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MOLECULAR TAXONOMY AND PHYLOGENETICS OF SOME SPECIES OF AUSTRALIAN PALAEMONID SHRIMPS

Nicholas P. Murphy and Christopher M. Austin

School of Ecology and Environment, Deakin University, P.O. Box 423, Warrnambool 3280, Australia (e-mail: npmurphy@deakin.edu.au)

A B S T R A C T

The evolutionary history and classification of the palaemonid shrimps has been the subject of constant speculation and debate. At present, all major systematic treatments have been based on morphological characteristics. To help resolve the phylogenetic relationships, and thus enable the creation of a classification system that reflects evolutionary history, a region of the 16S mitochondrial rRNA gene was sequenced for a number of Australian Palaemonidae. The resulting phylogenetic analyses indicated the presence of major anomalies in the current classification of Australian Palaemonidae. Significantly, three species belonging to three separate genera, *Macrobrachium intermedium*, *Palaemon serenus*, and *Palaemonetes australis*, are closely related, with genetic differences more characteristic with that of congeneric species. The results also demonstrate non-monophyly in Australian palaemonids with respect to both *Palaemonetes* and *Macrobrachium.*

The Palaemonidae family of shrimps is a very successful group of decapods, inhabiting marine, estuarine, and freshwater environments throughout the world. The Palaemonidae are currently divided into four subfamilies, the Palaemoninae, Pontoniinae, Euryrhynchinae, and Typhlocaridinae, with the first containing perhaps the most familiar palaemonid genera: *Macrobrachium*, *Palaemon*, and *Palaemonetes*. Whilst it is generally considered that the family Palaemonidae represents a natural group (Pereira, 1997), the relationships within the family are far from clear.

There have been many acknowledged problems associated with the classification of the Palaemonidae. These occur at the specific (Lindenfelser, 1984; Short, 2000), generic (Holthuis, 1952; Fincham, 1987; Short, 2000), and family (Boulton and Knott, 1984; Pereira, 1997) levels. The problems have been attributed to difficulties in the interpretation of the significance of morphological characteristics used to classify palaemonid shrimp and have hindered the development of stable classification systems. Holthuis (1950, 1952) established the most widely used classification of the Palaemonidae based on a number of morphological characteristics. However, this scheme has been criticized for not accurately reflecting evolutionary relationships within the group (Chace, 1972; Pereira, 1997).

Recently, attempts have been made to develop hypotheses for the evolutionary history of the Palaemonidae, both within and between genera. The only phylogenetic study to focus on the palaemonids as a whole was by Pereira (1997), who performed a cladistic analysis of morphological characteristics. The majority of other studies have focused on the genus *Macrobrachium*, the largest within the subfamily Palaemoninae (containing approximately 65% of the species within this subfamily) (Hedgecock *et al*., 1979; Lindenfelser, 1984; Mashiko and Namuchi, 1993; Short, 2000; Murphy and Austin, unpublished). Of these, Short (2000) presents the most comprehensive phylogenetic study using morphological, biological, and ecological characters to assess the evolutionary relationships of 30 Australian and non-Australian species of *Macrobrachium* and nine species of related genera of the Palaemoninae. Both Short (2000) and Pereira (1997) found evidence for anomalies within the current taxonomic classification of the palaemonids. Pereira (1997) concluded that although the family Palaemonidae represents a monophyletic group, several major lineages within this family are, in fact paraphyletic. Short (2000) provided further evidence that the current classification does not accurately reflect evolutionary history, by identifying polyphyletic relationships within the genus *Macrobrachium*.

Until recently, all major systematic treatments of palaemonids have been based on morphological characteristics alone (Holthuis, 1950; Riek, 1951; Holthuis, 1952; Bray, 1976;

Choy, 1984; Fincham, 1987; Chace and Bruce, 1993; Pereira, 1997), with the exception of Short (2000), who included some biological and ecological characters. A few allozyme-based studies have been undertaken (Trudaeu, 1978; Boulton and Knott, 1984; Lindenfelser, 1984; Chow and Fujio, 1985a, b), but no phylogenetic studies utilizing DNA-based data have been published. This is surprising, given the worldwide distribution of the family and the fact that several species, including the giant freshwater prawn *Macrobrachium rosenbergii*, are important commercially, particularly in developing countries. In contrast, other important decapod crustacean groups, such as penaeid prawns, freshwater crayfish, and marine lobsters, have been much more extensively studied using molecular genetic techniques (Palumbi and Benzie, 1991; Bouchon *et al*., 1994; Harding *et al*., 1997; Ovendon *et al*., 1997; Grandjean *et al*., 1998; Sarver *et al*., 1998; Crandall *et al*., 1999). The use of DNA sequence data has the great advantage that it allows the creation of a phylogeny that enables the testing of morphologically based systematic hypotheses and for the independent assessment of morphological evolution.

In Australia, palaemonids are widespread, with representatives inhabiting marine, estuarine, and freshwater environments. In preliminary work on the phylogenetics of Australian *Macrobrachium* sp. using 16S mitochondrial DNA (mtDNA) sequences, Murphy and Austin (2002) showed evidence for the non-monophyly within Australian *Macrobrachium*. Murphy and Austin (2002) found that *Macrobrachium intermedium* did not share a close evolutionary relationship with other Australian *Macrobrachium* and instead was found to share a closer affinity with *Palaemon serenus*. Boulton and Knott (1984), in a study of palaemonid species in the Swan River Estuary, Western Australia, also found inconsistencies between the current morphologically based classification system and genetic relationships between the species studied using allozyme data. Boulton and Knott (1984) showed *M. intermedium* to be closely allied to another species, *Palaemonetes australis*, suggesting that they may be congeners. Short (2000) and Pereira (1997) also found evidence that *M. intermedium* is polyphyletic with respect to *Macrobrachium*.

The biogeographic relationships and the origin of Australian palaemonids have been long debated, with a particular focus upon the representatives of the genus *Palaemonetes* in southwest Australia. *Palaemonetes* is represented by two species in Australia, the largely estuarine *Palaemonetes atrinubes* and the freshwater species *Palaemonetes australis*. (Bishop, 1967) suggested that *Palaemonetes* originated in Australia from larvae carried across the Indian Ocean from Africa. It has been said that the palaemonids, in general, appear to be in transition from marine to freshwater environments (Hedgepeth, 1957). However Boulton and Knott (1984) suggest, based on allozyme data, that the likely ancestor of *Palaemonetes australis* is a *M. intermedium*-like form.

To further explore the taxonomic, phylogenetic, and biogeographic questions pertaining to Australian palaemonids, sequencing of the 16S mtDNA gene region was undertaken. The 16S mtDNA gene region has been found to be extremely useful for studying taxonomic questions and phylogenetic relationships within a number of decapod crustacean groups (Bucklin *et al*., 1995; Crandall and Fitzpatrick, 1996; Ovendon *et al*., 1997; Kitaura *et al*., 1998; Tam and Kornfield, 1998; Crandall *et al*., 1999). The 16S rRNA gene has both fast- and slow-evolving regions and therefore can provide useful information across a broad taxonomic spectrum from the population to the family level.

The object of this paper is to essentially follow up Boulton and Knott's (1984) allozyme study of Palaemonidae in the Swan River Estuary, Western Australia, using powerful DNA-based analyses. In addition to the samples examined by Boulton and Knott, specimens of *Palaemon serenus* and *M. intermedium* from eastern Australia and *M. australiense* and *M. rosenbergii* are included for comparative purposes.

Materials and Methods

Taxa

The four Australian palaemonid species studied by Boulton and Knott (1984), *Macrobrachium intermedium* (Stimpson, 1860); *Palaemon serenus* (Milne-Edwards, 1837); *Palaemonetes australis* (Bray, 1976); and *Palaemonetes atrinubes* (Dakin, 1915) from the Swan River estuary, Western Australia, were included in this study. Also included were *M. intermedium* and *P. serenus* from Warrnambool, Victoria, to allow for intraspecific comparisons, and *M. australiense* (Ortman, 1891) from inland Australia and *M. rosenbergii* (De Man, 1879) from Papua New Guinea to allow for species-level comparisons with

Macrobrachium. *Macrobrachium intermedium* is found in estuarine and some marine waters around the southern Australian coastline from Perth, W.A., to the central Queensland coast; *P. serenus*, a marine species, has a similar distribution. The two *Palaemonetes* species are found only in Western Australia; *Palaemonetes australis* is generally found in freshwater and upper estuaries in south-west Western Australia, whilst *Palaemonetes atrinubes* inhabits coastal embayments and lower estuarine environments along eastern and western Australian coastlines and has also been recorded in New Caledonia. The atyid shrimp *Paratya australiensi*, was included as the outgroup species. As the evolutionary affinities of the species studied are relatively unknown, it was decided that the outgroup should initially be somewhat removed from the studied group. The atyids and the palaemonids are both members of the infraorder Caridea.

An abbreviated description of the main morphological features currently used to separate the three genera studied are as follows:

1. Hepatic spine absent, branchiostegal spine present 2
– Hepatic spine present, branchiostegal spine absent . . . . . *Macrobrachium*
2. No mandibular palp present . . . . . *Palaemonetes*
– Mandibular palp present . . . . . *Palaemon*

Sample Collection

Macrobrachium intermedium was collected from seagrass beds in the estuarine sections of the Fitzroy River in southwest Victoria and Swan River, Perth, Western Australia. *Palaemon serenus* was obtained from rock pools off Griffith Island, Port Fairy, Victoria, and Mandurah, Western Australia. *Palaemonetes australis* was obtained from the upper Swan River, whilst *Palaemonetes atrinubes* was collected from the lower Swan River estuary. *Macrobrachium australiense* was collected from the Murray River, N.S.W., and *Macrobrachium rosenbergii* was collected from a small river near Madang, Papua New Guinea. All specimens were collected using a dipnet and were either frozen in liquid nitrogen immediately and stored in 95% ethyl alcohol or transported live back to the laboratory and stored at −80°C. Voucher specimens were stored in 70% ethanol and are currently housed on site (Deakin University, School of Ecology and Environment, Molecular Ecology Lab), until the completion of this study, whereupon they will be housed in an appropriate Australian museum.

DNA Extraction and Amplification

DNA was extracted from abdominal muscle tissue using a high salt precipitation method (Crandall *et al*., 1999). A fragment of the 16S rRNA mitochondrial gene was amplified by PCR using universal 16S mtDNA primers (16Sar and 16Sbr). Double-stranded PCR products were obtained in a total reaction volume of 50 µl, containing 5 µl of 10X PCR buffer, 0.4 mM of each dNTP, 0.8 µM of each primer, 4 mM $MgCl_2$, 1 unit of Taq polymerase and 2 µl of DNA extract. The PCR amplification was carried out in a Corbett Palm-Cycler using the following temperature regime: an initial denaturation step of 95°C for 3 min, followed by 30 cycles of 95°C for 30 seconds (s), an annealing temperature of 50°C for 30s, and an extension temperature of 72°C for 30s. This was then followed by an additional extension of 72°C for 3 min. The PCR products were purified using a QIAGEN QIAquick PCR Purification Kit, with final elution volumes of 50 µl per individual. The DNA concentrations were approximated against a Promega DNA/Hae 111 marker on a 2% agarose/TAE gel containing ethidium bromide and viewed under UV light.

Samples were sent to the Australian Genome Research Facility (AGRF), University of Queensland, for sequencing. Sequencing reactions followed the protocol of Perkin Elmer, using an ABI big dye terminator reaction with custom primers. For each sample, sequencing was performed in both directions.

Phylogenetic Reconstruction

Sequence chromatograms were viewed and edited manually using a combination of EditView and MacClade (Maddison and Maddison, 2000). Once edited, multiple alignments were performed using Clustal X (Thompson *et al*., 1997) with multiple alignment parameters of gap penalty equal to 10–15, and gap extension penalty equal to 3–5. Alignments were then checked and verified manually using MacClade; positions of uncertain alignment were excluded to produce a stable data set (Gatesy *et al*., 1993) and ensure that orthologous characters were compared. Sequences were then imported into PAUP 4.0b4a (Swofford, 1998) for phylogenetic analysis. Three methods of tree building were used: maximum-likelihood, maximum parsimony, and neighbor joining. Heuristic searches were employed for both maximum-likelihood and maximum parsimony using random sequence addition to eliminate any bias from sequence addition; confidence in all trees was calculated by nonparametric bootstrapping.

The appropriate model of evolution for maximum-likelihood (ML) analysis was obtained via testing alternative modes of evolution using Modeltest (Posada and Crandall, 1998). Modeltest obtains a starting tree via neighbor joining; different models of evolution are then tested with this tree topology, and likelihood scores are calculated and compared statistically. On the basis of these tests, the TrN + G model of sequence evolution was chosen, and the parameters specified by this model were used to estimate a tree via the maximum-likelihood method. Specifically the parameters used were a rate matrix of (A-C) = 1.0000, (A-G) = 4.5195, (A-T) = 1.0000, (C-G) = 1.0000, (C-T) = 9.6383, (G-T) = 1.0000; base frequencies of A = 0.306, C = 0.108, G = 0.216, T = 0.371; gamma distribution parameter = 0.1542. Heuristic searches were used with 1,000 sequence additions, whilst bootstrappings consisted of 100 replications with 100 sequence additions. Maximum-parsimony analyses were performed with gaps treated as missing, heuristic searches as per maximum-likelihood analyses; however, 1,000 bootstrap replicates were performed each with 1,000 sequence additions. A neighbor-joining tree was constructed with distances calculated under the Tamura-Nei model of nucleotide sequence evolution; bootstrapping was performed with 1,000 replicates. A distance matrix was also created using the above model.

Phylogenetic Hypothesis Testing

To test phylogenetic hypotheses, comparisons were made between optimal trees and trees developed using topological constraints. The non-parametric test of Templeton (1983) was used to compare maximum parsimony trees. Maximum-parsimony estimates were carried out in PAUP* for alternative phylogenetic hypotheses by enforcing topological constraints developed in MacClade. A one-tailed Templeton (1983) test was carried out in PAUP* to

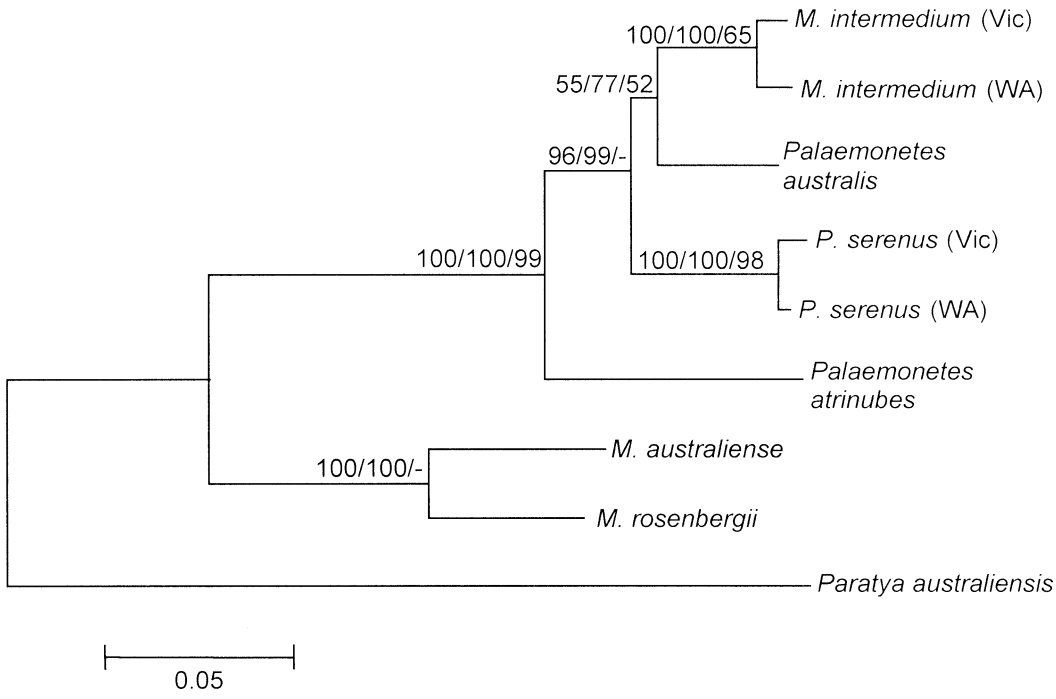

Fig. 1. Consensus phylogram* from maximum-parsimony, neighbor-joining, and maximum-likelihood analyses. Bootstrap values for each analysis are shown in the order above (− means < 50% bootstrap support) (* actual topology shown is that of neighbor joining analysis).

determine significant differences in tree length. An analysis of maximum-likelihood hypotheses was carried out via parametric bootstrapping and subsequent comparisons of likelihood ratios (Hillis *et al*., 1996; Huelsenbeck and Crandall, 1997; Goldman *et al*., 2000). A parametric bootstrap was implemented by comparing the likelihood ratio between the optimal tree created by PAUP* and the null hypothesis tree with likelihood ratios of optimal and null hypothesis trees from simulated data sets created essentially via Monte Carlo simulation of DNA sequence evolution (Rambaut and Grassly, 1997). One hundred replicate data sets with the same tree topology and branch lengths as the null hypothesis tree were generated using the same parameters and model of sequence evolution as estimated for the observed data (Jarman *et al*., 2000b) and with the same number of sites.

Results

The sequence alignment, after the removal of regions of ambiguous alignment, yielded a total of 494 sites for phylogenetic analysis, of which 206 were variable. The mean total nucleotide composition was A = 30%, T = 35%, C = 13% and G = 22%, indicating that the 16s RNA region of the mtDNA is adenosine- and thymine-rich in the palaemonids. No significant differences in base composition across all taxa were detected (χ^2 = 9.517, *d.f.* = 27, *P* = 0.99). Resulting sequences have been submitted to Genbank (AF439515–AF439523).

Table 1. The Tamura-Nei pairwise distances (below) and number of nucleotide substitutions (above) among the specimens studied.

	1.	2.	3.	4.	5.	6.	7.	8.	9.
1. *M. intermedium* (Vic)	—	8	29	41	42	59	108	105	160
2. *M. intermedium* (WA)	0.017	—	30	43	43	60	107	105	159
3. *Palaemonetes australis*	0.063	0.065	—	39	38	61	108	104	160
4. *P. serenus* (Vic)	0.091	0.096	0.087	—	5	53	107	111	162
5. *P. serenus* (WA)	0.093	0.096	0.084	0.010	—	50	105	110	158
6. *Palaemonetes atrinubes*	0.135	0.137	0.140	0.145	0.137	—	116	109	156
7. *M. australiense*	0.269	0.266	0.269	0.266	0.263	0.293	—	43	138
8. *M. rosenbergii*	0.261	0.261	0.257	0.278	0.275	0.273	0.097	—	145
9. *Paratya australiensis*	0.445	0.441	0.445	0.456	0.439	0.429	0.370	0.392	—

Phylogenetic Analyses

The construction of phylogenetic trees resulted in three equivalent topologies (Fig. 1). Bootstrap confidence levels were generally high for all nodes within the tree; the neighbor joining and maximum parsimony trees showing strong support for two clades. One contains *Macrobrachium intermedium*, *Palaemonetes australis*, and *Palaemon serenus*, with *Palaemonetes atrinubes* in the most basal position. A second clade containing *M. australiense* and *M. rosenbergii* was also strongly supported; there was no support for a relationship between these two species and *M. intermedium* apparent. The maximum-likelihood analysis identified the same major clades but with generally lower levels of bootstrap support, especially in relation to the position of *P. serenus* (< 50% support).

The Tamura-Nei pairwise distance matrix and absolute base difference (Table 1) shows the degree of divergence between *M. intermedium*, *Palaemonetes australis*, and *P. serenus* to be very similar (0.063–0.093), whilst *Palaemonetes atrinubes* is equally distant from all of these species (~ 0.150). The degree of divergence between the two other *Macrobrachium* species, *M. australiense* and *M. rosenbergii* (0.097), is comparable with the genetic divergence between *M. intermedium*, *Palaemonetes australis*, and *P. serenus*, whilst the distance between these two clades is much larger (0.258–0.293).

Tests of Phylogenetic Hypotheses

Using the maximum-parsimony and maximum-likelihood procedures, the 16S mtDNA sequence data were used to test four phylogenetic hypotheses in relation to the species studied. The hypotheses tested were: a) that the three *Macrobrachium* species are monophyletic; b) that the two *Palaemonetes* species studied are monophyletic; c) the phylogenetic relationships suggested by Short (2000) (a *Palaemon*/*Palaemonetes* clade and *Macrobrachium* polyphyletic with respect to *M. intermedium*); and d) the relationships suggested by Pereira (1997) (*M. intermedium* the most basal group). These hypotheses are represented as cladograms in Fig. 2.

From Table 2, it can be seen that these hypotheses are all rejected ($P < 0.001$) using Templeton's test and maximum parsimony. Parametric bootstrapping of the log-likelihood ratios (δ) (Huelsenbeck and Crandall, 1997; Goldman *et al.*, 2000) (Fig. 3) also led to all hypotheses being rejected ($P < 0.001$). Thus, all phylogenetic hypotheses tested resulted in trees with topologies highly inconsistent (either longer tree length or smaller Ln-likelihood) with the optimal trees.

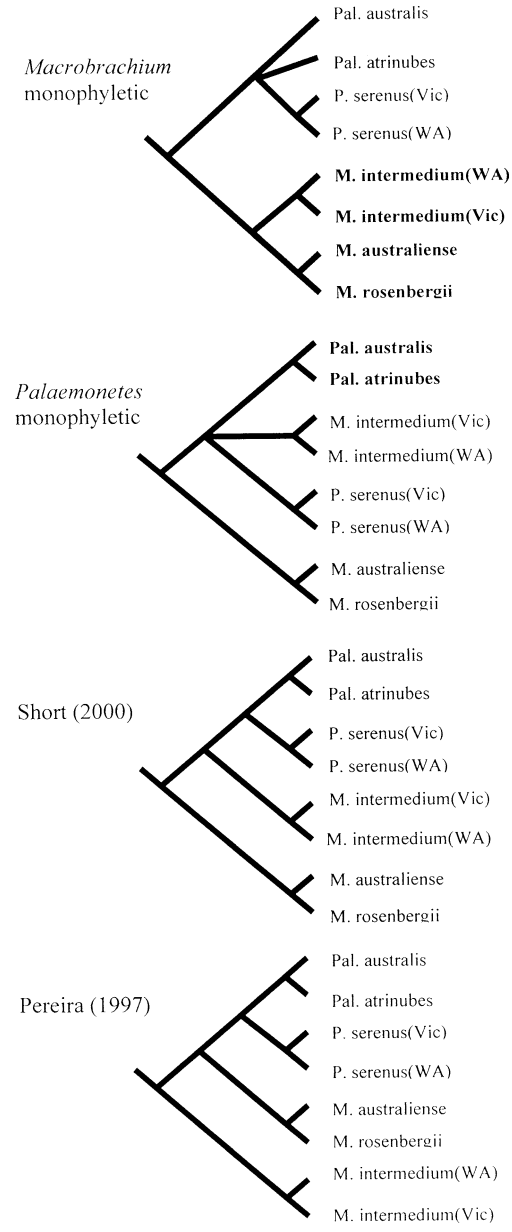

Fig. 2. Some phylogenetic hypotheses based on the species studied. Short (2000) and Pereira (1997) indicate representative topologies based on their more extensive studies.

Table 2. Templeton (1983) tests for significant differences in maximum parsimony tree lengths for the optimal tree *versus* constraint trees.

Constraint	Length	Rank sums	*n*	*Z*	*P*
Optimal	355				
Macrobrachium	389	877.5 −112.5	44	−5.1257	<0.0001*
Palaemonetes	365	157.5 −52.5	20	−2.2361	0.0253*
Short (2000)	365	133.0 −38.0	18	−2.3570	0.0184*
Pereira (1997)	393	927.5 −107.5	45	−5.1632	<0.0001*

* Significance at $\alpha = 0.05$.

Discussion

The results of this study indicate that the current classification of Australian Palaemoninae is questionable and that both *Macrobrachium* and *Palaemonetes* are not monophyletic. It also appears that *Macrobrachium intermedium*, *Palaemonetes australis*, and *Palaemon serenus* form a natural group. Genetic divergences among these species, when compared with the genetic divergences between *M. australiense* and *M. rosenbergii* and those of other studies of the 16S mtDNA region in Crustacea (Suno-Ughi *et al*., 1997; Ponniah and Hughes, 1998; Jarman *et al*., 2000a; Tong *et al*., 2000), are typical of those found between congeneric species. This reinforces Boulton and Knott's (1984) view that *Palaemonetes australis* is congeneric with *M. intermedium*, based on allozyme electrophoresis. Therefore, morphological features currently used to separate these three genera (i.e., hepatic/branchiostegal spine and mandibular palp) are phylogenetically unreliable and plastic.

These results are not altogether surprising and are consistent with other studies suggesting problems with the current classification of Australian palaemonids. (Boulton and Knott, 1984; Fincham, 1987; Short, 2000). On the basis of allozyme electrophoresis, Boulton and Knott (1984) found *Palaemonetes australis* to be closely related to *Macrobrachium intermedium*, but the wider relationships of these species could not be determined, as no other species of *Macrobrachium*, for example, were included in their data set. Other studies have found *Macrobrachium* to be polyphyletic with respect to *M. intermedium* (Pereira, 1997; Short, 2000; Murphy and Austin, 2002).

The wider evolutionary affinities of the *P. serenus*/*Palaemonetes australis*/*M. intermedium* group are uncertain. It may be a distinct Australian lineage of coastal palaemonids or part of a more widespread lineage. The position of *Palaemonetes atrinubes* within the Palaemonidae is also unclear. Bray (1976) claimed that *Palaemonetes atrinubes*, with the exception of one character linking this species with *Palaemonetes australis*, did not appear closely related to other Australian species. The results of this study are consistent with this observation indicating it is phylogenetically distinct from the three other "coastal palaemonid" species Increased taxon sampling of palaemonid shrimps within and outside Australia is currently being undertaken to clarify phylogenetic questions pertaining to palaemonid species worldwide. A better representation of species, including type species of these genera, will allow for a better understanding and may well change the relationships between the taxa studied.

These findings have implications for previous evolutionary analyses of morphological, life history, and physiological traits in these shrimps. For example, many comparisons of larval life cycles across *Macrobrachium* shrimp have been made, many of which have included *M. intermedium* (Holthuis, 1952; Williamson, 1972; Greenwood *et al*., 1976). Our results indicate it would be more appropriate to compare *M. intermedium* larval life stages and life history with those of *Palaemon serenus* and *Palaemonetes australis*. Similarly, in relation to morphology, it appears that undue emphasis has been placed on the taxonomic significance of the presence and absence of the hepatic and branchiostegal spines in defining the genera *Macrobrachium*, *Palaemo*, and *Palaemonetes*.

Phylogenetic analyses are also necessary for a proper understanding of the biogeographic history of organisms. The presence of the

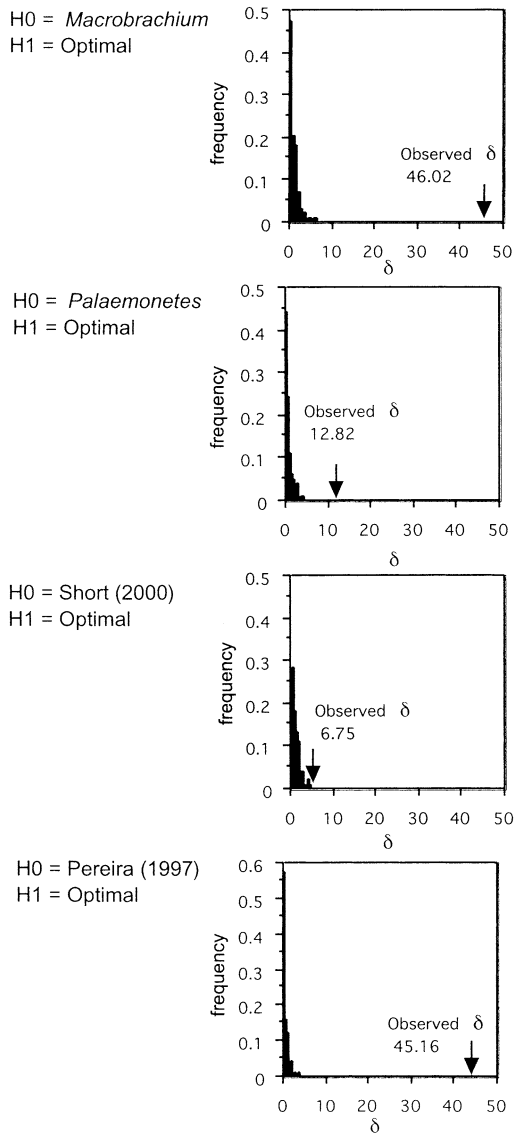

Fig. 3. Likelihood-ratio (δ) tests for monophyly. The observed value of δ for trees constrained to equal phylogenetic hypotheses and optimal trees compared to the distribution of δ generated via 100 Monte Carlo simulations.

Palaemonetes australis in southwest Western Australian rivers has long been recognized as a biogeographic conundrum. The results of this study support Boulton and Knott's (1984) theory of the invasion of unexploited freshwater habitat by a nearshore marine ancestor, probably of a *M. intermedium*-like form. While *M. intermedium* occurs along the entire southern Australian coastline, there is no closely related palaemonid species in freshwater systems along the coastal fringe of southeastern Australia. This is most likely due to the presence of the atyid shrimp *Paratya australiensis* in eastern coastal freshwater systems, which appears to exploit a similar freshwater niche. The origin of Australian *Macrobrachium* is a similarly debated topic. Bishop (1967) suggested that they most likely originated from Asia and then dispersed throughout eastern Australia, whilst Williams (1981) proposed that some Australian species may have evolved relatively recently from *in situ* marine ancestors. It is certain from the results of this study that if Williams' (1981) theory is correct, then the marine ancestral form of Australian *Macrobrachium* is not *M. intermedium*.

This study provides clear evidence that current generic classification of Australian Palaemoninae is invalid. As a consequence, it raises some intriguing questions regarding the taxonomic validity of these genera more widely and the evolutionary affinities and biogeographic relationships of Australian palaemonid shrimps. Thus, there is much scope for further study of the molecular phylogenetics of palaemonid shrimps. This should not be restricted purely to Australian species, but needs to encompass species worldwide. Such studies would allow for greater understanding of the taxonomic and phylogenetic relationships of these species and generate new perspectives on the evolutionary, morphological, and life history variation in these shrimps.

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