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Decapod Crustacean Phylogenetics

edited by

Joel W. Martin, Keith A. Crandall, and Darryl L. Felder

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Edited by

Joel W. Martin
Natural History Museum of L. A. County
Los Angeles, California, U. S. A.

Keith A. Crandall
Brigham Young University
Provo, Utah, U. S. A.

Darryl L. Felder
University of Louisiana
Lafayette, Louisiana, U. S. A.

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Contents

Molecular Genetic Re-Examination of Subfamilies and Polyphyly in the Family Pinnotheridae (Crustacea: Decapoda)

EMMA PALACIOS-THEIL[1], JOSÉ A. CUESTA[2], ERNESTO CAMPOS[3] & DARRYL L. FELDER[1]

[1] *University of Louisiana at Lafayette, Department of Biology and Laboratory for Crustacean Research, Lafayette, Louisiana, U.S.A.*

[2] *Instituto de Ciencias Marinas de Andalucía, Consejo Superior de Investigaciones Científicas (ICMAN-CSIC), Avenida República Saharaui, Puerto Real (Cádiz), Spain*

[3] *Universidad Autónoma de Baja California, Facultad de Ciencias, Ensenada, Baja California, México*

ABSTRACT

The family Pinnotheridae de Haan, 1833 is a highly adapted group of largely symbiotic species distributed among 49–56 genera, some of debatable status. Many species remain to be described, a task complicated by the confused state of systematics in the group. Despite a massive taxonomic literature base, illustrations of morphology are of limited scope and quality, hampering morphologically based phylogenetic comparisons. Striking post-planktonic changes in ontogeny, related to unique life histories, can occur among subadults, and different stages of the same species have occasionally been named independently. Polyphyly of the Pinnotheridae has been previously suggested in our own preliminary analyses that combined findings from adult and larval morphology with molecular genetic data. While some issues of polyphyly center at the generic level, questions also remain as to how family and subfamily ranks should be applied to reflect monophyletic clades. The present molecular analysis was based on combined sequence data for the partial mitochondrial large subunit 16S rRNA gene, the tRNA-Leu gene, and the partial mitochondrial gene for NADH1, primarily to examine generic assignments. The results of mitochondrial gene analyses are relatively unambiguous, with strong support values for transfer of Xenophthalminae and Asthenognathinae out of Pinnotheroidea. The family Pinnotheridae is partitioned between two primary clades representing the subfamilies Pinnothereliinae and Pinnotherinae, and smaller clades may justify one or more additional subfamilies. Members of several genera within these subfamilies require taxonomic revision. Analyses based upon the 18S nuclear gene, while supporting morphologically and mitochondrial gene-based definition of the Pinnothereliinae, did not clarify relationships between most other pinnotherid genera and were thus not incorporated into our analysis.

1 INTRODUCTION

Crabs of the family Pinnotheridae de Haan, 1833, the pea crabs, are typically symbiotic crustaceans found with ascidians, annelids, other crustaceans, echiurans, echinoderms, or molluscs (Schmitt et al. 1973) and are rarely free living. Their adaptation to this variety of host organisms likely accounts for their diversity. By the most commonly used current taxonomy, there are about 313 described species, or 287 if excluding Asthenognathinae and Xenophthalminae (Ng et al. 2008). These are distributed among a maximum of 56 genera (49 according to Ng et al. 2008), and some are of debatable generic assignment (Zmarzly 1992; Manning 1993a; Campos 1996a). The largest genera

are *Pinnotheres* (71 spp.), *Pinnixa* (56 spp.), and *Arcotheres* (20 spp.), while the other genera contain fewer than 10 species each, and 23 of those are monotypic. Since description of the first pinnotherid, *Nepinnotheres pinnotheres* (described as *Cancer pinnotheres* Linnaeus, 1758), discovery and description of new species have continued almost unabated. From the Gulf of Mexico alone, we estimate our present holdings to include no fewer than 20 undescribed species. In addition to increasing numbers of species and genera, taxonomy has become very unstable over recent decades. Some genera and species have been excluded from the family, species have been reassigned from one genus to another, and many synonymies have been recommended (e.g., Campos 1989; Manning 1993b; Ahyong & Ng 2007; Ng et al. 2008).

Complicating the taxonomic problems even further, post-planktonic development in pinnotherids can involve more complex metamorphoses than in most other brachyurans, often involving several morphologically distinct subadult stages during the postlarval ontogeny of a single species. Changes can involve carapace shape, abdominal morphology, and development of the pleopods, many of these altering characters used for morphological diagnoses of genera. As noted by Campos (1989), taxonomists have on some occasions assigned separate names to two different stages of the same species.

Classification of the pinnotherids has been the object of multiple revisions, especially since the late 1980s (Griffith 1987; Manning & Felder 1989; Campos 1996a, b; Coelho 1997; Campos 2006; Ahyong & Ng 2007; Ng et al. 2008). Most of these were partial revisions, limited to a certain subfamily or genus, or confined to a limited geographic region. However, even when only partial revisions, they often defined species and genera that remain of uncertain phylogenetic placement in the group.

Polyphyly of the Pinnotheridae in its present composition (sensu Schmitt et al. 1973) has already been supported in several studies based upon morphological analyses (Marques & Pohle 1995; Campos 1996b, 1999; Števčić 1996; Pohle & Marques 1998; Campos & Manning 2000), as well as in preliminary molecular analyses (Cuesta et al. 2001). Very recently, new arrangements at family and subfamily levels have been proposed (Cuesta et al. 2005; Števčić 2005; Ng et al. 2008; Campos 2009).

We herewith provide molecular phylogenetic analyses that bear on recently proposed revisions. In so doing, we evaluate clade relationships in a tree based upon the partial 16S rRNA gene, the tRNA-Leu gene, and the partial NADH1 gene from the mitochondrial genome. We also attempt phylogenetic analyses based upon the nuclear 18S rRNA for potential clarification of relationships at the subfamily and family levels.

2 MATERIALS AND METHODS

2.1 *Specimens used in analyses*

We attempted to include as many pinnotherid genera as possible, but especially those representing diverse morphologies or taxa that have been questionably placed in the past. Specimens represented the four putative subfamilies Pinnothereliinae, Pinnotherinae, Xenophthalminae, and Asthenognathinae, thus excluding only the monospecific Anomalifrontinae previously included in the family by Schmitt et al. (1973). Sequences were obtained from our own extractions, supplemented by some from GenBank (Table 1). For outgroups, we chose species from other brachyuran families of putative close or distant relationship to pinnotherids for which comparable 16S or 18S sequences were available (Table 2). In mitochondrial sequence analyses, we included a single member of Xenophthalminae, two species of two genera assigned to Asthenognathinae, 21 species representing three genera assigned to Pinnothereliinae, and 19 species of 16 genera recognized by Schmitt et al. (1973) as members of Pinnotherinae. For *Clypeasterophilus stebbingi*, *Clypeasterophilus rugatus*, *Tunicotheres moseri*, and *Zaops ostreum*, we sequenced specimens from more than one geographic location. In addition, we included two undescribed species that are morphologically assignable to

Table 1. Species used in molecular phylogenetic analyses of the family Pinnotheridae (*sensu* Schmitt et al. 1973). For collection catalog numbers (Cat. No.), abbreviations are as follow: CBM-ZC = Natural History Museum and Institute, Zoology, Crustacea, Chiba, Japan; CBR-ICM = Colección Biológica de Referencia, Instituto de Ciencias del Mar, Barcelona, Spain; RMNH = Rijksmuseum van Natuurlijke Historie, Nationaal Naturhistorisch Museum, Leiden; SMF = Senckenberg Museum, Frankfurt a.M., Germany; ULLZ = University of Louisiana at Lafayette Zoological Collections; USNM = U.S. National Museum of Natural History, Smithsonian Institution, Washington, D.C.

			GenBank Accession No.	
Species	**Location**	**Cat. No.**	**16S**	**18S**
Family PINNOTHERIDAE de Haan, 1833				
Pinnotherid sp. 1	Bahía de los Ángeles, México	ULLZ 9337	EU934955	EU934919
Pinnotherid sp. 2	Northern Gulf of Mexico	ULLZ 5582	EU934991	
Subfamily XENOPHTHALMINAE Alcock, 1900				
Xenophthalmus pinnotheroides White, 1846	Hiroshima Bay, Seto Is. Sea, Japan	CBM-ZC 7784	EU934951	EU934922
Subfamily ASTHENOGNATHINAE Stimpson, 1856				
Asthenognathus atlanticus Monod, 1933	Mauritania, off Banc d'Arguin	RMNH 40008	EU934952	
Tritodynamia horvathi Nobili, 1905	Aitsu Mar. Biol. St., Japan	ULLZ 5585	EU934953	EU934950
Subfamily PINNOTHERINAE de Haan, 1833				
Austinotheres angelicus (Lockington, 1877)	San Felipe, México	ULLZ 9601	EU935002	
Calyptraeotheres granti (Glassell, 1933)	San Felipe, México	ULLZ 9599	EU934979	
Clypeasterophilus rugatus (Bouvier, 1917)	Twin Keys, Belize	ULLZ 9511	EU934981	
Clypeasterophilus rugatus (Bouvier, 1917)	East Coast Florida, USA	ULLZ 5546	EU934980	EU934924
Clypeasterophilus stebbingi (Rathbun, 1918)	Praia do Leste, Brazil	ULLZ 5543	EU934984	EU934941
Clypeasterophilus stebbingi (Rathbun, 1918)	Is. Margarita, Venezuela	ULLZ 5545	EU934983	
Dissodactylus crinitichelis Moreira, 1901	Praia do Sul, Isla Anchieta, Ubatuba, Brazil	ULLZ 5561	EU934982	EU934942
Dissodactylus latus Griffith, 1987	East Coast Florida, USA	ULLZ 5548	EU934985	
Fabia subquadrata Dana, 1851	California, USA	ULLZ 5575	EU935000	EU934947
Limotheres sp.	off southeastern USA	ULLZ 9176	EU934996	EU934923
Holothuriophilus pacificus (Poeppig, 1836)	Bahía de Concepción, Cocholque, Chile	ULLZ 5569	EU934997	EU934948
Juxtafabia muliniarum (Rathbun, 1918)	San Felipe, México	ULLZ 9600	EU934990	
Nepinnotheres pinnotheres (Linnaeus, 1758)	Bahía de Cádiz, Spain	CBR-ICM pending	EU935001	
Orthotheres barbatus (Desbonne, 1867)	Los Roques, Venezuela	ULLZ 5559	EU934999	EU934921

Table 1. continued.

Species	Location	Cat. No.	GenBank Accession No.	
			16S	18S
Pinnaxodes chilensis (H. Milne Edwards, 1837)	Caleta Coquimbo, Chile	ULLZ 5570	EU934998	EU934949
Pinnotheres pisum (Linnaeus, 1767)	Regensburg, Germany (mussel import)	SMF 30947	AM180694	
Scleroplax granulata Rathbun, 1893	Bodega Bay, California, USA	ULLZ 5576	EU934972	EU934930
Tumidotheres maculatus (Say, 1818)	Praia do Lazaro, Ubatuba, Brazil	ULLZ 9512	EU934986	
Tumidotheres maculatus (Say, 1818)	Isla Coche, Venezuela	ULLZ 5534	EU934945	
Tumidotheres margarita (Smith, 1869)	Bahía Margarita, Baja California Sur, México	ULLZ 5533	EU934987	EU934946
Tunicotheres moseri (Rathbun, 1918)	Tampa Bay, Florida, USA	ULLZ 4516	EU934988	EU934925
Tunicotheres moseri (Rathbun, 1918)	Isla Margarita, Venezuela	ULLZ 5536	EU934989	EU934926
Zaops ostreum (Say, 1817)	Fort Pierce, Florida, USA	ULLZ 5537	EU934994	EU934943
Zaops ostreum (Say, 1817)	Isla Margarita, Venezuela	ULLZ 5535	EU934995	
Subfamily PINNOTHERELIINAE Alcock, 1900				
Austinixa aidae (Righi, 1967)	Praia do Perequê Açú, Ubatuba, Brazil	ULLZ 5538	EU934966	EU934936
Austinixa behreae (Manning & Felder, 1989)	Mustang Is., Texas, USA	ULLZ 5541	EU934956	EU934939
Austinixa chacei (Wass, 1955)	Navarre, Florida, USA	ULLZ 4405	EU934957	EU934940
Austinixa cristata (Rathbun, 1900)	Fort Pierce, Florida, USA	ULLZ 5556	EU934967	
Austinixa felipensis (Glassell, 1935)	San Felipe, Baja California Norte, México	ULLZ 5558	EU934969	EU934927
Austinixa gorei (Manning & Felder, 1989)	Islas del Rosario, Colombia	ULLZ 5586	EU934965	EU934920
Austinixa hardyi Heard & Manning, 1997	Blood Bay, Tobago, Trinidad and Tobago	USNM 284177	AF503185	
Austinixa patagoniensis (Rathbun, 1918)	Praia do Araçá, São Sebastião, Brazil	ULLZ 5549	EU934970	EU934935
Pinnixa chaetopterana Stimpson, 1860	Fort Pierce, Florida, USA	ULLZ 5553	EU934961	EU934937
Pinnixa cylindrica (Say, 1818)	Corpus Christi Bay, Texas, USA	ULLZ 5560	EU934963	EU934929
Pinnixa faba (Dana, 1851)	State of Washington, USA	ULLZ 5571	EU934976	EU934933
Pinnixa franciscana Rathbun, 1918	Bodega Bay, California, USA	ULLZ 5624	EU934974	
Pinnixa littoralis Holmes, 1894	Tahuya, Washington, USA	ULLZ 5572	EU934975	EU934932
Pinnixa monodactyla (Say, 1818)	Fort Pierce, Florida, USA	ULLZ 8713	EU934964	
Pinnixa pearcei Wass, 1955	Tampa Bay, Florida, USA	ULLZ 5557	EU934971	EU934934
Pinnixa rapax Bouvier, 1917	São Sebastião, Brazil	ULLZ 5568	EU934959	
Pinnixa retinens Rathbun, 1918	Fort Pierce, Florida, USA	ULLZ 9347	EU934992	

Table 1. continued.

Species	Location	Cat. No.	GenBank Accession No.	
			16S	18S
Pinnixa sayana Stimpson, 1860	Fort Pierce, Florida, USA	ULLZ 5620	EU934962	
Pinnixa schmitti Rathbun, 1918	Japonski Is., Stika, Alaska, USA	ULLZ 5574	EU934978	EU934931
Pinnixa tomentosa Lockington, 1877	Brown's Beach, Baranof Is., Sitka, Alaska	ULLZ 5522	EU934977	
Pinnixa tubicola Holmes, 1894	Brown's Beach, Baranof Is., Sitka, Alaska	ULLZ 5521	EU934973	
Pinnixa valerii Rathbun, 1931	Estero Corrientes, Nicaragua	ULLZ 9336	EU934993	
Pseudopinnixa carinata Ortmann, 1894	Moji, Fukuoka prefecture, Japan	ULLZ 5628	EU934954	EU934944
Austinixa sp. 1	Nagualapa, Nicaragua	ULLZ 5566	EU934958	EU934938
Austinixa sp. 2	Las Enramadas, Cosigüina, Nicaragua	ULLZ 5564	EU934968	EU934928
Pinnixa sp.	Tampa Bay, Florida, USA	ULLZ 8126	EU934960	

Austinixa (*Austinixa* sp. 1 and sp. 2) and two more undescribed species that are morphologically questionable as to placement among the Pinnotheridae (Pinnotherid sp. 1 and Pinnotherid sp. 2; Table 1).

For the 18S gene, we extracted DNA from a single species of each of the subfamilies Xenophthalminae and Asthenognathinae, 12 species of Pinnothereliinae (representing the genera *Austinixa*, *Pinnixa* and *Pseudopinnixa*), and 13 species of Pinnotherinae representing 11 genera, all of which were also included in the mitochondrial analyses. In this case we obtained sequences from different locations for two species (*Tumidotheres maculatus* and *Tunicotheres moseri*). The above-mentioned undescribed species of *Austinixa* again were used, and one of the questionably placed undescribed pinnotherid species was included (Pinnotherid sp. 1).

2.2 *DNA extraction and PCR*

Total genomic DNA was extracted from muscle tissue with a DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA) following the manufacturer's protocol or with the standard DNA extraction protocols (Robles et al. 2007). Polymerase chain reaction (PCR) was conducted to amplify a fragment of the mitochondrial genome that extends from the gene for the large ribosomal subunit 16S rRNA through the tRNA-Leu to and including part of the protein coding region of the mitochondrial nitrogen dehydrogenase subunit 1 (NADH1). For this fragment we used the primers 16SH2 (5'-AGA TAG AAA CCA ACC TGG-3') (Schubart et al. 2000, equivalent to the primer 1472 described in Crandall & Fitzpatrick 1996), 16SL2 (5'-TGC CTG TTT ATC AAA AAC AT-3'), 16SL6 (5'-TTG CGA CCT CGA TGT TGA AT-3') (developed by JAC and C. Schubart), and NADH1 (5'-TCC CTT ACG AAT TTG AAT ATA TCC-3'). We also used five internal primers designed specifically for pinnotherids, including PH1 (5'-CGC TGT TAT CCC TAA AGT AAC-3'), PH2 (5'-CCT GGC TCA CGC CGG TCT GAA-3'), PH3 (5'-AAT CCT TTC GTA CTA AAA-3'), PL1 (5'-AAC TTT TAA GTG AAA AGG CTT-3'), and PL2 (5'-TTA CTT TAG GGA TAA CAG CG-3').

For 18S rRNA the primers developed by Medlin et al. (1988) were used, including 18SC (5'-CGG TAA TTC CAG CTC CAA TAG-3'), 18SL (5'-AGT TAA AAA GCT CGT AGT TGG-3'),

Table 2. Outgroup sequences from GenBank used in phylogenetic analyses based upon mitochondrial 16S rRNA and the nuclear 18S rRNA genes.

			GenBank accession no.	
Superfamily	**Family**	**Species**	**18S**	**16S**
Heterotremata				
Majoidea	Majidae	*Maja crispata* Risso, 1827		EU000852
		Maja squinado (Herbst, 1788)	DQ079758	EU000851
	Oregoniidae	*Chionoecetes opilio* (Fabricius, 1788)		AB188684
Portunoidea	Portunidae	*Carcinus maenas* (Linnaeus, 1758)	DQ079757	
		Necora puber (Linnaeus, 1767)	DQ079759	
Potamoidea	Potamidae	*Geothelphusa* sp.	DQ079750	
Xanthoidea	Panopeidae	*Panopeus herbstii* H. Milne Edwards, 1834		AJ130815
	Xanthidae	*Xantho poressa* (Olivi, 1792)		AM076937
Thoracotremata				
Grapsoidea	Gecarcinidae	*Cardisoma crassum* Smith, 1870		AJ130805
		Gecarcinus lateralis (Freminville, 1835)		AJ130804
	Grapsidae	*Pachygrapsus marmoratus* (Fabricius, 1787)	DQ079763	
		Pachygrapsus transversus (Gibbes, 1850)		AJ250641
	Plagusiidae	*Euchirograpsus americanus* A. Milne-Edwards, 1880		AJ250648
		Plagusia dentipes de Haan, 1835		AJ308421
	Sesarmidae	*Sesarma reticulatum* (Say, 1817)		AJ130799
	Varunidae	*Cyrtograpsus altimanus* Rathbun, 1914		AJ487319
		Gaetice depressus (de Haan, 1835)	AY859577	
		Helice tridens tientsinensis Rathbun, 1931	Z70526	
		Varuna litterata (Fabricius, 1798)		AJ308419
Ocypodoidea	Dotillidae	*Dotilla wichmani* De Man, 1892		AB002126
		Scopimera globosa (de Haan, 1835)		AB002124
	Macrophthalmidae	*Macrophthalmus banzai* Wada & K. Sakai, 1989		AB002132
		Macrophthalmus japonicus (de Haan, 1835)	EU284156	
		Macrophthalmus latifrons Haswell, 1882		Z79669
	Ocypodidae	*Minuca minax* (LeConte, 1855)		Z79670
		Ocypode quadrata (Fabricius, 1878)	AY743942	Z79679

18SO (5'-AAG GGC ACC ACC AGG AGT GGA G-3'), 18SY (5'-GTT GGT GGA GCG ATT TGT CTG-3'), and 18SB (5'-AGG TGA ACC TGC GGA AGG ATC A-3'). Instead of primer 18SA indicated by Medlin et al. (1988), we used the slight variant 18SEF (5'-CTG GTT GAT CCT GCC AGT-3') (Hillis & Dixon 1991), which is three basepairs (bp) shorter at the 5' end.

2.3 *Phylogenetic analyses*

Sequences for each gene region were assembled and edited with Sequencher 4.7 (Genecodes, Ann Arbor, MI). Preliminary alignments were checked for accuracy with BioEdit 7.0.9.0 (Hall 1999) and then aligned with MUSCLE (Edgar 2004) on the website of the European Bioinformatics Institute (www.ebi.ac.uk). Outgroup sequences of 18S rRNA and 16S rRNA were obtained from GenBank (Table 2). Once all the sequences were added and aligned, regions where primers were located were trimmed to avoid artefacts. In addition, poorly aligned and gapped positions were removed after

identification with Gblocks (v. 0.91b, Castresana 2000). The resulting sequence lengths were 786 bp for the combined mitochondrial sequences and 1625 bp for the 18S sequences.

The combined mitochondrial sequence data were tested for partition homogeneity (Bull et al. 1993), as implemented in PAUP* 4.0 beta 10 (Swofford 2002) with 1000 replicates. PAUP* was also used for determining base composition, pattern of substitution for pairwise comparison, and analysis of variability along the 16S rRNA and 18S rRNA fragments. The alignment file was submitted for processing with RAxML version 7.0.4 (Stamatakis et al. 2008) and with bootstrapping at the Cyberinfrastructure for Phylogenetic Research (CIPRES) Web Portal (www.phylo.org). We used this program for a maximum likelihood search (ML), selecting the option of automatically determining the number of necessary bootstrapping runs. Once we obtained the results, the trees were analyzed and edited with Mega 4 (Tamura et al. 2007). In addition to ML analysis, Bayesian (BAY) phylogenetic analyses were performed using MrBayes for the mitochondrial combined data. Before conducting BAY analysis, the model of evolution that best fit the data was estimated with the computer program MODELTEST (Posada & Crandall 1998).

The phylogenetic analysis was conducted sampling one tree every 500 generations for 1,000,000 generations, starting with a random tree. We obtained 2001 trees, of which we discarded 4%. In a previous analysis we could determine that stasis was reached after approximately 35,000 generations, so we discarded the first 40,000 generations, or, in other words, the 81 first trees sampled. With the remaining trees we obtained 50% majority rule consensus trees by means of PAUP* 4.0 (see above).

Support values for analyses based on the 18S nuclear gene were in general so low that phylogenetic trees based upon these sequence data were not reproduced in the present manuscript. Where the 18S analyses did support phylogenetic groupings based on the combined mitochondrial sequence data or morphology, mention is made in the following sections.

3 RESULTS

3.1 *Utility of the combined mitochondrial analyses for the Pinnotheridae*

The concatenated BAY analysis of mitochondrial genes resulted in a well-resolved consensus tree (Fig. 1). Topologies for the Pinnotheridae in the separate ML and BAY trees (not shown) were virtually the same, with only minor differences. While in the ML tree *Zaops* was grouped with low support values into Clade IIA, it was in the BAY tree grouped at low support values into Clade IIC. Also, while the ML tree shows Clade III to include Pinnotherid sp. 2 with weak support, it was placed external to this group in the BAY tree. Aside from these differences, both analyses define the same membership in Clades I, II, and III.

3.2 *Restriction of the Pinnotheridae in the mitochondrial phylogenetic analyses*

In our molecular phylogeny, *Xenophthalmus pinnotheroides*, *Asthenognathus atlanticus*, and *Tritodynamia horvathi* are by ML and BAY analysis positioned among outgroup families rather than among other putative pinnotherids (Fig. 1). *Asthenognathus atlanticus* and *T. horvathi* are placed in both analyses with high support values into a common clade with the two outgroup species of the family Varunidae. On the other hand, *X. pinnotheroides* is grouped with strong support with representatives of the family Dotillidae. With the exception of *Pseudopinnixa carinata*, all other putative pinnotherids that were included in these analyses are joined together into a well-supported single clade, which is in turn subdivided into two major and one minor clade. The enigmatic *Pseudopinnixa carinata* is positioned basally to all other putative pinnotherid groups, but in a poorly resolved polytomy. It is clearly excluded from a highly supported node that groups Clades I, II, and III of the Pinnotheridae in our ML and BAY analyses. Among these clades, Clade III is of most limited membership, grouping *Pinnixa valerii*, *P. retinens*, and, with modest support, an undescribed species

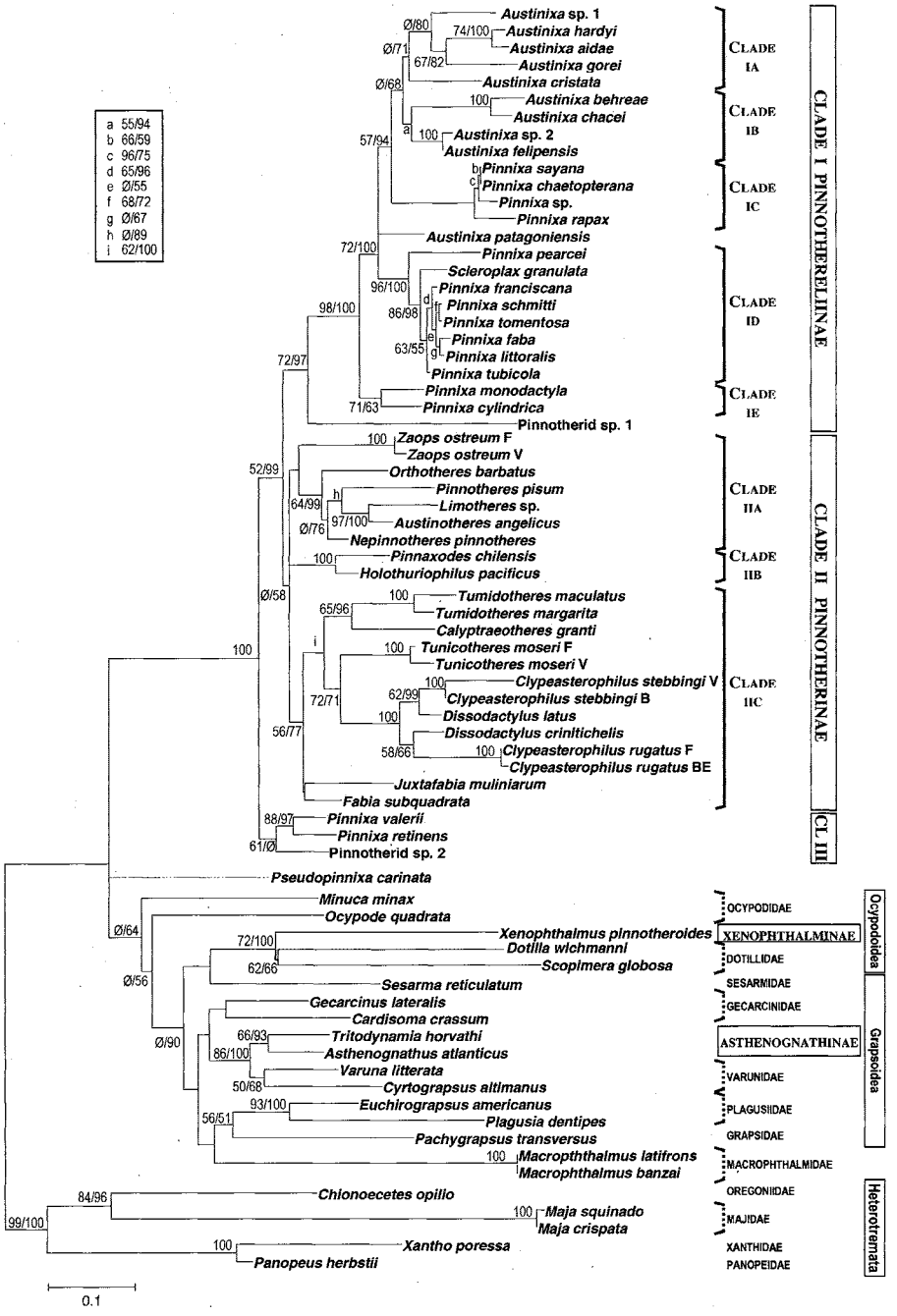

Figure 1. Phylogeny for species of the family Pinnotheridae, superimposed on classification of Schmitt et al. (1973), inferred from a maximum likelihood (ML) analysis of 786 bp of the mitochondrial genes for 16S rRNA (604 bp), tRNA-Leu, and NADH1 (together 182 bp). Bootstrap values for ML and Bayesian posterior probabilities are shown (ML bootstrap value first); ø means value < 50%. Where value is the same for both, only one number is shown; no number is shown if both values < 50%. Letters follow some species names to separate conspecific populations from Brazil (B), Belize (BE), Florida (F), and Venezuela (V).

(Pinnotherid sp. 2) of uncertain generic assignment. Given the polyphyletic stature of *Pinnixa* in the overall analysis, proper generic placement of all species grouped into this clade must be open to question.

3.3 *Definition of pinnotherid subfamilies in the mitochondrial phylogenetic analyses*

Two major groups of the putative pinnotherids included in our analyses are segregated in the molecular phylogenetic tree, and these are supported in both the ML and BAY analyses. The more strongly supported of these groups (Clade I) encompasses those pinnotherids that current taxonomy assigns to the subfamily Pinnothereliinae, thus including analyzed members of the genera *Austinixa* and *Pinnixa* but in this case also the species *Scleroplax granulata* (placed in Pinnotherinae instead of Pinnothereliinae by Schmitt et al. 1973). Clade I also includes a basally positioned undescribed species (Pinnotherid sp. 1) that is pending generic assignment. A less well-resolved second major group (Clade II) encompasses taxa currently assigned by most workers to the taxonomically diverse subfamily Pinnotherinae, thus including *Austinotheres*, *Limotheres*, *Orthotheres*, *Pinnotheres*, *Nepinnotheres*, *Zaops*, *Holothuriophilus*, *Pinnaxodes*, *Fabia*, *Juxtafabia*, *Calyptraeotheres*, *Tunicotheres*, *Tumidotheres*, *Clypeasterophilus*, and *Dissodactylus*.

3.4 *Subfamily Pinnothereliinae*

The Pinnothereliinae of Clade I are subdivided into five subgroups, two of which consist exclusively of species assignable to the genus *Austinixa*. The 8 species originally included in this genus (Heard & Manning 1997) were all represented in our analysis, in addition to two new species pending description. Additional congeners, *A. bragantina* and *A. leptodactyla*, placed in the genus by Coelho (1997, 2005), were not available for inclusion. As presently constituted, *Austinixa* appears to be polyphyletic. While 7 of the 8 named species, including the type species of the genus, *A. cristata*, share a common lineage (Clades IA–C), *Austinixa patagoniensis* is separated from this group in a poorly resolved polytomy.

Other members of *Austinixa* (Clade IA plus IB) are positioned as a sister clade to a grouping of four species (Clade IC) that are presently treated under *Pinnixa*, though these are not grouped in our analysis with the type species of that genus, *P. cylindrica*. With support only in BAY analysis, Clade IA includes *A. hardyi*, *A. aidae*, *A. gorei*, *Austinixa* sp. 1, and *A. cristata*, while Clade IB includes the closely related species *A. behreae* and *A. chacei* along with *A. felipensis* and the undescribed species *Austinixa* sp. 2 from eastern Pacific waters of Central America. Clade IC encompasses the very closely related sister species *P. chaetopterana* and *P. sayana*, along with *P. rapax* and an undetermined *Pinnixa* sp. from Tampa Bay, Florida. A fourth clade (ID) within the apparent Pinnothereliinae includes almost all remaining members of the genus *Pinnixa* that we analyzed (having previously excluded *Pinnixa valerii* and *P. retinens* from both subfamilies), along with *Scleroplax granulata*. However, *Pinnixa cylindrica*, type species of the genus *Pinnixa*, and *P. monodactyla* (Clade IE) are with strong support grouped separately from both Clade IC and ID, thus segregating them from all present congeners included in this analysis and underscoring polyphyly of this genus as presently recognized.

3.5 *Subfamily Pinnotherinae*

Clade II of our phylogeny (Fig. 1) includes a diverse set of genera that broadly represents the present subfamily Pinnotherinae, albeit without the previously affiliated genus *Scleroplax*, as noted above. While a number of its encompassed lower subclades are well supported, support for grouping of the subfamily overall is very limited and found only in the BAY analysis. Clades IIA–B are separated only as a polytomy. Without support, topology of our tree positions populations of *Zaops*

ostreum basally in Clade IIA, which contains a well-supported grouping of *Orthotheres barbatus*, *Pinnotheres pisum*, *Limotheres* sp., *Austinotheres angelicus*, and *Nepinnotheres pinnotheroides*. A second clade (IIB) defines the highly supported grouping of *Holothuriophilus pacificus* and *Pinnaxodes chilensis*, while a third clade (IIC) groups our included species of *Fabia*, *Juxtafabia*, *Calyptraeotheres*, *Tumidotheres*, *Tunicotheres*, *Clypeasterophilus*, and *Dissodactylus*.

As noted above, the BAY tree (not shown) also groups *Zaops* here, rather than with Clade IIA, but only with low support. While *Clypeasterophilus* and *Dissodactylus* are expectedly grouped together with high support within Clade IIC, neither of these genera appears to be monophyletic in our analyses, their constituent species being in both cases distributed between alternative sister subclades.

4 DISCUSSION

4.1 *Exclusions from Pinnotheridae, and exceptional members of the group*

While long affiliated with Pinnotheridae by some workers (see Schmitt et al. 1973), Asthenognathinae and Xenophthalminae have been the subject of several recent re-examinations. The subfamily Asthenognathinae was proposed by Števčić (2005) for elevation to the family level and transfer to the superfamily Grapsoidea. On the other hand, the subfamily Xenophthalminae was elevated by the same author to family level, though he retained it within the Pinnotheroidea. Based in part on a preliminary report (Cuesta et al. 2005), Ng et al. (2008) have instead recently placed both of the asthenognathine species that we analyzed (*Asthenognathus atlanticus* and *Tritodynamia horvathi*) among the varunids, and our present findings clearly offer further support for this placement. Thus, Ng et al. moved some present members of the subfamily Asthenognathinae to the family Varunidae H. Milne Edwards, 1853, but concluded that the genus *Tritodynamia* is polyphyletic, to the point that some of its members may warrant assignment to separate families. In their opinion, *T. horvathi* appears related to the varunids, but its congeners are more closely related to macrophthalmids. They thus transferred most members of *Tritodynamia* to the Macrophthalmidae Dana, 1851, as Tritodynamiinae Števčić, 2005. Among the species presently assigned to *Tritodynamia*, only *T. horvathi* was available for inclusion in our analysis, and therefore we can provide no support for division of the genus *Tritodynamia* as suggested. Studies with more members of this genus are thus warranted to support the proposed new classification.

In the case of Xenophthalminae, Ng et al. (2008) recommended the elevation of this group to the family level as Xenophthalmidae Stimpson, 1858, with the two subfamilies Anomalifrontinae Rathbun, 1931, and Xenophthalminae Stimpson, 1858, placing them in the superfamily Ocypodoidea. At least from our analysis of *Xenophthalmus pinnotheroides*, we can support this revision, as the species clearly is not placed by molecular genetics among other members of the family Pinnotheridae; rather, our molecular data and larval morphology suggest the close relationship of *X. pinnotheroides* with the family Dotillidae. Future molecular analyses should ideally include another member of *Xenophthalmus* White, 1846 (*X. wolffi*), the two species of the genus *Neoxenophthalmus* Serène & Umali, 1972, and the only representative of the subfamily Anomalifrontinae.

Pseudopinnixa carinata is presently considered a pinnotherid belonging to the subfamily Pinnothereliinae (Schmitt et al. 1973; Ng et al. 2008). Our results show this monospecific genus to be excluded from the highly supported grouping of Pinnothereliinae (Clade I) and Pinnotherinae (Clade II), being affiliated with neither of these major clades nor our newly defined Clade III of the Pinnotheridae. *Pseudopinnixa* is left in a poorly resolved basal polytomy, but given the distance by which it is separated from other putatively pinnotherid groups, it may warrant eventual treatment as a separate family of the Pinnotheroidea. Further molecular analyses must examine the relationship of *Pseudopinnixa* Ortmann, 1894, to a full array of both heterotrematan and thoracotrematan families of the Brachyura. Larval morphology suggests relationships with Grapsoidea, especially the family Macrophthalmidae, a proposed sister family of

Varunidae (Cuesta et al. 2005), but our present analysis does not lend any clear support to this hypothesis.

Several other taxa also do not easily resolve as to their exact relationships with other included taxonomic groups of the pinnotherids, some because of morphology and others because of their placement in the present molecular analyses, though we confidently conclude they are members of the family on the basis of molecular characters. Pinnotherid sp. 1 (currently in description as a new genus by EC) exhibits unique morphological characters that could perhaps justify assignment to a unique subfamily. However, it is unambiguously placed as the most basal branch of Clade I (Pinnothereliinae) in our analysis. Two other taxa (*Pinnixa valerii* and *P. retinens*) and more questionably Pinnotherid sp. 2 (external to this grouping in the separate BAY tree) form a well-supported clade that may also deserve separate subfamilial treatment. Of these, detailed morphological study has been completed only for *P. valerii*, which is pending assignment to a new genus (DLF and JAC in description). There appear to be clear morphological similarities of *Pinnixa valerii* with both *Pinnixa retinens* Rathbun, 1918, and *Alarconia seaholmi* Glassell, 1938, along with some evidence that these three species share characters of the carapace, sternum, abdomen, and third maxilliped that are distinct from other members of the Pinnotheridae. Should further molecular and morphological study support this grouping, these three species and Pinnotherid sp. 2 may deserve assignment to the tribe Alarconiini Števčić, 2005, which in turn could be rediagnosed for elevation to subfamily level.

4.2 *The Pinnotheridae restricted, two major subfamilies and more*

Clade I corresponds remarkably well to generally accepted membership of the current subfamily Pinnothereliinae. With the exception of species already pending assignment to new genera, including those in our Clade III (see above), its molecular definition includes all species of *Pinnixa* for which specimens were available in our analyses and all available specimens of the genus *Austinixa*, but it surprisingly also included *Scleroplax granulata*. Members of the subfamily Pinnothereliinae are characterized by a third maxilliped with the ischium not fused to the merus, which is oriented longitudinally or is skewed toward a longitudinal orientation. The palp is comparatively large, occasionally as wide as the ischiomerus; the carapace is ovoid in outline, usually much wider than long; and the fifth walking leg is often reduced (Balss 1957). In contrast to other members of Clade I, *Scleroplax* has been assigned previously to the Pinnotherinae by Schmitt et al. (1973). However, this genus does share with the genera *Pinnixa* and *Austinixa* a wider than long carapace and a distinct lateral exopod lobe on the third maxilliped (Campos 2006), characters that may be of more significance than previously thought.

In Clade II we find representatives of a restricted subfamily Pinnotherinae. Morphological characters typical of this subfamily are a third maxilliped ischium that is not distinguishable from, or is at least rudimentarily fused with, the merus, which usually lies transversely or is skewed toward a transverse orientation. The palp is not as wide as the ischiomerus and the carapace usually does not have a clearly transverse rectangular shape (Balss 1957). For the most part, our results agree with the reorganization adopted by Ng et al. (2008), which leaves two subfamilies within the Pinnotheridae, namely Pinnothereliinae Alcock, 1900, and Pinnotherinae de Haan, 1833. However, contrary to their placements, the monotypic genus *Scleroplax* belongs instead among the Pinnothereliinae, supported both by our results and by morphological characters (Campos 2006). Also, it does not appear that either of these two subfamilies encompasses at least one other minor clade (Clade III) that is well-supported in our molecular phylogenetic analyses.

Clearly, our molecular phylogenetic analysis contradicts a close monophyletic grouping of the genera *Pinnixa*, *Fabia*, and *Juxtafabia* that was previously postulated on the basis of larval morphology (Marques & Pohle 1995), as these genera represent members of separate subfamilies that are divergent at a basal node. The molecular data suggest that ostensibly synapomorphic larval features of the abdominal somites are instead best regarded as convergences. Adult morphological

differences between the *Pinnixa/Austinixa/Scleroplax* group and *Fabia/Juxtafabia* group would also support present molecular evidence indicating that these two groups of genera do not have a close sister relationship (see Campos 1993, 1996a, 2006).

4.3 *Constituents of the subfamily Pinnothereliinae*

Within Clade I, the subfamily Pinnothereliinae, five internal clades were distinguished. Clades IA and IB included most species of *Austinixa* in our analysis, with only *Austinixa patagoniensis* distinctly excluded from these groups. The character that differentiates members of this genus (formerly treated as the "*Pinnixa cristata* complex," Manning & Felder 1989) from others in the subfamily Pinnothereliinae is a complete (side to side) transverse ridge or carina across the cardiac region of the carapace (Heard & Manning 1997). In previous molecular genetic studies of species assigned to *Austinixa*, varied trees were based upon analyses of 16S and COI mitochondrial genes, and slight differences from our outcomes were evident in some (Harrison 2004). As in our present results, *A. aidae* and *A. hardyi* were resolved in at least some of those previous analyses as distinct but closely related species, forming a sister group to *A. gorei*. Placement of the undescribed *Austinixa* sp. 1 into this clade suggests yet other members of this grouping remain to be named. Our results also agree with the previous report of Harrison (2004) in placing *A. behreae* and *A. chacei* as sister species, and in both cases *A. cristata* is somewhat separated from the two aforementioned clades, being in our analysis basally positioned in Clade IA. Differences arise in that *A. patagoniensis* occupies a basal position within the genus *Austinixa* in the earlier analysis (Harrison 2004), but it must be noted that this earlier work included only two species of the subfamily Pinnothereliinae. Thus, the position of *A. patagoniensis* relative to varied members of the genus *Pinnixa* could not be robustly evaluated. But also of potential impact, *Scleroplax granulata* was used in this previous analysis as an outgroup, while present evidence suggests it is in fact a member of the subfamily Pinnothereliinae.

Our own phylogeny suggests that revisions may be justified for the genus *Austinixa*. Whether or not one were to split Clades IA and IB into separate genera, a separate genus does appear warranted for *A. patagoniensis*, which differs morphologically from all other present members of the genus in having branchial ridges that extend fully to the orbits (Manning & Felder 1989). The positions of *A. bragantina* and *A. leptodactyla* relative to the clades we have defined remain unknown, since these species were not available for inclusion. At the specific level, it has been recommended recently that *A. hardyi* and *A. aidae* be treated as synonyms (Harrison & Hanley 2005) on the bases of mitochondrial genetic and morphometric analyses. However, the genetic distances we observed for these two species had high support values and were not smaller than others shown by different species pairs, as, for example, between some of the species within Clade ID for *Pinnixa* or between *Pinnixa chaetopterana* and *P. sayana* (Clade IC). While Harrison & Hanley (2005) reported a genetic distance of only 0.28% within the COI region, and no differences at all for the 16S region they analyzed, we found a genetic distance of 1.53% for the 16S region we studied (8 mutations in the 16S region, since the genes for tRNA-Leu and NADH1 were not included in the sequence for *A. hardyi* we obtained from GenBank). Our differing outcomes are not readily explained, but we also find no ambiguity in applying diagnostic morphological characters (*sensu* Heard & Manning 1997) to the separation of these species. Clearly, additional analyses would be welcomed, but for now we must recommend treatment of *A. hardyi* and *A. aidae* as separate species.

A third internal clade (IC) of the subfamily Pinnothereliinae included four species of the genus *Pinnixa* (*P. rapax*, *P. chaetopterana*, *P. sayana*, and the undescribed *Pinnixa* sp.), while a fourth clade (ID, dominated by northeastern Pacific species of *Pinnixa*) was also formed along with the northeastern Pacific *Scleroplax granulata* as previously discussed. With good support for most branches among species of *Pinnixa* in our analyses, the topology strongly suggests that this genus is polyphyletic and requires revision. However, our present representation of this largest genus of the Pinnotheridae includes but a fraction of its almost 60 presently named species. Furthermore, only

one other species of the genus aligned closely with the type species, *Pinnixa cylindrica*, which was basally positioned within the subfamily Pinnothereliinae; this suggests that most species presently assigned to the genus would better be treated under some other generic name. In addition, no readily apparent morphological character sets have been found to support most of the branch groupings among species of *Pinnixa* that were here defined by molecular methods. Morphological and further molecular analyses of *Pinnixa* sensu lato are in progress, and revision of the genus must follow.

Finally, it is imperative that *Pinnotherelia*, type genus of Pinnothereliinae, eventually be included in molecular phylogenetic analyses. This genus is morphologically very different from all putative members of the Pinnothereliinae included in our present analysis, and may require restricted application of this subfamily name. The genera we have treated do indeed form a morphologically and molecularly defined group, but one that may instead warrant recognition as a separate subfamily, perhaps equivalent to the tribe Pinnixini of Števčić (2005).

4.4 *Constituents of the subfamily Pinnotherinae*

Within Clade II, the subfamily Pinnotherinae, three internal clades were recognized, with one of them (IIA) questionably including *Zaops* with a well-defined grouping of the genera *Orthotheres*, *Pinnotheres*, *Limotheres*, *Austinotheres*, and *Nepinnotheres*. The composition of this clade is particularly of interest in that it lends provisional support to a revised classification recently proposed by one of us (Campos 2009) on the basis of adult and larval morphological characters. Under this pending revision, 25 genera (8 tentatively) are proposed to constitute a restricted, monophyletic subfamily Pinnotherinae in which all members share a soft, thin carapace and a unique protuberance on the basal antennal article. Of the 25 genera so grouped, to date we have been able to represent only the aforementioned six in our molecular analyses, but they may indeed be definable as in a single clade. To this end, additional analyses with more representative genera will be essential, especially to resolve the questionable placement of genera like *Zaops*.

The remaining genera that were treated as Pinnotherinae in the Schmitt et al. (1973) classification (excepting *Scleroplax*, as earlier noted) but excluded from the subfamily by Campos (2009) are grouped into at least two other clades (IIB and IIC), which again generally conform with Campos' revised grouping of subfamilies. Separated as Clade IIB, under strong support values, are the genera *Pinnaxodes* and *Holothuriophilus*, which have long been regarded as close relatives, with species having been transferred back and forth between them and remaining debate as to the proper assignment of species for each (see Manning 1993b; Ng & Manning 2003). Members of both these genera use holothurians as hosts and exhibit very similar morphology in the third maxilliped (Ng & Manning 2003).

Clade IIC, by contrast, encompasses a more complex topology, with some internal subgroupings that appear to reflect morphological similarities. Considering that *Clypeasterophilus* was originally erected to receive some members of *Dissodactylus* by Campos & Griffith (1990), it is not surprising to see these genera positioned closely in our phylogeny, given that they share adaptive synapomorphies such as bifid walking leg dactyls and a similar fusion of abdominal somites. However, it is also evident that our present molecular phylogenetic analysis does not support monophyly in either of these genera. Both *Clypeasterophilus* and *Dissodactylus* may warrant further subdivision and/or revisionary reassignments in membership.

A sister clade to the *Clypeasterophilus/Dissodactylus* group is formed by *Tunicotheres*, while *Tumidotheres* and *Calyptraeotheres* are strongly grouped as a more basal branch. At least some support for these groupings may be found in morphology, though it is not entirely congruent with proximities suggested by molecular phylogenetics. Some species of the *Clypeasterophilus/Dissodactylus* group share a two-segmented third maxilliped palp with *Tunicotheres*, though shape of the palp articles in the latter genus differs. Morphology in the former genera appears nearer that of *Calyptraeotheres*, which contains species with very similar third maxillipeds (and other features), even though they may bear a two- or three-segmented palp. It is noteworthy that members of the genus

Tumidotheres most resemble *Fabia* in this character (Campos 1996a, b). Zoeal morphology of the *Clypeasterophils/Dissodactylus* group and of at least the type species of *Calyptraeotheres* is very similar, even though it has not been formally described (but see Marques & Pohle 1995). On the other hand, *Tumidotheres*, *Calyptraeotheres*, and *Tunicotheres* are morphologically and ecologically very different from one another. The only shared feature presently apparent among them is the dactylus of the walking leg 4 (pereopod 5), which is larger than the others, a character that develops in the adult female. Thus, we cannot at present offer a set of morphological features that uniquely groups all of these genera to support the genetically defined Clade IIC. Present knowledge of larval and adult morphology would suggest a closer relationship of the *Clypeasterophilus/Dissodactylus* group to *Calyptraeotheres* than to other genera of Clade IIC.

Finally, we note a highly supported separation between the included populations of *Clypeasterophilus stebbingi* from Brazil and Venezuela, respectively. Distances between these two populations suggest they likely represent separate species.

4.5 *Limited utility of the nuclear 18S rRNA in phylogenetic analysis of the Pinnotheridae*

The nuclear gene for the large ribosomal subunit 18S rRNA has been used previously for phylogenetic studies of many crustacean groups at varied phylogenetic levels, including studies of decapods at the level of family and above (e.g., Kim & Abele 1990; Crandall et al. 2000; Oakley 2005; Porter et al. 2005). Initially, our analyses of this gene looked promising for study of pinnotherid genera, as the genetic variation that we found among the first set of genera that we analyzed appeared to be larger than that reported previously among genera of other decapod families (Crandall et al. 2000). However, while 18S rRNA sequences served to differentiate among pinnotherid genera, and in some cases even species, it does not allow us to infer a well-supported phylogeny within the family. While the overall topology of the pinnotherids and their putative relatives by ML (not shown) approximated the phylogeny based upon our mitochondrial sequences, bootstrap values generally did not exceed the 50% majority consensus rule. Nonetheless, it provided a definition of the subfamily Pinnothereliinae that grouped the included species of *Austinixa*, *Pinnixa* (*P. valerii* not included in analysis), and *Scleroplax granulata*, as inferred from the combined genes 16S rRNA, tRNA-Leu, and NADH1, albeit with somewhat different internal topology. This adds evidence for reassignment of *Scleroplax* to this subfamily. It is also of interest that *Pinnixa cylindrica* is separated in the 18S ML analysis at high support values from the other included members of *Pinnixa* (*P. monodactyla* not available for inclusion).

Membership of the subfamily Pinnotherinae (sensu Schmitt et al. 1973) is not resolved by the 18S analyses. Some taxa like *Xenophthalmus pinnotheroides* were peculiarly placed among the pinnotherine genera, perhaps because of long-branch attraction. While positioned external to the pinnotherids among representatives of the outgroup families as in our mitochondrially based analysis, the asthenognathine genus *Tritodynamia* is not definitively affiliated to any one grapsoid family in the 18S analysis; this should be expected, as there was no strong support for separation of these families from one another in the 18S analysis, at least based on our presently limited sampling. Yet, as in our mitochondrially based phylogeny, *Zaops* and *Limotheres* were grouped, and *Dissodactylus*, *Clypeasterophilus*, and *Tumidotheres* were grouped, in both cases at moderate levels of support. *Pinnaxodes* and *Holothuriophilus* were also grouped together, and *Pseudopinnixa* was positioned basally, both as in the 16S analysis, but in both cases at low support values.

We must conclude that genetic variability in the 18S rRNA gene within the members of the family Pinnotheridae is not high enough to allow general resolution of the relationships among most of its constituent genera or thus a bootstrap-supported topology of its subfamilies. Indicative of this is the difference between the overall mean distance for the mitochondrial pinnotherid sequences (0.17) and those for 18S (0.013). Limitations of 18S analyses have been previously noted (Hillis & Dixon 1991; Aleshin & Petrov 1999). While this gene can be informative, its utility is apparently defined

not only by the phylogenetic level at which it is applied but also by unique evolutionary histories of the taxonomic group under investigation.

4.6 *Perspectives for the future*

While present results from our analyses of mitochondrial genes allow a number of conclusions, work is under way to confirm and refine these results. On one front, we will integrate additional sequence data into our analyses, including at least the nuclear 28S rRNA gene and two more mitochondrial genes, the cytochrome oxidase subunit I (COI) and the 12S rRNA gene. We are also expanding taxonomic coverage in these analyses, seeking to more comprehensively represent a greater diversity of named and pending pinnotherid genera. We are also continuing to add coverage at the species level in our analyses, especially in large genera like *Pinnixa*, to undertake taxonomic revisions that appear to be warranted, and to define ecologically informative clades. At the other extreme, we seek to integrate all of these data into a comprehensive analysis of phylogeny of brachyuran decapods that will provide improved resolutions at the family and superfamily level. As possible, we are integrating further efforts in our respective labs to draw upon multiple genes in our molecular phylogenies as well as adult and larval characters in morphological analyses.

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