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ON THE PHYLOGENETIC POSITIONING OF THE SOUTH AMERICAN FRESHWATER CRAYFISH GENERA (DECAPODA: PARASTACIDAE)

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A B S T R A C T

South America contains three endemic genera of parastacid freshwater crayfishes (i.e., *Parastacus* Huxley, *Samastacus* Riek, and *Virilastacus* Hobbs), with a current total of ten described species. A previous author has argued that each of these genera has closer affinities with genera from Australia than to each other. We sequenced approximately 500 nucleotides of the 16S gene from mitochondrial DNA to estimate phylogenetic relationships among the South American genera of freshwater crayfishes and determined their phylogenetic positioning relative to the Australian genera. We sampled seven species representing all three genera from South America with 19 individuals. These sequences were combined with other Australasian crayfishes for a total representation of 54 sequences covering 13 genera. Our results indicate that the South American genera form a well-supported monophyletic group closely related to a subset of the Australasian crayfishes (*Paranephrops* and *Parastacoides*). Our results also provide strong support for the currently recognized generic designations.

R E S U M E N

En Sud América se encuentran tres géneros endémicos de camarones parastácidos, i.e., *Parastacus* Huxley, *Samastacus* Riek, y *Virilastacus* Hobbs, que reunen a diez especies en total. En publicaciones previas se ha argüido que cada uno de estos géneros tiene afinidades más estrechas con géneros australianos que con alguno otro sud-americano. Con el fin de estimar las relaciones filogenéticas entre los géneros sudamericanos y determinar, además, su relación con los géneros australasiáticos, se secuenciaron aproximadamente 500 nucleótidos del gen 16S del DNa mitocondrial de siete de las diez especies de parastácidos sudamericanos. Las muestras de DNA se obtuvieron de 19 especímenes. La información obtenida se combinó con información previa acerca de los géneros australasiáticos hasta completar 54 secuencias que involucraron a 13 géneros. Los resultados indican que los tres géneros sudamericanos forman un grupo monofilético estrechamente relacionado con un subgrupo de géneros australasiáticos (*Paranephrops* y *parastacoides*). Además, los resultados respaldan la actual asignación de géneros sudamericanos.

The parastacid freshwater crayfishes of South America are represented by ten species restricted to southern Chile and southern Brazil/Uruguay (Fig. 1). Of these ten recognized species, eight are placed in the genus *Parastacus* (with two species in Chile and six in Brazil) with each of the other two genera (*Samastacus* and *Virilastacus*) containing only a single species restricted to southern Chile, near Valdivia. The South American crayfishes are generally burrowing species occurring in fields and along river banks. It has been suggested that the limited number of crayfish species and their near exclusion from streams is due to their interactions with freshwater crabs from the family Aeglidae (Riek, 1971).

The crayfishes of South America were originally placed in a single genus and later divided into two genera, *Samastacus* and *Parastacus,* based on the orientation of the chelae (Riek, 1971). *Parastacus* contained species with the chelae moving vertically. *Samastacus,* on the other hand, contained crayfishes with chelae moving horizontally. Riek described these genera in terms of ecological habitat as well, with *Parastacus* being burrowers and *Samastacus* being stream and lake inhabiting species. However, there are *Parastacus* inhabiting streams and *Virilastacus araucanius* (Faxon, 1914) is a burrowing species. Hobbs (1991) later partitioned the two species in the genus *Samastacus* into two separate genera, *Samastacus* and *Virilastacus,* based on morphological differences between the two species.

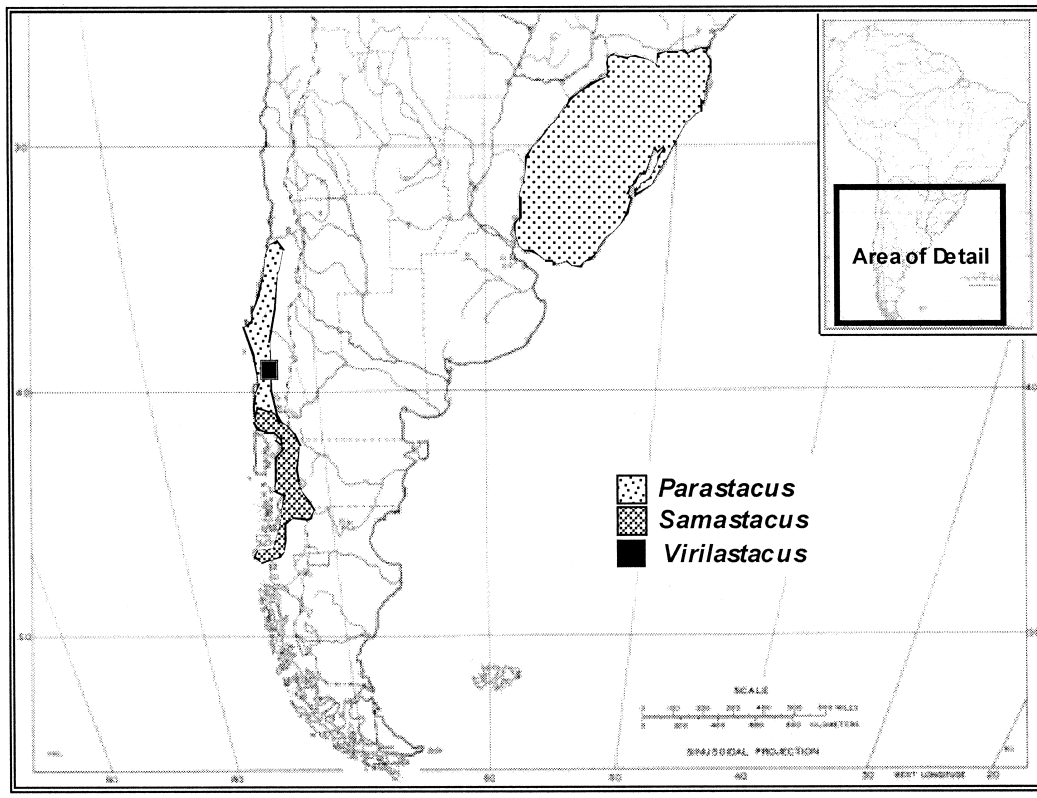

Fig. 1. Geographic distributions of the South American genera of freshwater crayfishes.

Although recent reviews of the genera of South America have been offered (Buckup and Rossi, 1980; Hobbs, 1991; Buckup and Rossi, 1993), the hypothesis of their relationships to other Southern Hemisphere crayfishes has not been well explored. Riek (1972) was one of the few astacologists to venture an opinion on the phylogenetic positioning of the South American crayfishes. Based on chelae orientation, Riek suggested *Samastacus* was more closely related to *Geocharax* and *Gramastacus,* whereas *Parastacus* belonged in a clade with *Engaeus, Engaewa,* and *Tenuibranchiurus* (Fig. 2A). Crandall *et al.* (1999) have shown that the ecological characters and morphological characters associated with ecological traits used by Riek provide inconsistent estimates of phylogeny relative to nucleotide sequence data. In addition, Starobogatov (1995), while not proposing an explicitly phylogenetic hypothesis, recently suggested that the family Parastacidae be subdivided into two subfamilies with one, Parastacinae, containing *Parastacus, Virilastacus, Samastacus,* and *Paranephrops* (an

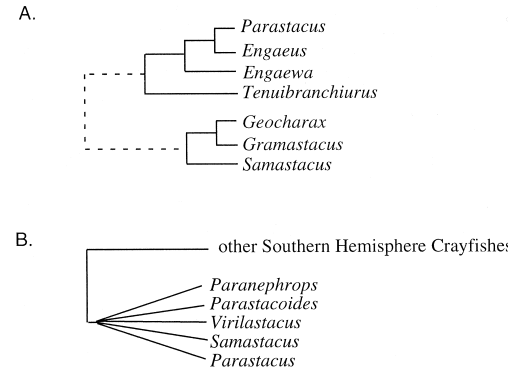

Fig. 2. Alternative hypotheses concerning the relationships among the South American genera relative to the Australasian genera. A. Riek's (1972) hypothesis suggesting South American genera are not a monophyletic group, but *Samastacus* (and *Virilastacus* by implication) are more closely related to *Gramastacus* and *Geocharax*, whereas *Parastacus* is more closely related to *Engaeus;* B. Starobogatov's (1995) hypothesis suggesting a close affinity of the South American crayfishes with one another and *Paranephrops* from New Zealand.

Table 1. South American freshwater crayfish species examined in this study and corresponding GenBank accession numbers.

Species	*n*	Location	KAC #	Accession numbers
Parastacus brasiliensis	2	Stream SW of Porto Alegre, Brazil.	1520	AF175244
			1521	AF175245
Parastacus defossus	2	Burrows on farm SW of Porto Alegre, Brazil.	1515	AF175243
		30°20.642′S, 51°33.878′W	1516	AF175242
Parastacus nicoleti	3	Drain ditches on the Univ. Austral de Chile campus,	1405	AF175231
		Valdivia, Chile.	1408	AF175233
		39°48′S, 73°14′W	1409	AF175234
Parastacus nicoleti	1	From burrows, Mafil, Chile.	1406	AF175232
Parastacus pilimanus	2	From burrows in a pasture, 200 m from coast, Brazil.	1536	AF175247
		32°29.383′S, 52°34.924′W	1537	AF175246
Parastacus pugnax	2	Cholchol, NW of Temuco, Chile.	1419	AF175237
		38°36′S, 72°51′W	1420	AF175238
Parastacus pugnax	1	Parral, Chile.	1429	AF175239
		36°09′S, 71°50′W		
Samastacus spinifrons	2	Rio Cruces at Puente Negro North of Mafil, Chile.	1449	AF175240
		39°27′S, 72°46′W	1450	AF175241
Virilastacus araucanius	4	Found in drain ditches on Univ.	1400	AF175229
		Austral de Chile campus, Valdivia, Chile.	1401	AF175230
		39°48′S, 73°15′W	1415	AF175235
			1416	AF175236

endemic genus from New Zealand). This suggestion clearly supposes a closer relationship among the genera of South America relative to those in Australia (Fig. 2B). We tested these hypotheses using nucleotide sequence data from the 16S region of the mitochondrial genome (mtDNA). We have sequenced representatives from all three South American genera, including five species of *Parastacus* from both Brazil and Chile. These sequences are combined with our previously published sequences from the Australasian crayfishes, representing all nine genera from Australia plus an endemic genus from New Zealand (Crandall *et al.,* 1999). With this enlarged data set of 54 sequences, we estimated the phylogenetic relationships of the South American genera relative to the Australasian genera.

Materials and Methods

Crayfish Samples.—Crayfishes were collected by hand, dipnet, or trap in November 1997 (Chile) and 1998 (Brazil). Abdomen and gill tissues were dissected and frozen in liquid nitrogen for DNA extraction. The remaining specimens were preserved in 70% EtOH and are housed in the crustacean collection at the Monte L. Bean Life Science Museum, Brigham Young University. We sampled between two and four individuals per species for a total of seven of the ten species within the genus (Table 1). Those species not sampled include *Parastacus laevigatus, Parastacus saffordi,* and *Parastacus varicosus.*

Crayfish DNA was extracted from the frozen tissues using the methods described in Crandall *et al.* (1999). The polymerase chain reaction (PCR) was carried out using 16S primers previously described (Crandall and Fitzpatrick, 1996). Standard PCR conditions were used on a Perkin-Elmer 9600 machine and consisted of the following: an initial denaturation at 96°C for 3 min followed by 45 cycles of 95°C for 30 sec, 41°C for 45 sec, 72°C for 1 min and 45 sec followed by an extension at 72°C for 5 min. Successful PCR products were purified using a GeneClean® II kit (Bio 101). Automated sequences were generated in both directions on an ABI 377XL automated sequencer using the ABI Big-dye Ready-Reaction kit, following the standard cycle sequencing protocol but using a quarter of the suggested reaction size.

Phylogenetic Analyses.—Nucleotide sequences were aligned using Clustal X (Thompson *et al.,* 1994) and then adjusted by eye. Phylogenetic relationships were estimated using maximum parsimony, neighbor-joining (Saitou and Nei, 1987), and maximum likelihood (Felsenstein, 1981). Results from these three optimality criteria are presented not as an assessment of confidence in resulting relationships but to acknowledge the diversity of opinions about how phylogeny reconstruction should be performed. All phylogeny reconstruction methods assume a model of evolution. Maximum parsimony implicitly as-

Table 2. Likelihood ratio tests of models of molecular evolution (Huelsenbeck and Crandall, 1997; Posada and Crandall, 1998). Due to the performance of multiple tests, the significance level of rejection of the null hypothesis should be adjusted via the Bonferroni correction to $\alpha = 0.0083$.

Null hypothesis	Models compared	$-\ln L_0$	$-\ln L_1$	$-2\ln\lambda$	*d.f.*	*P*
Equal base frequencies	H_0: JC69 H_1: F81	7,983	7,878	210	3	<0.000001
Equal ti/tv rates	H_0: F81 H_1: HKY85	7,878	7,634	488	1	<0.000001
Equal ti rates	H_0: HKY85 H_1: TrN	7,634	7,628	12	1	0.000577
Equal tv rates	H_0: TrN H_1: GTR	7,628	7,566	124	3	<0.000001
Equal rates among sites	H_0: GTR H_1: GTR+Γ	7,566	6,832	1,468	1	<0.000001
Proportion of invariable sites	H_0: GTR+Γ H_1: GTR+Γ+I	6,832	6,812	40	1	<0.000001

sumes that all character changes are equally likely. Maximum likelihood and neighbor-joining, on the other hand, make explicit assumptions about the relative likelihoods of character change using a model of evolution (Huelsenbeck and Crandall, 1997). Therefore, for those methods making explicit use of models of evolution, the choice of model must be justified relative to the data at hand. This can be easily accomplished within the likelihood framework (Felsenstein, 1988; Goldman, 1993; Huelsenbeck and Crandall, 1997). We used the approach outlined by Huelsenbeck and Crandall (1997) to test hypotheses relating to the molecular evolution of the nucleotide sequences examined in this study. This approach estimates a starting tree using neighbor-joining assuming a Jukes and Cantor model of evolution. With this tree, likelihood scores are calculated for a variety of models of evolution that incorporate different assumptions about the types of changes involved (e.g., base frequencies are equal or not). The likelihood scores are then compared statistically using a chi-square test (Posada and Crandall, 1998). The model choice is then dictated by the null hypotheses rejected. The following null hypotheses were tested: 1) nucleotide frequencies are equal, 2) transition rate equals transversion rate, 3) transition rates are equal, 4) transversion rates are equal, 5) rate homogeneity across sites, 6) no significant proportion of invariable sites. The likelihood values associated with these models were estimated in PAUP* (Swofford, 1999). The statistical tests were performed using Modeltest Version 2.0 (Posada and Crandall, 1998).

Once a model was selected, phylogenetic relationships were estimated via maximum likelihood and neighbor-joining incorporating this model of evolution. Maximum likelihood and maximum parsimony searches were heuristic. As such, they are subject to biases associated with the order of taxon addition (Templeton, 1992) and multiple tree islands (Maddison, 1991). To avoid these biases, 10 random addition heuristic searches were performed for both the likelihood and parsimony analyses. Confidence in the resulting relationships was assessed using the bootstrap procedure with 100 replications (Felsenstein, 1985) for maximum likelihood and 1,000 replications for neighbor-joining and maximum parsimony. Because each maximum likelihood bootstrap replication took, on average, more than one day to run, only 100 replications were made using this optimality criterion. We feel it is more important to do full heuristic searches with random addition for 100 bootstrap replications rather than to use the "Fast step-wise" search strategy for more replicates. Likelihood, neighbor-joining, and parsimony searches, as well as the bootstrap analyses, were executed in PAUP* (Swofford, 1999).

RESULTS

Our sequencing efforts resulted in 19 new 16S mtDNA sequences from seven different species of South American crayfishes representing all three endemic genera (Table 1). These sequences were combined with the 35 sequences from our previous study on the Australasian crayfishes for a total data set of 54 sequences representing 13 of the 14 genera within the family Parastacidae. The only genus yet to be sampled is *Astacopsis* from Madagascar. The alignment for these 54 sequences can be downloaded from our lab webpage (http://bioag.byu.edu/zoology/crandall_lab/cranlabpubs.htm#JCB99). The new sequences have been deposited in GenBank under the accession numbers shown in Table 1.

The maximum likelihood hypothesis testing procedure resulted in the rejection of all six null hypotheses tested (Table 2). Nucleotide frequencies were significantly different from being equal with A = 0.33, C = 0.11, G = 0.21, and T = 0.35. Transition rates and transversion rates were not equal and a different estimated rate was used for each of the six reversible rates of change ($R_1 = 1.55$, $R_2 = 6.16$, $R_3 = 0.970$, $R_4 = 1.04$, $R_5 = 10.20$, and $R_6 = 1.00$). This corresponds to the general-time-reversible (GTR) model of molecular evolution (for a thorough discussion of models of evolution, see Swofford *et al.,* 1996). There was also significant rate heterogeneity in these data. Rate heterogeneity

is taken into account in models of evolution using a gamma distribution with the shape parameter of the distribution (α) estimated from the data via maximum likelihood (Yang, 1996). The estimated shape parameter for the gamma distribution for these data was $\alpha = 0.716$. There was also a significant proportion of invariable sites in these data estimated at 31.5%. This model assumes 31.5% of the sites are incapable of accepting substitutions, perhaps due to functional constraints in this gene region (Swofford *et al.,* 1996). Thus, our justified model was the general-time-reversible model plus gamma distributed rate heterogeneity plus a significant proportion of invariable sites (GTR + Γ + I).

Incorporating this model of molecular evolution, we estimated phylogenetic relationships among these taxa using maximum likelihood. Ten random sequence addition searches resulted in the same maximum likelihood tree (Fig. 3). The resulting phylogeny clearly shows the South American genera forming a monophyletic group with a sister relationship to *Paranephrops*/*Parastacoides* clade (Fig. 3). The South American genera form a distinct monophyletic clade with 62% bootstrap support and well-supported genera (97% *Parastacus,* 93% *Virilastacus*, and 100% *Samastacus*).

The neighbor-joining search also incorporated this same maximum likelihood model of evolution. The resulting tree, again, clearly supports the monophyly of the South American genera (82% bootstrap support) and the monophyly of each genus (each with 100% bootstrap support) (Fig. 4). Furthermore, the neighbor-joining tree also supports the sister relationship between the South American genera and the *Paranephrops*/*Parastacoides* clade with 86% bootstrap support (Fig. 4).

Unlike maximum likelihood and neighbor-joining methods for which all characters are "phylogenetically informative", maximum parsimony limits phylogenetically informative characters to synapomorphic character changes. Our data set consisted of 243 parsimony informative characters. The initial maximum parsimony analysis resulted in two islands of most parsimonious trees with tree length 1,412 steps. Therefore, the number of random sequence additions was increased to 100 to more thoroughly explore the tree space. This search resulted in 52 most parsimonious trees residing in three different islands of most parsimonious trees. The majority rule consensus tree of these 52 maximum parsimony trees supports the monophyly of the South American genera (Fig. 5). Indeed, the only conflict relative to the South American genera is in the positioning of lineages within the *Virilastacus* clade. The remaining ambiguity is associated with the Australian taxa and has been discussed elsewhere (Crandall *et al.,* 1999). The maximum parsimony bootstrap analysis gave similar results to the likelihood analysis with strong support for the South American genera forming a monophyletic group (73% bootstrap support) and each genus forming a monophyletic group as well (98% *Parastacus,* 100% *Virilastacus,* 100% *Samastacus*) (Fig. 6). Furthermore, all analyses gave strong support for the sister group relationship of *Virilastacus* and *Samastacus* (90% likelihood bootstrap, 98% neighbor-joining bootstrap, and 94% parsimony bootstrap).

Discussion

Our analysis shows clear support for the monophyly of the South American crayfish genera and their sister group relationship to *Paranephrops,* as suggested by Starobogatov (1995), or (*Paranephrops, Parastacoides*). The relationships proposed by Riek (1972) separating the South American genera as sister group to two different genera from Australia is clearly not supported by our analyses. Indeed, we have statistically tested the Riek hypothesis *versus* that presented by the maximum likelihood analysis. Both the association of *Parastacus* with *Engaeus* and *Samastacus* with (*Geocharax, Gramastacus*) can be rejected ($P < 0.0004$ and $P = 0.008$, respectively, using a two-tailed sign test). These two components of the Riek hypothesis, taken together, can be strongly rejected ($P < 0.0004$, two-tailed sign test). Thus, the strong bootstrap support, coupled with the statistical rejection of the Riek hypothesis, offers strong evidence for the monophyly of the South American genera of freshwater crayfish and their sister group relationship to *Paranephrops* or (*Paranephrops, Parastacoides*).

In Hobbs' (1991) evaluation of the South American genera, he was somewhat concerned about the establishment of a new genus (*Virilastacus)* with only a single species representing that genus. This division was

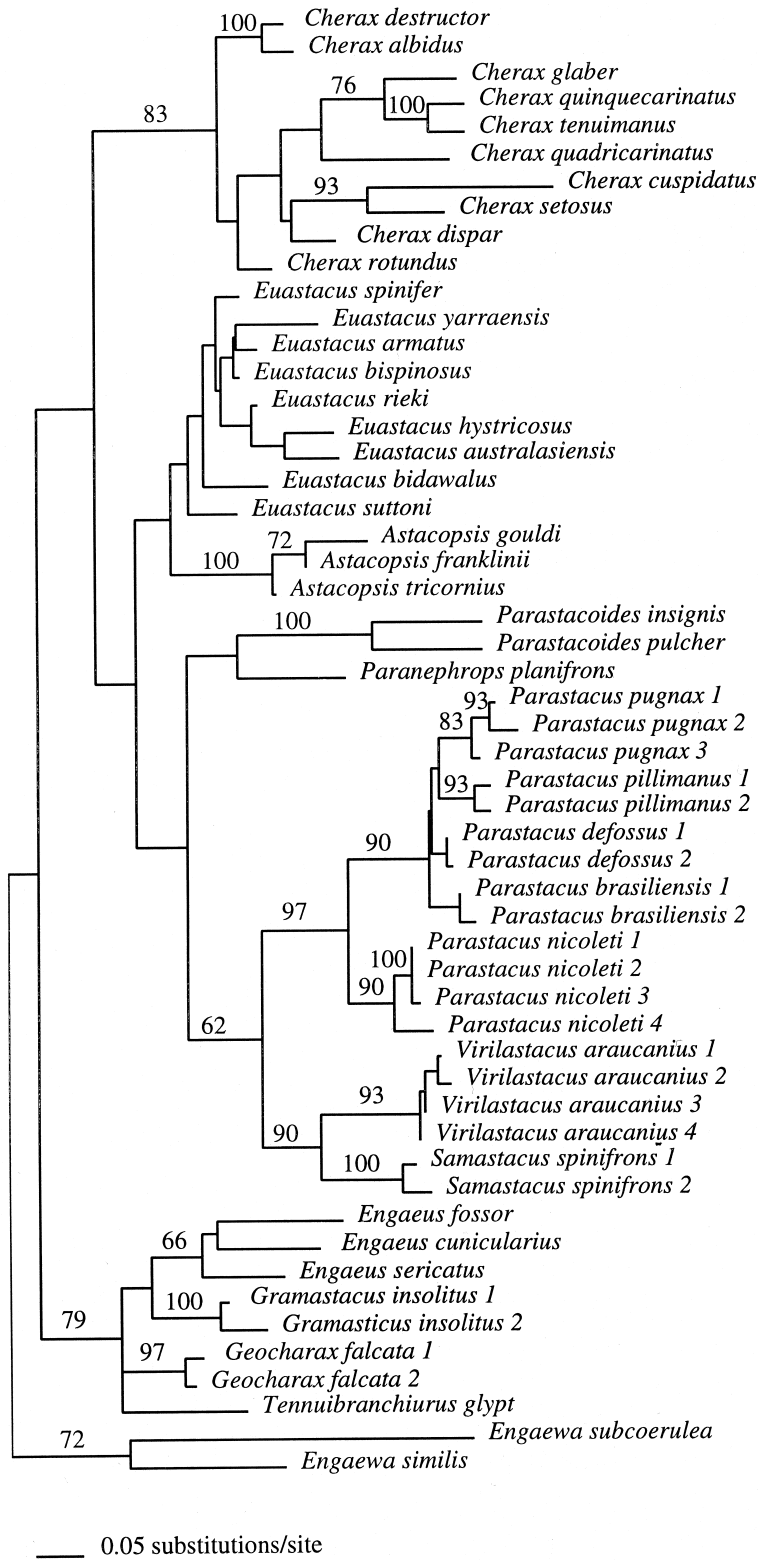

Fig. 3. The maximum likelihood estimate of phylogenetic relationships among the South American and Australasian freshwater crayfishes assuming the GTR + Γ + I model of evolution (Table 2). Branch lengths are shown proportional to the amount of change along the branches. Bootstrap values are shown as percentages and are based on 100 bootstrap replications.

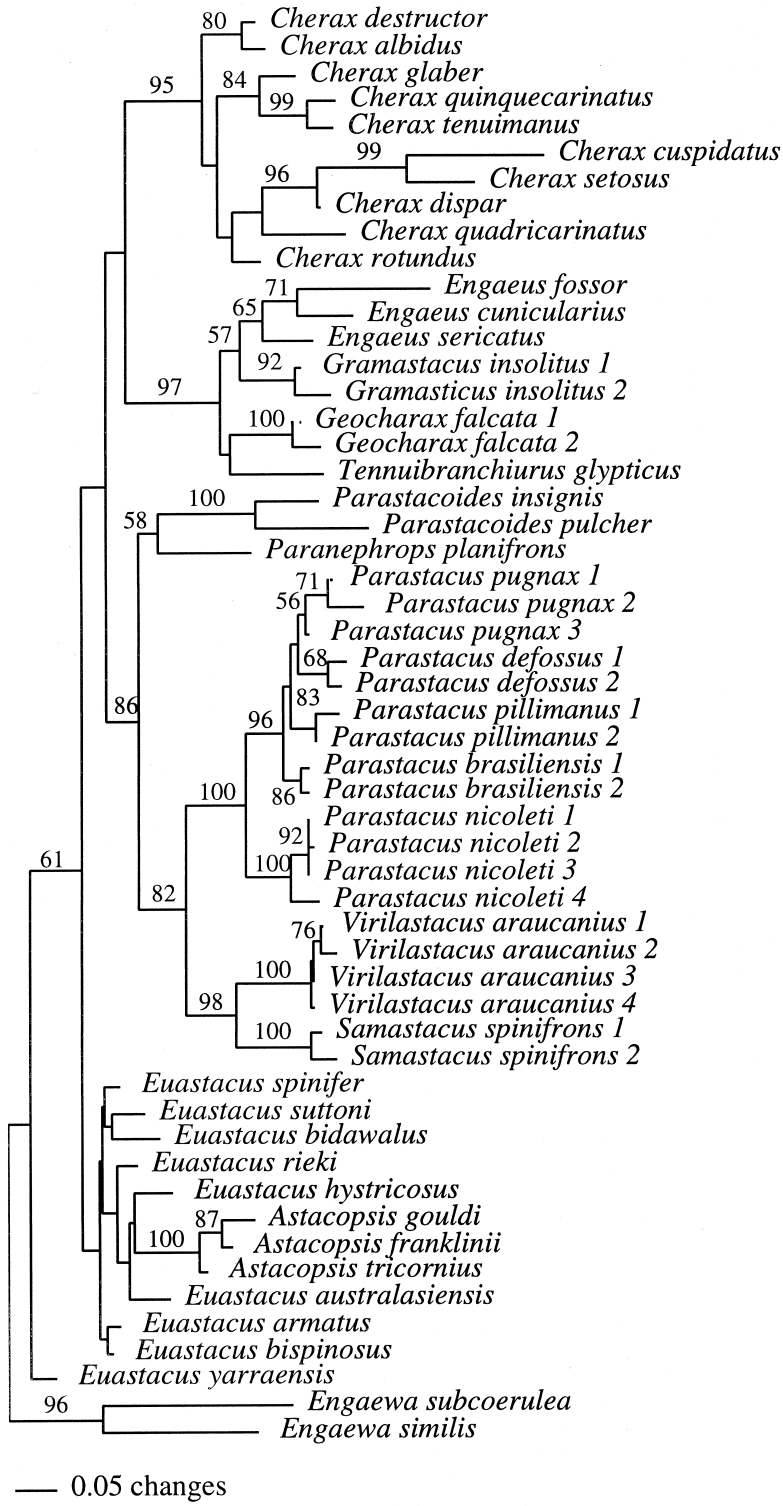

Fig. 4. The neighbor-joining estimate of phylogenetic relationships incorporating the justified GTR + Γ + I model of evolution (Table 2). Branch lengths are shown proportional to the amount of change along the branches. Bootstrap support is labeled on individual nodes where support was greater than 50% based on 1,000 bootstrap replications.

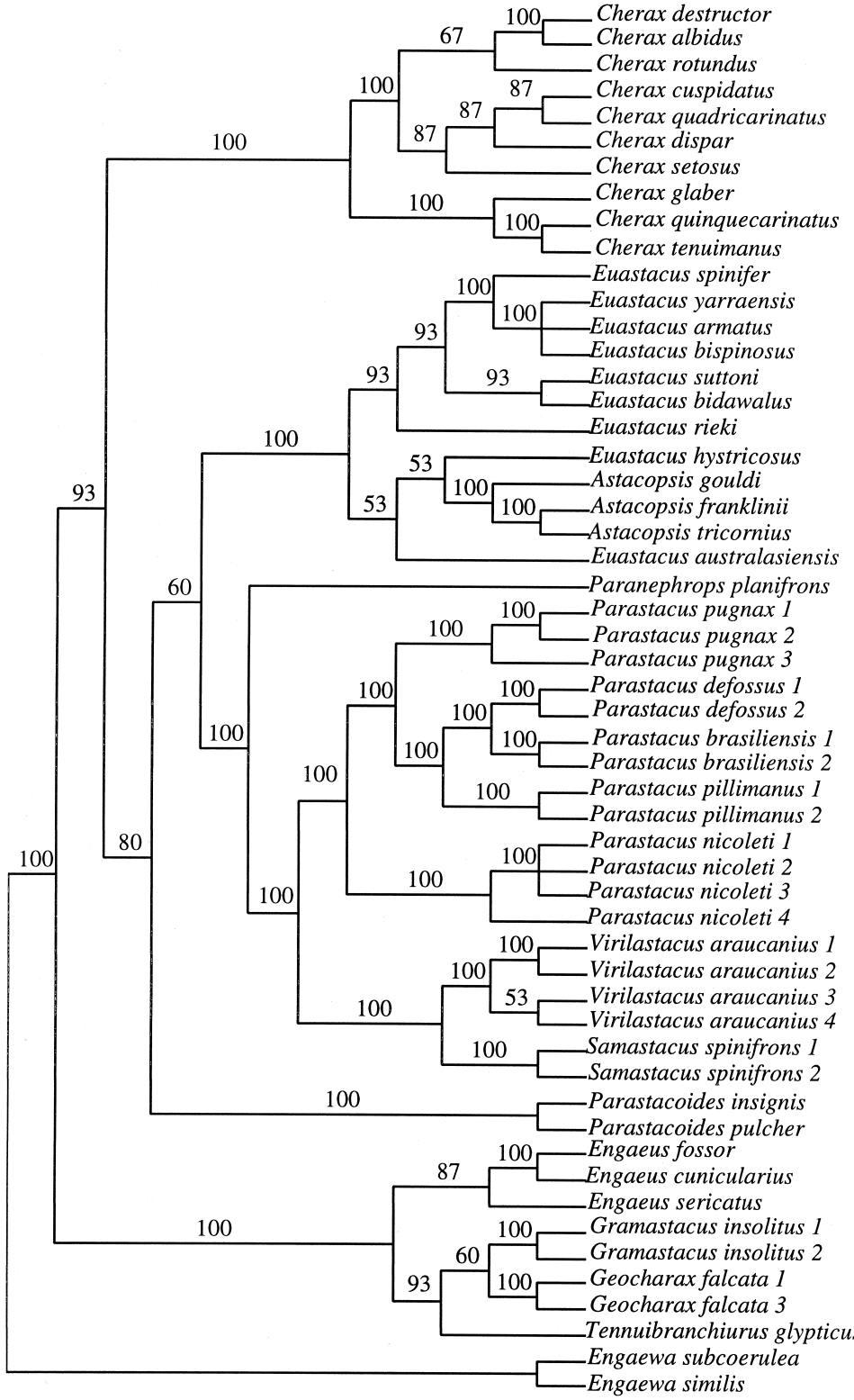

Fig. 5. The majority-rule consensus tree of the 52 most parsimonious trees found after 100 random addition searches. These 52 trees were found in 3 distinct islands of most parsimonious trees.

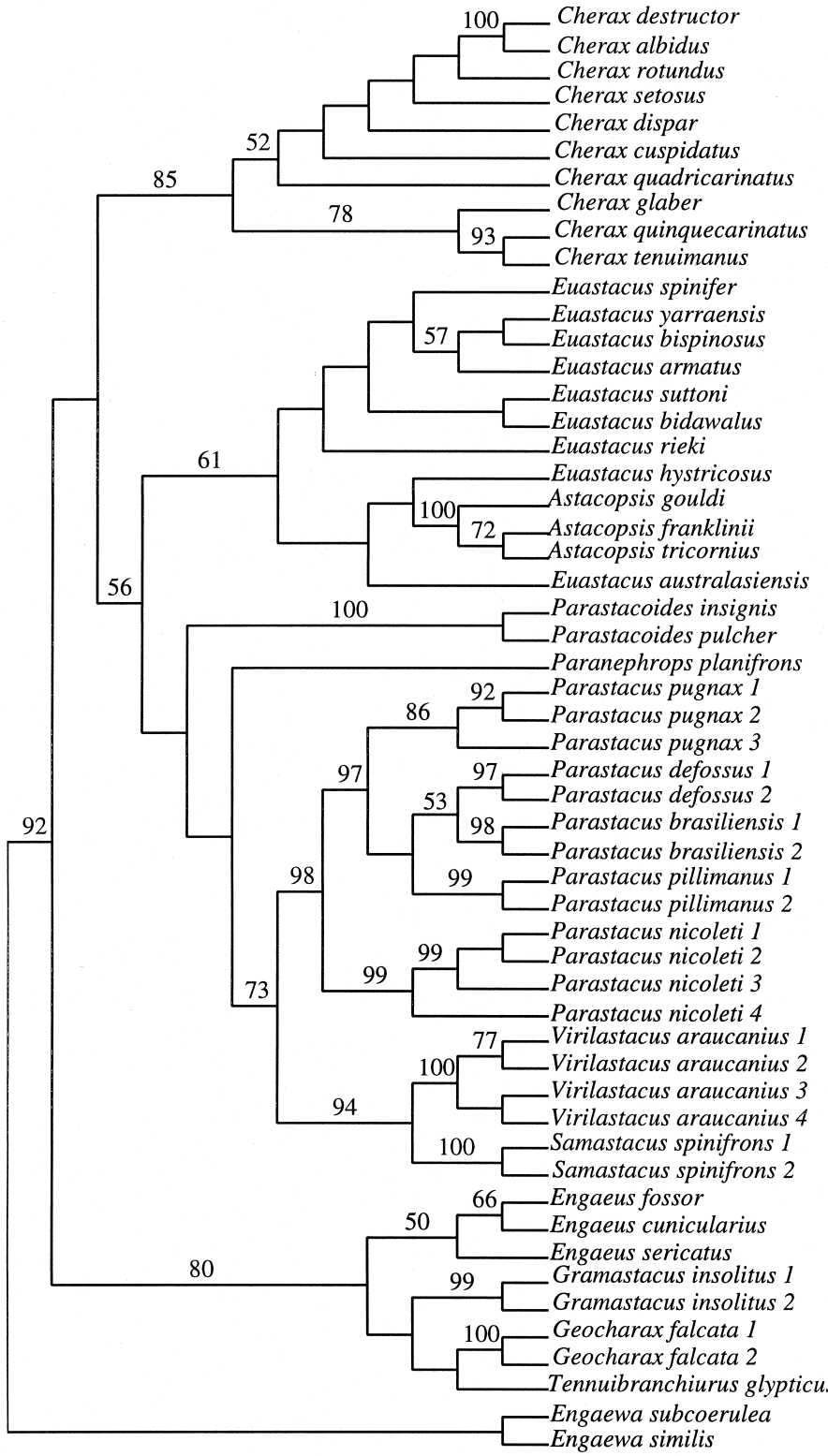

Fig. 6. The maximum parsimony estimate of phylogenetic relationships among the South American and Australasian freshwater crayfishes. Branch lengths are shown proportional to the amount of change along the branches based on a single representative of the most parsimonious trees. Bootstrap values are shown as percentages and are based on 100 bootstrap replications.

supported not only by Hobbs' study of mandibular characters and a host of others (see his Table 2) but also by previous analyses of Jara (1983) and Rudolph and Rivas (1988). Our nucleotide sequence data support this separation of *Samastacus* and *Virilastacus* from both a cladistic and genetic distance standpoint. These genera clearly form two distinct clades, and these clades are well separated in genetic distance (Figs. 3, 4). Indeed, their separation is on the order of the separation of other sister genera (e.g., *Parastacoides, Paranephrops*; *Engeaus, Gramasticus*; *Euastacus, Astacopsis*). These separations and the sister group relationships are all supported by extremely high bootstrap values (Figs. 3, 4, 6).

The other interesting relationship of note is the clear distinction of two subclades within *Parastacus.* One would expect, from biogeographic arguments, this separation to be between the Brazilian and Chilean representatives of the genus. However, the two endemic species from Chile are split in these two clades, with the one major clade being made up solely of *Parastacus nicoleti* and the second clade representing the sampled species from Brazil plus *Parastacus pugnax* from Chile. Again, the division of these subclades is strongly supported by high bootstrap values for both the likelihood (Fig. 3) and parsimony (Fig. 6) analyses. It will be of great interest to obtain data from the remaining species of *Parastacus* to thoroughly explore the relationships among species within this genus.

We hope that our hypothesis of the phylogenetic relationships among these genera and species will set the stage for comparative analyses of ecological, morphological, and developmental analyses of these understudied and interesting crayfishes. For Hobbs' description of the genus *Virilastacus,* only three specimens were available for analysis. Our collection has tripled the world's holdings of this species. Nearly nothing is known about the ecology and morphology of these animals, although progress is starting to be made (e.g., Almeida and Buckup, 1997). This phylogeny will provide the phylogenetic framework needed for advances in our understanding of the comparative biology of parastacid freshwater crayfishes.

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APPENDIX. AUTHORITIES OF THE SPECIES.

Astacopsis gouldi Clark, 1936
Astacopsis franklinii Gray, 1845
Astacopsis tricornius Clark, 1936
Cherax albidus Clarke, 1936
Cherax cuspidatus Riek, 1969
Cherax destructor Clark, 1936
Cherax dispar Riek, 1951
Cherax glaber Riek, 1967
Cherax quadricarinatus von Martens, 1868
Cherax quinquecarinatus Gray, 1845
Cherax rotundus Clark, 1941
Cherax setosus Riek, 1951
Cherax tenuimanus Smith, 1912
Engaeus cunicularius (Erichson, 1846)
Engaeus fossor (Erichson, 1846)
Engaeus sericatus Clark, 1936
Engaewa similis Riek, 1967
Engaewa subcoerulea Riek, 1967
Euastacus armatus von Martens, 1866
Euastacus australasiensis Milne-Edwards, 1837
Euastacus bidawalus Morgan, 1986
Euastacus bispinosus Clarke, 1936
Euastacus hystricosus Riek, 1951
Euastacus rieki Morgan, 1997
Euastacus spinifer Heller, 1856
Euastacus suttoni Clark, 1941
Euastacus yarraensis McCoy, 1888
Geocharax falcata Clark, 1936
Gramastacus insolitus Riek, 1972
Paranephrops planifrons White, 1842
Parastacoides insignis Clark, 1939
Parastacoides pulcher Riek, 1967
Parastacus brasiliensis (von Martens, 1869)
Parastacus defossus Faxon, 1898
Parastacus laevigatus Buckup and Rossi, 1980
Parastacus nicoleti (Philippi, 1882)
Parastacus pillimanus (von Martens, 1869)
Parastacus pugnax (Poeppig, 1835)
Parastacus saffordi Faxon, 1898
Parastacus varicosus Faxon, 1898
Samastacus spinifrons (Philippi, 1882)
Tennuibranchiurus glypticus Riek, 1951
Virilastacus araucanius (Faxon, 1914)