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Molecular approach to the phylogenetics of sea spiders (Arthropoda: Pycnogonida) using partial sequences of nuclear ribosomal DNA☆

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Abstract

The phylogenetic relationships among major evolutionary lineages of the sea spiders (subphylum Pycnogonida) were investigated using partial sequences of nuclear DNA, 18S, and 28S ribosomal genes. Topological differences were obtained with separate analyses of 18S and 28S, and estimates of phylogeny were found to be significantly different between a combined molecular data set (18S and 28S) and a subset of a morphological data matrix analyzed elsewhere. Colossendeidae played a major role in the conflicts; it was closely related to Callipallenidae or Nymphonidae with 18S or 28S, respectively, but related to Ammotheidae according to morphological characters. Austrodecidae was defined as a basal taxon for Pycnogonida by these molecular data. The 18S sequences were surprisingly conserved among pycnogonid taxa, suggesting either an unusual case of slow evolution of the gene, or an unexpected recent divergence of pycnogonid lineages. Notwithstanding difficulties such as non-optimal taxon sampling, this is the first attempt to reconstruct the pycnogonid phylogeny based on DNA. Continued studies of sequences and other characters should increase the reliability of the analyses and our understanding of the phylogenetics of sea spiders.

1. Introduction

Pycnogonids or sea spiders are a distinct group of exclusively marine arthropods found from the intertidal to abyssal depths in all the seas of the world. Pycnogonids range in size from tiny midgets of less than 2 mm up to 700 mm in leg span. They are recognized as voracious predators of various sessile and slow moving invertebrates (Arango, 2001; Arango and Brodie, 2003; Mercier and Hamel, 1994; Rogers et al., 2000), and potential "catchers" of metabolites and toxins from their prey (Sheerwood et al., 1998). Most species are epibenthic (but see Child and Harbison, 1986) and some are described as parasites (Arnaud, 1978; Benson and Chivers, 1960; Russell and Hedgpeth, 1990). There are about 1100 described species distributed in 80 genera (Munilla, 1999) and it is believed there are many deep-sea species still to be discovered. Pycnogonids are scarcely studied perhaps because they are considered a small group of aberrant arthropods, usually occurring in low abundance and well camouflaged, and without any economic importance. Pycnogonids display a range of peculiar traits such as taxa with extra body segments (Hedgpeth, 1947), offspring attached to the male parent (see revision in Arnaud and Bamber, 1987) and chemical compounds not present in any other animal group (Tomaschko, 1997). From an evolutionary point of view, pycnogonids are critical to the study of arthropod evolution in general, as evidenced by the controversy surrounding their position within Arthropoda (see Edgecombe et al., 2000; Giribet and Ribera, 2000) and their proposed sister-group relationship to all other extant euarthropods (Giribet et al., 2001). Alternatively, pycnogonids have been proposed as chelicerates based on phylogenetic analyses of nuclear protein-encoding genes (Regier and Shultz, 2001; Shultz and Regier, 2000).

Pycnogonids are morphologically diverse but relatively simple. They are unified by a number of unique

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derived characters, the most conspicuous being the presence of an external proboscis, a ventral pair of appendages or ovigers used by males to carry fertilized eggs, and an extreme reduction of the abdomen (Boudreaux, 1979). Traditionally, the classification of families of Pycnogonida has been based on the presence and absence of the cephalic appendages (chelifores, palps, and ovigers) in adults, and it has been assumed that a reductive trend is the direction of the evolution within the group (Arango, 2002; Hedgpeth, 1947; Munilla, 1999; Stock, 1994). Chelifores, palps, and ovigers, also show a wide range of variation in number of segments and other external features, and this is true even within some genera and among ontogenetic stages. A gradual reduction of the appendages of pycnogonids as an evolutionary hypothesis has been traditionally and intuitively assumed, but phylogenetic analyses to investigate this assumption have been scarcely attempted. A recent cladistic analysis of 36 morphological characters and 38 taxa has shown the possibility of reduction and loss of appendages as parallel evolutionary events in this group (Arango, 2002).

Cladistic analyses of the high-level phylogeny of pycnogonids using morphology present certain difficulties associated with large amounts of inapplicable data due to the coding of absences. Additional difficulties are due to the external morphological simplicity of the pycnogonids and the lack of informative characters not related to the "unstable" structures. These obstacles encourage the search for different sets of characters that support or reject the affinities suggested by external morphology.

Molecular data have not been used to explore the phylogenetic history of pycnogonids. Although a few sea spiders have been characterized for molecular studies of arthropod phylogeny (Colgan et al., 1998; Giribet and Ribera, 2000; Regier and Shultz, 1997; Wheeler and Hayashi, 1998), the internal relationships of the Pycnogonida have not been studied. Here, I present a molecular phylogenetic study of the high-level relationships of the Pycnogonida or sea spiders, using 18S and 28S rDNA partial sequences from 11 species. "Traditional" classifications are not supported and patterns shown solely by morphological analysis are challenged by this first insight into molecular phylogenetics of pycnogonids.

2. Materials and methods

2.1. Sampling of the molecular data

Molecular data were obtained for representative genera of six of the eight lineages or families of pycnogonids. Most of the specimens were collected during a taxonomic study of pycnogonids from shallow waters of North Queensland, Australia (Arango, 2003). *Pallenopsis schmitti* was collected from subtidal soft-bottoms of the Colombian Caribbean (see Arango, 2000), and other samples (e.g., *Colossendeis megalonyx* from the deep-sea) were made available by collaborators abroad. Partial sequences of the 18S were obtained for nine pycnogonid taxa including sequences available in GenBank (accession numbers in Table 1). Six families were represented and three genera of Ammotheidae were included. A few chelicerate and arthropod-related taxa were included as outgroups based on results of arthropod phylogenetic studies (Giribet and Ribera, 2000). Partial sequences of 28S rDNA were obtained from 11 species representing all the families of sea spiders except Pycnogonidae and Rhynchothoracidae. The fragment D4–D7 of 28S rDNA is not commonly sequenced for chelicerates or closely related groups and was not available for outgroup taxa. Sequences available are mostly for Diptera (Friedrich and Tautz, 1997), which is too divergent.

2.2. Preparation of molecular samples

DNA samples were obtained from fresh material or 90–95% ethanol-preserved specimens when possible, although older samples preserved in 70% ethanol were used when fresh specimens were not available. Whole individuals or a piece of tissue from the legs was used for the extractions depending on the size of the individuals, following the protocol for the Chelex extraction (Walsh et al., 1991). The V4 region of the 18S rDNA was PCR-amplified using the following primers designed for prostigmatid mites (Black et al., 1997; Otto and Wilson, 2001): Mite18S-F (5′ ATATTGGAGGGCAAGTCTGG 3′) and mite18S-R (5′ TGGCATCGTTTATGGTTAG 3′). Amplifications were carried out in 20 μl reactions with 0.5 U of *Taq* Polymerase (Qiagen), 2 μM dNTPs, and 10μM of each primer. The PCR amplification consisted of a 2 min denaturation step at 94 °C followed by 35 cycles at 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min 30 s, and a final extension at 72 °C for 10 min. On a few occasions a "touchdown" PCR program (from 51 to 49 °C) was used. The 28S fragment from the D4 to the D7 region was amplified using the primer pair 28SD3N-5′ TAGTA GCTGGTTCCTTCCG 3′ [the complementary reverse form of 28Sb in Whiting et al. (1997)], and 28SD7C-5′ GACTTCCCTTACCTACAT 3′, used in a high-level phylogeny study of Diptera (Friedrich and Tautz, 1997). Amplifications were carried out in 20–25 μl reactions and similar conditions to those used for amplification of 18S sequences. The basic PCR program included a 2 min denaturation step at 94 °C and 40 amplification cycles (92 °C for 45 s, 50 °C for 1 min, and 72 °C for 1 min 30 s) and 72 °C for 10 min at the end. Most of the PCR products were purified using the Qiagen PCR Purification Kit (Qiagen). Alternatively, 70% isopropanol precipitation method was carried out according to manufacturer's

Table 1
Species included in the study and the type of data used for each of them

	18S	28S	Morphology
Pycnogonida			
AMMOTHEIDAE			
Achelia assimilis	—	AF448557	X
Achelia echinata	AF005438*	—	—
Ammothella sp. (*appendiculata-group*)	AF448552	AF448558	X
Ascorhynchus ramipes	AF448555	—	X
COLOSSENDEIDAE			
Colossendeis megalonyx	—	AF448562	X
Colossendeis sp.	AF005440*	—	—
AUSTRODECIDAE			
Austrodecus childi	AF448554	AF448559	X
NYMPHONIDAE			
Nymphon micronesicum	AF448556	AF448560	X
CALLIPALLENIDAE			
Callipallene novaezealandiae	—	AF448563	X
Callipallene sp.	AF005439*	—	—
FAMILY UNCERTAIN			
Pallenopsis schmitti	—	AF448564	X
PHOXICHILIDIIDAE			
Anoplodactylus proliferus	AF448565	—	X
Anoplodactylus tenuicorpus	—	AF448533	—
Endeis biseriata	—	AF448561	X
Endeis laevis	AF005441*	—	—
Outgroup taxa used only for 18S analysis			
Xiphosura			
Limulus polyphemus	U91490		
Scorpiones			
Androctonus australis	X74761	—	—
Araneae			
Hypochilus pococki	AF062951	—	—
Ricinulei			
Pseudocellus pearsei	U91489	—	—
Myriapoda			
Polyxenus lagurus	X90667	—	—

Morphological characters from Arango (2002).

For purposes of comparison between genes and for the combined analysis, the 28S sequence of *Colossendeis megalonyx* has been paired with the 18S sequence of *Colossendeis* sp. The 28S sequence of *Callipallene novaezealandiae* has been paired with the 18S sequence of *Callipallene* sp. The 28S sequence of *Anoplodactylus proliferus* has been paired with the 18S sequence of *Anoplodactylus tenuicorpus*. The 28S sequence of *Achelia assimilis* has been paired with the 18S sequence of *Achelia echinata*. The 28S sequence of *Endeis biseriata* has been paired with the 18S sequence of of Endeis laevis.

*Sequences reported in Giribet and Ribera (2000).

instructions (ABI). The samples were directly sequenced in both forward and reverse directions using an automated ABI prism 377 DNA sequencer using Dye Terminator Sequence Kit (Applied Biosystems). Each cycle of sequencing reaction was carried out in a 20 μl reaction containing 8 μl of BDT mix, 10 ng/ml of PCR product, and 1 μM of primer and dH_2O to 20 μl. The products were precipitated with 70% isopropanol, centrifuged for 20 min at 13,000 rpm. The pellet was rinsed with 500 μl of 70% isopropanol and spin-dried at 1300 rpm for 5 min.

DNA sequences were aligned using Clustal W (Thompson et al., 1994) with the default alignment parameters. Alignments were then checked and adjusted using MacClade (Maddison and Maddison, 1992) to conform with the secondary structure model (Black et al., 1997). Primer sequences were excluded from analyses. Base frequencies and corrected average pairwise sequence divergence were calculated between taxa for each data set under complete deletion of gaps in PAUP*4 V4.0b8 (Swofford, 2000) and MEGA 2.1 (Kumar et al., 2001). Alignments can be found as supplementary material available online on the Molecular Phylogenetics and Evolution web site: www.academicpress.com/mpe.

2.3. Sampling of the morphological data

Morphological data for the 13 genera included in the molecular analyses were extracted from a previous study (Arango, 2002) to be analyzed in a simultaneous analysis with the molecular data. Details of the taxon sampling, collection localities, and deposited material for the morphological study are described in Arango (2002). The taxa used in the combined analysis are indicated in Table 1.

2.4. Phylogenetic analysis

Sequences were analyzed using both maximum parsimony (MP) and maximum likelihood (ML) criteria. MP and ML analyses were performed using branch and bound searches in PAUP*. ML analyses were run with parameter settings identified as optimal for the data set by Modeltest (Posada and Crandall, 1998), which compares the model goodness-of-fit by using hierarchical likelihood ratio tests (hLRTs) and the Akaike Information Criterion (AIC) (Posada and Crandall, 2001). ML analyses were implemented in PAUP* using heuristic search and tree bisection–reconnection (TBR) as a branch swapping algorithm (Swofford, 2000). Transition–transversion ratios and other parameters were estimated during the ML analysis. Both parsimony and maximum likelihood were applied to both complete (aligned in Clustal W) and reduced versions (after excluding highly variable regions) of each of the data sets. 18S sequence data were analyzed with and without outgroup taxa. Only parsimony-informative characters were used to calculate parsimony indexes and all sites were included in the ML analyses for both 18S and 28S data.

I analyzed the 18S and 28S data sets separately, and also combined them in a single data matrix. A controversy on combining data sets for phylogenetic analyses has been extensively reviewed (Bremer, 1996; Huelsenbeck et al., 1996; Miyamoto and Fitch, 1995; Page and Holmes, 1998, among others). However, it is widely accepted that a combined analysis is an appropriate method for phylogenetic reconstruction and also that there is no conflict in analyzing the data sets separately as well (Bremer, 1996). The Incongruence Length Distance Test (Farris et al., 1995) was performed to examine heterogeneity of the two molecular data sets. It was run in PAUP* (Partition Homogeneity Test) using a branch-and-bound search with 1000 replicates for each case. Analyses included the molecular partitions combined in a single matrix in which taxa lacking either 18S or 28S data were coded as missing values or excluded, to see the effects of missing values on the results. Finally, I combined morphological data (see Arango, 2002) for the taxa used in the molecular analysis with the sequence data in a single matrix for maximum parsimony analysis. Non-parametric bootstrapping was run in PAUP* with number of replicates set to 1000 for all the analyses performed.

3. Results

3.1. Alignment and nucleotide variation of 18S fragment

A total of 487 sites of the 18S were aligned for nine pycnogonids and five outgroup taxa: *Limulus polyphemus* (Xiphosura), *Hypochilus pococki* (Araneae), *Androctonus australis* (Scorpiones), *Pseudocellus pearsei* (Ricinulei) and *Polyxenus lagurus* (Myriapoda). The sequence of *Peripatopsis* sp. (Onychophora) showed a large divergence compared to pycnogonid sequences (>25%) making the alignment very difficult, then the onychophoran sequence was not included in the analyses. The 18S fragment selected was alignable with positions 561–1165 of the 18S rDNA sequences of sea spiders and other arthropod taxa (Giribet and Ribera, 2000) (GenBank accession numbers in Table 1), located between the V3 and V5 domains in Black et al. (1997).

There were a total of 134 (27%) variable characters of which 54 (11%) were parsimony informative. Overall base composition was statistically homogeneous across taxa ($\chi^2 = 3.72$, df = 39, and $P = 1.0$; PAUP* results), the average observed base frequencies being: $A = 25.57\%$, $C = 20.66\%$, $G = 27.15\%$, and $T = 26.66\%$. Uncorrected sequence pairwise divergence (p-distance) within pycnogonid species calculated with complete deletion of gaps (Swofford et al., 1996) was very small, ranging from 1 to 3% (total of 19 parsimony informative sites). Mean sequence divergence between outgroups and pycnogonids was 9%.

3.2. Phylogenetic analysis of 18S ribosomal DNA

Maximum parsimony searches based on the informative characters of 18S, yielded three most-parsimonious trees (Fig. 1; $L = 203$, CI = 0.62, and RC = 0.57) differing in the internal relationships among chelicerate outgroups (*Limulus*, *Hypochilus*, *Androctonus*, and *Pseudocellus*). The diplopod *Polyxenus* showed the highest sequence divergence overall compared to chelicerate and pycnogonid sequences (14–15%) and was placed as the root of the trees. Monophyly of the Pycnogonida was fully supported by 100% bootstrap support. Nymphonidae appeared related to Colossendeidae + Callipallenidae. Phoxichilidiidae (including *Endeis*) joined the clade basally, although weakly supported. The ammotheid clade *Ammothella* + *Achelia* was better supported (>68% bootstrapping) (Fig. 1), however, monophyly of Ammotheidae was not clear. *Austrodecus* was shown basal to all the pycnogonids.

For 18S, a TrNef model with a discrete approximation of the Γ-distribution (TrNef + Γ; $\Gamma = 0.27$) was preferred under the hLRTs. According to this criterion, the assumption of equal base frequencies (JC vs. F81) was met, there were equal rates for transversions (TrNef vs. TIMef), and no significant proportion of invariant sites, but the assumptions of an equal ratio of transition and transversion rates (JC vs. K80), equal rates for transitions (K80 vs. TrNef), and equal rates among sites (TrNef vs. TrNef + Γ) were all rejected. These assumptions slightly changed under the AIC, which indicated two unequal rates for transversions (TrNef vs. TIMef), a significant

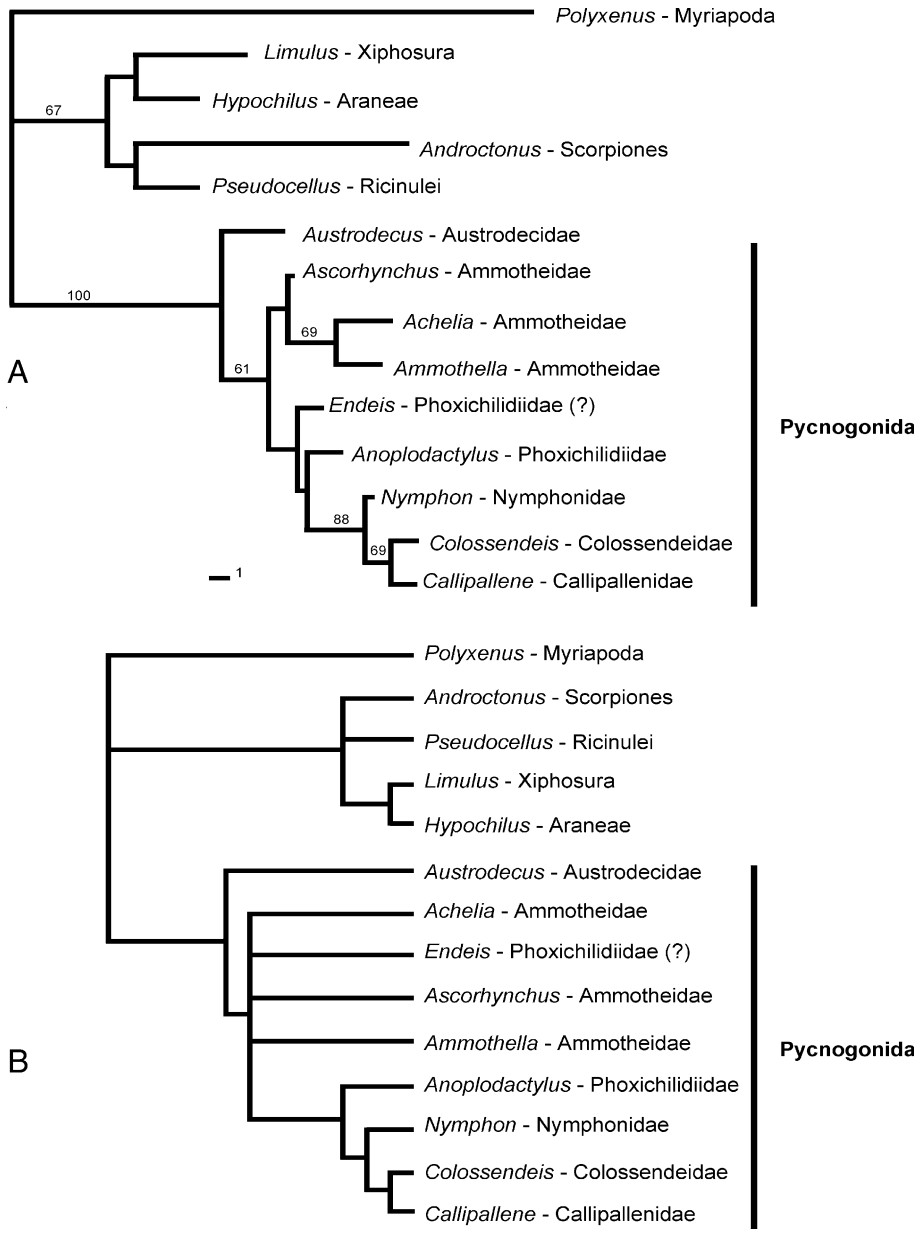

Fig. 1. Phylogenetic tree of the Pycnogonida based on 18S. (A) Same topology obtained under maximum parsimony (1/3 trees, $L = 203$, CI = 0.62, and RC = 0.57) and maximum likelihood based on TIMef + I + G model, −Ln likelihood = 1697.25. Bootstrap values above the branches are based on the MP analysis. Branch lengths proportional to character changes (same for all figures). (B) Strict consensus tree of seven MP trees ($L = 172$) obtained after excluding hyper-variable regions of the 18S.

proportion of invariant sites ($I = 0.36$) and a higher value for the Γ-distribution ($\Gamma = 0.64$). The best tree obtained under ML settings using the model preferred by the AIC (TIMef + I) did not differ from the one produced with hLRTs parameters. The ML tree was obtained from a heuristic search using the MP strict consensus topology as starting tree. Both the ML and the MP trees showed the same pattern of relationships among pycnogonid lineages (Fig. 1A). Appropriate references and descriptions for each of the models mentioned in this study can be found in Page and Holmes (1998) and Posada and Crandall (1998, 2001).

Two small hyper-variable regions were identified in the positions 123–137 and 216–219. These fragments consist of indels and are alignable among subsets of taxa; the former contained a peculiar 15-site insertion only present in Pycnogonida and Myriapoda. I carried out the same analysis excluding these regions to explore what influence these sections had on the topologies obtained, but less resolution was obtained [total parsimony informative characters = 45 (9.6%)]. Only (Nymphonidae (Colossendeidae + Callipallenidae)) and the basalmost Austrodecidae, were supported in the strict consensus tree (Fig. 1B, details in Table 2).

Table 2
Summary of results obtained with each of the data sets used to infer phylogenetic affinities of the Pycnogonida

	18S with outgroups	18S (E) with outgroups	18S pycnogonids	18S (E) pycnogonids	28S	28S (E)	Combined 18S + 28S	18S + 28S + morphology
Number of taxa	9 P, 5 O	9 P, 5 O	9 P, 1 O	9 P, 1 O	9 P	9 P	10 P, 1 O	10 P, 1 O
Characters included	487	487	487	471	877	810	1363	1399
Characters excluded	—	19	—	15	—	67	—	—
Variable	134	116	68	57	177	133	245	278
Parsimony-informative	54	45	24	18	61	40	85	111
Length MPT	203	172	89	75	239	171	334	423
Number MPT	3	7	1	4	1	2	1	1
CI	0.62	0.80	0.67	0.65	0.74	0.76	0.70	0.65
RC	0.57	0.59	0.57	0.55	0.61	0.66	0.57	0.47
Model hLRTs	TrNef + Γ	TrNef + Γ	TrNef + Γ	TrNef + Γ	HKY + Γ	HKY + Γ	—	—
	$\Gamma = 0.27$	0.30	$\Gamma = 0.01$	$\Gamma = 0.27$	$\Gamma = 0.22$	$\Gamma = 0.22$		
–Ln L	1693.80	1451.36	1169.77	1536.36	2474.80	2085.13		
Model AIC	TIMef + I + Γ	TrN + I + Γ	TrN + I	TrN + I + Γ	HKY + Γ	HKY + Γ	—	—
	$I = 0.36$; $\Gamma = 0.64$	$I = 0.39$; $\Gamma = 0.76$	$I = 0.79$	$I = 0.41$; $\Gamma = 0.75$	$\Gamma = 0.22$	$\Gamma = 0.22$		
–Ln L	1690.96	1445.99	1166.28	1531.63	2474.80	2085.13		

(E) represents sets for which hyper-variable regions were excluded. Number of taxa includes pycnogonids (P) and outgroup taxa (O). CI, consistency index; RC, rescaled consistency index (calculated without uninformative characters). Model selected by hierarchical likelihood tests is "model hLRTs," model selected by the Akaike information criterion is "model AIC."

A separate set of data excluding the outgroup taxa except *Limulus* was used for subsequent analyses to inspect the interaction of outgroups with pycnogonid relationships resulting from the 18S analysis. The MP search produced a single shortest tree that showed exactly the same pattern of pycnogonid relationships to the one found with outgroups included (Fig. 2A; $L = 90$, CI = 0.79, and RC = 0.43). The model selected and the parameter settings selected by hLRTs remained the same as those selected for the complete data set, but under the AIC the data rejected the assumption of equal base frequencies (JC vs. F81) and accepted to assume equal transversion rates (TrN vs. GTR). Regardless of the criterion followed, the topology was the same under both sets of conditions (shown in Fig. 2B). This tree differed from the one produced by the complete set of taxa in the basal position of *Endeis* and the inclusion of Austrodecidae with the ammotheids (see Table 2 for details of the analyses).

3.3. Alignment and nucleotide variation of 28S fragment

A total of 877 sites of 28S sampled from nine genera of pycnogonids were included in the analysis. Of these, 177 sites were variable (20%) and 61 were parsimony-informative (7.0%). Average base frequencies $A = 19.7\%$, $C = 32.15\%$, $G = 25.6\%$, and $T = 22.4\%$, were statistically homogeneous across taxa ($\chi^2 = 7.6$, df = 24, and $P = 0.9$). Uncorrected p-distance with complete deletion of gaps ranged from 1.4 to 10.9% among pycnogonid taxa, *Colossendeis* showing the highest values of divergence (7.12–10.3%) compared to other pycnogonid taxa.

3.4. Phylogenetic analysis of 28S ribosomal DNA

Branch-and-bound parsimony analysis of 877 sites produced a single MP tree (Fig. 3A; $L = 239$, CI = 0.74, and RC = 0.61). Colossendeidae + Nymphonidae, a strongly supported clade, was the most divergent from other pycnogonid taxa. Unlike the 18S analysis, Callipallenidae was a sister taxon to *Anoplodactylus* + *Pallenopsis*, both nodes being well supported. Unexpectedly, *Endeis* joined the ammotheid clade and *Achelia* did not appear as sister taxon of *Ammothella* as shown by the analysis of 18S.

The model HKY with a discrete approximation of the Γ-distribution (HKY + Γ; $\Gamma = 0.22$) provided the best fit for the data according to the hLRTs and the AIC. Assumptions of equal base frequencies (JC vs. F81), equal ratio of transition and transversion rates (F81 vs. HKY) and equal rates of substitutions among sites (HKY vs. HKY + Γ) were rejected by the data. Transition and transversion rates were assumed equal (HKY vs. TrN and HKY vs. K81, respectively), Ti/Tv was 0.76, and no significant proportion of invariant sites (I)

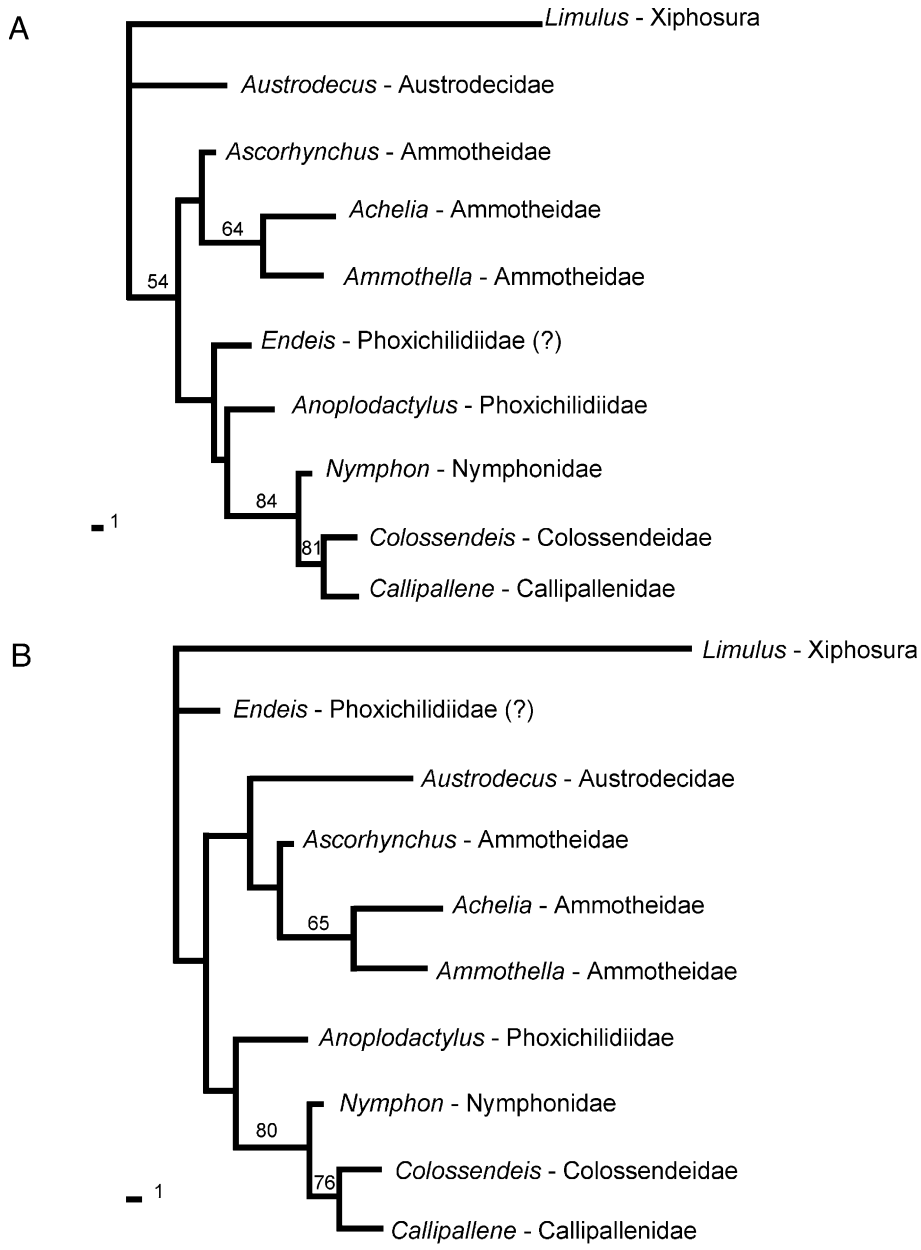

Fig. 2. Phylogeny based on 18S after exclusion of outgroups (except *Limulus*). (A) Single tree obtained under maximum parsimony analysis ($L = 90$, CI = 0.79, and RC = 0.43). (B) Tree obtained with maximum likelihood analysis based on the TrN + *I* model, −Ln likelihood = 1166.28.

was found. The resulting topology of the ML analysis is shown in Fig. 3B.

A great part of the variation in the 28S sequences was concentrated in positions 251–280 and 508–544. Although these two regions were difficult to align unambiguously, there were insertions that could be aligned for subsets of taxa. When these highly variable positions were excluded, the number of variable sites was 133 (16%) of which 40 (5%) were parsimony-informative sites. Neither the statistics for homogeneity of base frequencies nor the settings indicated by hLRTs and AIC for maximum likelihood analyses changed after the omission of variable regions (see Table 2). The ML topology was the same as that obtained with all the characters included (Fig. 3B), but the two resulting MP trees (not shown; $L = 171$, CI = 0.76, and RC = 0.66) showed support only for the nodes in (Callipallenidae (*Anoplodactylus* + *Pallenopsis*)).

3.5. Combined analysis of the 18S and 28S ribosomal DNA

The topology produced by the 28S data set is not the same as that obtained with the 18S sequences. However, the two estimates of phylogeny (Figs. 2B and 3B, respectively) did not appear significantly different

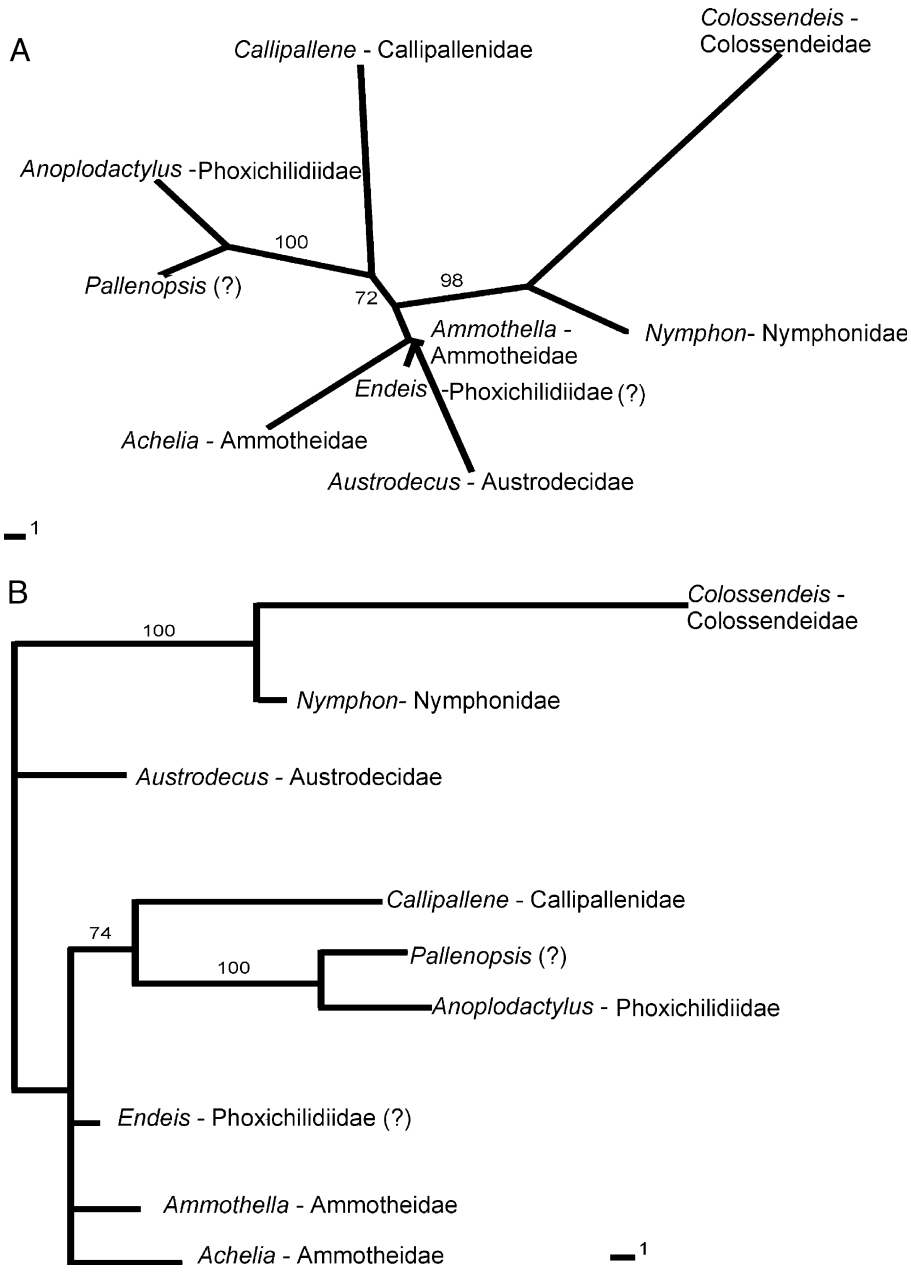

Fig. 3. Phylogeny based on 28S. (A) Single phylogram obtained under maximum parsimony ($L = 239$, CI = 0.74, and RC = 0.61). (B) Maximum likelihood analysis, tree rooted at midpoint, based on the HKY + G model. −Ln likelihood = 2473.26. Same topology obtained after exclusion of hyper-variable regions.

according to the homogeneity test (nreps = 10,000 and $p = 0.0662$; PAUP* results). Disagreement between 18S and 28S was concentrated in the unexpected position of *Endeis* related to ammotheids according to 28S, and the strong affinity between Callipallenidae and Colossendeidae shown by 18S. Callipallenidae, sister-taxon of *Pallenopsis* and *Anoplodactylus* in the 28S analysis, is in some agreement with conventional classifications (see Child, 1992). Unfortunately, the position of *Pallenopsis* could not be confirmed with the 18S because its sequence was not available.

Sequences of 18S and 28S of 11 taxa were combined in a single matrix using *Limulus* as outgroup introducing missing values for the 28S fragment. Missing values were also included for 28S of *Ascorhynchus* and the 18S fragment of *Pallenopsis*. This procedure did not have any effect on the topology and it is preferred over excluding available information (Wiens and Reeder, 1995). The combined data set comprised 1363 sites, of which 486 were 18S and 877 were 28S. Branch-and-bound search under parsimony criterion of the combined data set resulted in a single MP tree (Fig. 4; $L = 334$,

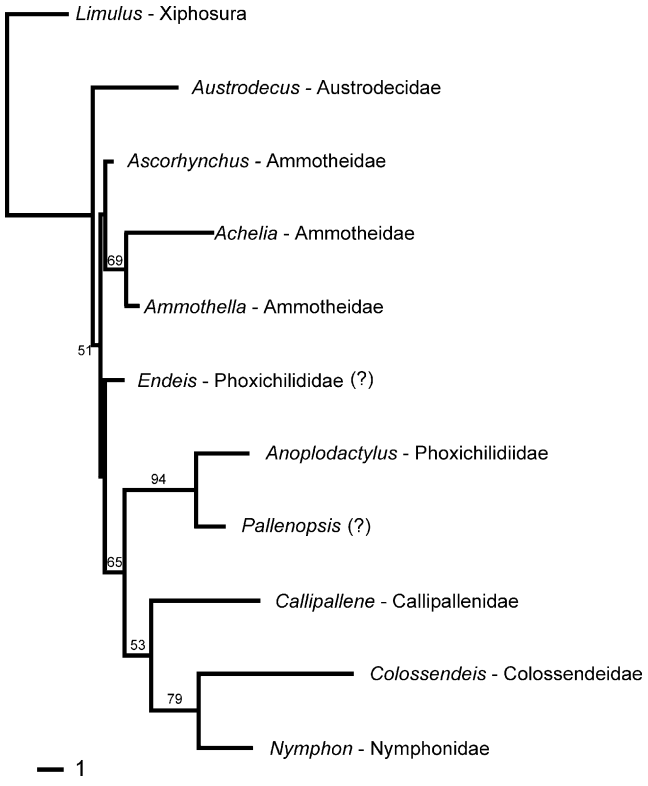

Fig. 4. Estimate of phylogeny based on 18S + 28S + morphology. Maximum parsimony analysis, all characters equally weighted ($L = 423$, CI = 0.64, RC = 0.45). Same topology obtained with weighting of morphological characters 1.3/1 ratio and also with 18S + 28S alone ($L = 335$, CI = 0.69, RC = 0.54).

CI = 0.69, and RC = 0.56). Nymphonidae + Colossendeidae were related to *Callipallene* with *Anoplodactylus* + *Pallenopsis* as a sister clade. The ammotheid genera form a monophyletic group diverging earlier, but not strongly supported, and *Austrodecus* remained at the base of the tree.

3.6. Combined morphological and molecular data

A significant heterogeneity between the molecular and the morphological partitions was shown by the ILD test (nreps = 10,000 and $P = 0.0001$). Additionally, a significant difference between the molecular topology (18S and 28S) and the morphology-based topology was detected (KH test $P = 0.003$). Despite the differences found, data combination was explored using an assembling of the three data partitions (morphology, 28S, and 18S). Table 1 shows the data included for each of the partitions and Table 2 includes a summary of results. A single shortest tree was obtained with the maximum parsimony criterion (Fig. 4; $L = 423$, CI = 0.67, and RC = 0.45). The tree remained the same after excluding highly variable regions from the DNA partitions and also under differential weighting of the morphological characters based on total number of characters (1.3:1). The best-supported clades were *Anoplodactylus* + *Pallenopsis*, Nymphonidae + Colossendeidae, and *Achelia* + *Ammothella*. *Austrodecus* remained as the basal taxon (Fig. 4).

4. Discussion

4.1. Outgroup relationships

Chelicerate representatives and the basal millipede *Polyxenus* were included as outgroups in the analysis of 18S sequences. These taxa on one occasion or the other, depending on the parameters used in analyses of arthropod phylogeny, had been shown as relatives of pycnogonids (Giribet and Ribera, 2000). The monophyly of the Pycnogonida was strongly supported as it was expected due to their peculiar morphological autapomorphies (Boudreaux, 1979). Pycnogonids showed 12 molecular autapomorphies compared to chelicerates and *Polyxenus*. The rejection of assumptions of equal rates of transitions and transversions in the 18S fragment is explained by the lower internal variation among pycnogonids (average of Ti in outgroups = 5.6%, Ti in pycnogonids = 1.4%; Tv in outgroups = 3.6%, Tv in pycnogonids = 0.5%). The rate of variation of this 18S segment within Pycnogonida is rather low and it might be considered too conserved to look at internal relationships of the group compared to rates observed in similar studies for other arthropod groups. The sequence of the millipede *Polyxenus* was highly divergent compared to pycnogonid sequences. However, the 15-site insertion in the V4 domain of the 18S shared between most pycnogonids and *Polyxenus* represents a particular character worthy of examination under conditions of analysis not subject to ambiguity of the alignments [e.g., the "direct optimization" method (Wheeler, 1996; Giribet and Ribera, 2000)].

4.2. Ingroup relationships

This first attempt to propose phylogenetic relationships among the families of pycnogonids based on molecular data supports some patterns of affinities that are not evident according to morphological data. The details of some of these patterns shown by the present study are discussed below.

4.3. Colossendeis in conflict

According to the molecular data, Colossendeidae is closely related to Nymphonidae and Callipallenidae. This result contrasts with the position of Colossendeidae attached to ammotheid genera in the results of a morphological analysis (Arango, 2002) and also according to traditional classifications (Hedgpeth, 1947). A strong

affinity between Nymphonidae and Colossendeidae is suggested by the 28S sequences, while 18S shows Colossendeidae closely related to Callipallenidae. The combined analysis of the two molecular partitions shows the three lineages grouped in a single clade. Colossendeidae is a family of distinct sea spiders in which the adults have no chelae and their proboscis is enormously large in relation to the trunk while Callipallenidae and Nymphonidae show contrasting morphological features to those of Colossendeidae. Thus, morphological characters do not yield the patterns shown by DNA and despite the strong support shown with molecular data, they cannot yet be explained on morphological grounds. Colossendeidae appears as a long unstable branch in both molecular and morphological analyses and only the search for additional characters could help to solve the conflict.

4.4. Austrodecidae: highly divergent

Austrodecidae appears at the base of the topologies in the 18S analysis and the combined analysis of the two molecular data sets. This particular lineage shows a number of molecular apomorphies (18S = 6 autapomorphies and three plesiomorphies; 28S = 14 autapomorphies) and quite particular morphological features including a pipette-like proboscis and extreme reduction in the size of the ovigers. This taxon has never been mentioned as a possible early form of sea spiders, and its position here contrasts with its derived position related to the small-sized ammotheids according to morphology. However, it also showed a number of autapomorphies suggesting it might be a divergent lineage within the evolution of sea spiders represented by an unstable branch in the analyses (Arango, 2002).

Nymphonidae or Ammotheidae are usually referred to as primitive taxa due to their similarity to other chelicerates in terms of number of appendages of the adults. However, the position of Austrodecidae as a basal taxon could be further examined looking at its affinities with ammotheid genera including Nymphonidae and Colossendeidae as outgroup taxa.

4.5. Pallenopsis and Endeis: are they phoxichilidiids?

The problematic genus *Pallenopsis* seen as a transitional form between Callipallenidae and Phoxichilidiidae is defined by molecular data as the sister-group of *Anoplodactylus*, the type genus of Phoxichilidiidae. This agrees with Stock's intuitive classification (Stock, 1965) of including *Pallenopsis* within Phoxichilidiidae but opposes other classifications in which *Pallenopsis* is regarded as a callipallenid (Child, 1992; Hedgpeth, 1947). There is morphological resemblance between *Pallenopsis* and *Anoplodactylus*, but the presence of ovigers in females of *Pallenopsis* is in conflict with a synapomorphy of the Phoxichilidiidae, that is, the females lacking ovigers. In the morphological analysis (Arango, 2002) *Pallenopsis* is basal to the non-ammotheid taxa: (Nymphonidae + Callipallenidae + Phoxichilidiidae + Pycnogonidae). Although morphological data have not been decisive regarding the position of *Pallenopsis*, DNA data have given additional evidence of proximity between *Pallenopsis* and *Anoplodactylus* as suggested by Stock (1965).

Endeis is proposed here as a divergent branch according to the separate and combined analyses of 18S and 28S, and it is not supported as sister taxon of any of the other families. A closer relationship between *Endeis* and Phoxichilidiidae would be expected according to few morphological synapomorphies shared by the two taxa (Arango, 2002).

4.6. Ammotheidae paraphyletic?

Ammotheidae is one of the most diverse families of pycnogonids in both number of taxa and morphological features. The three genera included in this study are grouped together, *Achelia* and *Ammothella* strongly supported as a single clade, *Ascorhynchus* attached to them but not well supported. This weak support for *Ascorhynchus* + (*Achelia* + *Ammothella*) in the DNA analyses can be related to the paraphyletic appearance of the family already suggested by morphology (Arango, 2002). Although a limited taxon sampling of the molecular study does not allow making strong inferences on the monophyly of the families, it indicates that more extensive sampling is needed for a complete molecular analysis of the relationships within this lineage.

4.7. Confronting morphology and DNA

Separate analyses of nuclear ribosomal DNA and morphological data show disagreement between the cladograms proposed. Most of the incongruence centers on the lineage Colossendeidae, which is related to Nymphonidae and/or Callipallenidae according to DNA but is related to Ammotheidae genera according to morphology. Maximum parsimony and maximum likelihood analyses resulted in very similar topologies in most cases, thus the conflict seems to be more related to the type of data used rather than to a method of analysis.

The lack of congruence among different data sets is a common result in phylogenetic studies at various levels, and usually a mismatch between the gene phylogeny and species phylogeny is mentioned as a possible source of incongruence among data sets (Wiens and Hollingsworth, 2000). In the case of pycnogonids, a robust species phylogeny has not yet been defined and there are problems related to nonindependence of characters and the lack of appropriate outgroups for analysis. A solid

morphological phylogeny is still a main task to be approached that could be aided by further studies to identify additional morphological, ecological and palaeontological characters.

Taxon sampling in high-level phylogeny studies is a relevant issue usually limiting a complete phylogenetic analysis of a group of organisms (Swofford, 2000). The analysis of morphological characters for a subset of taxa (selected according to molecular data available) yielded a different topology (not shown) from the tree obtained in the complete analysis of 38 taxa (Arango, 2002). This difference suggests that inclusion of Pycnogonidae and Rhynchothoracidae and more representatives from other lineages might change the pattern observed in a future outcome. Difficulties in finding material of many rare pycnogonid taxa have prevented more comprehensive studies. However, this study encourages more extensive sampling at all taxonomic levels, expanding the collections to deeper habitats and different latitudinal areas. Thus, the data set could be enhanced and more solid conclusions drawn about pycnogonid phylogeny.

4.8. Divergence times

The analysis of the V4 region of 18S revealed a low level of sequence divergence (<4%) in pycnogonids. This rather low rate of variation of this portion of 18S within Pycnogonida is similar to results obtained for the phylum Ctenophora, believed to have the lowest level of sequence variability at the level of the 18S rDNA gene (<5%) in any metazoan phylum (Podar et al., 2001). It was expected that the V4 region would give representative variation, as has been the case of other arthropod groups. However, the result of an absolute average distance value among lineages of pycnogonids of 10 (2.8%), contrasts with a much higher value (37.5; 8%) among genera of ticks, family Ixodidae (sequences from Black et al., 1997) and among orders of Chelicerata (20; 6.1%). Pycnogonids have been assumed to be an ancient group of animals and recently presented at the base of the arthropod tree (Giribet et al., 2001).

The fossil records suggest they have existed since the Cambrian (Walossek and Dunlop, 2002). Being such an old group, the fact that 18S sequences are so homogeneous among pycnogonids suggest a possible recent divergence of the extant lineages with a high rate of extinction of taxa preceding them. Alternatively, an unusually extreme slow rate of evolution of the 18S would explain the low rate of variation.

The possibility that the extant lineages of sea spiders are relatively young has not been considered since Hedgpeth (1947) when he argued that metameric instability and "transitional" forms could be features of a recent group "...still undergoing active evolution." A few years later, describing a Devonian fossil, Hedgpeth retracted and preferred the hypothesis of pycnogonids as a primitive branch of the arthropods (Hedgpeth, 1955). In a similar situation to that exposed for Ctenophora (Podar et al., 2001), the pycnogonids might be so ancient (see Giribet et al., 2001) that it is difficult to establish their position within Arthropoda, but the extant taxa may have evolved from a recent common ancestor. The notion is supported in this study by a long, well-supported branch leading to Pycnogonida, but very short branches within the group. A more extensive approach using the complete sequences of 18S and other molecular markers is desired, to evaluate a possible recent divergence of extant lineages of pycnogonids.

This study provides a first step in the reconstruction of pycnogonid phylogeny based on molecular data. Although far from optimal, it has shown possible affinities not proposed before, challenging traditional views by morphologists. It is necessary that other genes are sequenced and the number of taxa sampled is increased, not only to test various phylogenetic hypotheses but also to provide some insight into their evolution and the timing of major divergences within the group.

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