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PHYLOGENETIC POSITION OF THE FRESHWATER ANOMURAN FAMILY AEGLIDAE

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A B S T R A C T

We sequenced approximately 2,000 nucleotides of the 18S ribosomal DNA gene to test previous morphological hypotheses concerning family and superfamily relationships within the Anomura. Twelve new sequences from the superfamilies Galatheoidea, Paguroidea, Hippoidea (all Anomura), and Callianassoidea (Thalassinidea) were generated, and these were combined with three previously published sequences from GenBank to estimate phylogenetic relationships among these taxa. Our results show a clear separation of the Aeglidae from the other galatheoid families, which form a sister group with the Paguroidea. Within the Galatheoidea, chirostylids and porcellanids are sister groups. Hippoidea was revealed as the most basal taxon within the anomurans.

The extant Aeglidae Dana, 1852, are freshwater decapod crustaceans consisting of the single genus *Aegla*. They are unique ecologically (the only anomuran family restricted to freshwater), biogeographically (endemic to temperate South America), and morphologically [e.g., trichobranchiate gill structure (terminology after Dana, 1852, and as is described in Martin and Abele, 1988) and presence of sutures on the caparace]. Taxonomically, the Aeglidae are usually placed in the Galatheoidea, along with the Galatheidae, Chirostylidae, and Porcellanidae. However, based on morphological differences (e.g., transverse dorsal suture of the carapace), aeglids and porcellanids were originally placed in different sections (Dana, 1852; Fig. 1A). Several hypotheses have also been proposed concerning the relationships among the families within the Galatheoidea (see Fig. 1), although most of them consider the Aeglidae the most basal group (Fig. 1A, B, and D). The phylogenetic position of the Galatheoidea itself is not clear within the Anomura; it is clustered either with the Paguroidea (Milne-Edwards and Bouvier, 1894, Fig. 1B; Tudge, 1997), the Paguroidea + Lomoidea (Martin and Abele, 1986, Fig. 1D), the Hippoidea (Scholtz and Richter, 1995, Fig. 1E), or the Hippoidea + Paguroidea (Morrison *et al.*, 2002, Fig. 1F).

We tested different hypotheses about the taxonomic positioning of the Aeglidae and the phylogenetic relationships among the Anomura superfamilies (Fig. 1) using the 18S ribosomal DNA (rDNA) gene. Spears and Abele (1988) demonstrated the utility of this gene for phylogeny reconstruction within the Anomura. We follow the classification proposed by McLaughlin (1983), which excludes thalassinids from the Anomura.

MATERIALS AND METHODS

"Crab" Samples.—Marine and freshwater anomuran crabs were collected by hand, dipnet, or trawl fishing from September 1999 to February 2000 (Table 1). Abdomen and gill tissues were dissected and preserved in 100% EtOH for DNA extraction. The remainder of the 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 one or two species from three of four Anomura superfamilies, including

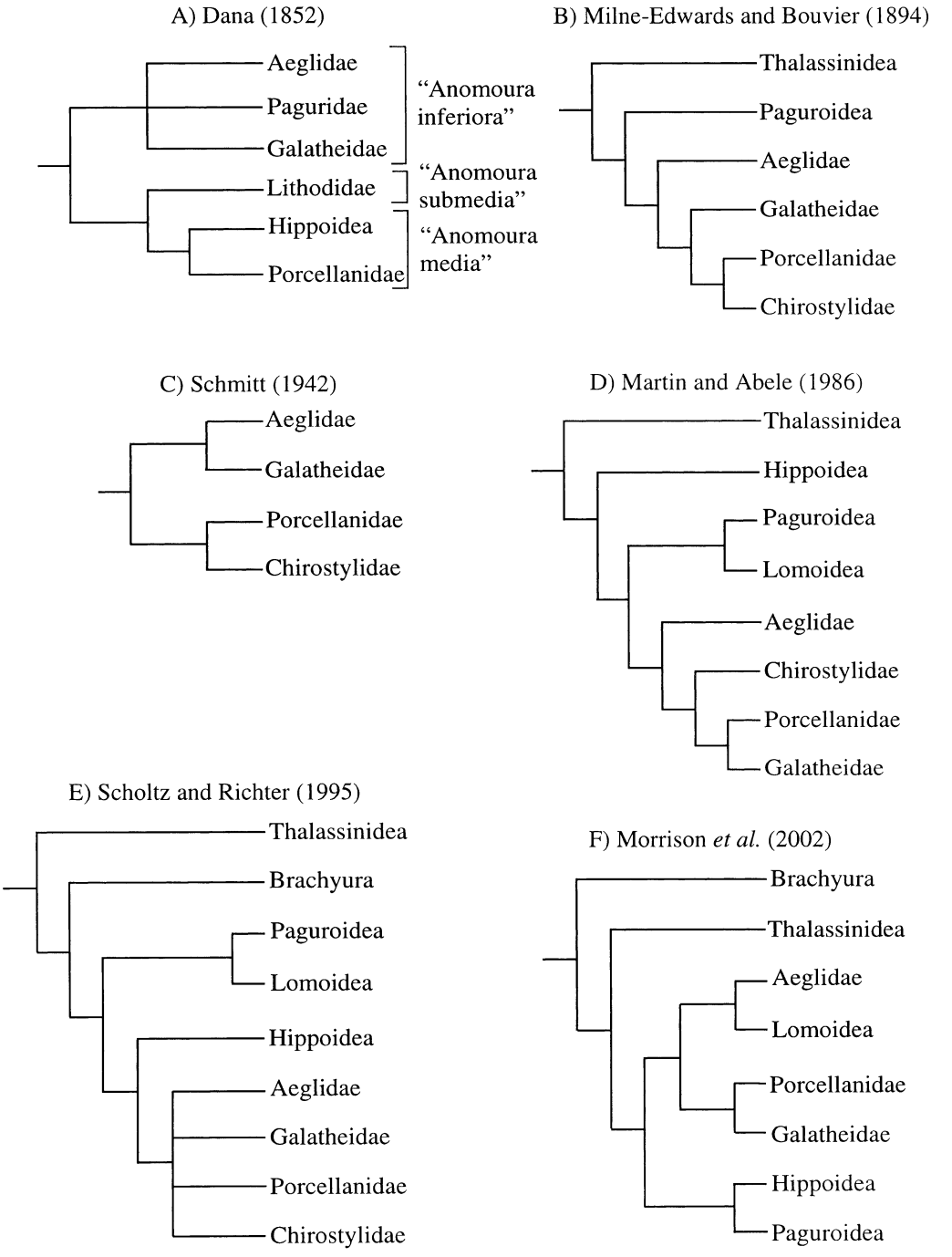

Fig. 1. Alternative hypotheses concerning the relationships among the families of the Galatheoidea and the superfamilies of the Anomura. The section "Anomoura superiora" is not shown in the hypothesis of Dana (1852). The Thalassinidea in the hypothesis of Morrison *et al.* (2002) does not include *Callichirus* and *Neotrypaea.*

Table 1. Taxa examined in this study.

Infraorder-superfamily-family/species	Location	Coordinates
Anomura-Galatheoidea-Porcellanidae		
Pachycheles haigae Rodrigues da Costa, 1960	Tramandaí, Brazil	29°55′S, 50°00′W
Petrolisthes laevigatus (Guérin, 1835)	La Misión, Valdivia, Chile	39°47′S, 73°24′W
Anomura-Galatheoidea-Chirostylidae		
Uroptychus parvulus (Henderson, 1885)	Corral, Valdivia, Chile	40°04′S, 74°02′W
Uroptychus nitida (A. Milne-Edwards, 1880)	Florida, U.S.A.	28°16′N, 86°28′W to 28°14′N, 86°21′W
Anomura-Galatheoidea-Galatheidae		
Munida subrugosa (White, 1847)	Quellón, Chiloé, Chile	43°06′S, 73°40′W
Munida longipes A. Milne-Edwards, 1880	Brazil	14°28′S, 38°52′W
Anomura-Galatheoidea-Aeglidae		
Aegla abtao Schmitt, 1942	Rupanco Lake, Chile	40°46′S, 72°36′W
Aegla rostrata Jara, 1977	Riñihue Lake, Chile	39°46′S, 72°27′W
Anomura-Paguroidea-Lithodidae		
Lithodes santolla (Molina, 1782)	Corral, Valdivia, Chile	40°04′S, 74°02′W
Oedignathus inermis (Stimpson, 1860)	GenBank Z14062	
Anomura-Hippoidea-Hippidae		
Emerita brasiliensis Schmitt, 1935	Tramandaí, Brazil	29°55′S, 50°00′W
Emerita analoga Stimpson, 1857	La Misión, Valdivia, Chile	39°47′S, 73°24′W
Thalassinidea-Callianassoidea-Upogebiidae		
Upogebia affinis (Say, 1818)	Galveston, Texas, U.S.A.	29°18′N, 94°59′W
Brachyura-Grapsoidea-Varunidae		
Helice tridens shenei Sakai, 1939	GenBank Z70525	
Brachyura-Leucosioidea-Leucosiidae		
Philyra pisum De Haan, 1841	GenBank Z25817	

representatives from all of the Galatheoidea families (Porcellanidae, Chirostylidae, Galatheidae, and Aeglidae), and we obtained one sequence from GenBank corresponding to the paguroid *Oedignathus inermis* (Table 1). The only anomuran superfamily that could not be sampled was Lomoidea, which includes a single species, *Lomis hirta* (Lamarck, 1810), with a restricted distribution. The thalassinid *Upogebia affinis* was also collected to be used as the outgroup in combination with two previously published sequences from GenBank corresponding to the brachyurans *Helice tridens shenei* and *Philyra pisum* (Table 1).

DNA Extraction and Sequencing.—DNA was extracted from preserved tissues using the methods described in Crandall and Fitzpatrick (1996). Polymerase-chain-reaction (PCR; Saiki *et al*., 1988) products for the 18S rDNA gene (~2,000 bp) were amplified using the primers from Whiting *et al*. (1997). Standard PCR conditions (5 µl 10× *Taq* buffer; 6 µl 25 mM Mg_2Cl; 8 µl 10 mM dNTPs; 5 µl each of two 10 mM primers; 1.25 U *Taq*; ≈ 20 µl ddH_2O) were used on a Perkin-Elmer 9600 machine and consisted of the following: an initial denaturation at 96°C for 3 min followed by 50 cycles of 95°C for 1 min, 50°C for 1 min, 72°C for 1 min followed by an extension at 72°C for 5 min. After visualization by agarose (1.5%) gel electrophoresis, successful PCR products were purified using a GeneClean® II kit (Bio 101). Sequences were generated in both directions on an Applied Biosystems (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*., 1997) and then adjusted by eye. Regions of doubtful homology (~200 bp total) in the alignment were deleted. Phylogenetic relationships were estimated using maximum parsimony and maximum likelihood. Both phylogeny reconstruction methods assume a model of evolution. Maximum parsimony implicitly assumes that all character changes are equally likely. Maximum likelihood (Felsenstein, 1981), on the other hand, makes explicit assumptions about the relative likelihoods of character change using a model of evolution (Huelsenbeck and Crandall, 1997). Therefore, for the method 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 (Saitou and Nei, 1987) assuming a Jukes and Cantor (1969) 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). Then, using a likelihood-ratio test, the likelihood scores from each model are compared in a hierarchical hypothesis-testing framework (Posada and Crandall, 1998) that includes the following null hypotheses: 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 model of choice will be the model for which the null hypothesis (i.e., assumptions) has not been rejected. The likelihood values associated with these models were estimated in PAUP* (Swofford, 2000). The statistical tests were performed using Modeltest 3.06 (Posada and Crandall, 1998).

Maximum-likelihood searches were heuristic, but maximum-parsimony searches were performed under the branch-and-bound exact method. Heuristic searches are subject to

Table 2. Likelihood-ratio tests of models of molecular evolution (Huelsenbeck and Crandall, 1997; Posada and Crandall, 1998). Only the hypothesis equal transversion (tv) rates was not rejected.

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	6,890	6,883	14	3	0.0037
Equal ti/tv rates	H_0: F81 H_1: HKY85	6,883	6,814	138	1	<0.000001
Equal ti rates	H_0: HKY85 H_1: TrN	6,814	6,797	34	1	<0.000001
Equal tv rates	H_0: TrN H_1: K81uf	6,797	6,796	1	2	0.4084
Equal rates among sites	H_0: TrN H_1: TrN + Γ	6,797	6,562	470	1	<0.000001
Proportion of invariable sites	H_0: TrN + Γ H_1: TrN + Γ + I	6,562	6,558	8	1	0.0035

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 likelihood. Confidence in the resulting relationships was assessed using the bootstrap procedure (Felsenstein, 1985) with 100 replications for maximum likelihood and 1,000 replications for maximum parsimony. Likelihood and parsimony searches as well as the bootstrap analyses were executed in PAUP*. Phylogenetic signal within the data set was assessed using the g_1 statistic calculated in PAUP*. One-hundred-thousand random trees were evaluated in the analysis, and the resulting frequency distribution of the tree scores was examined for skewness (g_1 statistic) and compared to the critical values by Hillis and Huelsenbeck (1992). To correct for strong contribution in signal of the best supported clades in the tree, clades with bootstrap values higher than 90% were collapsed, and g_1 was recalculated.

To root the trees, the thalassinid *Upogebia affinis* and the two brachyurans *Helice tridens shenei* and *Philyra pisum* were used as the outgroup. Thalassinids have been considered the sister group of anomurans based both on larval (e.g., MacDonald *et al*., 1957) and adult (e.g., Martin and Abele, 1986) morphological evidence. However, in recent cladistic morphological analyses (e.g., Scholtz and Richter, 1995) brachyurans and anomurans were suggested as sister groups, with thalassinids being the closest relative to this clade. Thus, we have used representatives from both groups in our phylogeny reconstructions.

To test the Galatheoidea monophyly, alternative maximum-likelihood tree topologies were searched using heuristic searches in PAUP*. These alternative phylogenetic hypotheses were tested for significant differences using the Shimodaira and Hasegawa (1999) method implemented in PAUP*. This test is a more conservative modification of the Kishino and Hasegawa (1989) test that allows for comparison of topologies specified *a posteriori*. We performed 100 bootstrap replicates reoptimizing all the parameters for each tree.

Results

Our sequencing efforts resulted in twelve new 18S rDNA sequences from eleven anomuran and one thalassinidean species. The alignment for these sequences can be downloaded from our lab webpage (http://zoology.byu.edu/zoology/crandall_lab/cranlabpubs.htm). The new sequences have been deposited in GenBank under the accession numbers AF439381–AF439392.

Phylogenetic signal (g_1) within this data set ranged between −0.90 and −1.26 ($P < 0.01$). The maximum-likelihood hypothesis testing procedure resulted in the rejection of all six null hypotheses tested except transversion rates are equal (Table 2). Nucleotide frequencies were significantly different from being equal with $A = 0.27$, $C = 0.22$, $G = 0.25$, and $T = 0.26$. Transition rates were not equal, and transversion rates were equal; thus, identical estimated rates ($R = 1.00$) were used for each of the six reversible rates of change except A↔G ($R_2 = 1.93$) and C↔T ($R_5 = 3.53$). There was also significant rate heterogeneity in these data. Rate heterogeneity was taken into account by using a gamma distribution with the shape parameter of the distribution ($\alpha = 0.88$) estimated from the data via maximum likelihood (Yang, 1996). There was also a significant proportion of invariable sites in these data estimated at 48.8%. Thus, our justified model was the TrN model (Tamura and Nei, 1993) plus gamma-distributed rate heterogeneity plus a significant proportion of invariable sites (TrN + Γ + I).

Incorporating the TrN + Γ + I model of molecular evolution, we estimated pairwise sequence divergence between ingroup and outgroup (3.4–20.3%), within the ingroup (0.0–23.4%), and within the outgroup (1.3–5.8%). Phylogenetic relationships among anomurans were estimated using maximum likelihood and maximum parsimony. Ten random sequence-addition searches resulted in the same maximum-likelihood tree (Fig. 2). This tree supported the monophyly of the Paguroidea and

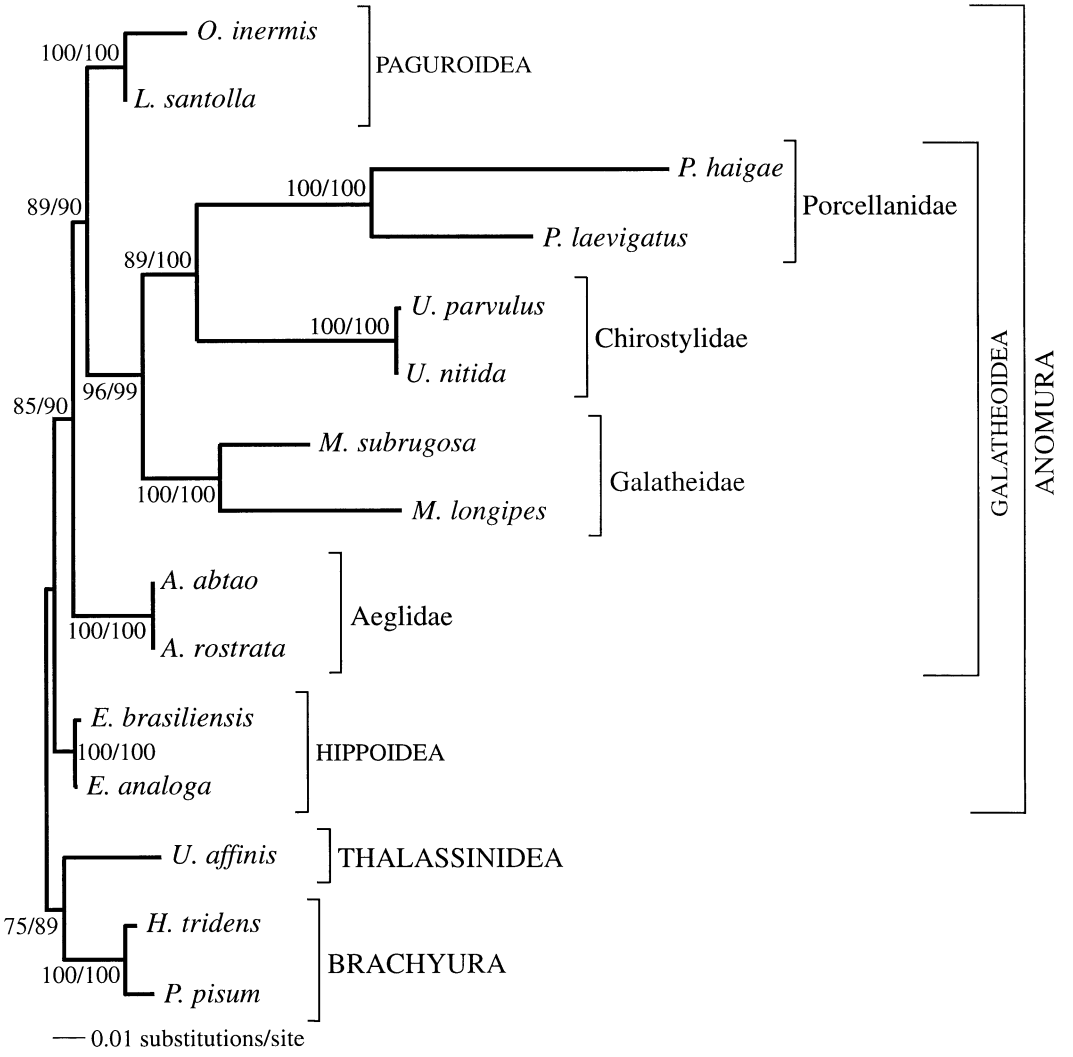

Fig. 2. The maximum-likelihood (ML) and maximum-parsimony (MP) estimates of phylogenetic relationships among anomurans. Branch lengths are shown proportional to the amount of change along the branches assuming the TrN + Γ + I model of evolution within a likelihood framework (Table 2). Bootstrap values are shown as percentages on the tree branches (ML/MP) and are based on 100 bootstrap replications for ML and 1,000 bootstrap replications for MP.

the Hippoidea superfamilies (100% bootstrap values), but suggested that the Galatheoidea were paraphyletic. The Aeglidae were clearly apart from the other Galatheoidea (85% bootstrap support), which formed a sister group with the Paguroidea (89% bootstrap support). Within this likelihood framework, we tested the nonmonophyly of the Galatheoidea. Alternative maximum-likelihood tree topologies constraining the Galatheoidea to be monophyletic were evaluated using heuristic searches in PAUP*. This search resulted in a single maximum-likelihood tree that was compared to the tree in Fig. 2 using the Shimodaira and Hasegawa (1999) test. The log-likelihood (lnL) for the monophyletic hypothesis was −6,566, the difference in lnL between both alternatives was 8, and the P was 0.044 after 100 bootstrap replicates. Thus, the Galatheoidea formed statistically significant nonmonophyletic relationships for the alternative monophyletic hypothesis tested. Excluding the Aeglidae, the other Galatheoidea families were shown to be a monophyletic clade (96% of bootstrap support), with Porcellanidae and Chirostylidae being sister groups (89% bootstrap support).

Unlike maximum-likelihood methods for which all characters are "phylogenetically informative," maximum parsimony limits informative characters to synapomorphic character changes. Our data set consisted of 277 parsimony-informative characters. The maximum-parsimony analysis resulted in a single most parsimonious tree with a tree length of 792 steps. This maximum-parsimony search resulted in the same phylogenetic relationships shown in the maximum-likelihood tree (Fig. 2). The maximum-parsimony bootstrap analysis also gave the same results as the likelihood analysis, but with stronger support for some clades (Fig. 2).

Discussion

Our analyses showed clear support for the separation of the extant Aeglidae from the other galatheoid families (Fig. 2). This result is also supported by several morphological features: the "eclosion from eggs" (postlarval in the aeglids and zoeal in the galatheids; although this is likely coupled with the aeglid adaptation to a freshwater environment); the pleopods (vestigial in the male aeglids and well developed in the male galatheids); the gill structure (trichobranchiate in the aeglids and phyllobranchiate in the other families); the absence of a *linea anomurica* (particularly obvious in the other Galatheoidea); the presence in the Aeglidae of weakly calcified lines that divide the caparace into discrete regions (see Martin and Abele, 1988); and the particular sperm structure of the Aeglidae (Tudge and Scheltinga, 2002). However, although these morphological features have suggested common ancestry between the aeglids and the hermit crabs (Paguroidea) (e.g., Martin and Abele, 1988), the Aeglidae are still included within the Galatheoidea. This view has gone almost unchallenged since Latreille (1803) first described an *Aegla* under the name *Galathea*. Only Dana (1852) placed them in a different section ("Anomoura inferiora"), along with the subtribes Paguridea and Galatheidea (Fig. 1A). Therefore, based on our molecular results and previous morphological evidence, we suggest that Aeglidae may represent a distinct superfamily. This same proposition has been recently suggested by Tudge and Scheltinga (2002) based on sperm structure evidence. To confirm our and Tudge and Scheltinga's hypothesis, the single species within the Lomoidea (*Lomis hirta*) — already included in their analysis — and representatives from other Paguroidea and Hippoidea families should be included in future analyses, as well as more extensive sampling from the Aeglidae.

Among the other anomurans, our molecular trees (Fig. 2) revealed the hippoids as the most basal group (75% ML and 89% MP bootstrap support, respectively). Hippoidea is usually considered to be a distinct lineage within the Anomura, with its closest links to the Galatheoidea (Makarov, 1962; Scholtz and Richter, 1995, Fig. 1E; Paul, 1989). However, evidence from both gills and abdominal sterna (Martin and Abele, 1986, Fig. 1D) suggests a basal position for this group within the Anomura. Our molecular data support this latter hypothesis.

The two lithodid species (Paguroidea) *Lithodes santolla* and *Oedignathus inermis* formed a well-supported sister group with the Galatheoidea, excluding the Aeglidae. This supports the Milne-Edwards and Bouvier (1894) (Fig. 1B) and Martin and Abele (1986) (Fig. 1D) hypotheses about the relationships between these superfamilies. However, our data do not agree with previous molecular (Morrison *et al.*, 2002, Fig. 1F) and morphological (McLaughlin, 1983) studies which suggest the Paguroidea and the Hippoidea were more closely related.

Different phylogenetic hypotheses have been proposed concerning the relationships among the Galatheoidea families (see Fig. 1). Besides the fact that our molecular analyses do not place the Aeglidae within this superfamily, relationships among the other three families agree with the Milne-Edwards and Bouvier (1894) and Schmitt (1942) morphological hypotheses (Figs. 1B and 1C). Our data do not support the Martin and Abele (1986) hypothesis (Fig. 1D) or the Tudge (1997) spermatological phylogeny. Tudge considers these three families polyphyletic, with porcellanids being the sister group of paguroids and the remaining galatheoids. Moreover, chirostylids are widely placed in his analysis and are closely associated with a variety of paguroids, and galatheids do not form a monophyletic clade.

Although a vast amount of work has been done on the Anomura over recent years, there have been relatively few studies on the phylogeny of the group as a whole and virtually nothing based on molecular data. We hope our results will provide the phylogenetic framework needed for advances in our understanding of anomuran evolution. Our current collecting efforts are focused on this question.

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