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The monophyletic origin of freshwater crayfish estimated from nuclear and mitochondrial DNA sequences

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Despite their widespread use as model organisms, the phylogenetic status of the around 520 species of freshwater crayfish is still in doubt. One hypothesis suggests two distinct origins of freshwater crayfish as indicated by their geographical distribution, with two centres of origin near the two present centres of diversity; one in south-eastern United States and the other in Victoria, Australia. An alternative theory proposes a single (monophyletic) origin of freshwater crayfish. Here we use over 3000 nucleotides from three different gene regions in estimating phylogenetic relationships among freshwater crayfish and related Crustacea. We show clear evidence for monophyly of freshwater crayfish and for the sister-group relationship between crayfish and clawed lobsters. Monophyly of the superfamilies Astacoidea and Parastacoidea is also supported. However, the monophyly of the family Cambaridae is questioned with the genus *Cambaroides* being associated with the Astacidae.

Keywords: crayfish; phylogeny; 18S rDNA; 28S rDNA; 16S mtDNA; maximum likelihood; models of evolution

1. INTRODUCTION

Since the publication of Huxley's (1880) *The crayfish*, freshwater crayfish have served as model organisms in zoological studies. In particular, crayfish have played a central role in vision research (Wald 1967; Crandall & Hillis 1997) and neural physiology (Yeh *et al*. 1996; Edwards *et al*. 1999) and an increasing role in studies of molecular evolution (Bode *et al*. 1992; Crandall & Cronin 1997) and ecology (Lodge 1993; Garvey *et al*. 1994). However, their phylogenetic status continues to be questioned.

The standard classification of freshwater crayfish is within the infraorder Astacidea including three superfamilies, i.e. Astacoidea (Northern Hemisphere crayfish), Nephropoidea (clawed lobsters) and Parastacoidea (Southern Hemisphere crayfish). The Astacoidea are divided into two families, the Cambaridae and Astacidae. The Cambaridae are distributed in North America east of the Rocky Mountains, north into southern Canada and south through Mexico and in Asia (figure 1). The largest number of species of freshwater crayfish occurs in this family with over 350 described species. The Astacidae are distributed west of the Rocky Mountains (mainly in the Pacific North-West) and in Europe (figure 1). The superfamily Parastacoidea contains a single family Parastacidae with 14 genera and around 180 species. Nine out of these 14 genera are found in Australia and three genera are distributed in southern South America, while New Zealand and Madagascar each contain an endemic genus belonging to this family (figure 1).

Freshwater crayfish are typically presented as a monophyletic group relative to the clawed lobsters (Hobbs 1974; Brusca & Brusca 1990) (figure 2*a*). Yet the relationships between these groups have remained enigmatic. Huxley (1880) originally proposed two distinct origins of freshwater crayfish (figure 2*b*) with two centres of origin near the two present centres of diversity; one in south-eastern United States and the other in Victoria, Australia. An alternative hypothesis was offered by Ortmann (1902) who proposed a single (monophyletic) origin of the freshwater crayfish. Recently, the monophyletic origin hypothesis has been supported by a few morphological characters with very limited taxon sampling (Jamieson 1991; Scholtz 1993, 1998; Scholtz 1995). Scholtz & Richter (1995) further suggested that there are no morphological characters uniting freshwater crayfish with the clawed lobsters and that the mud shrimps (Thalassinida) might actually be more closely related to crayfish (figure 2*c*).

In order to investigate the origin of freshwater crayfish, we sampled each superfamily, family and subfamily of crayfish with representatives of each of the proposed major clades within subfamilies (Hobbs 1988). In addition, we sampled the closely related clawed lobsters (Nephropoidea), mud shrimps (Thalassinidea) and spiny lobsters (Palinura) as well as Brachyuran and Anomuran crabs (table 1).

We collected nucleotide sequence data from the 18S (1954 base pairs (bp)) and 28S (965 bp) regions of rDNA and the 16S (517 bp) region of mitochondrial DNA (mtDNA). From these data, we estimated phylogenetic relationships between freshwater crayfish and their decapod relatives. Using these phylogenetic trees, we examined the hypothesis of crayfish monophyly (figure 2*a*) versus the alternative of Nephropoidea falling between the Northern and Southern Hemisphere crayfish (figure 2*b*). We also examined the Scholtz & Richter (1995) hypothesis by constraining the Thalassinidea to be a sister group to the crayfish and the crayfish monophyletic (figure 2*c*). Thus, we examined the monophyly of freshwater crayfish and their positioning within the decapod crustaceans.

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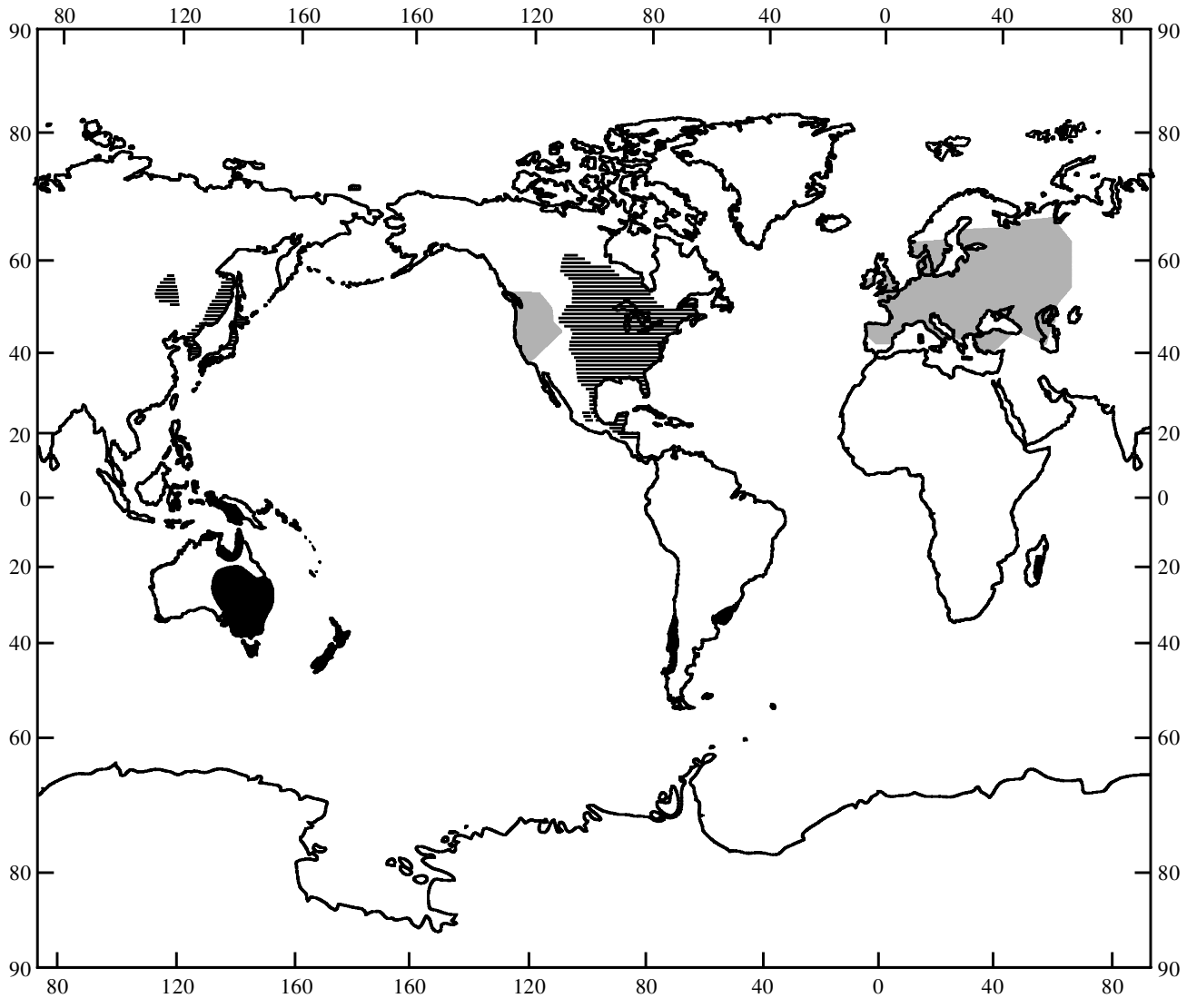

Figure 1. Geographical distribution of the > 520 species of freshwater crayfish representing two superfamilies: Parastacoidea (Parastacidae, solid areas) and Astacoidea (Astacidae, hatched areas and Cambaridae, horizontally lined areas) (Hobbs 1988).

2. METHODS

(a) *Samples*

Crayfish were collected by hand, dip-nets or traps and tissues were dissected and placed in liquid nitrogen. The tissues were then transported to the laboratory and stored at −80 °C. The remainder of the specimen was preserved in 70% ethanol and housed at the Monte L. Bean Life Science Museum, Brigham Young University, USA. Total genomic DNA was extracted from the frozen tissues using standard protocols (Crandall & Fitzpatrick 1996). DNA was then dried and resuspended in Tris-EDTA buffer. Polymerase chain reaction (PCR) products were amplified using the following primers: 18S (Whiting *et al*. 1997), 16S (Crandall & Fitzpatrick 1996) and 28S Rdla 5′-CCCSCGTAAYTTAAGCATAT-3′ Rd4b 5′-CCTTGGTCCGTGTTTCAAGAC-3′ (M. F. Whiting, personal communication). Standard PCR reactions were carried out on a Perkin-Elmer 9600 machine with 35 cycles of 92 °C for 30s, 50 °C for 30s and 72 °C for 30s followed by 72 °C for 5 min, except for the 16S primers which used an annealing temperature of 42 °C. Successful PCR products were purified using a GeneClean II kit (Bio 101; www.bio101.com). Automated sequences were generated in both directions on an ABI 377XL automated sequencer using the ABI Big-dye Ready-Reaction kit (Perkin-Elmer; www.pecorporation.com) following the standard cycle sequencing protocol, but using one-quarter of the suggested reaction size.

(b) *Phylogeny reconstruction*

Sequences were aligned using Clustal X (Thompson *et al*. 1997). Some adjustments were made by eye. They were then imported into PAUP* (Swofford 1999) for phylogenetic analyses. When estimating phylogenetic relationships between sequences, one assumes a model of evolution regardless of the optimality criteria employed. Determining which model to use given one's data is a statistical problem (Goldman 1993). We used the approach outlined by Huelsenbeck & Crandall (1997) in order to test alternative models of evolution, employing PAUP* and Modeltest (Posada & Crandall 1998). A starting tree was obtained using neighbour joining. Likelihood scores for 56 different models of evolution were calculated with this tree and then compared statistically using a χ^2-test with degrees of freedom equal to the difference in the free parameters between the models being tested. The null hypotheses tested in this way included (i) equal nucleotide frequencies, (ii) equal transition

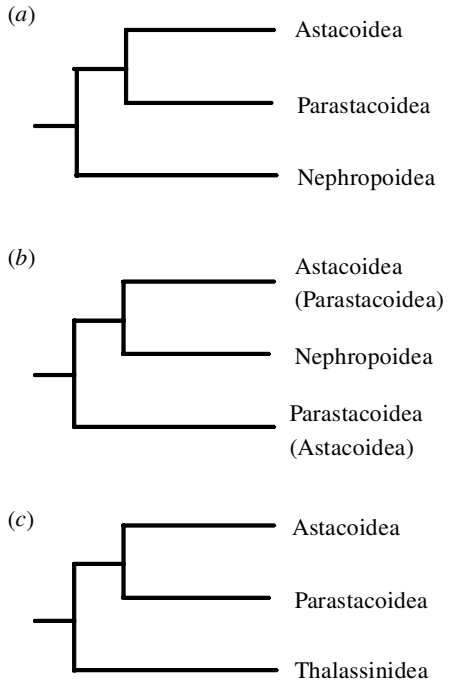

Figure 2. Alternative hypotheses of the relationships between freshwater crayfish and clawed lobsters. (*a*) Ortmann's (1902) hypothesis of a monophyletic grouping of freshwater crayfish relative to the clawed lobsters (Nephropoidea). This theory has recently been supported by only three characters (Scholtz & Richter 1995). (*b*) Huxley's (1880) hypothesis of two independent origins of freshwater crayfish. This theory has been supported by other authors based mainly on the discontinuous geographical distribution and differences in secondary sexual characteristics between Northern and Southern Hemisphere crayfish (Bott 1950; Hobbs 1974; Albrecht 1983). Clawed lobsters falling in between the Northern (Astacoidea) and Southern (Parastacoidea) Hemisphere groups of freshwater crayfish indicate two independent origins of freshwater crayfish. (*c*) A monophyletic grouping of the freshwater crayfish, but sister to the mud shrimps (Thalassinidea) instead of the clawed lobsters.

rates to transversion rates, (iii) equal transition rates, (iv) equal transversion rates, (v) rate homogeneity within the data set, and (vi) no significant proportion of invariable sites.

Once a model of evolution was selected by this approach, it was used to estimate a tree with the maximum-likelihood (ML) optimality criterion. Trees were also estimated using maximum parsimony (MP) (assuming equal weights for all changes). Because a tree estimated by an ML or MP search can be influenced by the ordering of taxa in a data set (Templeton 1992), we used random sequence addition in order to eliminate this bias in the addition of taxa. We present the results from both of these optimality criteria not as an assessment of confidence in relationships, but in recognition of a diversity of philosophies concerning phylogeny reconstruction.

Confidence in the resulting nodes was assessed using the bootstrap approach (Felsenstein 1985). Bootstrap values for the ML and MP trees were based on 1000 bootstrap replications. Data sets were analysed independently with models optimized for each data set (results not shown). We then performed a partition homogeneity test in order to see whether we were justified in combining data sets (Farris *et al.* 1994). This test was implemented in PAUP* using 1000 replicates.

3. RESULTS

We obtained 15 new complete 18S rDNA and 16 partial 28S and 16S rDNA sequences from crayfish, clawed lobsters, spiny lobsters and mud shrimps (table 1). Resulting sequences have been submitted to GenBank (AF235983–AF235992 for 16S, AF235959–AF235972 for 18S AF235973 to AF235982 for 28S). The alignment of these data is available at our Web site in NEXUS format (http://bioag.byu.edu/zoology/crandall_lab/cranlabpubs.htm). Initially, we examined the 18S rDNA as this is the slowest evolving gene and, therefore, we could include more distant outgroups without problems of saturation. The tree was rooted using *Stenopus hispidus*. Using Modeltest (Posada & Crandall 1998), we concluded that the Tamura–Nei model with a gamma-distributed rate heterogeneity model and an estimated proportion of invariable sites was the most appropriate model of evolution for these data (table 2). A ten-replicate heuristic search using random sequence addition with this model produced a single ML tree of score $-\ln$ 8326.74 (figure 3). MP searches were also carried out and support for nodes was estimated using the bootstrap technique (figure 3).

A partition homogeneity test (Farris *et al.* 1994) indicated that our data from these three distinct gene regions were not significantly heterogeneous ($p = 0.618$), thereby justifying the combining of sequence data. We therefore carried out an analysis combining sequences from the 18S, 28S and 16S gene regions because they offer resolving power across a broad range of evolutionary time. *Nephrops norvegicus* and *Homarus americanus* were used to root the trees for this analysis. Again using Modeltest (Posada & Crandall 1998), we concluded that the transversion model (TVM) of evolution with a gamma-distributed rate heterogeneity model and an estimated proportion of invariable sites was the most appropriate model of evolution for these data (table 2). A ten-replicate heuristic search using random sequence addition with this model produced a single ML tree of score $-\ln$ 11 820.57 (figure 4). MP searches were also carried out and support for nodes was estimated using the bootstrap technique (figure 4).

4. DISCUSSION

Using 18S sequences from the freshwater crayfish and other crustaceans, the monophyly of the freshwater crayfish was strongly supported as was the hypothesis of the Nephropoidea (the clawed lobsters) as the sister group to freshwater crayfish (figure 3). This justifies our use of them as an outgroup in the analyses including all three gene regions. This analysis again strongly supported the monophyly of freshwater crayfish (figure 4). The phylogenies estimated independently from morphological data also strongly support the monophyly of freshwater crayfish (Ortmann 1902; Hobbs 1988).

Three families are widely accepted within the crayfish, the Astacidae, Cambaridae and Parastacidae (Hobbs 1988). Our analysis, which was based on all three gene regions, strongly supports a grouping of Astacidae and Cambaridae (100% bootstrap support in ML and MP analyses) and a monophyletic Parastacidae. However, the genus *Cambaroides* is suggested to be a sister species to

Table 1. *Specimens and associated sequences*

species	gene	GenBank accession	reference
Astacoidea (Northern Hemisphere crayfish)			
Astacidae			
Astacus astacus (701)	18S	AF235959	this study
	28S	AF235973	this study
	16S	AF235983	this study
Austropotomobius torrentium (JF135)	18S	AF235960	this study
	28S	AF235974	this study
	16S	AF235984	this study
Pacifastacus leniusculus (1736)	18S	AF235961	this study
	28S	AF235975	this study
	16S	AF235985	this study
Cambaridae			
Cambarellus shufeldtii (1211)	18S	AF235962	this study
	28S	AF235976	this study
	16S	AF235986	this study
Cambaroides japonicus (695)	18S	AF235963	this study
	28S	AF235977	this study
	16S	AF235987	this study
Cambarus maculatus (63)	18S	AF235964	this study
	28S	AF235978	this study
	16S	AF235988	this study
Orconectes virilis (95)	18S	AF235965	this study
	28S	AF235979	this study
	16S	AF235989	this study
Procambarus leonensis	18S	M34363	Kim & Abele (1990)
Procambarus leptodactylus (1398)	28S	AF235980	this study
Procambarus clarkii (837)	16S	AF235990	this study
Parastacoidea (Southern Hemisphere crayfish)			
Parastacidae			
Cherax quadricarinatus (720)	18S	AF235966	this study
	28S	—	—
	16S	AF135975	Crandall *et al*. (1999)
Euastacus bispinosus (628)	18S	AF235967	this study
	28S	AF235981	this study
	16S	AF235991	this study
Geocharax gracilis (627)	18S	AF235968	this study
	28S	AF235982	this study
	16S	AF235992	this study
Parastacus pugnax (1419)	18S	AF235969	this study
	28S	—	—
	16S	AF175237	Crandall *et al*. (2000)
Viriliastacus araucanius (1415)	18S	AF235970	this study
	28S	—	—
	16S	AF175235	Crandall *et al*. (2000)
Nephropoidea (clawed lobsters)			
Nephropidae			
Homarus americanus ('HA')	18S	AF235971	this study
	28S	—	—
	16S	U11238	I. Kornfield, Y. K. J. Tam and P. Moran (unpublished results)
Nephrops norvegicus ('N')	18S	Y14812	Winnepenninckx *et al*. (1998)
	28S	—	—
	16S	U96083	Tam & Kornfield (1998)
Palinura (spiny lobsters)			
Palinuridae			
Jasus edwardsii (725)	18S	AF235972	this study
	28S	—	—
	16S	—	—
Panulirus argus	18S	U19182	H. G. Trapido-Rosenthal, K. A. Coates, A. Kinloch, L. McDowell and K. M. Halanych (unpublished results)
	28S	—	—
	16S	—	—

(*Cont.*)

Table 1. (*Cont.*)

species	gene	GenBank accession	reference
Anomura (hermit crabs)			
Coenobitoidea			
Clibanarius vittatus	18S	M91051	Spears *et al.* (1992)
Clibanarius vittatus	28S	—	—
	16S	—	—
Paguroidea			
Oedignathus inermis	18S	Z14062	Kim *et al.* (1992*b*)
	28S	—	—
	16S	—	—
Branchyura (short-tailed crabs)			
Grapsoidea			
Helice tridens	18S	Z70526	Y. Do and W. Kim (unpublished results)
	28S	—	—
	16S	—	—
Majoidea			
Pugettia quadridens	18S	Z22518	Kim *et al.* (1992*a*)
	28S	—	—
	16S	—	—
Stenopodidae			
Stenopus hispidus	18S	M34361	Kim & Abele (1990)
	28S	—	—
	16S	—	—
Thalassinidae			
Callichirus sp.	18S	—	T. Spears and L. G. Abele (unpublished results)
	28S	—	—
	16S	—	—
Upogebia affinis	18S	—	T. Spears and L. G. Abele (unpublished results)
	28S	—	—

Table 2. *Likelihood ratio tests of models of molecular evolution* (*Huelsenbeck & Crandall 1997; Posada & 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 $\alpha = 0.0083$. Ti, transition; Tv, transversion.)

null hypothesis	models compared	$-\ln L_0$	$-\ln L_1$	d.f.	p
18S					
equal base frequencies	H_0, JC69; H_1, F81	8858	8848	3	< 0.000001
equal Ti/Tv rates	H_0, F81; H_1, HKY85	8848	8798	1	< 0.000001
equal Ti rates	H_0, HKY85; H_1, TrN	8798	8769	1	< 0.000001
one or two Tv rates	H_0, TrN; H_1, TIM	8769	8768	1	0.974098
equal rates among sites	H_0, TrN; H_1, TrN + G	8769	8375	1	< 0.000001
proportion of invariable sites	H_0, TrN + G; H_1, TrN + G + I	8375	8356	1	0.000127
18S + 28S + 16S					
equal base frequencies	H_0, JC69; H_1, F81	12 719	12 701	3	< 0.000001
equal Ti/Tv rates	H_0, F81; H_1, HKY85	12 701	12 595	1	< 0.000001
equal Ti rates	H_0, HKY85; H_1, TrN	12 595	12 594	1	0.190611
one or two Tv rates	H_0, HKY85; H_1, K81uf	12 595	12 580	1	< 0.000001
two or four Tv rates	H_0, K81uf; H_1, TVM	12 580	12 540	2	< 0.000001
equal rates among sites	H_0, TVM; H_1, TVM + G	12 540	11 856	1	< 0.000001
proportion of invariable sites	H_0, TVM + G; H_1, TVM + G + I	11 856	11 821	1	< 0.000001

Pacifastacus so that neither Astacidae nor Cambaridae were monophyletic in our analyses (figure 4). Morphological characters also support a close relationship between holarctic crayfish (Astacidae and Cambaridae). *Cambaroides* is usually considered the most basal member of the Cambaridae, and Hobbs (1988) noted that 'the Asiatic *Cambaroides* share more in common with astacids than do the American cambarids, but there is little, if any, reason to assume they represent an arrested transitory stage between the two families' (p. 76). The short branches in our analysis within the lineage containing Astacidae and Cambaridae suggest that more sequence information will be needed in order to assess the monophyly of these families accurately.

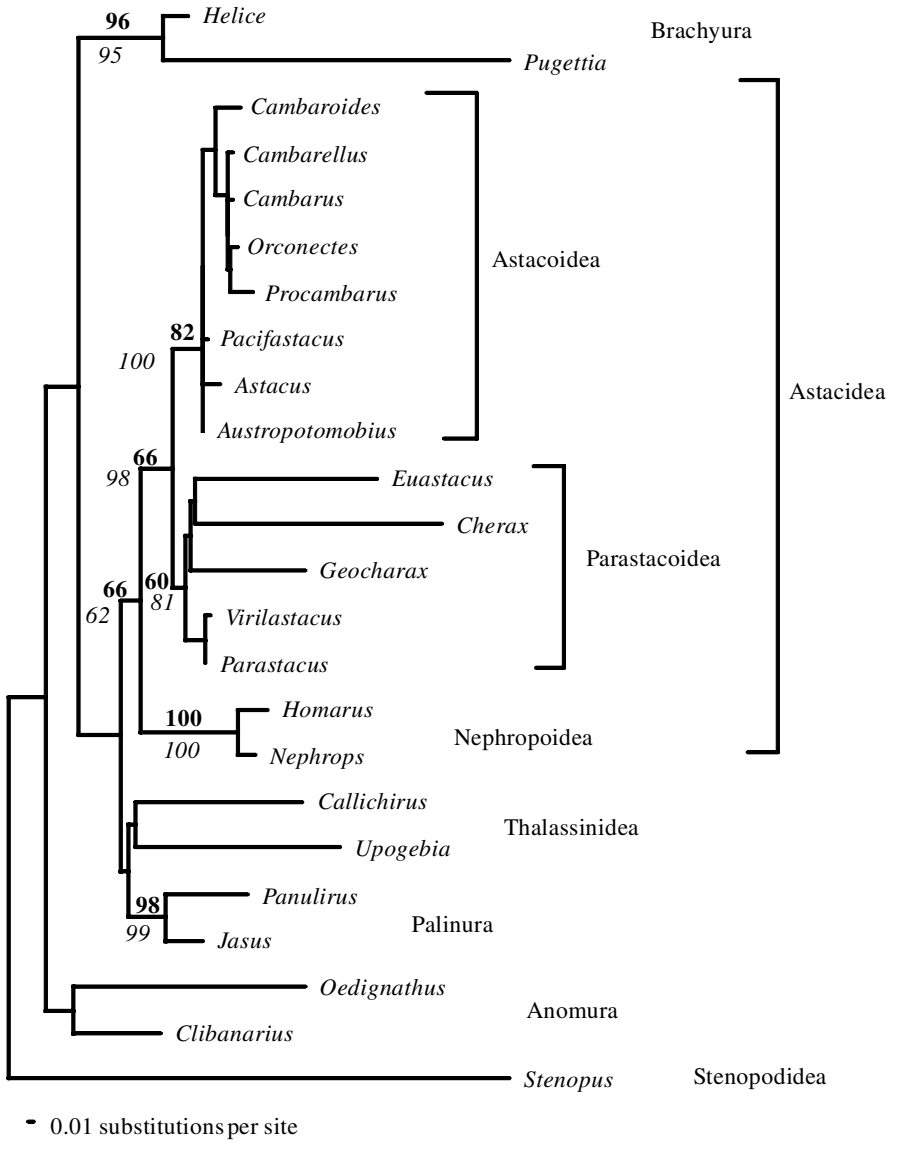

Figure 3. A maximum-likelihood topology of the 18S sequences from freshwater crayfish and other crustaceans with bootstrap percentages shown for both parsimony (bold) and maximum-likelihood (italics) analyses based on 1000 bootstrap replications.

The firm establishment of freshwater crayfish as a monophyletic group with morphological and molecular data allows researchers to use a comparative approach when studying diverse questions with this model organism (Harvey & Pagel 1991). This framework also allows one to explore the timing of origin of the freshwater crayfish. Given the geographical distribution of this group (figure 1) and the strong support for a monophyletic origin (figures 3 and 4), the crayfish must have originated in Pangaea by the Triassic period (185–225 million years (Myr) ago). The separation of the two crayfish superfamilies represents the splitting of Pangaea into northern (Laurasia) and southern (Gondwana) land masses *ca.* 185 Myr ago. This separation is clearly seen in the crayfish phylogenies supported with high bootstrap values (figures 3 and 4). The antiquity of the crayfish is supported by recent fossil evidence from Colorado and Utah with fossil crayfish and burrows associated with Permian and Early Triassic (265 Myr ago) deposits (Hasiotis & Mitchell 1993), and from Antarctica where the fossils date back to 280 Myr ago (Babcock *et al.* 1998). Furthermore, the phylogenic connection of the Southern Hemisphere crayfish represented in southern South America, Madagascar and Australia (with fossils from Antarctica) corresponds to the distribution patterns of the predatory dinosaur group Abelisauridae (Sampson *et al.* 1998). Thus, the crayfish offer further support for the hypothesis suggesting extended contact between these land masses via Antarctica (Sampson *et al.* 1998) and the antiquity of the freshwater crayfish lineage (Hobbs 1988; Hasiotis & Mitchell 1993; Scholtz & Richter 1995). The branch lengths in the phylogeny of the freshwater crayfish (figure 4) suggest that the divergence between genera within this Southern Hemisphere group is much older than the divergences between genera within the Northern Hemisphere crayfish, consistent with the fossil evidence from Antarctica versus Colorado.

Finally, our study demonstrates the usefulness of multiple gene regions with different rates of evolution in resolving phylogenetic relationships across a broad range of evolutionary time. The 18S sequence data place the freshwater crayfish as sister to the clawed lobsters, yet provide little resolution within the Northern Hemisphere

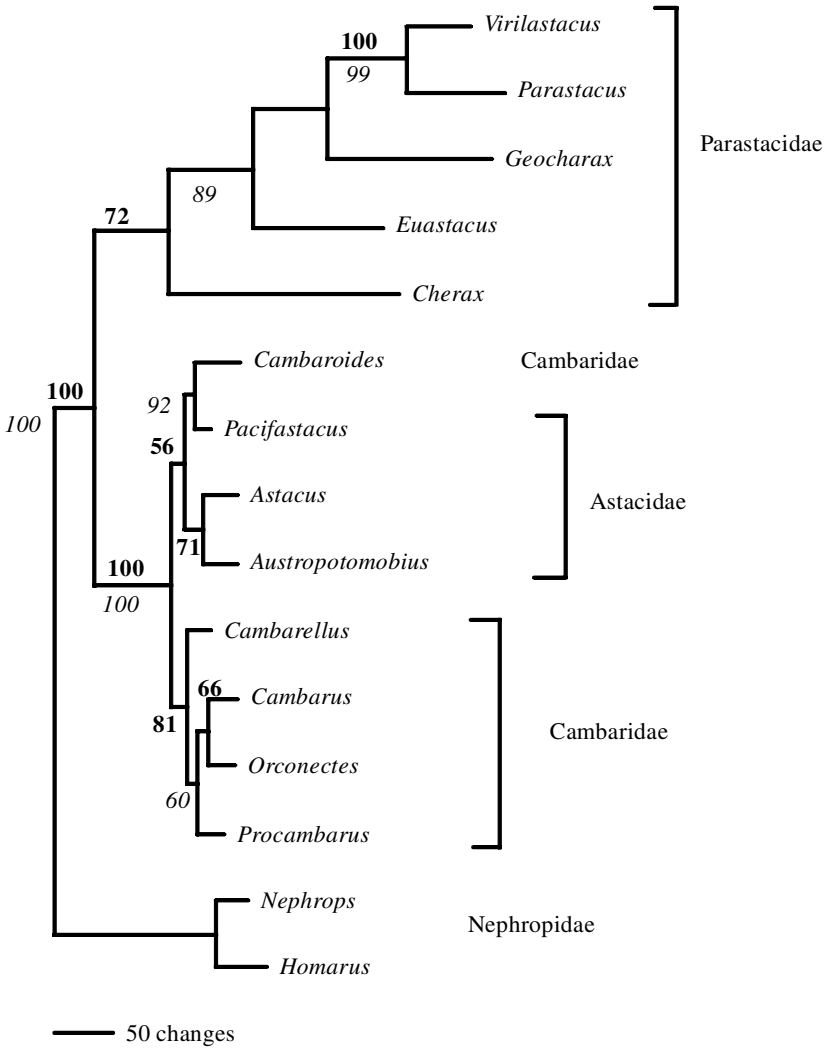

Figure 4. A maximum-likelihood topology of all sequences from freshwater crayfish and other crustaceans with boot-strap percentages shown for both parsimony (bold) and maximum-likelihood (italics) analyses based on 1000 bootstrap replications.

clade. The 28S sequences provide good phylogenetic information for the Northern–Southern Hemisphere crayfish and some resolution among genera within these superfamilies. The 16S sequences provide stronger evidence for genus-level relationships. These three genes combined provide a broad spectrum of inference and have provided great insights into the evolutionary history of freshwater crayfish.

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