

First Molecular Data on the Phylum Loricifera – An Investigation into the Phylogeny of Ecdysozoa with Emphasis on the Positions of Loricifera and Priapulida

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Recent progress in molecular techniques has generated a wealth of information for phylogenetic analysis. Among metazoans all but a single phylum have been incorporated into some sort of molecular analysis. However, the minute and rare species of the phylum Loricifera have remained elusive to molecular systematists. Here we report the first molecular sequence data (nearly complete 18S rRNA) for a member of the phylum Loricifera, *Pliciloricus* sp. from Korea. The new sequence data were analyzed together with 52 other ecdysozoan sequences, with all other phyla represented by three or more sequences. The data set was analyzed using parsimony as an optimality criterion under direct optimization as well as using a Bayesian approach. The parsimony analysis was also accompanied by a sensitivity analysis. The results of both analyses are largely congruent, finding monophyly of each ecdysozoan phylum, except for Priapulida, in which the coelomate *Meiopriapulid* is separate from a clade of pseudocoelomate priapulids. The data also suggest a relationship of the pseudocoelomate priapulids to kinorhynchans, and a relationship of nematodes to tardigrades. The Bayesian analysis placed the arthropods as the sister group to a clade that includes tardigrades and nematodes. However, these results were shown to be parameter dependent in the sensitivity analysis. The position of Loricifera was extremely unstable to parameter variation, and support for a relationship of loriciferans to any particular ecdysozoan phylum was not found in the data.

Key words: Loricifera, Kinorhyncha, Priapulida, Ecdysozoa, metazoan phylogeny

INTRODUCTION

Since the advent of molecular phylogenetics in the study of metazoan evolution (e.g., Field *et al.*, 1988), investigators have gradually produced sequence data for virtually all the major metazoan lineages. After sequences were obtained for species of the newest discovered animal lineages Cycliophora (Winnepeinninckx *et al.*, 1998) and Micrognathozoa (Giribet *et al.*, 2004), to date only the members of one animal phylum remain unsequenced—those of Loricifera (see Halanych, 2004). The exclusively marine phylum Loricifera constitutes a group of tiny metazoans described in 1983 by Danish zoologist R. Møbjerg Kristensen. Since the description

of the first loriciferan, *Nanalaricus mysticus*, more than one-hundred species have been discovered (Nielsen, 2001); however only few have been formally described. The phylum consists of two very different families, Pliciloricidae and Nanalaricidae (Higgins and Kristensen, 1986). Species of Pliciloricidae are common in the deep sea, whereas those of Nanalaricidae are usually found interstitially in coarse sediments at shallower depths. The Loricifera are hypothesized to belong to the clade Introverta (Nielsen, 1995; 2001; Nielsen *et al.*, 1996; Schmidt-Rhaesa, 1997/98; Sørensen *et al.*, 2000), and a relationship to Priapulida and Kinorhyncha to form the group Scalidophora has found broad morphological support (Neuhaus, 1994; Nielsen *et al.*, 1996; Neuhaus *et al.*, 1997; Sørensen *et al.*, 2000). However, the phylogenetic position of Loricifera is far from settled. Warwick (2000) recently postulated that loriciferans are simply paedomorphic priapulids, while others have considered Loricifera a member of

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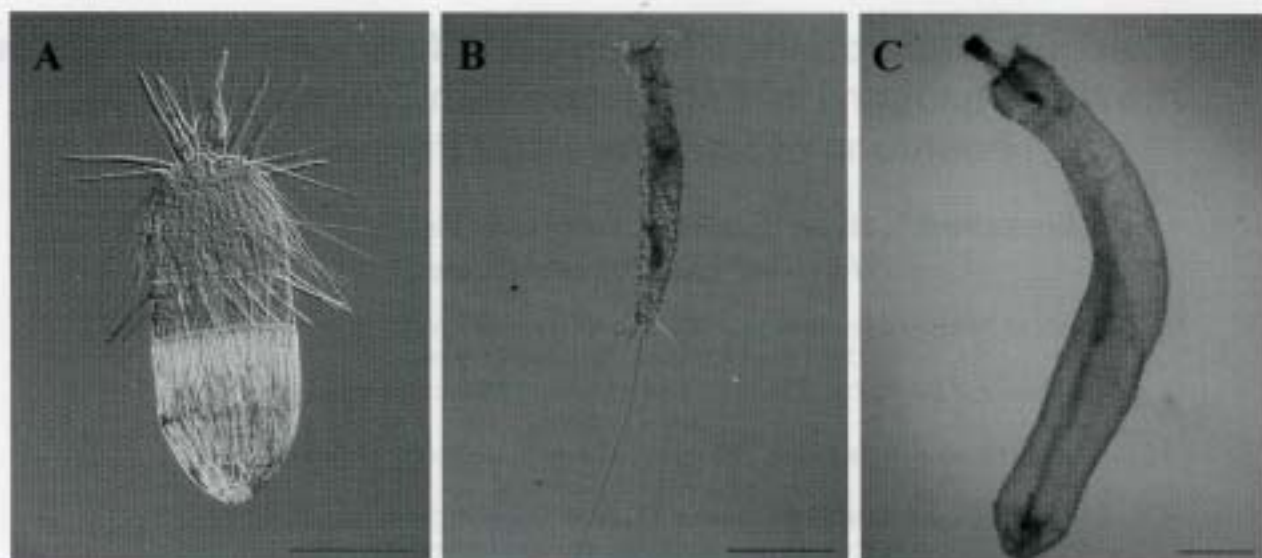


Fig. 1. Photomicrographs of the species for which 18S rRNA was sequenced in this study. (A) *Pliciloricus* sp. (scale bar = 50 μ m); (B) *Zelinkaderes* sp. (scale bar = 200 μ m); (C) *Melopriapulus* sp. (scale bar = 500 μ m).

a phylum named Cephalorhyncha, which also includes Nematomorpha, Priapulida, and Kinorhyncha (Malakhov and Adrianov, 1995; Adrianov and Malakhov, 1999). Nielsen (e.g. 1995, 2001) used the term Cephalorhyncha as a supraphyletic category that includes the phyla Loricifera, Kinorhyncha and Priapulida.

According to most morphological analyses published so far, loriciferans belong with other members of the clade Invertebrata (Schram and Ellis, 1994; Nielsen et al., 1996; Zrzavý et al., 1998; Sørensen et al., 2000; Peterson and Eernisse, 2001), which constitutes one of the major ecdysozoan lineages. Ecdysozoa (Aguinaldo et al., 1997) has emerged as a widely accepted group of animals supported by both morphological and molecular data (Schmidt-Rhaesa et al., 1998; Zrzavý et al., 1998; Giribet and Ribera, 1998; Giribet et al., 2000; Zrzavý, 2001; Giribet, 2003a), though Wägele et al. (1999), Wägele and Misof (2001), and Scholtz (2002) presented an opposite view. In this study we present the first DNA sequence data for an undescribed member of the phylum Loricifera collected from shallow depths in Korea (Fig. 1). We also present novel data on other invertebrate phyla such as Priapulida and Kinorhyncha from Korea and Greenland. Due to the limited amount of molecular data available for Loricifera, restricted to a single locus (nearly complete 18S rRNA), we attempt to study the phylogenetic relationships of Loricifera with other members of Ecdysozoa, using the non-ecdysozoan Chaetognatha as outgroup. Although we recognize the putative pitfalls of phylogenetic approaches based on single

genes (e.g., Giribet, 2002a), it was not possible to amplify other genes for Loricifera. Still, addition of molecular data for this last animal phylum may shed new light on the position of this ubiquitous but seldom-found invertebrate group.

MATERIALS AND METHODS

Sampling and DNA Sequencing

To attempt to reconstruct the phylogenetic relationships of Loricifera with other major ecdysozoan groups, nearly complete 18S rRNA sequences of four representative species of scalidophoran phyla (Loricifera, *Pliciloricus* sp.; Kinorhyncha, *Zelinkaderes* sp., and *Echinoderes aquilonius*; and Priapulida, *Melopriapulus* sp.) were determined (Table 1). *Pliciloricus* sp., *Zelinkaderes* sp., and *Melopriapulus* sp. were collected by SCUBA diving in subtidal zones of Korea and fixed in 95% ethanol. *Echinoderes aquilonius* was collected from sediment samples in Greenland. A single animal for each of the species was rinsed with distilled water several times, and DNA was extracted. Table 1 lists sampling locations, and Table 3 illustrates the GenBank accession numbers for all species utilized in this study. Total genomic DNA was extracted from single individuals with a modification of standard phenol-chloroform methods, or by using the DNEasy Tissue Kit from Qiagen with the protocol provided by the manufacturer. The small subunit rRNA (SSU rRNA) of the loriciferan species was amplified by polymerase chain reaction (30 cycles of 94°C for 1 min, 50°C for 40 s, and 72°C for 2 min, followed by 72°C for 10 min) with the primer set 328/329 designed on the basis of sequences of the conserved regions at the 5' and 3' ends of eukaryotic homologs (Nelles et al., 1984). More information on PCR and the sequencing primers used in this study is given in Table 2. Negative controls were also performed in every PCR reac-

Table 1. Sampling locations of the new scalidophoran species sequenced for this study.

Phylum	Species	Locality
Loricifera	<i>Pliciloricus</i> sp.	Korea: Imwon-Gun, Gangwon-do Province (N 37°13'78", E 129°20'47")
Kinorhyncha	<i>Zelinkaderes</i> sp.	Korea: Guryongpo, Gyeongsangbuk-do Province (N 35°59'30", E 129°34'91")
	<i>Echinoderes aquilonius</i>	Greenland: Igloik, Disko Is., West Greenland (N 69°17.3', E 053°13.1'), 195-214 m depth
Priapulida	<i>Melopriapulus</i> sp.	Korea: Marado Is., Jeju-do Province (N 33°06'41", E 126°16'03")

Table 2. Oligonucleotide primers used in this study.

Primer name	Sequence (5'-3')	Position ^a
328 (forward)	TAC CTG GTT GAT CCT GCC AG	1-20
A- (forward)	TGG AGG GCA AGT CTG GTG	553-570
A (reverse)	CTG GCA CCA GAC TTG CCC T	570-553
G- (forward)	AGA GGT GAA ATT CTT GGA	909-926
G (reverse)	TCC AAG AAT TTC ACC TCT	926-909
I- (forward)	AAA CTT AAA GGA ATT GAC GGA	1142-1162
I (reverse)	TCC GTC AAT TCC TTT AAG TTT	1162-1142
D- (forward)	TGT GAT GCC CTT AGA	1441-1455
D (reverse)	TCT AAG GGC ATC ACA	1455-1441
329 (reverse)	TAA TGA TCC TTC CGC AGG TT	1791-1810

^aNucleotide position in the *Artemia salina* 18S rRNA sequence of Nelles et al. (1984).

tion. PCR products were isolated from 1% agarose gels, purified with a GeneClean (Bio 101) Nal/glass-powder Kit, and cloned into pGEM[®]-easy vector (Promega Co.). Cloned inserts were cycle-sequenced with a Big Dye Terminator Cycle-Sequencing Kit (Applied Biosystems), and the sequencing products were purified by alcohol precipitation and electrophoresed on an ABI 3100 automated DNA sequencer. The total sequence product was 1,855 bp for *Pliciloricus* sp. (Loricifera), 1,774 bp for *Zelinkaderes* sp. (Kinorhyncha), and 1,835 bp for *Meiopriapulus* sp. (Priapulida).

Data Analysis

The data were analyzed in the computer programs POY v. 3.0 (Wheeler et al., 2002) and MrBayes v. 3.0 (Huelsenbeck and Ronquist, 2003). In total, we analyzed almost complete 18S rRNA sequences for 54 ecdysozoan taxa, using a chaetognath as an outgroup. In using a single outgroup, we were not attempting to test monophyly of Ecdysozoa, but merely testing monophyly of each ecdysozoan phylum and the interrelationships among the phyla. The 18S rRNA locus was divided into 48 sequence fragments that correspond to secondary structure features (as in Giribet, 2001), and from those, four fragments (ecd18s7.seq, ecd18s21.seq, ecd18s39.seq, and ecd18s46.seq) were excluded from all subsequent analyses due to their extreme length variation.

Initially the data were analyzed under parsimony with the direct optimization method (Wheeler, 1996) for 100 random replicates using subtree pruning and regrafting (spr), tree bisection and reconnection (tbr), ratcheting, and tree fusing (tf) as branch-swapping algorithms. Up to 10 trees were retained per replicate, and up to 500 trees after all replicates had been completed. This analysis was reproduced for a total of 12 parameter sets that varied in the gap-cost and the transversion/transition-cost ratios (Wheeler, 1995; Giribet, 2003b). The results of all these analyses were pooled into a file and used for a new round of tree fusing—also called sensitivity analysis tree fusing (sattf)—and analyzed under the 12 initial parameter sets. This second analytical round with sattf may help to find shorter trees. The results of these analyses were taken as our optimal trees, and the results of the sensitivity analysis are used to discuss several aspects of the phylogenetic affinities of the ecdysozoan phyla. Nodal support was measured via jackknifing with a probability of deletion of e^{-1} (Farris, 1997). Since we used a single data partition for this analysis, we did not perform a congruence analysis, and we could not use congruence as an optimality criterion for choosing our preferred parameter set (Wheeler, 1995; Giribet and Wheeler, 1999; Giribet, 2003b). Therefore, we present the results of all analyses in several ways, including the strict consensus of trees from all parameter sets, the tree under equal weights, the tree with the highest jackknife support, and Navajo rugs illustrating some of the most important relationships obtained.

An implied alignment (Wheeler, 2003; Giribet, 2005) was generated for parameter set 211 (gap cost = 2; transversion = 1; tran-

Table 3. Species employed in this study and GenBank accession numbers for the sequences utilized. Asterisks indicate new sequences. ¹*Echinoderes aquilonius* has two GenBank accession numbers because the sequences were separately submitted in two non-overlapping fragments.

Phylum	Species	GenBank accession no.
Chaetognatha	<i>Sagitta</i> sp.	AY922316*
Loricifera	<i>Pliciloricus</i> sp.	AY746986*
Kinorhyncha	<i>Zelinkaderes</i> sp.	AY746985*
	<i>Pycnophyes greenlandicus</i>	AY428820
	<i>Echinoderes aquilonius</i> ¹	AY922317*, AY922318*
Nematomorpha	<i>Nectonema agile</i>	AF421767
	<i>Chordodes morganii</i>	AF036639
	<i>Gordius albopunctatus</i>	U88337
	<i>Paragordius</i> sp.	AY428819
Priapulida	<i>Meiopriapulus</i> sp.	AY746984*
	<i>Halicyptus spinulosus</i>	AF342790
	<i>Priapulus caudatus</i>	AF025927
	<i>Tubiluchus corallicola</i>	AF119086
Nematoda	<i>Trichinella spiralis</i>	U60231
	<i>Plectus</i> sp.	U61761
	<i>Ascaris suum</i>	U94367
	<i>Paracanthorhynchus caecus</i>	AF047888
	<i>Desmodora ovigera</i>	Y16913
	<i>Diplogaster lethieri</i>	AF036643
	<i>Daptonema procerus</i>	AF047889
	<i>Myrionchulus arenicolus</i>	AF036596
	<i>Brumptiaemilus justini</i>	AF036589
	<i>Brugia malayi</i>	AF036588
	<i>Caenorhabditis sonoroae</i>	AF083026
	<i>Meloidogyne incognita</i>	U81578
Onychophora	<i>Peripatoides novaezealandiae</i>	AF342794
	<i>Peripatopsis capensis</i>	AF119087
	<i>Euperipatoides leuckarti</i>	U49910
Tardigrada	<i>Thulinia stephaniae</i>	AF056023
	<i>Hypsibius</i> sp.	Z93337
	<i>Macrobiotus hufelandi</i>	X81442
	<i>Mimesium tardigradum</i>	U49909
	<i>Echiniscus viridissimus</i>	AF056024
Arthropoda	<i>Colossendeis</i> sp.	AF005440
	<i>Endeis laevis</i>	AF005441
	<i>Limulus polyphemus</i>	U91490
	<i>Belisarius xambeui</i>	AF005442
	<i>Nipponopsalis abei</i>	AF124948
	<i>Atrax</i> sp.	AF370784
	<i>Mastigoproctus giganteus</i>	AF005446
	<i>Scutigera coleoptrata</i>	AF173238
	<i>Lithobius obscurus</i>	AF334271
	<i>Scolopendra cingulata</i>	U29493
	<i>Polyxenus fasciculatus</i>	AF173235
	<i>Proteronius fuscus</i>	AF173236
	<i>Hutchinsoniella macracantha</i>	AF370801
	<i>Artemia</i> sp.	X01723
	<i>Triops longicaudatus</i>	AF144219
	<i>Nebalia</i> sp.	U81945
	<i>Allomachilis froggatti</i>	AF370788
	<i>Tricholepidion gertschi</i>	AF370789
	<i>Thermobia domestica</i>	AF370790
	<i>Calibaetis ferrugineus</i>	AF370791
	<i>ferrugineus</i>	
	<i>Locusta migratoria</i>	AF370793

sition = 1), which resulted in the highest overall jackknife support. This implied alignment was used for the Bayesian analysis.

Due to the instability of the position of Loricifera in the direct

optimization approach—in part due to the uniqueness of the loriferan (and other ecdysozoan) sequences, which are often referred to as “long branches”—we analyzed the data using a model-based approach. A Bayesian approach was selected due to its efficiency in searching tree space, although we are aware of several of the

problems with this method, including overestimation of support when the model is unrealistic (Suzuki et al., 2002) and the problem of using flat priors (Simmons et al., 2004). The Bayesian approach also allows a more direct comparison to the results of Mallatt et al. (2004), who studied ecdysozoan relationships using 18S rRNA and

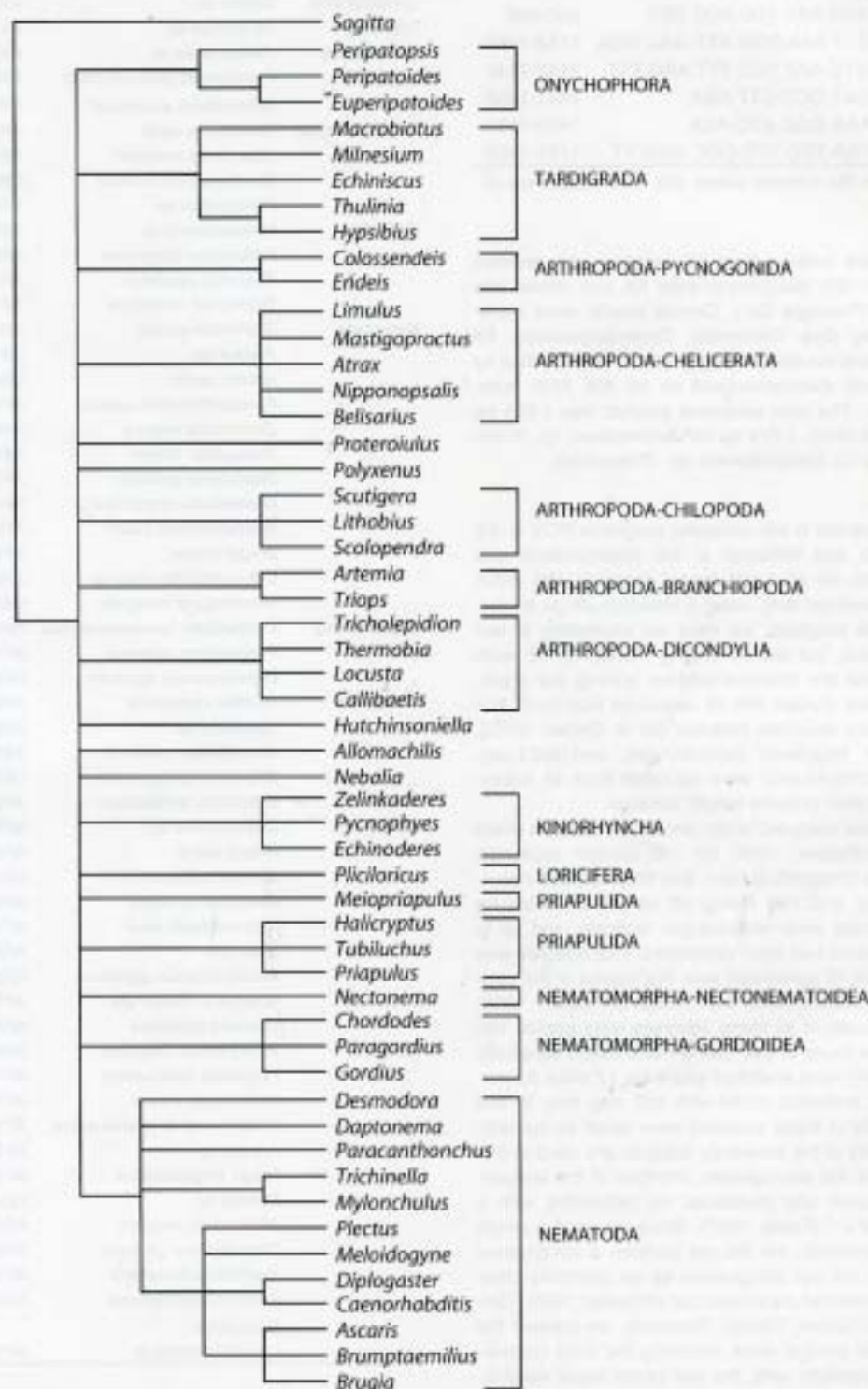


Fig. 2. Strict consensus of all trees obtained under the 12 parameter sets for which the data were analyzed (parsimony analysis under direct optimization).

28S rRNA sequence data.

In order to estimate the best-fit substitution model for this alignment, we employed the Akaike information criterion using Modeltest version 3.6 (Posada and Crandall, 1998). We utilized the estimated best-fit model for a Bayesian estimate of phylogeny implemented in MrBayes 2.01 (Huelsenbeck and Ronquist, 2003). Four Markov Chain Monte Carlo (MCMC) chains were run for 10^6 generations

and sampled every 100 generations. The stationary phase of the chains was achieved after the first 10^5 generations, and thus 1,000 trees were discarded. Bayesian posterior probabilities were given as the percentages of samples recovering a particular clade. We accepted a clade in the Bayesian tree at around 95% posterior probability (Murphy et al., 2001; Mallatt et al., 2004).

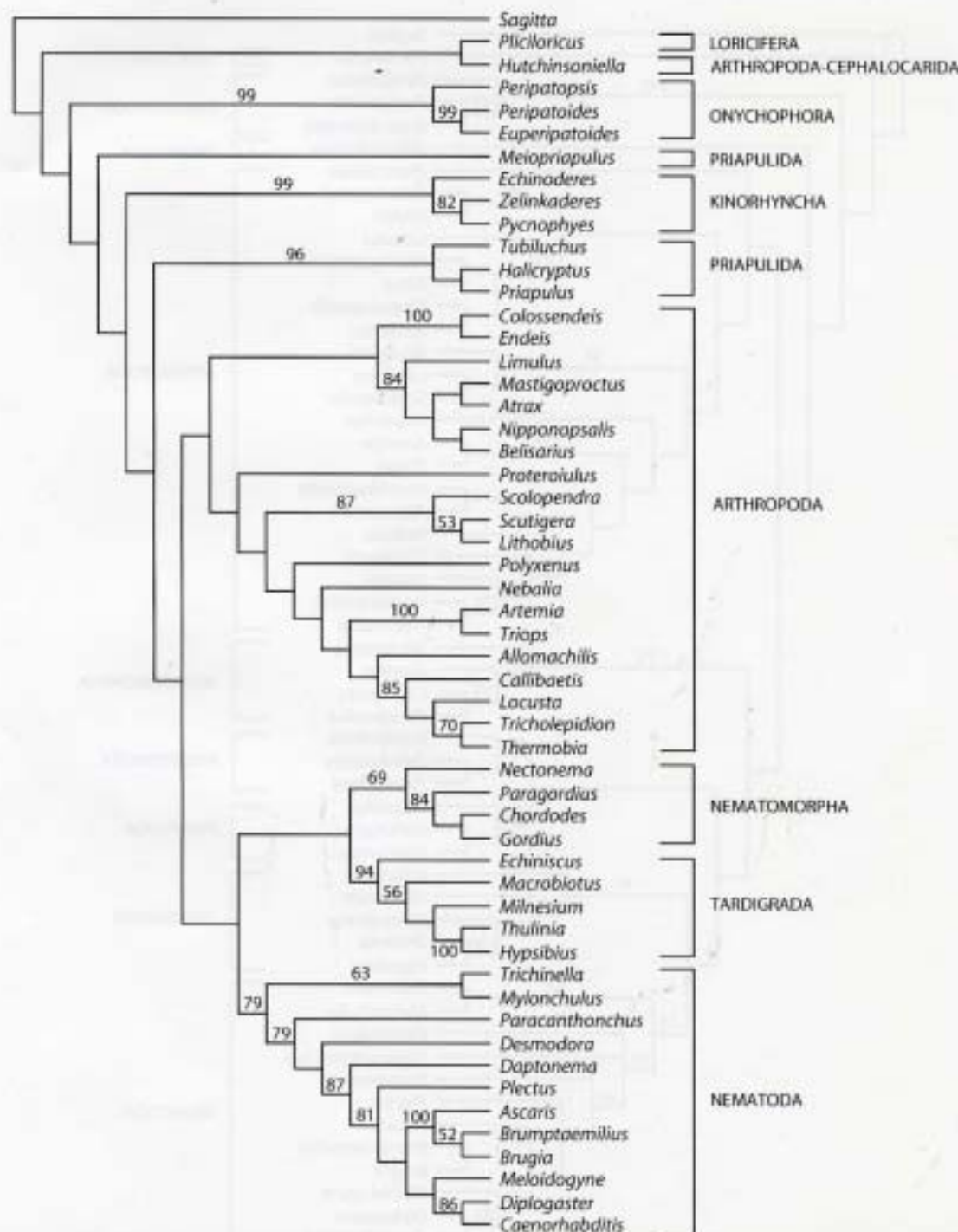


Fig. 3. Single most parsimonious tree obtained with parameter set 111 (equal weights; length = 4,767 steps). Numbers above branches show nodal support measured with jackknifing resampling.

RESULTS AND DISCUSSION

Few scalidophorans were previously known from shallow waters of Korea, perhaps due to the little taxonomic work conducted in this region. The loriferan species we found is clearly a new species of the genus *Pliciloricus* (Fig. 1A), closely related to the Atlantic species *P. dubius* found

at 294 m depth (Higgins and Kristensen, 1986).

The kinorhynch genus *Zelinkaderes* Higgins, 1990 was originally described from sandy mud off the coast of Fort Pierce, Florida (Higgins, 1990). The type species, *Z. floridensis*, and a second species, *Z. submersus* (formerly *Cateria submersa* Gerlach, 1969), were included in the new family Zelinkaderidae. The *Zelinkaderes* specimen we used

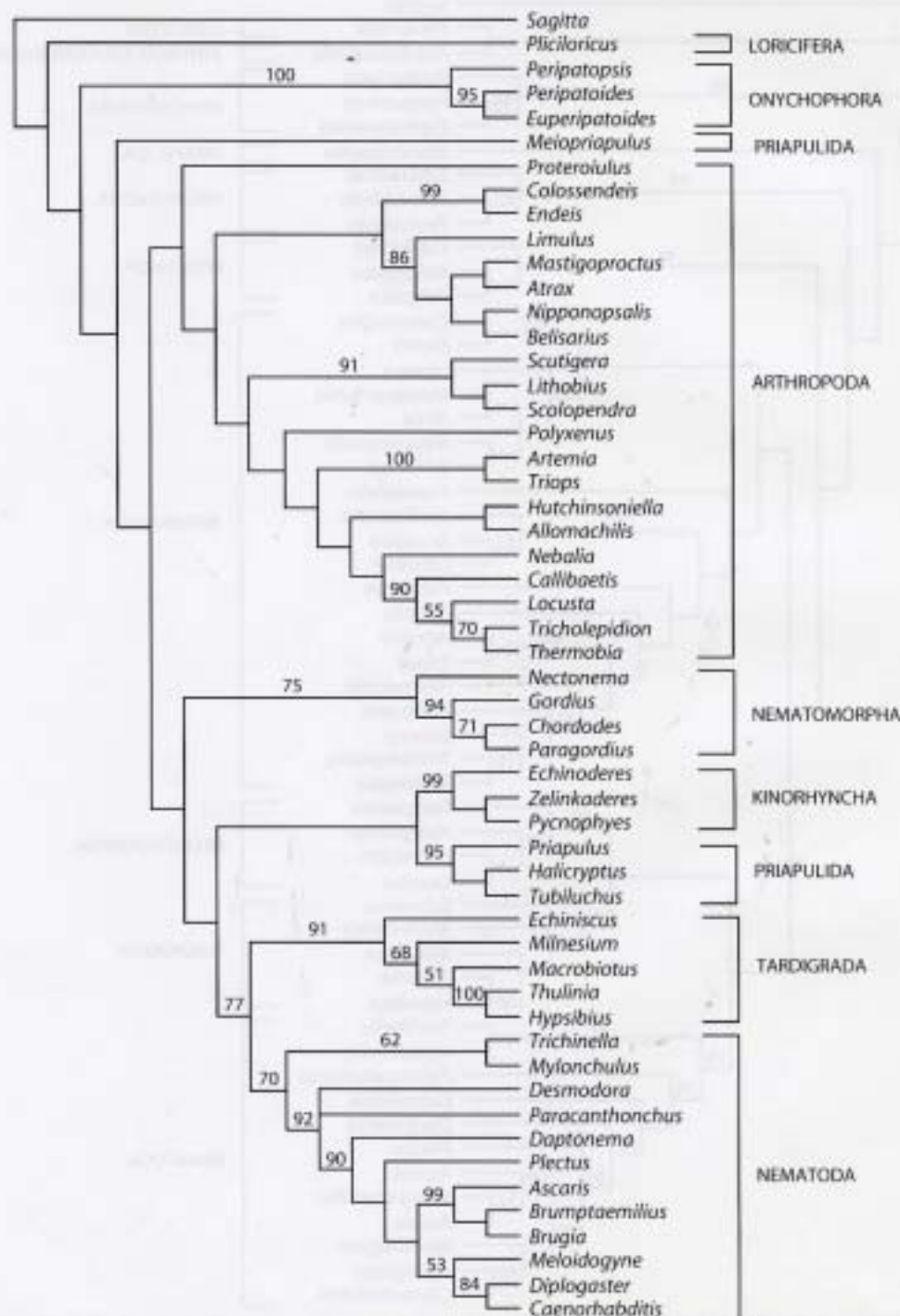


Fig. 4. Strict consensus of the two most parsimonious trees obtained with parameter set 211 (gaps cost was double that of transversion or transition cost). Length = 5,142 weighted steps. Numbers above branches show nodal support measured with jackknifing resampling. The implied sequence alignment from one of these trees was employed for the Bayesian analysis.

for sequencing clearly differed from these other two species (Fig. 1B). It is characterized by having (1) an extremely long midterminal spine (the ratio of midterminal spine length to trunk length expressed as percent (MTS/TL) is about 98%), (2) middorsal spines on segments 6, 8, 10–13, (3) cuspidate spines on segments 4, 7, 10, 11 and (4) small subdorsal spines on segment 12. The Korean specimen of *Zelinkadere* seems to be closely related to the type species, *Z. floridensis*.

The interstitial genus *Meiopriapulid* has until now remained monotypic, with the single species *M. fijiensis* Morse, 1981 known from its type locality, Korolevu Beach on Viti Levu, Fiji Islands (R.M.K. collected specimens of *M. fijiensis* from this coral beach in 1996) and from several specimens from a coral reef on South Andaman Island, Andaman Sea (Westheide, 1990). The Korean specimen (Fig. 1C) used in our study is clearly a member of the genus *Meiopriapulid*; however, it may be a new species. Like *M. fijiensis*, it has a cylinder-shaped body without a tail, it has a circle of small anal spines posteriorly on the trunk, and the conical-shaped posterior end bears the terminal anus (cloaca). The bulbous anterior introvert (head) has the eight elongate clavoscalids (sensory scalids of the first row) typical of *M. fijiensis*. The mouth cone ("everted pharynx") bears pharyngeal teeth. The specimen is clearly a species of *Meiopriapulid* rather than of the only other interstitial genus of priapulid, *Tubiluchus*, members of which always have a long tail and the anus not terminal.

Direct Optimization analyses

Phylogenetic analysis of the sequence data under 12 parameter sets that varied indel/transversion ratios and transversion/transition ratios resulted in trees fundamentally different in the relationships among the phyla represented. The strict consensus of all trees found—the most conservative estimate of the phylogeny—is presented in Fig. 2, which indicates the taxa that are monophyletic under all analytical conditions. Table 4 lists the number and weighted steps of the trees obtained for each parameter set. Jackknife support values greater than 50% for all parameter sets are listed in Table 5.

Analyses conducted with different parameter sets (and

Table 4. Number of weighted steps (Length) and number of trees (N trees) found for each parameter set (Par) explored using tree fusing and SATF in the search strategy described.

Par	Length	N trees
110	2232	3
111	4767	1
121	6856	2
141	10702	1
210	2557	6
211	5142	2
221	7516	2
241	12067	1
410	2990	16
411	5616	3
421	8412	1
441	13828	1

jackknifing analyses) were congruent in showing monophyly of most animal phyla under most analytical conditions. The phyla Onychophora, Tardigrada, Kinorhyncha, and Nematoda were monophyletic in all analyses (Fig. 2), even though jackknife support was less than 50% under some analytical parameters. Support tended to be especially low for nematodes (Table 5). Monophyly of Nematomorpha was found under several parameter sets (e.g., Figs. 3, 4) but was parameter-dependent (Fig. 5). Support for this node greater than 50% was only found under three analytical parameter sets (Table 5). Monophyly of Arthropoda was also found under some but not all parameter sets (Fig. 5), and never received jackknife support above 50%, in contrast to studies that included more taxa and more loci (e.g., Mallatt and Giribet, 2006). Arthropod monophyly was also parameter-dependent (Figs. 3–5), because under certain parameter sets the cephalocarid *Hutchinsoniella* was placed near the root of the tree. The only phylum that was not monophyletic under any conditions was Priapulida. *Meiopriapulid*, the only priapulid genus with a true coelom (Storch et al., 1989) and direct development (Higgins and Storch, 1991), clustered outside the priapulids that have a loricate larva. The clade of pseudocoelomate priapulids was found under all analytical conditions (Fig. 2), with jackknife support values of up to 95% (Table 5). The data also showed resolution within most of the phyla included. Several internal clades were recovered under most analytical conditions (see Fig. 2) and received jackknife support values above 50% for at least some parameter sets (Table 5, Figs. 3, 4). In summary, our 18S rRNA analyses across a range of selected parameter sets supported relationships both among animal phyla and at lower taxonomic ranks, as shown in the stability and jackknife support of many clades.

Clades contradicting morphological hypotheses, although frequent in several trees, did not receive support values much higher than 50%. In Table 5, we have labeled with an asterisk all clades strictly contradicted by morphology. A clade supporting the monophyly of *Scutigera* and *Lithobius* was found under parameter sets 111 (jackknife support 53%) and 241 (jackknife support 66%), and under parameter sets 110, 121, 221, 241, and 421 with jackknife support below 50% (but see Edgecombe and Giribet, 2004; Giribet and Edgecombe, 2006). Another surprising clade composed of *Zygentoma* and the orthopteran *Locusta* was found under parameter set 211, with 55% jackknife support.

We could conclude little with respect to the relationships among the ecdysozoan phyla, especially the position of Loricifera, due to lack of support from the data here analyzed. No relationship among two animal phyla received jackknife support above 50% under a single parameter set. The relationships among selected phyla are illustrated in Fig. 5. For example, Onychophora and Tardigrada form a clade under five parameter sets; the pseudocoelomate priapulids form a clade with Kinorhyncha under five parameter sets; Tardigrada and Nematoda form a clade under three parameter sets; and Nematoida (= Nematoda + Nematomorpha) appears only under one analytical condition. This finding may support the idea of Malakhov (1980), that Nematoda and Nematomorpha are not sister groups, but that Nematomorpha is more closely related to Priapulida and Kinorhyncha (the so-called Cephalorhyncha); this contra-

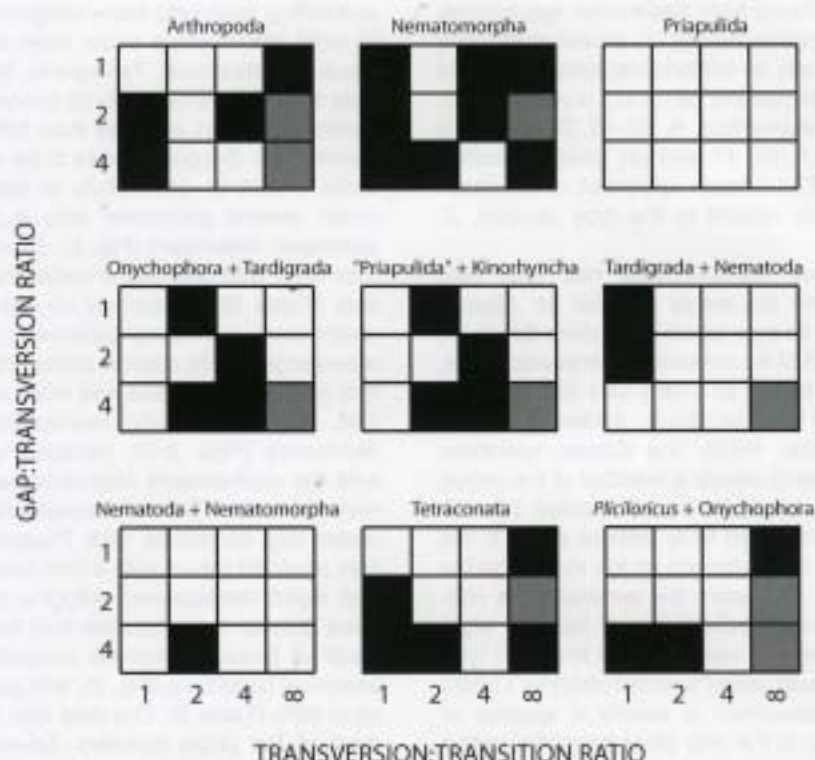


Fig. 5. Navajo rugs showing monophyly (black squares), non-monophyly (white squares), or monophyly under some of the optimal trees (gray squares) for selected clades under different analytical parameters. "Priapulida" refers to the members of the phylum Priapulida exclusive of *Meiopriapulidus*.

Table 5. Jackknife resampling values for nodes obtained under different parameter sets (110 to 441). Nodes with less than 50% support for all parameter sets have been excluded from the table.

Phylum	Node	110	111	121	141	210	211	221	241	410	411	421	441
Onychophora	Onychophora	99	99	100	91	95	100	95			85		98
	<i>Paripatoides</i> + <i>Euperipatoides</i>	98	99	89		80	95	61			57		84
Tardigrada	Tardigrada	87	94	94	55	78	91	85		53	84	53	84
	Eutardigrada	65	56	66	52	64	68	79			63	72	61
Arthropoda	<i>Thulinia</i> + <i>Hypsibius</i>	97	100	100	90	97	100	99	68	89	100	95	93
	Pycnogonida	95	100	98	85	87	99	89			81		87
	Chelicerata		84	68			86	67			68		
	Chilopoda	60	87	82	57	68	91	85		61	83	70	81
	<i>Scutigera</i> + <i>Lithobius</i> *		53						66				
	Branchiopoda	93	100	98	76	92	100	97	76	80	99	92	97
	Dicondylia	58	85	73		61	90	78			82	53	73
	<i>Locusta</i> + <i>Zygentoma</i> *						55						
Priapula	Zygentoma		70				70						
	Priapula (excluding <i>Meiopriapulidus</i>)	75		93	60	78	95	93	76	81	95	92	89
Nematoda	Priapulidae								53			61	55
	Nematoda		79	65			77	55					
	<i>Trichinella</i> + <i>Myonchulus</i>			75			62	59					
	Nematoda (minus <i>Trichinella-Myonchulus</i>)	75	79	74			70	55					
	<i>Diplogaster</i> + <i>Caenorhabditis</i>	51	86	78		53	84	71			64	53	
	<i>Ascaris</i> + <i>Brugia</i> + <i>Brumptsemitius</i>	80	100	97	72	81	99	94	52	56	97	76	79
	7 nematode-clade	73	81	81		77	90	75			62		65
	7-clade + <i>Daptonema</i>		87	72			92	75			66		
Kinorhyncha	Kinorhyncha	88	99	96	59	83	99	97			93	70	86
	<i>Zelinkaderes</i> + <i>Pycnophyes</i>		82	70				54			51		
Nematomorpha	Nematomorpha		69				75				58		
	Gordiidae	94	84	90	67	92	94	94		79	84	85	89
	<i>Chordodes</i> + <i>Paragordius</i>	64		53		70	71	65		68	62	63	70

dicts Schmidt-Rhaesa (1997/98). Malakhov and co-workers later also included Loricifera in their Cephalorhyncha concept (for a review, see Andrianov and Malakhov (1999)).

The position of Loricifera is especially intriguing. None of the analyses suggested a position of Loricifera within Priapulida, rejecting Wärrick's (2000) idea that loriciferans are

paedomorphic priapulids. Five out of 12 parameter sets identified a possible relationship to Onychophora (Fig. 5). Another parameter set identified the loriciferan species as a sister group to the cephalocarid crustacean *Hutchinsoniella* (Fig. 3). Two parameter sets clustered the loriciferan with the marine nematode *Nectonema*, contradicting the sev-

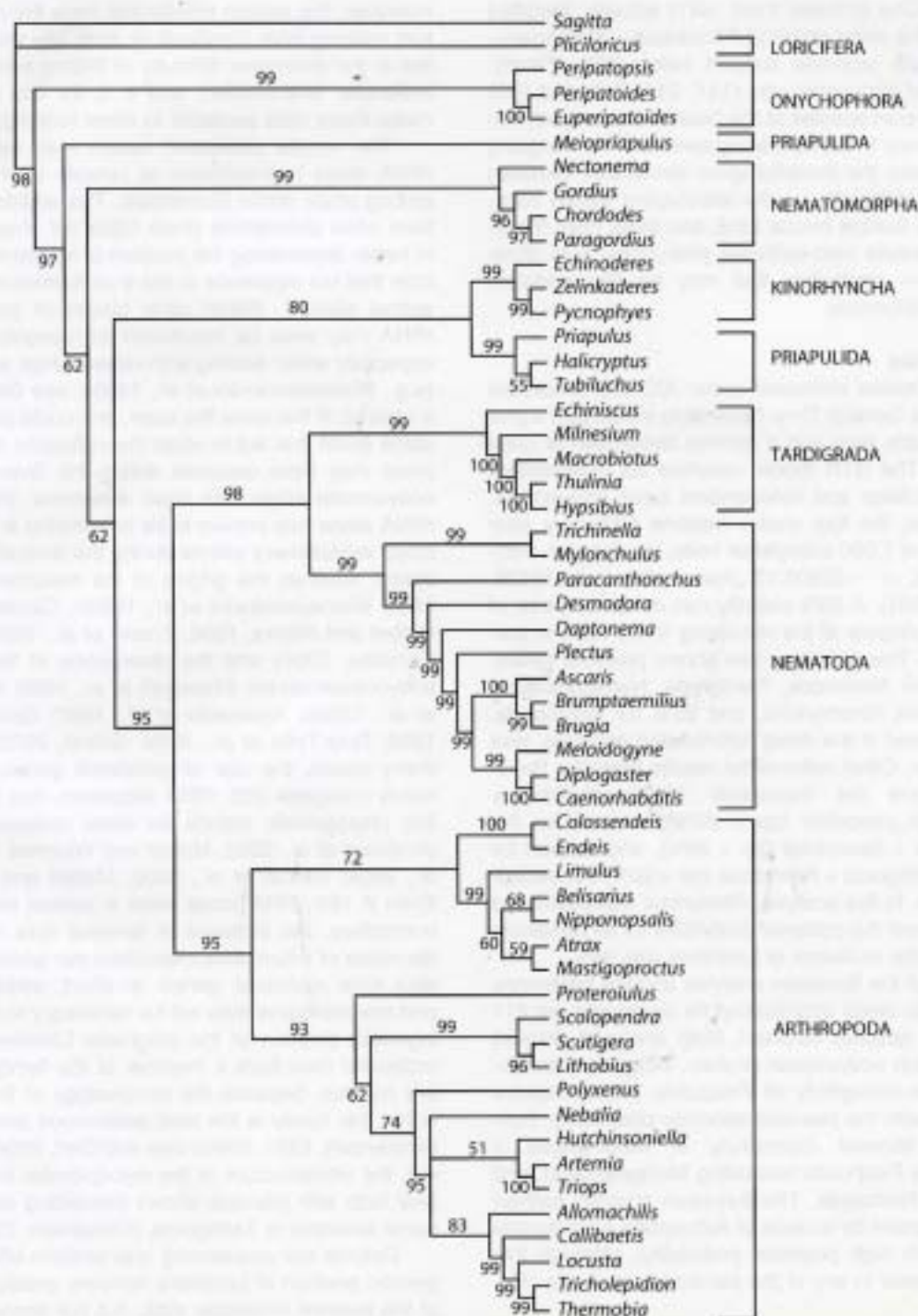


Fig. 6. Bayesian tree representing the 50% majority-rule consensus of trees obtained using the implied sequence alignment generated under parameter set 211 in the direct optimization analysis. The consensus tree was obtained under model GTR+I+ Γ , and the analysis was run for 10^6 generations with four chains and a sampling frequency of 1% (burn-in asymptote, 1,000 trees). Numbers above branches indicate posterior probabilities.

eral parameter sets that indicated monophyly of the Nematomorpha. This is not that surprising, since Kristensen (1983) noted the morphological similarities between the marine nematode larva of *Nectonema munidae* and adult *Nanalaricus mysticus* in his original description of the phylum Loricifera. Yet two other parameter sets (441 and 410) placed the loriciferan with the coelomate priapulid *Meiopriapulid*. One of these trees (441) actually identified *Nectonema* as the sister group of *Pliciloricus* + *Meiopriapulid*, although with jackknife support below 50%. Finally, another series of parameter sets (141, 211, 241, and 210) placed the loriciferan species at the base of the ecdysozoan tree, as sister group to the remaining species. This intriguing result may explain the morphological similarities between Loricifera and Tardigrada: — the telescoping mouth cone, annulation of the flexible buccal tube, and three rows of placoids in the triradial myo-epithelial pharyngeal bulb (Kristensen, 2003) — similarities that may be plesiomorphic characters for Ecdysozoa.

Bayesian analysis

The best-fit model estimated under AIC with Modeltest version 3.6 was a General Time-Reversible model with a proportion of invariable sites and a gamma distribution of rates (GTR + I + Γ). The GTR model assumes six independent transition probabilities and independent base frequencies. Under this model, the four chains became stationary after removal of the first 1,000 suboptimal trees, reaching an arithmetic mean $-\ln L = -22301.12$ (mean $\ln L = 0.218425$, mean $\alpha = 0.615691$). A 50% majority-rule consensus tree of the Bayesian phylogeny of the remaining 9,000 trees is presented in Fig. 6. This summary tree shows posterior probabilities of 99% for Nematoda, Tardigrada, Nematomorpha, Onychophora, and Kinorhyncha, and 95% for Arthropoda. Priapulida, as found in the direct optimization analyses, was not monophyletic. Other noteworthy results from the Bayesian analysis are the monophyly of Kinorhyncha + pseudocoelomate priapulids (pp = 80%), support for the clade Tardigrada + Nematoda (pp = 98%), and support for Arthropoda + Tardigrada + Nematoda (pp = 95%) but excluding Onychophora. In this analysis, *Pliciloricus* was positioned next to the root, and the posterior probability for all remaining ecdysozoans to the exclusion of Loricifera was 98%.

The results of the Bayesian analysis and the parsimony analysis based on direct optimization for parameter set 211 (Fig. 4) are not radically different. Both analyses showed monophyly of each ecdysozoan phylum, including Nematomorpha, but non-monophyly of Priapulida (*Meiopriapulid* does not group with the pseudocoelomate priapulids). Both analyses also showed monophyly of Kinorhyncha + pseudocoelomate Priapulida (excluding *Meiopriapulid*), and of Tardigrada + Nematoda. The Bayesian analysis furthermore showed support for a clade of Arthropoda + Nematoda + Tardigrada with high posterior probability, although this result did not appear in any of the parsimony analyses (Fig. 6).

CONCLUSIONS

The loriciferan species was highly unstable in phylogenetic position and tended to group with taxa having aberrant sequences, such as onychophorans and cephalocarids, or

with "misplaced" members of other phyla, such as *Meiopriapulid* or *Nectonema*, when these did not cluster with other members of their respective phyla. None of these results was significantly more stable than others, nor showed jackknife support or significant posterior probabilities. Due to the high degree of autapomorphy of the loriciferan sequence, little can be concluded until more species are sequenced. However, the reason loriciferans were the only animal phylum missing from GenBank or from any molecular analysis lies in the enormous difficulty of finding animals suitable for molecular examination, and it is for this reason that we make these data available to other investigators.

The results presented herein may suggest that 18S rRNA alone is insufficient to reliably explain relationships among phyla within Ecdysozoa. The addition of sequences from other protostome phyla (data not shown) did not help in better determining the position of loriciferans, but did indicate that our sequence is not a contaminant from any other animal phylum. Within other clades of protostomes, 18S rRNA may even be insufficient to recognize animal phyla, especially when dealing with relationships among spiralian (e.g., Winnepenninckx et al., 1995a; see Giribet (2002a) for a review). If this were the case, one could postulate that the same event that led to rapid diversification of spiralian body plans may have occurred during the diversification of the ecdysozoan phyla. We must remember, though, that 18S rRNA alone has proven to be informative in resolving some major evolutionary events during the diversification of metazoans, such as the origins of the mesoderm (Field et al., 1988; Winnepenninckx et al., 1995b; Carranza et al., 1997; Giribet and Ribera, 1998; Zrzavý et al., 1998; Peterson and Eernisse, 2001) and the divergence of the spiralian and ecdysozoan clades (Halanych et al., 1995; Winnepenninckx et al., 1995b; Aguinaldo et al., 1997; Giribet and Ribera, 1998; Ruiz-Trillo et al., 1999; Giribet, 2002b). However, in many cases, the use of additional genes, especially the nearly complete 28S rRNA sequence, has helped to stabilize phylogenetic results for deep metazoan splits (e.g., Jondelius et al., 2002; Mallatt and Winchell, 2002; Mallatt et al., 2004; Giribet et al., 2006; Mallatt and Giribet, 2006). Even if 18S rRNA alone were a perfect marker devoid of homoplasy, the increase in terminal taxa may result in a decrease of informative characters per taxon, thus requiring data from additional genes. In short, additional molecular and morphological data will be necessary to resolve the phylogenetic position of the enigmatic Loricifera. Additionally, molecular data from a member of the family Nanalaricidae are needed, because the morphology of the three genera within this family is the best understood among loriciferans (Kristensen, 1991; Kristensen and Gad, 2004). In these genera, the ultrastructure of the myoepithelial triradial pharyngeal bulb with placoids shows interesting similarities to the same structure in Tardigrada (Kristensen, 2003).

Despite our sequencing and analytic efforts, the phylogenetic position of Loricifera remains unstable on the basis of the present molecular data, but our analyses serve as a first test of the monophyly of Scalidophora using molecular data. The new analyses also provide the first comprehensive molecular test for the monophyly of all ecdysozoan phyla. The data strongly suggest non-monophyly of Priapulida, which is divided into two clades, one including

pseudocoelomate species with a loricate larva and another containing the single coelomate species without a loricate larva. After all, this fundamental anatomical difference—the presence of a true coelomic cavity in *Meiopriapulidus* but not in any other priapulid genera—together with the difference in development, may indicate the polyphyly of the phylum Priapulida. Further study of the members of this phylum should shed light on this challenging hypothesis.

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