branches in all of the molecular trees. The genetic distinctiveness of these species supports the conclusions from previous morphological

Table 2. Results of hierarchical likelihood-ratio tests.

Null hypothesis	Models tested	−lnL H_0	−lnL H_1	−2lnL	*d.f.*	*P*
Equal base frequencies	H_0: JC69 H_1: F81	2690.58	2671.90	37.36	3	<0.001
Equal ti/tv rates	H_0: F81 H_1: HKY85	2671.90	2599.48	144.84	1	<0.005
Equal ti and equal tv rates	H_0: HKY85 H_1: GTR	2599.48	2553.86	91.24	3	<0.001
Equal rates among sites	H_0: GTR H_1: GTR + G	2553.86	2341.89	423.93	1	<0.001
Proportion of invariable sites	H_0: GTR + G H_1: GTR + G + I*	2341.89	2330.97	21.84	1	<0.001

* Six substitution rate-matrix values (r_1 = 1.92, r_2 = 30.20, r_3 = 9.81, r_4 = 5.41 e − 08, r_5 = 31.25, r_6 = 1.00), α = 0.50, I = 0.52, four rate categories, and empirical base frequencies were used.

studies (Geddes, 1981; Timms, 2001). However, molecular support for the morphological species groups proposed by Geddes (1981) was limited to Group III (see Fig. 1). In all analyses, a clade composed of *B. pinnata*, *B. arborea*, *B. frondosa*, *B. wellardi*, and an undetermined population from Queensland (*B.* sp. Clr3QLD) was resolved with good support (bs = 75–77%, di = 4). Two other species (*B. dubia*, *B. basispina*) were placed in this group by Geddes (1981), but were not examined in this study. Their analysis in future studies would be critical to fully confirm the validity of this species assemblage. In contrast, Group I was not supported by the 16S data because in all analyses *B. n. hattahensis* and *B. n. buchananensis* (and by implication *B. nichollsi*) were positioned well away from its other members (*B. australiensis*, *B. occidentalis*). Enforcing the monophyly of these species based on Geddes' (1981) scheme increased the tree length by 9–11 steps relative to that presented in Fig. 1. The 16S data indicate a close association between *B. lyrifera* (a species Geddes (1981) did not assign to any of his species groups) and *B. australiensis*. Moreover, the genetic data indicate that these species are sister to *B. occidentalis*. The precise nature of the relationships of these three species could not, however, be established due to poor branch support. The close affinity of these species is intriguing because *B. lyrifera* is small-bodied, whereas *B. australiensis* and *B. occidentalis* are both large. Size differences among these taxa are probably correlated with a shift in their feeding behavior, as *B. occidentalis* is a predator of other fairy shrimp and *B. australiensis* is a partial predator (Rogers and Timms, unpublished data), whereas *B. lyrifera* is a filter feeder. The 16S data suggest that within *B. nichollsi*, *B. n. hattahensis*, and *B. n. buchananensis* deserve subspecies status, as proposed by Geddes (1981) on morphological grounds. These two taxa are separated by molecular distances of 1.7–1.9%, which are higher than those found between most conspecific individuals within a population, but less than those between species whose taxonomic status is clear. It is imperative, however, to obtain the 16S sequence for *B. n. nichollsi* in order to assess the extent of sequence divergence between the nominate form and the other subspecies.

The validity of Group II is also refuted by the 16S data because in all analyses *B. longirostris* and *B. probiscida* were well separated from *B. affinis*, the only other Group II species that we examined. Constraining these species to form a monophyletic clade required 16–22 more steps than the most-parsimonious solution. The identification of *B. affinis* as being more closely allied to *B. budjiti* (bs = 90%, di = 4) than it is to *B. longirostris* and *B. probiscida* further argues against the validity of Geddes' (1981) Group II. To obtain more insights on species affinities in this group, it is critical to sequence other Group II species (e.g., *B. denticulata*, *B. latzi*, *B. apophysata*) that were not analyzed in this study. Imposing Geddes' (1981) overall scheme on the molecular phylogeny required the addition of 27 steps, and gave the poorest estimate of relationships relative to the MP and ML trees based on Shimodaira and Hasegawa's (1999) test ($P < 0.05$).

Our study has confirmed that two of the recently discovered species, *B. campbelli* and *B. budjiti*, are genetically distinct, exhibiting divergences ranging 6–9% and 4–6%, respectively, from other members of the genus. This

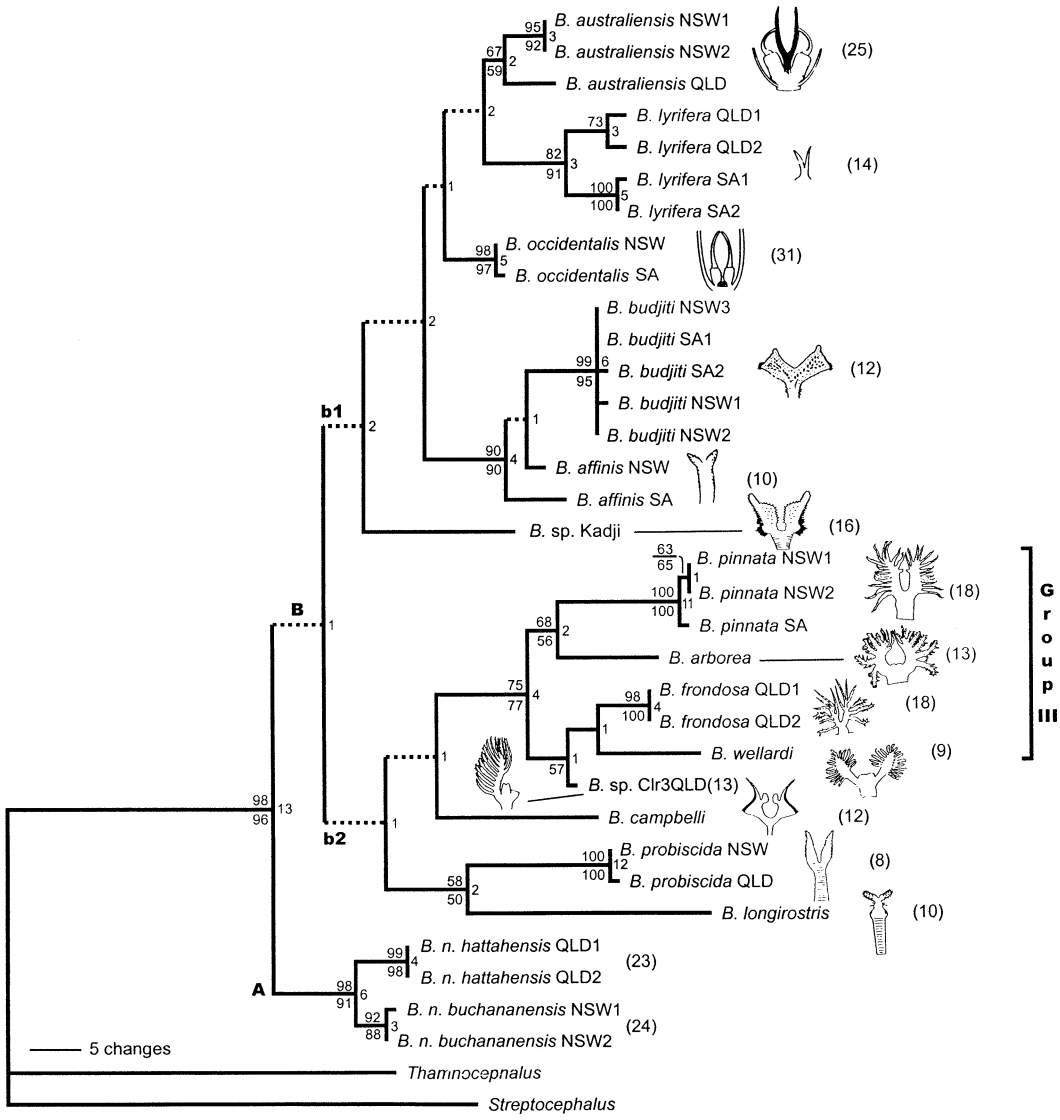

Fig. 1. Phylogram of the shortest MP tree. Numbers above and below the horizontal lines are bootstrap scores from MP and ML analyses, respectively. Only clades with ⩾50% bootstrap support are indicated. Numbers on the right of the vertical lines are decay indices. Sketches of the frontal appendage [all are from Geddes (1981; used with permission from CSIRO Publishing, Australia), except those of *B. campbelli*, *B. budjiti*, *B.* sp. Kadji, and *B.* sp. Clr3QLD] typical among species examined in which it is present are mapped on the tree. Note that only in *B. australiensis* and *B. occidentalis* are the frontal appendages (shaded) shown attached to the second antennae. Numbers in parentheses are mean body lengths (in mm) typical of adult males, obtained from Geddes (1981), Timms (2002), and Timms and Sanders (2002), and this study. See Table 1 for definitions of abbreviations of collection sites following taxon names.

finding is consistent with discernible differences in the frontal appendage between these species and the other taxa, as well as in other morphological features (Timms, 2001). The same result applies to the undetermined population from Kadji Kadji (designated *B.* sp. Kadji in Fig. 1), which showed divergences of 5–7% from the other branchinellids. This is also reflected in notable differences in its frontal appendage. The identification of *B. affinis* as being more closely allied to *B. budjiti* (bs = 90%, di = 4) than it is to *B. longirostris* and *B. probiscida* further argues against the validity of Geddes' (1981) Group II.

Disparities between molecular and morphological change exist between certain species.

For example, although there are notable morphological differences between *B. budjiti* and *B. affinis* (Fig. 1; Timms, 2001; Timms and Sanders, 2002), they show a level of genetic divergence (2–3%) similar to that between some conspecific populations. Similarly, among species comprising Group III, the unique branching pattern of each species' broadly feather-like frontal appendage is evident (Fig. 1), but levels of sequence divergence separating these species (2–4%) are low. More detailed molecular and morphological investigations are required to determine the specific status of these species.

Phylogenetic analyses revealed that statistical support for internal branches, especially at deeper levels, was poor, regardless of the method of phylogenetic reconstruction used. These branches were depicted as polytomies in bootstrap analyses constrained to collapse branches receiving less than 50% support. This finding may be the due to rapid cladogenesis, resulting in very few or no derived character states in the common ancestor of the inferred species clades. Additional evidence for this comes from our analyses of sequences from the mt protein-coding gene, COI, for nine species of *Branchinella*. This gene, which evolves about two times as fast as the 16S based on comparisons of average levels of sequence divergence, also failed to depict with confidence relationships at deeper parts of the phylogeny (unpublished data). Sequence data from a nuclear locus, e.g., the ribosomal ITS region, would be crucial in confirming whether the "hard" polytomy, i.e., the species evolved at brief intervals, as implied by the mt gene sequences is real. It is also possible, however, that the unresolved relationships at these levels are artifacts of limited taxonomic coverage. Future studies would therefore benefit from both analyses of other genes and additional sampling of taxa to distinguish between these alternative possibilities. In particular, data from a relatively conserved molecule (e.g., the nuclear ribosomal ITS), in combination with the 16S sequences, might provide a better opportunity for resolving deeper nodes.

The frontal appendage, a character frequently used to infer affinities among anostracan species, appears to be subject to considerable homoplasy, except among those species showing the greatest structural complexity (i.e., group III). Thus, this trait is of limited taxonomic value. It remains to be ascertained whether the absence (e.g., in *B. n. hattahensis*, *B. n. buchananensis*) or presence of a frontal appendage is an ancestral character owing to weak statistical support for deeper branches. The pattern of evolution of this character is further complicated by the presence in *B. occidentalis* of a rudimentary frontal appendage.

With 27 species presently known from Australia (Geddes, 1981; Timms, 2001, 2002; Timms and Geddes, in press) and 13 species from other continents (Belk and Brtek, 1995), *Branchinella* is one of the most speciose of anostracan genera. Because many of these species co-occur with others in the same genus and because 14 species are known from a small area in New South Wales and Queensland (Timms and Sanders, 2002), ecological differentation among the species is undoubtedly strong. A study in the Paroo catchment of New South Wales and Queensland (Timms and Sanders, 2002) has shown that habitat factors (e.g., turbidity, salinity, and length of the wet phase) serve to differentiate species. Furthermore, among species that co-exist, size differences are often substantial. For instance, *B. occidentalis* (mean male length = 31 mm) often co-occurs with either *B. lyrifera* (14 mm) or *B. probiscida* (8 mm). Similarly, *B. australiensis* (25 mm) co-exists with either *B. budjiti* (12 mm) or *B. arborea* (13 mm) (see Fig. 1). On a broader scale, there is latitudinal separation among species, as evidenced by the restriction of some species to tropical settings and others to temperate settings in Western Australia (Timms, 2002). Most of the species characteristic of clear waters belong to Geddes' Group III (*B. pinnata*, *B. arborea*, *B. frondosa*, *B. wellardi*), and other clear-water species (*B. campbelli*, *B. longirostris*) are closely allied to this group. In contrast, species typical of turbid waters are more widely scattered in the tree, but most are grouped in the large clade b1 (*B. australiensis*, *B. lyrifera*, *B. occidentalis*, *B. budjiti*, *B. affinis*); however, other taxa are distinct (e.g., *B. probiscida*, *B. n. hattahensis*). Although species of Australian *Branchinella* hardly enter saline waters (which are occupied by many species of the endemic anostracan genus *Parartemia*), *B. n. buchananensis* does occur in more saline waters (i.e., 2–10 g/L) than the other species (Timms and Sanders, 2002). *Branchinella nichollsi*, *B. simplex*, and *B. compacta* also exhibit some salinity tolerance (i.e., 3–21 g/L) (Williams and Geddes, 1991; Timms, 2002). The high species diversity in Australian *Bran-*

chinella could therefore be explained by the species' successful occupancy of a variety of habitats. The elevated species diversity of fairy shrimp in Australian inland waters is not unique to *Branchinella*. Prior molecular work on members of the endemic halophilic brine shrimp genus *Parartemia* also revealed high species diversity (Remigio *et al*., 2001).

The present study lays the groundwork for a more detailed determination of species relationships within Australian *Branchinella*. Our results have shown that past attempts to categorize species based on complex morphological characters could be clarified using a molecular phylogenetic approach and are also consistent with the outcome of previous molecular work providing evidence for homoplasy in a number of morphological features in anostracans (e.g., Remigio and Hebert, 2000). Nonetheless, a well-resolved phylogeny for the genus would require both increased taxonomic coverage and analyses of other genes. Whether the extensive diversification shown by Australian branchinellids occurred *in situ* or was the product of independent colonization events could be tested by acquiring sequence data for species of *Branchinella* found outside the continent.

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