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Re-examination of the taxonomy of the *Macrobrachium australiense* Holthuis (Decapoda : Palaemonidae) species-complex: molecular evidence for a single species

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Abstract. The freshwater shrimp *Macrobrachium australiense* is distributed throughout the majority of inland, north-west, north-east and eastern drainages. Owing to the large amount of morphological divergence, both between and within catchments, this species has proven to be taxonomically difficult and, until recently, consisted of three separate species, each with subsequent subspecies. This study uses nucleotide sequences from the *16S* rRNA mitochondrial gene region to investigate the genetic relationships between populations and confirm the taxonomic status of *M. australiense*. The results from sequencing an approximately 450-bp fragment from this gene region from *M. australiense* sampled from 12 locations across inland, eastern and northern Australia identified very little variation. The variation found between *16S M. australiense* haplotypes is much less than that found between *Macrobrachium* species, indicating that it is in fact a single species. The results are concordant with a recent morphological revision of Australian species in which nominal taxa of the *M. australiense* complex were synonymised.

Introduction

The freshwater shrimp *Macrobrachium australiense* Holthuis, 1950 is one of the most widespread freshwater decapod crustaceans in Australia, occurring in the major inland drainages and in the majority of north-east, north-west and eastern coastal drainages (Short 2004). Over this enormous range, which encompasses several distinct biogeographic regions (Unmack 2001), *M. australiense* shows considerable morphological variation (McNeill 1929; Lee 1979; Short 2004). The taxonomic significance of this variation is difficult to interpret; consequently there has been considerable uncertainty over the number of taxa within what can best be referred to as the *M. australiense* species-complex and their geographic distribution.

Ortmann first described the species in 1891 as *Palaemon australis* (a name preoccupied by *P. australis* Guérin-Méneville, 1838). McNeill (1929) re-described the species in detail, based on several specimens from the Horton River, Pallal, New South Wales. McNeill (1929: 146) also noted the presence of 'many perplexing racial forms' within the species. Holthuis (1950) re-assigned the species to *Macrobrachium* and provided the replacement name, *M. australiense*. Soon after, Riek (1951) reviewed the taxonomy of Australian freshwater species of *Macrobrachium* and described several new species and subspecies, including new subspecies of *M. australiense*.

Lee (1979), using a much greater range of samples than previous workers, examined morphological variation within the subspecies of *M. australiense* and found that although some distinct geographic patterns in variation were apparent, considerable overlap in characters occurred among all subspecies. He concluded that the differences were not numerous or consistent enough to warrant taxonomically subdividing the species, although he postulated that there might be a complex of ecotypes or physiological races. These, he believed, may have been the result of geographical isolation and adaptation to local environments. He suggested a northern warm-water race with a short rostrum, an eastern coastal race with a long rostrum and an inland, southern race with an intermediate rostrum.

In an extensive morphological study on the taxonomy of Australian *Macrobrachium*, Short (2004) examined over 1700 specimens of the species-complex from localities throughout its broad geographic range, including type and topotypical material of all the nominal species and subspecies recognised by Riek (1951). He found that the holotypes of three of the taxa described by Riek (*M. adscitum adscitum, M. atactum atactum* and *M. atactum ischnomorphum*) were not fully developed males and the features used by Riek (1951) to distinguish these taxa from *M. australiense* were merely examples of normal developmental variation. These three taxa and *M. atactum sobrinum*

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Table 1. Sample locations and codes, current taxonomy, *16S* haplotype designation and GenBank accession numbers

Sample site	Location[1]	Designated taxonomy[2]	*16S* rRNA haplotype	*16S* GenBank no.
Thompson River	MUT	*M. atactum sobrinum*	A	AY316582
Georgina River	GEO	*M. atactum sobrinum*	A	AY316582
Coongie Lakes	CL	?	B	AY316581
Finke River	FIN	?	C	AY316588
Bulloo River	BUL	?	B	AY316581
Lake Alexandrina	LA	*M. adscitum* subsp.	D	AY316580
Murray River	MUR	*M. australiense cristatum*	D	AY316580
Namoi River	NAM	*M. australiense cristatum*	D	AY316580
Enoggera Creek	EN	*M. australiense australiense*	D	AY316580
Fishers Ck	FIS[3]	?	D	AY316580
Mary River	MAR	*M. atactum atactum*	E	AY316586
Carron River	CAR	?	F	AY316587

[1]See Fig. 1 for map of sampling locations. [2]Designated taxonomy based on distributions given by Riek (1951). [3]Dwarf form, as recognised by Short (2000).

Riek, 1951 were synonymised under *M. australiense*. In regard to the subspecies of *M. australiense* described by Riek, Short (2004) concluded:

> 'Although there is undoubtedly morphological divergence between catchments and across the broad geographic range of the species, delimiting subspecies is likely to be a difficult and impractical task, considering the large number of drainage basins involved. At this point in time it is hard to justify the continued usage of Riek's subspecies or to describe further subspecific taxa.'

Like Lee (1979), Short (2004) also noted general patterns in rostral variation but considered it difficult to delimit subspecies and link them to definite geographic areas based on rostral features. A recent molecular study of *M. australiense* (Cook *et al.* 2002) determined that there are significant genetic differences between the Darling River catchments and the Bulloo, Cooper and Georgina River catchments in western Queensland, but the taxonomic implications of these findings were not discussed.

The current study investigates relationships within the *Macrobrachium australiense* complex using DNA sequence data obtained from 12 different geographic sites. These sites cover most of the distributional range of the species-complex, with the exception of far northern populations. From this study, it should be possible to evaluate both Riek's (1951) classification of this complex into three species and several subspecies and Short's (2004) view that Riek's species/subspecies cannot be justified and that *M. australiense* should be synonymised. The study expands on preliminary DNA sequence data (Murphy and Austin 2002), which demonstrated that two nominal species of the complex, *viz. M. australiense* and *M. atactum* Riek, 1951, showed a high level of genetic similarity, suggesting that they may represent a single species.

Molecular data have proven very useful for clarifying the taxonomic relationships and defining species boundaries in morphologically conservative or highly variable groups of freshwater crustaceans. In particular, the *16S* rRNA mitochondrial (mtDNA) gene has proven effective for elucidating molecular phylogenetic and taxonomic relationships between crustaceans (Crandall and Fitzpatrick 1996; Tam and Kornfield 1998; Crandall *et al.* 1999; Ptacek *et al.* 2001; Murphy and Austin 2002).

Materials and methods

Sample collection

Samples representative of the *Macrobrachium australiense* species-complex were collected from inland, northern and eastern Australia (Table 1, Fig. 1). An attempt was made to include, where possible, species obtained from the type locations of Riek (1951), or from

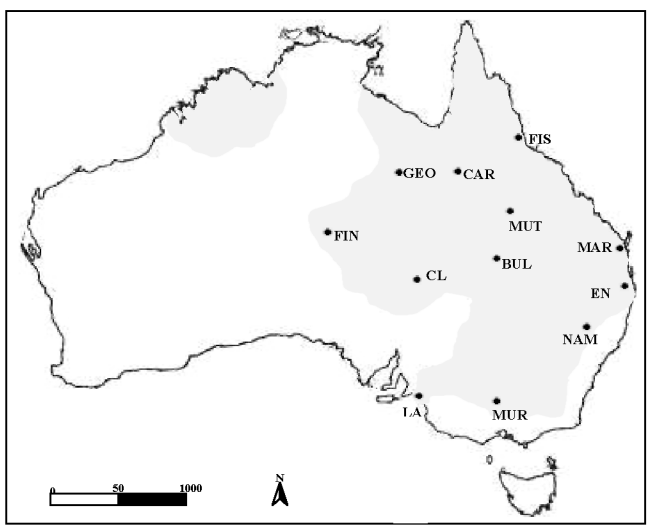

Fig. 1. Collection of localities for *Macrobrachium australiense* samples used in this study. Sample locations: MUT, Thompson River; GEO, Georgina River; CL, Coongie Lakes; FIN, Finke River; BUL, Bulloo River; LA, Lake Alexandrina; MUR, Murray River; NAM, Namoi River; EN, Enoggera Creek; FIS, Fisher Creek; MAR, Mary River; CAR, Carron River. Shaded area indicates species distribution.

locations within the distributional ranges given by Riek. Also included in this study is a sample from Fishers Creek (a tributary of the Johnstone River, northern Queensland), representing a dwarf form of *M. australiense* of uncertain taxonomic status (Short 2000).

Samples were collected via baited traps placed near sunken logs, by dip netting around weed beds and undercut banks and, in larger water bodies, by using seine nets. Samples were frozen immediately in liquid nitrogen and stored at –80°C on return to the laboratory. Additional samples were obtained from the Queensland Museum, Brisbane, Australia by removing a pleopod from specimens stored in 75% ethanol. Table 1 lists the samples analysed, together with the sample codes, geographic locations and taxonomic identity based on Riek (1951). In addition, four other Australian species, *M. rosenbergii* (De Man, 1879), *M. novaehollandiae* (De Man, 1908), *M. lar* (Fabricius, 1798) and *M. tolmerum* Riek, 1951 sampled from the east coast of Australia were also sequenced for comparative purposes.

Laboratory procedures

Deoxyribonucleic acid extraction and PCR amplification of the *16S* rRNA mtDNA gene were performed as described by Murphy and Austin (2002). Sequencing reactions were performed using ABI Big Dye Terminator Chemistry (Applied Biosystems, Foster City, CA, USA), with 6 pmol of each primer to amplify 30–50 ng of PCR product. The resultant sequencing reactions were sent to the Institute of Medical and Veterinary Science (IMVS), Adelaide, Australia for analysis on an ABI 3700 capillary sequencer (Applied Biosystems).

Data analysis

The *16S* rRNA sequences were examined with the inclusion of the four additional *Macrobrachium* species in order to determine interspecific divergence levels within the genus for this gene region. Sequences were aligned in ClustalX (Thompson *et al.* 1997) following the methods of Gatesy *et al.* (1993). After alignment, sequences were imported into PAUP* version 4.0b4 (Swofford 2000) for neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) phylogenetic analyses. Models of evolution for NJ and ML trees were chosen using the hierarchical likelihood-ratio test as implemented in ModelTest (Posada and Crandall 1998). For MP and ML analyses, heuristic searches were applied with 10 random stepwise additions and tree bisection–reconnection branch swapping. Statistical confidence in nodes was evaluated using nonparametric bootstrap support.

Results

Sequences from the *16S* rRNA gene were obtained for a fragment approximately 450 bp in length. A total of six haplotypes were found in samples of the *Macrobrachium australiense* complex from twelve sites throughout eastern and central Australia (Table 2). The haplotypes contained ten variable sites with no insertions or deletions and differed by 0.2 to 1.6% divergence. Three unique haplotypes (A, B and C) were found in the Lake Eyre Basin and a single haplotype (D) occurred in the Murray–Darling Basin and also Enoggera Ck and Fisher Ck (Queensland eastern coastal drainage). The other two unique haplotypes (E and F) were the most divergent, and were found at single locations in the Gulf of Carpentaria (CAR) and the east coast (MAR). Divergence levels (Table 2) among the four additional *Macrobrachium* species ranged from 8.4% to 13.4%. A dwarf form of *M. australiense* (FIS) identified by Short (2000) shares the same haplotype (D) as the east coast and Murray–Darling Basin populations and does not show any discernable divergence from the other *M. australiense* complex samples.

The trees produced by NJ and MP analyses were identical (Fig. 2), whereas the ML tree differed only in the arrangement of the outgroup species (not shown). Haplotypes representing the *M. australiense* species-complex form a tight monophyletic group in all analyses, with very short branch lengths compared with the much longer branch lengths observed for the other *Macrobrachium* species.

Discussion

Crustacean species within a single genus commonly exhibit significant differences in the *16S* rRNA mtDNA gene, ranging from 2% to 17% sequence divergence (Sarver *et al.* 1998; Crandall *et al.* 1999; Jarman *et al.* 2000; Schubart *et al.* 2000; Murphy and Austin 2002). Thus, the differences between the individual outgroup *Macrobrachium* species and between the outgroup species and the ingroup samples (Table 2, Fig. 2) are typical for those observed between crustacean species. In contrast, the much lower level of divergence found among the ingroup samples in this study is more typical of what is seen among populations within species. These findings reinforce Short's (2004) view, based

Table 2. The uncorrected pairwise distances (below diagonal) and number of nucleotide substitutions (above diagonal) between *16S* rRNA mtDNA sequences for *Macrobrachium australiense* haplotypes and *M. rosenbergii* (MROS), *M. tolmerum* (MTOL), *M. lar* (MLAR) and *M. novaehollandiae* (MNOV)

	HAP-A	HAP-B	HAP-C	HAP-D	HAP-E	HAP-F	MROS	MTOL	MLAR	MNOV
HAP-A	–	1	1	1	4	5	46	50	39	60
HAP-B	0.002	–	2	2	5	6	47	51	40	60
HAP-C	0.002	0.004	–	2	5	6	45	49	40	59
HAP-D	0.002	0.004	0.004	–	3	4	47	49	38	59
HAP-E	0.009	0.011	0.011	0.007	–	7	45	52	39	58
HAP-F	0.011	0.013	0.013	0.009	0.016	–	50	51	40	60
MROS	0.102	0.104	0.100	0.104	0.100	0.111	–	57	52	58
MTOL	0.112	0.114	0.109	0.109	0.116	0.114	0.127	–	48	58
MLAR	0.087	0.089	0.089	0.084	0.087	0.089	0.116	0.107	–	59
MNOV	0.134	0.134	0.132	0.132	0.129	0.134	0.129	0.130	0.132	–

on an analysis of morphological characters, that *M. australiense* should be considered a single species.

The failure to find evidence of cryptic speciation within the *Macrobrachium australiense* complex contrasts with studies of inland fish species, which have revealed several cryptic species-complexes encompassing the Murray–Darling and Lake Eyre drainage basins (Ivantstoff *et al.* 1987; Musyl and Keenan 1992). The presence of a single species of *Macrobrachium* in inland Australia and the low level of variation at the *16S* rRNA gene suggest that *M. australiense* has only recently invaded the waters of inland Australia.

The relationships between drainages, both within catchments and, more particularly, between them, can provide insights into the evolution of this species and the historical biogeography of Australian waterways. The phylogenetic tree suggests a disparity between the haplotypes from the two major inland catchments, Lake Eyre Basin (MUT, GEO, CL, FIN) and Murray–Darling Basin (MUR, NAM, LA), which cover virtually the entire eastern half of the continent. Instead, a close relationship is evident between the Murray–Darling Basin and eastern coastal drainages (EN, FIS, MAR), with the Enogerra Creek (EN) and Fisher Creek (FIS) samples sharing the same haplotype with the Murray–Darling populations. This close relationship between samples from the eastern coastal drainages and the Murray–Darling Basin suggests that *M. australiense* has managed to disperse across the Great Dividing Range more recently than it has dispersed between the inland drainages. This is despite Lake Eyre and the Murray–Darling system being geographically adjacent, with little significant topological relief between the basins (Cook *et al.* 2002). Despite the apparent barrier (represented by the Great Dividing Range) to dispersal of aquatic organisms, there are a significant number of species inhabiting both the inland drainages and eastern coastal drainages and a close genetic relationship has been found for freshwater fish species between the Murray–Darling Basin and eastern coastal rivers (Musyl and Keenan 1992; McGlashan and Hughes 2001). Further studies are currently being undertaken to examine the phylogeographic structure of *M. australiense* using the more quickly evolving *ATPase 6* gene and the systematics and zoogeography of *Macrobrachium* in general using the *16S* and *28S* rRNA genes.

The complete lack of agreement between Riek's (1951) taxonomy and the results of this study and Short (2004) may be due to several factors. First, shrimps belonging to the genus *Macrobrachium* appear to be inherently difficult taxonomically. Much of the current classification of *Macrobrachium* worldwide is a result of the work by Holthuis (1950, 1952). Holthuis (1952) lists several reasons for the difficult taxonomy of the genus: the restricted number of characters available for identification, with many features common to all species; the high variability of characters within species; strong sexual dimorphism; and age- (size) or maturity-related morphological variation. Specifically in relation to *M. australiense*, McNeill (1929), Lee (1979) and Short (2004) all commented on the high degree of morphological variability within and between populations and agree that delimiting subspecific variation is a difficult task based

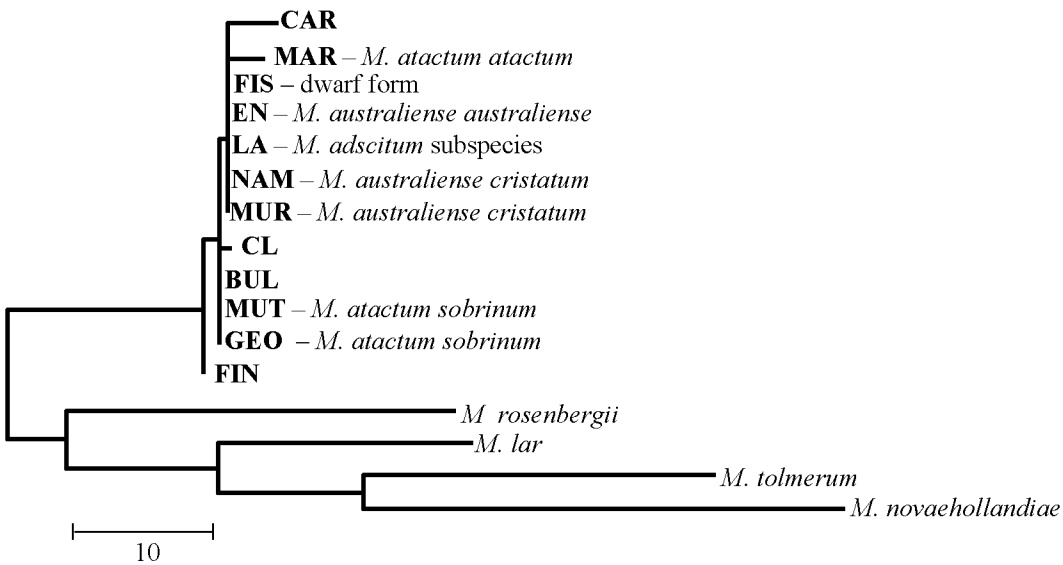

Fig. 2. Neighbour-joining estimate of phylogenetic relationships based on *16S* rRNA mtDNA sequences among *Macrobrachium australiense* haplotypes and other Australian *Macrobrachium* species. Sample locations: MUT, Thompson River; GEO, Georgina River; CL, Coongie Lakes; FIN, Finke River; BUL, Bulloo River; LA, Lake Alexandrina; MUR, Murray River; NAM, Namoi River; EN, Enoggera Creek; FIS, Fisher Creek; MAR, Mary River; CAR, Carron River.

on morphological analysis alone. It is also clear that morphological taxonomic studies on *Macrobrachium* shrimps need to be based on thorough sampling of different populations, so that character variation can be appropriately quantified and its significance reliably interpreted.

Molecular data, like other kinds of data, have limitations, especially if only a restricted number of samples or characters are examined. In addition, different kinds of molecular data suffer from specific limitations. For example, taxonomic conclusions derived from mtDNA sequences may be erroneous owing to the inadvertent amplification of nuclear translocated genes (Nguyen *et al.* 2002), or when gene trees do not accurately mirror evolutionary relationships (Nichols 2001). Conclusions can be drawn with greater confidence if data derived from different sources are congruent. In this case, congruence between the current study and the morphological work of Short (2004) provides compelling support for the conclusion that *M. australiense* represents a single species.

In conclusion, this study extends the molecular systematic studies of Murphy and Austin (2002, 2003) and provides convincing evidence, based on mtDNA sequences, that nominal taxa of the *M. australiense* species-complex, described by Riek (1951), represent a single species. This supports a recent morphological revision of Australian *Macrobrachium* in which these taxa were synonymised under *M. australiense* (Short 2004).

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