



Phylogenetic relationships between spiny, slipper and coral lobsters (Crustacea, Decapoda, Achelata)

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ABSTRACT

Molecular data can aid in the resolution of conflicting hypotheses generated through difficulties in the interpretation of morphological data and/or an incomplete fossil record. Moreover, the reconstruction of phylogenetic relationships using molecular data may help to trace back the origin of morphological innovations which had a major impact on the radiation of a taxonomical group. In this work, different nuclear (18S, 28S, and H3) and mitochondrial (16S and COI) gene regions were sequenced in a total of 35 Achelatan species to test conflicting hypotheses of evolutionary relationships within the Achelata infraorder and solve the taxonomic disagreements in the group. The combined molecular dataset strongly supports the hypothesis that Achelata is a monophyletic group composed of two main families: Palinuridae and Scyllaridae. Synaxidae is found to be a polyphyletic group, which should be included within Palinuridae. Consequently, our results indicate that the origin of the stridulating organ occurred only once during Achelata evolution. Finally, the two main clades found within the Scyllaridae are in agreement with previous inferences based on adult morphological data. The dating of divergence of Achelata obtained with a relaxed-clock model is compatible with previous hypotheses of a Triassic origin of the Achelata.

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1. Introduction

It is increasingly apparent that systematics and taxonomy are interconnected and that advances in one area can bring substantial benefits to the other. Traditional classification has focused mainly on morphological comparisons of organisms, sometimes resulting in conflicting hypotheses when the homology of morphological characters was difficult to establish or when there was not enough evidence of shared ancestry from the fossil record (Moritz and Hillis, 1996; Garcia-Machado et al., 1999). Molecular systematics provides a powerful approach to study phylogenetic relationships among organisms, which may help to resolve taxonomic uncertainties even for deep-phylogenetic levels. Combining both fast and slow-evolving genes is recommended when inferring phylogenetic relationships across a broad evolutionary framework using molecular data (Yang, 1998). Slowly-evolving genes may help to infer deep-level phylogeny because of the presence of a higher proportion of phylogenetically reliable characters (Graham and Olmstead, 2000; Wortley et al., 2005) and, given sufficient taxon

sampling, rapidly evolving regions can also be phylogenetically informative (Borsch et al., 2003; Hilu et al., 2004). Moreover, it has been shown that for typical data sets, the number of genes utilized may be even more important for phylogenetic accuracy than the number of taxa analyzed (Rokas and Carroll, 2005).

An example of taxonomic controversies at different phylogenetic levels is found in Achelatan lobsters. Even though the monophyly of the infraorder Achelata is generally accepted, deep-phylogenetic relationships within the group still remain uncertain (Holthuis, 1991; Patek and Oakley, 2003). The Achelata contains three main families, namely Palinuridae (spiny lobsters), Scyllaridae (slipper lobsters), and Synaxidae (furry or coral lobsters). These families share a unique larval phase called phyllosoma and lack chelae on their first pair of pereopods (Scholtz and Richter, 1995; Dixon et al., 2003). Spiny lobsters are one of the most commercially important groups of decapod crustaceans (Palero and Abelló, 2007; Follesa et al., 2007) and have received great attention during recent years, including numerous studies on their ecology, phylogeography, and molecular phylogeny (Díaz et al., 2001; Patek and Oakley, 2003; Palero et al., 2008a). However, the slipper and coral lobsters have been the subject of much less research, probably because they do not include many species of commercial interest (Holthuis, 1991). Two controversial hypotheses have been proposed with respect to the evolution of Achelatan

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lobsters based on paleontological (Förster, 1973) and morphological (Bate, 1881; Holthuis, 1991) evidence. Förster's hypothesis based on Achelatan fossils (Fig. 1A) placed Scyllaridae within Palinuridae and hence this latter would be a paraphyletic group (Förster, 1973). The second hypothesis, which we call the Synaxidae hypothesis (Fig. 1B), is the most commonly accepted and assumes reciprocal monophyly of the three families Palinuridae, Synaxidae, and Scyllaridae (Martin and Davis, 2001; GBIF Data Portal). Recent morphologically-based reconstructed phylogenies for all Decapoda either presented a basal polytomy with the three previously established families (Schram, 2001) or supported closer relationships between Palinuridae and Scyllaridae, with Synaxidae being the most basal Achelata (Dixon et al., 2003). Finally, molecular-based phylogenies for all Decapoda also showed differing results, with low support for the intra-Achelata relationships being proposed (Ahyong and O'Meally, 2004; Porter et al., 2005; Tsang et al., 2008), presumably due to poor sampling within the Achelata as this was not the focus of these studies.

Within the Palinuridae, evolutionary relationships also present some uncertainties. Following Parker (1884), Palinurid genera are commonly divided into spiny lobsters with a stridulating organ or Stridentes (*Linuparus*, *Palinustus*, *Puerulus*, *Palinurus*, *Panulirus*, and *Justitia*) and without a stridulating organ or Silentes (*Jasus*, *Sagmariasus*, and *Projasus*). The stridulating organ allows Stridentes to make a "rasp" sound by rubbing an extension off the base of each antenna (the plectrum) over the antennular plate and represents an evolutionary adaptation for communication and defense (Moulton, 1957; Patek and Oakley, 2003). George and Main (1967) considered the pair of genera *Linuparus*/*Puerulus* as representatives of the ancestral stridentes stock based on the shape of the female pleopod on the second abdominal segment (Fig. 1C). Baisre (1994) found support for some of the phylogenetic relationships suggested in George and Main (1967) using larval characters, even though in this case *Puerulus* clustered with *Palinurus* (Fig. 1D). Furthermore, previous molecular studies were not able to find strong statistical support for the relationships among genera within Palinuridae and found some indications that the Silentes could occur within the Stridentes (Patek and Oakley, 2003). In a comprehensive review of the group, and including results from Patek and Oakley's genetic analysis, George (2006) placed the synaxid genera

within Palinuridae and pointed out the current uncertainties about the placement of the genus *Justitia* and the monophyly of the Silentes clade, while maintaining George and Main's (1967) clade *Linuparus*/*Puerulus* (Fig. 1E).

The Scyllaridae family is presently divided into four main subfamilies, and its morphology has been thoroughly revised during the last decades (Holthuis, 1985, 1991, 2002). Slipper lobsters are a clear monophyletic group characterized by having the antennal flagellum reduced to a flat plate which functions as a steer to help escape from predators (Spanier and Weihs, 1990). The established Scyllaridae subfamilies are Arctidinae (including the genera *Scyllarides* and *Arctides*), Ibacinae (*Ibacus*, *Evibacus*, and *Parribacus*), Theninae (*Thenus*) and Scyllarinae (including *Scyllarus* and 13 new genera proposed by Holthuis, 2002). However, no molecular phylogeny has been developed so far for Scyllaridae and our understanding of the evolution of scyllarid lobsters is only in its infancy (Schram, 2007).

Finally, the family Synaxidae includes only three rather rare species belonging to two different genera (*Palybithus* and *Palinurellus*). Even though they have been grouped together through shared characters such as a flat triangular rostrum, a carapace without frontal horns and a narrow thoracic sternum (Holthuis, 1991), some concerns have been raised regarding the validity of their taxonomic position (Davie, 1990). Patek and Oakley (2003) indicated in a recent molecular study that maybe both *Palybithus* and *Palinurellus* should be placed among the Palinuridae, even though their results were inconclusive. Under the traditional classification scheme, the presence of a stridulating organ in *Palybithus* and absence in *Palinurellus* would imply that either this specialized structure appeared twice (once in the Synaxidae lineage and again in the Stridentes) or that it disappeared twice (once in the Synaxidae lineage and again in the Silentes) (Holthuis, 1991).

The present study aims to provide new insights on conflicting hypotheses of the evolutionary relationships among taxa using both slow and fast-evolving molecular markers. A total of 5.4 kb sequence data from three nuclear and two mitochondrial genes has been analyzed in 35 species belonging to 14 different genera of the decapod crustacean Achelatan infraorder and including representatives of all known genera of the families Palinuridae and Synaxidae. This would allow us to trace back the origin of morpho-

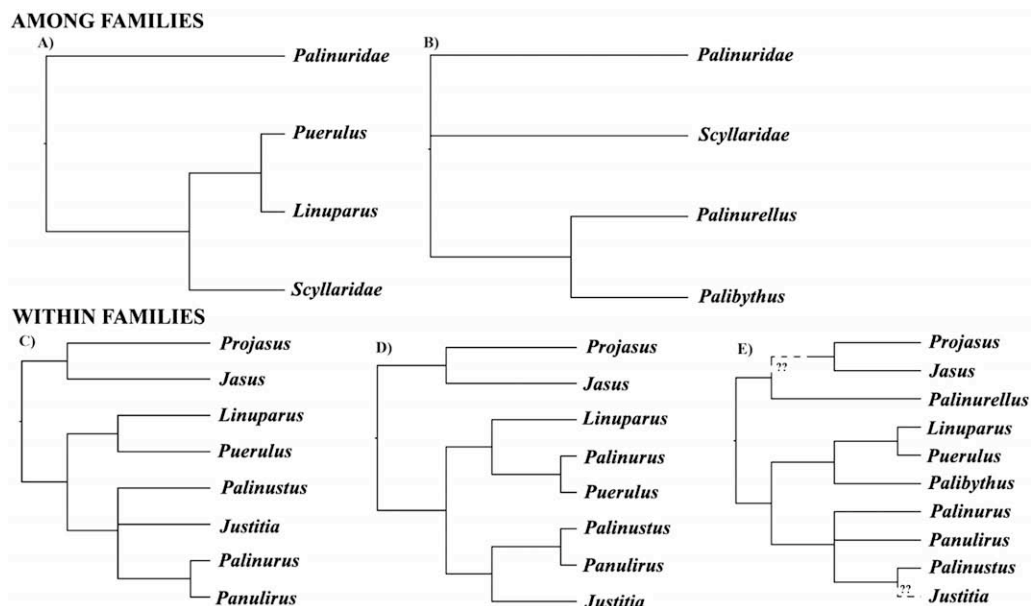


Fig. 1. Phylogenetic hypothesis of Achelata among-families relationships based on (A) paleontological evidence (Förster hypothesis), and (B) morphological evidence (Synaxidae hypothesis). Hypotheses of evolution within Palinuridae: (C) George and Main (1967), (D) Baisre (1994), and (E) George (2006).

logical innovations that had a major impact on the radiation of a taxonomic group (e.g., stridulating organ). We also date the divergence time among taxa using the molecular relaxed-clock and relate it to the available fossil record.

2. Materials and methods

2.1. Taxon sampling

A total of 35 species from 14 different genera of the infraorder Achelata were included in the analysis (Table 1). All known Achelatan genera were included except the scyllarid genera *Ibacus*, *Eviacus*, and *Arctides*, due to difficulties with sampling them. In order to avoid an outgroup selection effect on phylogenetic reconstruction, both closely and more distantly related outgroups were also included in the analysis. Two different genera of Nephropoidea (*Homarus* and *Nephrops*) were included as outgroups since multiple studies (Tam and Kornfield, 1998; Tsang et al., 2008) have suggested sister relationships between the Achelata and Astacidea (Table 1). *Polycheles typhlops* was also included in the analysis considering that Polychelidae used to be grouped with Achelatan lobsters in the infraorder Palinura; although they now belong to the infraorder Polychelida (Scholtz and Richter, 1995). By adding external outgroups, we expect to better define the relationships among Palinurid, Scyllarid, and Synaxid genera.

Tissue samples were preserved in 100% ethanol and total genomic DNA extraction was performed using the QIAamp DNA mini Kit (QIAGEN Inc). Amplification of a total of eight segments from five different genes, namely three nuclear (28S rDNA, 18S rDNA, histone H3) and two mitochondrial (16S rDNA, and COI) genes, was accomplished using either universal or newly designed primers (Table 2). Amplification was carried out with 30 ng of genomic DNA in a reaction containing 1 U of Taq polymerase (Amersham), 1× buffer (Amersham), 0.2 μM of each primer and 0.12 mM dNTPs. The PCR thermal profile used was 94 °C for 4 min for initial denaturation, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing temperature as indicated in Table 2 for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 4 min. Amplified PCR products were purified with QIA Quick Purification Kit (Qiagen Inc., Maryland, US) before sequencing the product. The sequences were obtained using the Big-Dye Ready-Reaction kit v3.1 (Applied Biosystems) on an ABI Prism 3770 automated sequencer from the Scientific and Technical Services of the University of Barcelona. The gene sequences obtained have been deposited in GenBank under accession numbers indicated in Table 1.

2.2. Phylogenetic analyses

Alignments of the five gene data sets were conducted using the program Muscle v3.6 (Edgar, 2004) with default parameters and were then checked by eye. To avoid alignment ambiguity for the three rDNA genes, gaps and hyper-variable regions were excluded from further analysis using GBlocks software v0.91b (Castresana, 2000). To examine possible incongruence between genes, we used the incongruence length difference (ILD) test (Farris et al., 1994) referred to as a partition homogeneity test in PAUP 4.0b10a (Swofford, 2003). A total of 1000 replicates of the ILD test with 10 random addition sequences were run. Before carrying out the likelihood-based analysis, model selection of nucleotide substitution was performed with MrAIC (Nylander, 2004) for each gene individually and for the global data set according to the AIC criterion. The combined mitochondrial and nuclear genes dataset was used to estimate maximum likelihood (ML) phylogenies using GARLI v0.951 (Zwickl, 2006). A total of four different runs were carried out in Garli using default parameters. Bootstrap branch support values were calculated with 100 ML replicates. Bayesian

inference (BI) was carried out with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) allowing the program to estimate parameters for each gene partition. The Bayesian posterior probabilities (BPP) were obtained by performing three separate runs with four Markov chains. Each run was conducted with 2,000,000 generations and sampled every 100 generations. Convergence was checked by examining the generation plot visualized with TRACER v1.4 (Rambaut and Drummond, 2007) and computing the potential scale reduction factor (Gelman and Rubin, 1992) with the sump command in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). A consensus tree was calculated after omitting the first 25% of the iterations as burn-in.

2.3. Testing alternative hypothesis

Besides the unconstrained search, MrBayes runs were carried out using the same conditions but including several constrained searches in order to test the two main hypothesis of the evolution of Achelatan lobsters. The first constrained search was made to test the Synaxidae hypothesis (Fig. 1B), sampling only topologies that included *Palinurellus* and *Palybithus* clustering together in a separate clade. In the second constrained search for testing the Förster hypothesis (Fig. 1A), scyllarid genera were made to cluster together with *Linuparus* and *Puerulus* in every sampled tree. When testing evolutionary hypotheses within Palinuridae, a constrained search was made to sample only topologies that included *Puerulus* and *Linuparus* clustered together in a separate clade (George and Main hypothesis, Fig. 1C–E) and the other constrained search sampling only topologies that included *Puerulus* and *Palinurus* clustered together in a separate clade (Baisre hypothesis, Fig. 1D). We employed the Bayes factor to compare the different models (Nylander et al., 2004), evaluating the hypothesis (H0) that our constrained and unconstrained BI topologies explain the data equally well, versus the alternative hypothesis (H1) that constrained BI searches provide a poorer explanation of the data. We calculated the Bayes factor as twice the difference in the harmonic mean $-\ln L$ scores ($2\ln B01$) between alternative hypotheses (Brandley et al., 2005) and compared these values to the framework provided by Kass and Raftery (1995) where <0 is evidence against H1, $0-2$ provides no evidence for H1, $2-6$ is positive support for H1, $6-10$ is strong support for H1, and >10 is very strong support for H1 (see Nylander et al., 2004 and Brandley et al., 2005).

2.4. Divergence time and biogeographical estimation

The relaxed-clock analysis was used to infer divergence times. It has been shown that the “uncorrelated relaxed-clock” models, in which the mutation rates in each branch are allowed to vary within particular constraints, perform better than strict molecular clock or the correlated models (Drummond et al., 2006). We used the Bayesian relaxed-clock uncorrelated lognormal approach as implemented in BEAST v1.4.7 (Drummond and Rambaut, 2007) with the corresponding model of sequence evolution previously inferred for each gene partition and a Yule process for the tree prior. We calibrated two nodes using a uniform prior distribution with minimum-age constraints obtained from Achelatan fossils that can be assigned to present groups. These fossils are from the Early Cretaceous: (i) *Palinurus palaciosi* (Vega et al., 2006) (110–125 My) for the Palinuridae clade, and *Scyllarella gardneri* (Woods, 1925) (100–115 My) for the Scyllaridae. It appears that true *Palinurus* only have recent fossils dating back to the Miocene or Oligocene (see George and Main, 1967; George, 2006), so there could be synonymy confusion in these fossil names. Therefore, *Palinurus palaciosi* was used as a minimum-age constraint for the whole Palinuridae lineage and not just the genus *Palinurus*. Markov-Chain Monte Carlo (MCMC) simulations were run for 10,000,000

Table 1
Species used and gene sequences included in the analysis.

Species	Voucher specimen	Distribution	Depth (m)	Sridulating organ	COI-Folmer	16SrDNA	18SrDNA	28S rDNA-A	28S rDNA-B	28S rDNA-C	H3
Palinuridae Latreille (1802)											
<i>Palinurus barbarae</i> Groeneveld et al. (2006)	FP0001	WIO	>100	Present	FJ174960	FJ174903	FJ174925	FJ036949	FJ174817	FJ174849	FJ174876
<i>Palinurus charlestoni</i> Forest and Postel (1964)	FP0002	EAO	50–300	Present	FJ174959	FJ174902	FJ174924	FJ036947	FJ174815	FJ174851	FJ174877
<i>Palinurus delagoae</i> Barnard (1926)	FP0003	WIO	0–400	Present	FJ174958	FJ174904	FJ174926	FJ036950	FJ174816	FJ174850	FJ174878
<i>Palinurus elephas</i> Fabricius (1787)	FP0004	MED EAO	5–160	Present	FJ174956	FJ174900	FJ174922	FJ036946	FJ174812	EU449505	FJ174881
<i>Palinurus gilchristi</i> Stebbing (1900)	KC3232	EAO WIO	55–360	Present	FJ174961	FJ174905	FJ174927	FJ036948	FJ174814	FJ174848	FJ174879
<i>Palinurus mauritanicus</i> Gruvel (1911)	FP0005	MED EAO	180–600	Present	FJ174957	FJ174901	FJ174923	FJ036945	FJ174813	EU449506	FJ174880
<i>Panulirus argus</i> Latreille (1804)	Coll. Sheila Patek	WAO	0–90	Present	FJ174969	AF502947	AY743955	–	–	–	–
<i>Panulirus homarus</i> Linnaeus (1758)	KC3216	WIO EIO EPO	0–5	Present	FJ174963	AF337962	AF498681	–	FJ174801	–	FJ174884
<i>Panulirus inflatus</i> Bouvier (1895)	KC3229	EPO	5–30	Present	FJ174964	AF337960	AF498680	–	–	–	FJ174885
<i>Panulirus japonicus</i> Von Siebold (1824)	KC3223	WPO	1–15	Present	FJ174968	AF337968	AF498670	–	–	FJ174846	FJ174886
<i>Panulirus penicillatus</i> Olivier (1791)	KC3220	WIO EIO EPO WPO	1–4	Present	FJ174970	AF337974	AF498671	–	–	FJ174844	FJ174887
<i>Panulirus regius</i> De Brito Capello (1864)	FP0006	EAO	5–15	Present	FJ174962	FJ174899	FJ174916	FJ036938	FJ174802	FJ174827	FJ174883
<i>Panulirus versicolor</i> Latreille (1804)	KC3217	WIO EIO EPO	5–15	Present	–	AF337978	AF498682	–	–	–	–
<i>Linuparus trigonus</i> (Von Siebold 1824)	KC3228	WPO WPO WPO	30–318	Present	–	AF502946	AF498675	–	FJ174808	FJ174830	FJ174874
<i>Palinustus waguensis</i> Kubo (1963)	KC3226	WPO WPO EIO	70–180	Present	–	AF502952	AF498667	–	FJ174826	FJ174847	FJ174866
<i>Puerulus angulatus</i> Bate (1888)	KC3227	WPO WPO WIO EIO	274–536	Present	FJ174954	AF502951	AF498668	–	FJ174811	FJ174845	FJ174882
<i>Justitia longimana</i> H. Milne Edwards (1837)	Jus-0101-01	WAO	1–300	Present	–	AF502953	AF498674	–	–	FJ174841	FJ174873
<i>Jasusedwardsii</i> Hutton (1875)	KC3211, KC3232	WPO	5–200	Absent	FJ174951	FJ174894	FJ174937	FJ036943	FJ174821	FJ174836	FJ174867
<i>Jasus lalandii</i> Milne Edwards (1837)	Jas-0302-01	EAO	0–46	Absent	AF192882	FJ174895	FJ174936	–	–	–	FJ174869
<i>Jasus tristami</i> Holthuis, (1963)	Jas-0601-01	EAO	0–200	Absent	AF192881	FJ174893	FJ174938	–	–	–	FJ174868
<i>Sagmariasus verreauxii</i> Milne Edwards (1851)	KC3212	WPO	20–150	Absent	FJ174952	FJ174896	FJ174933	–	FJ174820	FJ174833	FJ174870
<i>Projasus parkeri</i> Stebbing, (1902)	FP0007	EAO WIO WPO	370–841	Absent	FJ174953	FJ174898	FJ174935	FJ036944	FJ174819	FJ174835	FJ174872
<i>Projasus bahamondei</i> George (1976)	KC3207, KC3225	WPO	175–300	Absent	–	FJ174897	FJ174934	–	FJ174818	FJ174834	FJ174871
Synaxidae Bate (1881)											
<i>Palinurellus wienecki</i> De Man (1881)	Pal-0201-01	WPO WPO WIO EIO	9–27	Absent	–	AF502954	AF498672	–	–	–	–
<i>Palibythus magnificus</i> Davie (1990)	KC3221	WPO	220–300	Present	FJ174955	AF502950	AF498666	FJ036957	FJ174825	FJ174843	FJ174875
Scyllaridae Latreille (1825)											
<i>Scyllarus arctus</i> Linnaeus (1758)	ICMD 12/1995	MED EAO	4–50	Absent	FJ174966	FJ174911	FJ174930	FJ036955	FJ174805	EU449508	FJ174859
<i>Scyllarus caparti</i> Holthuis (1952)	ICMD 222/1998	EAO	21–109	Absent	–	FJ174909	FJ174928	FJ036953	FJ174806	FJ174839	FJ174860
<i>Scyllarus posteli</i> Forest (1963)	ICMD 218/1998	EAO	10–70	Absent	FJ174967	FJ174910	FJ174929	FJ036956	FJ174807	EU449510	FJ174864
<i>Scyllarus pygmaeus</i> Bate (1888)	ICMD 5/1995	MED EAO	5–100	Absent	FJ174965	FJ174908	FJ174931	FJ036954	–	EU449511	FJ174861
<i>Scyllarus subarcticus</i> Crosnier (1970)	ICMD 299/1991	EAO	126–155	Absent	–	FJ174912	FJ174932	–	–	FJ174840	FJ174865
<i>Parribaculus antarcticus</i> Lund (1793)	KC3231	WIO EIO WPO WAO	0–20	Absent	–	FJ174913	AF498676	–	FJ174822	FJ174832	–
<i>Thenus unimaculatus</i> Burton and Davie (2007)	FP0012	WIO EIO	10–50	Absent	FJ174950	FJ174915	FJ174942	FJ036952	FJ174810	FJ174838	FJ174858
<i>Thenus orientalis</i> Lund (1793)	FP0013	WIO EIO WPO	10–50	Absent	–	FJ174914	FJ174941	FJ036951	FJ174809	FJ174837	FJ174857
<i>Scyllarides herklotsii</i> Herklots (1851)	ICMD 230/1998	EAO	5–70	Absent	FJ174946	FJ174906	FJ174939	FJ036958	FJ174823	FJ174842	FJ174863
<i>Scyllarides latus</i> Latreille (1802)	FP0014	EAO	4–100	Absent	FJ174947	FJ174907	FJ174940	FJ036959	FJ174824	EU449509	FJ174862
Nephropidae Dana (1852)											
<i>Homarus americanus</i> Milne Edwards (1837)	FP0010			Absent	FJ174944	FJ174888	FJ174917	FJ036941	FJ174803	FJ174831	FJ174854
<i>Nephrops norvegicus</i> Linnaeus (1758)	ICMD-279/1991			Absent	FJ174945	FJ174889	FJ174918	FJ036942	FJ174804	EU449504	FJ174855
Polychelidae Wood-Mason (1874)											
<i>Polycheles typhlops</i> Heller (1862)	ICMD-295/1991			Absent	FJ174943	FJ174890	FJ174921	–	–	EU449507	FJ174856

Abbreviations: EIO-WIO, Eastern and Western Indian Ocean; EAO-WAO, Eastern and Western Atlantic Ocean; EPO-WPO, Eastern and Western Pacific Ocean; MED, Mediterranean. (–): no amplification.

Table 2
Loci and primers used in this study to amplify and sequence mtDNA and nucDNA in Achelata and related taxa.

Locus	Primer	Sequence	Source	Annealing temperature (°C)	Fragment size
COI	LCO1490	5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3'	Folmer et al. (1994)	45	650
	HCO2198	5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3'			
16S rDNA	16Sar-L	5'-CGC CTG TTT ATC AAA AAC AT-3'	Palumbi (1996)	50	450
	16Sbr-H	5'-CCG GTC TGA ACT CAG ATC ACG T-3'			
18S rDNA	18S 1f	5'-TAC CTG GTT GAT CCT GCC AGT AG-3'	Whiting (2002)	48	880
	18S b2.9	5'-TAT CTG ATC GCC TTC GAA CCT CT-3'			
	18S 5FrRNA	5'-CGG AAA GCA TTT GCC AAG AA-3'	Carranza et al. (1996)	50	900
	18S_9RrRNA	5'-GAT CCT TCC GCA GGT TCA CCT AC-3'			
28S rDNA	28SAF	5'-AGT AAG GGC GAC TGA AMM GGG A-3'	Palero et al. (2008c)	50	814
	28SAR	5'-CAC ATG TTG GAC TCC TTG GCC CG-3'			
	28SBF	5'-CGG GCC AAG GAG TCC AAC ATG TG-3'	This study	50	862
	28SBR	5'-CCC ACA GCG CCA GTT CTG CTT ACC-3'			
	NuriA	5'-GGT AAG CAG AAC TGG CGC TGT GGG-3'	Palero et al. (2008b)	50	468
	NuriB	5'-GGG ATC AGG CTT TCG CCT TGG G-3'			
Histone H3	H3AF	5'-ATG GCT CGT ACC AAG CAG ACV GC-3'	Colgar et al. (1998)	55	350
	H3AR	5'-ATA TCC TTR GGC ATR ATR GTG AC-3'			

* Fragment size: amplified band size in base pairs.

generations, with the first 2,000,000 discarded as burn-in. The lower and upper bounds of the 95% highest posterior density (HPD) interval were obtained for every node.

Finally, we used the software TreeFitter (Ronquist, 2001) in order to test for the presence of a cophylogenetic structure between the molecular-based phylogenetic tree and the area cladogram (Page and Charleston, 1998; Stevens, 2004). Parsimony methods for tree fitting are based on models recognizing different types of events and associating each of these events with a cost inversely related to the likelihood of the event. The overall cost of the area cladogram indicates which cladogram is the most parsimonious given the observed phylogenetic tree. In the four-event model implemented in TreeFitter, we recognize four different types of events: codivergence events (vicariance), duplication events (sympatric speciation or allopatric speciation in response to a temporary barrier), sorting events (partial extinction) and switching events (dispersal between isolated areas). Inferences about historical constraints or the number of events of a particular type are tested against inferences obtained from random data sets drawn from the original data by random permutation of the terminals. The three area cladograms being tested in the present study represent the hypotheses of Achelata originating from each of the main oceans occupied by the group (Indian, Atlantic, and Pacific Oceans), respectively. Default cost assignments were used for running 100,000 permutations.

3. Results

3.1. Achelata phylogenetics

A total of 4584 positions (84% of the original 5412 bp alignment) were kept after removing poorly aligned regions with GBlocks. A total of 3316 nucleotides were constant, 296 variable sites were parsimony-uninformative and 972 were parsimony-informative. The partition homogeneity test showed no evidence of a conflicting phylogenetic signal between the gene regions studied ($P = 0.55$). The GTR+I+G always gave the lowest score under the Akaike Information Criterion (AIC) for each gene partition and the combined dataset and therefore it was used for subsequent Bayesian and maximum likelihood searches. The analysis of the combined dataset under maximum likelihood or Bayesian inference showed a similar tree topology and hence only the Bayesian consensus is shown, but including both bootstrap and BPP values (Fig. 2). In both phylogenetic reconstructions, Achelata

was recovered as a monophyletic clade, subdivided in two main lineages separating the Palinuridae/Synaxidae genera from the Scyllaridae genera and thus, strongly supporting the reciprocal monophyly of both groups.

Within the Palinuridae/Synaxidae clade, both reconstruction methods gave good support values in clustering the Silentes palinurid clade (*Projasus/Sagmariasus/Jasus*) with the synaxid genus *Palinurellus* (clade 1) and in supporting the *Linuparus/Justitia* clade (clade 2). Genera belonging to clade 3 (*Palinurus/Panulirus/Palybithus/Puerulus/Palinustus*) also clustered together but with good support values only with BI. The main difference between ML and BI topologies regarded the placement of the *Linuparus/Justitia* clade within the Palinuridae. However, the placement of the *Linuparus/Justitia* clade remained unresolved both under ML (bootstrap value = 36) or Bayesian inference (BPP = 52). Within clade 3, two well-supported clades were also recovered, one grouping all *Palinurus* with good support from both reconstructions and the other clade including *Panulirus* and the synaxid genus *Palybithus*. The two lineages (Lineage 1 represented by *Panulirus argus/P. japonicus/P. penicillatus* and Lineage 2 represented by *P. versicolor/P. regius/P. inflatus/P. homarus*) recovered within *Panulirus* presented striking evolutionary differences, with Lineage 2 probably corresponding to a fast-evolving lineage. Finally, the Scyllaridae subfamilies were found to group in two main clades, the Arctidinae and Ibacinae clade showing very low support and a well-supported clade including the Theninae and Scyllarinae.

3.2. Testing different hypotheses of Achelatan evolution

In order to test the statistical support for previously established hypotheses (Fig. 1), Bayes factors were computed comparing the tree topology obtained under the unconstrained model (Fig. 2) against the constrained topologies (Table 3). Both the Synaxidae hypothesis and the results obtained from the unconstrained tree resulting from genetic data explain the data much better than the Förster hypothesis (see Section 4), lending further support for the Palinuridae and Scyllaridae as reciprocally monophyletic groups. Moreover, the Bayes factors between the unconstrained result and the Synaxidae hypothesis indicate that the Synaxidae are actually polyphyletic and give support to the presence (absence) of a stridulating organ over the presence (absence) of a rostrum as a taxonomically relevant character. Our results indicate that the family Palinuridae (now including Palinuridae and Synaxidae genera) contains three well-supported monophyletic clades. The first

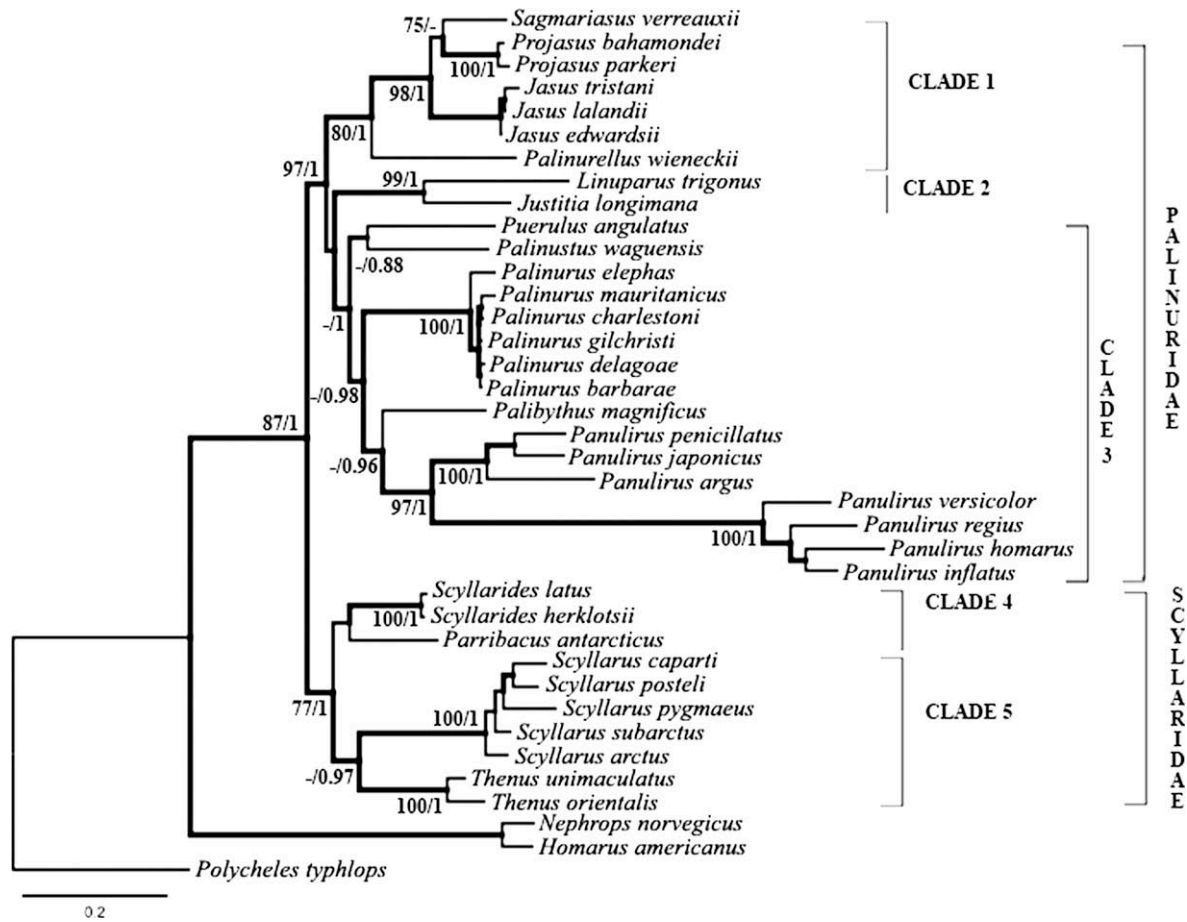


Fig. 2. Topology obtained using BI after alignment with muscle and pruning with Gblocks. Bootstrap branch support for ML analysis (before slash) and Bayesian posterior probabilities (after slash) are indicated above a cut off value of 70 and 0.70, respectively.

Table 3

Bayes Factors contrasting different phylogenetic hypotheses, calculated as twice the difference in the harmonic mean $-\ln L$ scores.

HO	Synaxidae	Förster	George and main	Baisre	Unconstrained
Synaxidae	-	-107.74	-76.2	-47.2	12.24
Förster		-	31.54	6.67	119.98
George and Main			-	-9.1	88.44
Baisre				-	53.32
Unconstrained					-
In L scores	-26923.68	-26977.55	-26961.78	-26970.88	-26917.56

one includes the synaxid *Palinurellus* plus Silentes (*Projasus*, *Sagmariasus*, and *Jасus*). The second clade includes the genera *Justitia* and *Linuparus*, and the third well-supported clade includes the rest of Stridentes (*Palinustus*, *Puerulus*, *Palinurus*, *Panulirus*) and the synaxid genus *Palybithus*, also showing a stridulating organ. Finally, the Bayes factors for the unconstrained versus George and Main or versus the Baisre hypotheses of relationships within Stridentes, showed that neither the *Linuparus*/*Puerulus* nor the *Palinurus*/*Puerulus* clades are well-supported by the molecular dataset.

Divergence dates calculated using a Bayesian 'relaxed molecular clock' method indicated that the Palinuridae and Scyllaridae families diverged around 143 Mya (95% HPD 125–178 My) (Fig. 3). The most recent common ancestor for the Palinuridae clade would be found around 124 Mya (95% HPD 120–160 My) and around 119 Mya (95% HPD 117–151 My) for the Scyllaridae. The origin of

the main clades within families is placed in the period spanning from the end of the Lower Cretaceous to the beginning of the Upper Cretaceous (approx. 85–115 Mya). Within Palinuridae, clade 1 appears to have originated around 75 Mya (95% HPD 55–125 My), clade 2 around 83 Mya (95% HPD 30–105 My) and clade 3 around 113 Mya (95% HPD 80–135 My). The origin of the Scyllarus/Thenus (clade 5) is placed at 93 Mya (95% HPD 64–129 My).

The test for a cophylogenetic structure between the molecular phylogenetic tree and the area cladograms using TreeFitter indicated that the area cladogram with the lowest cost corresponds to that of a basal clade including the Indian Ocean (Cost: 21), compared to the higher cost of an Atlantic-first (Cost: 24) or Pacific-first hypothesis (Cost: 29). The Indian Ocean-first cladogram showed that the distribution of the species on the Achelata phylogenetic tree is not random ($P = 0.008$) and that the observed number of duplications (range 20–22) is statistically significant ($P < 0.05$), indicating sympatric speciation or allopatric speciation in response to a temporary barrier. The number of codivergences (range 8–10), sorting events (range 11–15) and switching events (range 3–5) were not statistically significant ($P > 0.05$).

4. Discussion

4.1. Deep-phylogenetic levels: hypotheses of Achelata evolution

The inclusion of both slow and fast-evolving genes and a broad sampling of taxa including several outgroups has allowed us to solve the among-families uncertainties in Achelata with strong statistical support. This indicates that not only increasing the number

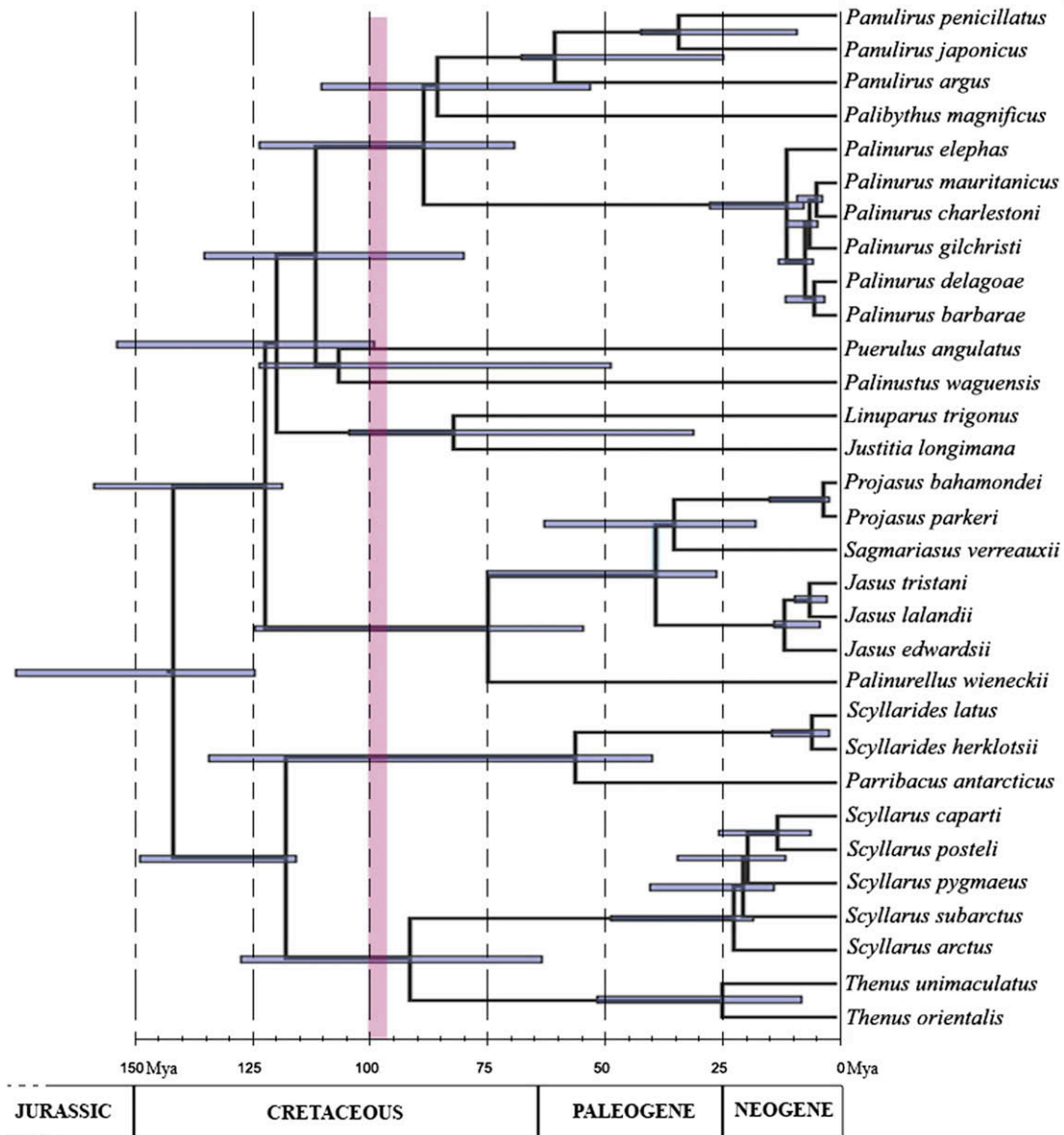


Fig. 3. Phylogenetic tree dating divergence among taxa with a relaxed-clock model, using *Palinurus palaciosi* (Vega et al., 2006) (110–125 My) and *Scyllarella gardneri* (Woods, 1925) (100–115 My) fossils as minimum-age constraints. Purple band indicates the Cenomanian stage, with the highest sea-level observed in the past six hundred million years (Gradstein et al., 2004).

of genes but including more taxa is important for resolving uncertainties in molecular phylogenetics (Rokas et al., 2005). Thus, the genetic dataset points conclusively to the reciprocal monophyly of the Achelatan families Palinuridae and Scyllaridae and therefore contradicts Förster proposal of Scyllaridae origin from a *Linuparus*-like ancestor (Förster, 1973). While the molecular dataset supports both Palinuridae and Scyllaridae as legitimate families, Synaxidae does not seem to be supported by the genetic evidence, since synaxids were always found to cluster within the Palinuridae, as tentatively seemed to point out Fig. 1E (George, 2006). Previous arguments suggested that the Synaxidae and the Palinuridae evolved separately from the Pemphicidae in the early Mesozoic (Glaessner, 1969; George and Main, 1967). However, each synaxid genus was consistently placed in different clades within Palinuridae, supporting previous morphological analyses that pointed to Synaxidae as a polyphyletic group (Davie, 1990). The relationships of *Palinurellus* and *Palibythus* inferred from our molecular dataset clearly confirm the proposal of Davie (1990) that Synaxidae should

be considered as a synonym of Palinuridae (also George, 2006). Thus, we formally recommend this taxonomy be adopted, namely that the Achelata consists of two families, Palinuridae and Scyllaridae and that the family Synaxidae now should be considered a synonym of Palinuridae.

When focusing on the within-family relationships, George and Main (1967) proposal of Silentes and Stridentes tribes within the Palinuridae is supported by the molecular evidence although with low support value for the Stridentes group. The Silentes clade is always found to be monophyletic with strong statistical support and inferred relationships among Silentes genera agree with morphological data, grouping all species without stridulating organ and pointing to *Palinurellus* as an earlier diverged lineage with *Projasus*, *Sagmariasus*, and *Jasus* originating later. However, inferred relationships among the Stridentes genera differ from those previously proposed (George and Main, 1967; Baisre, 1994; Patek and Oakley, 2003). Our molecular dataset shows clear support for a *Linuparus*/*Justitia* clade versus the previously proposed *Linuparus*/*Puerulus*

clade (George and Main, 1967; George, 2006). It is worth pointing out that not only molecular, but also morphological evidence supports the existence of this *Linuparus/Justitia* clade (clade 2), since these two genera are the only Palinuridae showing the male genitals on a chitinous tube-like extension off the base of the fifth pair of walking legs (Holthuis, 1991; Patek and Oakley, 2003). At the same time, the position of this clade (*Linuparus/Justitia*) relative to the Silentes and the other Stridentes genera remains unresolved. The very short unsupported branch separating Silentes from Stridentes could be indicative of a rapid radiation of the three main Palinuridae lineages, which makes it difficult to get strong statistical support for the inferred clades. As observed in several taxa, rapid radiation events are not easy to trace with phylogenetic reconstruction methods (Carreras-Carbonell et al., 2005; Rokas et al., 2005). Including more species belonging to clades 2 and 3 (e.g., *Puerulus* and *Palinustus*) could help resolve the monophyly of the Stridentes genera. In particular, increasing the sampling effort on the genus *Palinustus* would allow testing George's (2006) suggestion that *Palinustus* species have survived as relicts in the three major oceans following the Tethys Sea break-up.

Instead of a *Palinurus/Puerulus* clade (Baisre, 1994), the molecular dataset showed consistent support for the existence of a *Palinurus/Panulirus/Palibythus* clade (clade 3). This result is highly relevant, since the morphology of *Palibythus* is particularly divergent and its position within Palinuridae remained unsolved until now (Patek and Oakley, 2003; George, 2006). Furthermore, *Panulirus* and *Palibythus* are the only Stridentes showing large spines on the antennular plate (Davie, 1990; Holthuis, 1991; Patek and Oakley, 2003), which would be synapomorphies of this group. Finally, the two main clades found within the Scyllaridae are in agreement with previous inferences based on the adult morphological data (Holthuis, 1985, 1991). Even though the Arctidinae and Ibacininae clade showed very low support, both subfamilies share the presence of multiarticulated maxillipeds 1–3 while the Theninae and Scyllarinae lack flagella on the maxillipeds 1 and 3. Again, increasing sampling of species within this family could help increase support values for this clade. It should be considered that mutation saturations on the segments used in this study could also make them fail to resolve the phylogenetic relationships at the family level, even though excluding saturated COI 3rd positions did not change support values when using Bayesian phylogenetic inference as previously observed by Carreras-Carbonell et al. (2005).

4.2. Historical biogeography and the fossil record

The biogeographical analysis allowed us to find the most parsimonious hypothesis explaining a concordant area cladogram and phylogenetic tree. From a theoretical point of view, two phenomena have largely been ignored in biogeographical analyses: distributional noise (created by sampling errors) and the effect of geodispersal producing multiple signals (Upchurch, 2008). The result of these phenomena is a network of interactions between biotas, rather than a simple branching pattern produced by vicariance alone (Ronquist, 2001; Stevens, 2004). Since multiple biogeographical patterns may occur, we have used pattern-spotting techniques to untangle reticulate histories (Upchurch, 2008). Nevertheless, it should be pointed out first that the biogeographical analysis included in the present study could have several limitations, since most genera are widespread and there may not be enough species exemplars to accurately track species–area divergences, especially among the genus *Panulirus* and the very diverse scyllarids. The results obtained from our TreeFitter analysis are in agreement with an ancient Tethys (Indo-Pacific region) origin of the Achelata, since the lowest cost corresponds to that of a basal clade including the Indian Ocean (Cost: 21). Under the four-event model, the statisti-

cally significant more frequent duplication events in the phylogeny would correspond to sympatric speciation or allopatric speciation in response to a temporary barrier (Ronquist, 2001). In agreement with this, previous studies had proposed that temporary barriers to larval dispersal caused by a particular oceanographic feature could be responsible for the speciation pattern observed in some spiny lobsters of the genus *Panulirus* from the western Indian Ocean (Pollock, 1993). Nevertheless, adjusting fixed area cladograms to reflect the dynamic modification of geographical barriers through time is not straightforward and much research is needed to refine the current methods on historical biogeography reconstruction (Upchurch, 2008). It should be pointed out that these results are in accordance with a larger number of Achelatan species being found in the Indo-Pacific area (Table 1; George, 2006).

The dating of divergence of the two main Achelatan families (95% HPD node age: 125–178 My) obtained with the relaxed-clock model is compatible with previous hypothesis of a Triassic origin of Achelata (Porter et al., 2005; George, 2006). The Permian–Triassic extinction event (251 Mya), with up to 96% of all marine species becoming extinct, is supposed to have allowed a large turn-over of taxonomic groups occupying empty habitats (López-Gómez and Taylor, 2005). The first Achelatan-like fossils are found by the Triassic Period (250–190 Mya) [*Pemphix sueurii* (Meyer, 1839) and *Palinurus sueurii* (Desmarest, 1822)] and fossil representatives that can be confidently assigned to the Achelata are found in the early Jurassic [*Palinurina* (Munster, 1839), *Palaeopalinurus* (Bachmeyer, 1954) and *Cancrinus* (Munster, 1839)], coinciding with the early break-up of Pangaea about 180 million years ago (180 Mya). The separation of the two main families of Achelatan lobsters could be related to the origin of the Tethys Ocean; since Achelatan fossils from the Middle Cretaceous (Aptian and Albian strata, around 110–120 Mya) already show the divergence into the main two families of Palinuridae [*Astacodes falcifer* (Bell, 1863), *Palinurus palaciosi* (Vega et al., 2006)] and Scyllaridae [*Scyllarella gardneri* (Woods, 1925), *Scyllarides punctatus* (Woods, 1925)].

The origin of the main clades within families would therefore be located in the period spanning from the end of the Lower Cretaceous to the beginning of the Upper Cretaceous (Cenomanian, approx. 100 Mya). The late Cenomanian represents the highest sea-level observed in the past six hundred million years (approximately one hundred and fifty meters above present day sea-levels) (Gradstein et al., 2004). The Silentes/Stridentes divergence must have had a significant impact on the evolution of the Palinuridae. The acquisition of a complex structure such as the stridulating organ would have represented an increase in adaptive advantage by improving the chances of escape from a predator (Lewis and Cane, 1990). However, the monophyly of Stridentes was not strongly supported in our molecular analysis, not allowing us to conclude if the Stridentes clade (e.g., *Linuparus/Justitia*) represents an independent lineage. Nevertheless, the monophyly of the Silentes clade was unambiguously determined and their estimated divergence 75 Mya (95% HPD 55–125 My) is consistent with the fossil record (Glaessner, 1969). Consequently, the Silentes genera would have been able to radiate into the newly forming and geographically separating Southern Ocean habitats. Under such a scenario, the Eocene fossil genus *Archeocarabus* would represent a transitional form leading to the present *Jasus*, *Sagmariasus*, and *Projasus*; since its rostrum is about the same size as the supraorbital horns (Woods, 1931). Interestingly, our date estimate for the most recent common ancestor of *Jasus*, *Sagmariasus*, and *Projasus* (39 Mya) falls within the Eocene, even though *Archeocarabus* was not used for calibrating the clock.

Among the Stridentes genera, *Panulirus* is not well represented in the fossil record and therefore has traditionally been supposed to have appeared in the Miocene (approx. 20 Mya)

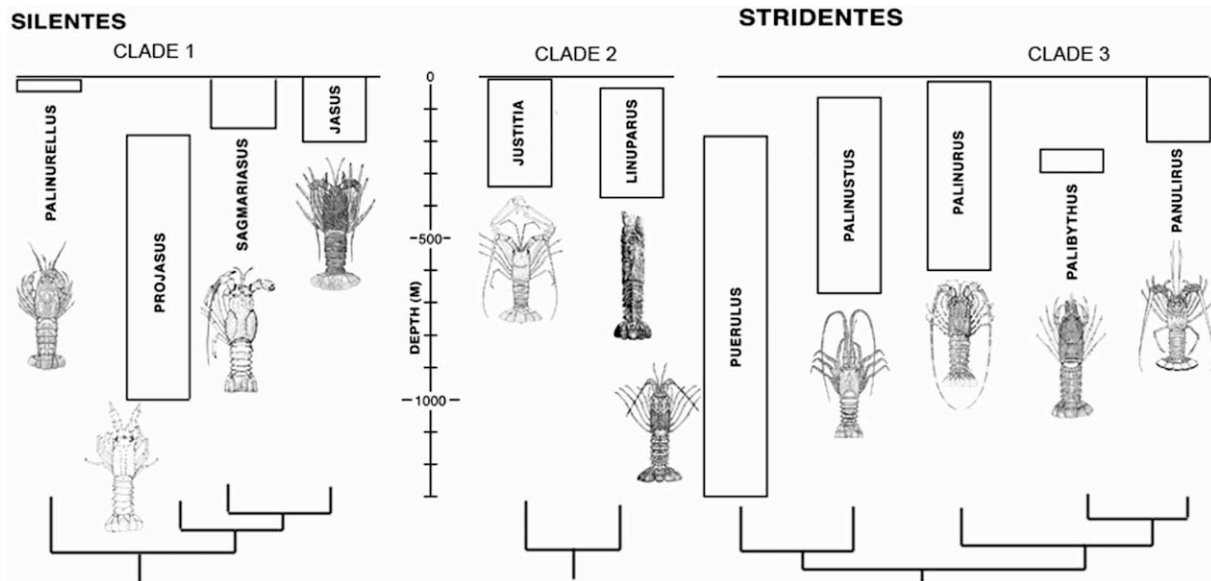


Fig. 4. Depth range and estimated clades found in Palinuridae. Figure modified from Holthuis (1991) and George (2006) according to our results.

from a *Palinurus*-like stock (George and Main, 1967; George, 2006). It has been inferred that the genus *Panulirus* probably originated in the Tethys Sea and both morphological and genetic evidence point to the Atlantic *P. argus* (Latreille, 1804) as most likely to represent the earliest species to diverge (Ptacek et al., 2001). Interestingly, our estimate for the divergence date between the *Panulirus/Palibythus* clade and *Palinurus* is about five times older than the assumed divergence time of the species and suggests that the genera may have split with the formation of the Atlantic Ocean around 100 million years ago. Our results do agree with previously obtained independent estimates using the alpha-type hemocyanins of *Palinurus elephas* and *Panulirus interruptus* (Kusche et al., 2003).

It has been proposed that, as the major continental plates moved northward fragmenting the Tethys Sea, *Panulirus*, the typically shallow water Stridentes radiated into the numerous newly formed habitats. In the remaining deeper water, older genera would have been less affected by the Tethys Sea fragmentation and today they would survive in relict habitats in the separate major oceans (George, 2006). Furthermore, George and Main (1967) suggested a habitat trend during palinurid evolution from deep to shallower waters and believed that the ancestral stocks are best represented by the deeper water species while the more specialized derived-species would live in shallower waters. However, Jablonski et al. (1983) pointed to the presence of shallow to deep water transition patterns in the evolution of Phanerozoic shelf communities. More evidence of an onshore to offshore shift in different marine communities was also provided by Sepkoski and Miller (1985), Bambach (1986), and Briggs (2003). However, our results do not seem to show a correlation between phylogenetic clades and depth range of the genera included in each clade (Fig. 4). In fact, the evolutionary direction in bathymetric distribution within the Palinuridae may be variable. For example, *Linuparus* spp. underwent a significant radiation in relatively shallow waters during the Cretaceous and early Tertiary (60–70 Mya) and yet most contemporary species are considered to be deep-water inhabitants (Glaessner, 1969; Holthuis, 1991).

In conclusion, our results indicate that Achelata forms a monophyletic group of decapod crustaceans composed of two main families: Palinuridae and Scyllaridae. The molecular analysis confirms Synaxidae as a polyphyletic group, which should be included with

in Palinuridae. The Palinuridae family presents three main lineages: (1) the Silentes, including the former synaxid genus *Palinurellus*; (2) a *Linuparus/Justitia* clade and (3) a main clade of Stridentes, including *Puerulus*, *Palinustus*, *Palinurus*, *Panulirus* and the rare former synaxid lobster *Palibythus*. Moreover, the two main clades found within the Scyllaridae are in agreement with current taxonomy based on adult morphological data. Finally, the dating of divergence of Achelata obtained with a relaxed-clock model is compatible with previous hypotheses of a Triassic origin of the Achelatan lobsters. The use of both slow and fast-evolving molecular markers and a broad taxa sampling proved to be effective in solving the uncertainties found in previous phylogenetic approaches and on obtaining consistent divergence time estimates even using an incomplete fossil record. The approach used in this study could be generalized to solve uncertainties in the classification in other taxonomic groups and to compare conflicting evolutionary hypotheses.

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