

## Phylogenetic Relationships of Acanthocephala Based on Analysis of 18S Ribosomal RNA Gene Sequences

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**Abstract.** Acanthocephala (thorny-headed worms) is a phylum of endoparasites of vertebrates and arthropods, included among the most phylogenetically basal triploblastic pseudocoelomates. The phylum is divided into three classes: Archiacanthocephala, Palaeacanthocephala, and Eoacanthocephala. These classes are distinguished by morphological characters such as location of lacunar canals, persistence of ligament sacs in females, number and type of cement glands in males, number and size of proboscis hooks, host taxonomy, and ecology. To understand better the phylogenetic relationships within Acanthocephala, and between Acanthocephala and Rotifera, we sequenced the nearly complete 18S rRNA genes of nine species from the three classes of Acanthocephala and four species of Rotifera from the classes Bdelloidea and Monogononta. Phylogenetic relationships were inferred by maximum-likelihood analyses of these new sequences and others previously determined. The analyses showed that Acanthocephala is the sister group to a clade including Eoacanthocephala and Palaeacanthocephala. Archiacanthocephala exhibited a slower rate of evolution at the nucleotide level, as evidenced by shorter branch lengths for the group. We found statistically significant support for the monophyly of Rotifera, represented in our analysis by species from the clade

Eurotatoria, which includes the classes Bdelloidea and Monogononta. Eurotatoria also appears as the sister group to Acanthocephala.

**Key words:** Acanthocephala — Archiacanthocephala — Eoacanthocephala — Palaeacanthocephala — Rotifera — Eurotatoria — Bdelloidea — Monogononta — 18S rRNA — Phylogeny

### Introduction

The phylum Acanthocephala includes approximately 1150 described species with indirect life cycles always involving arthropods as intermediate hosts and vertebrates as definitive hosts (Nickol and Crompton 1985). The phylum is divided into three classes—Archiacanthocephala, Palaeacanthocephala, and Eoacanthocephala—although a fourth class, Polyacanthocephala, has recently been proposed (Amin 1987). The classification of the phylum is based mostly on the location of the lacunar canals, the persistence of ligament sacs in females, the number and type of cement glands in males, the number and size of proboscis hooks, and the host taxonomy and ecology (Bullock 1969; Amin 1985).

Acanthocephala has been considered to be related to a number of other phyla, including Rotifera, Nematoda, Nematomorpha, Gastrotricha, Kinorhyncha, and Priapulida. Together, these phyla of pseudocoelomate organ-

isms are usually known as Aschelminthes (Hyman 1951; Marcus 1958; Clark 1979; Brusca and Brusca 1990; Rupert and Barnes 1994). However, a recent phylogenetic analysis using 18S rRNA gene sequences provided support for the hypothesis that Aschelminthes is polyphyletic and consists of at least three distinct clades (Winnepeninckx et al. 1995). Based on four synapomorphies derived from a morphological study, it has been suggested that Acanthocephala and Rotifera represent a monophyletic group (Lorenzen 1985). It has also been proposed that Acanthocephala is sister to the class Bdelloidea (Rotifera) and, hence, that Rotifera is paraphyletic based on analysis of 18S rRNA gene sequences (Garey et al. 1996, 1998). Finally, a recent study based on a protein-coding gene also suggests that acanthocephalans are rotifers (Mark Welch 2000), although the sisterhood between Acanthocephala and Bdelloidea is not supported.

We sequenced the near-complete 18S rRNA gene from nine species of Acanthocephala, from the classes Archiacanthocephala, Palaecanthocephala, of Eoacanthocephala, and four species of Rotifera, from the classes Bdelloidea and Monogononta. These two classes are generally included in a clade designated Eurotaroria (De Ridder 1956; Wallace and Colburn 1989; Melone et al. 1998; Mark Welch 2000). Maximum-likelihood analysis of sequences from these taxa, and others obtained from GenBank, including 12 Acanthocephalan species, 2 Rotifera species, and 10 species from other phyla, was conducted. Several phylogenetic hypotheses were tested: (1) Acanthocephala and Rotifera are monophyletic (Acanthocephala (Bdelloidea, Monogononta)); (2) Acanthocephala is the sister group to Bdelloidea and composes a clade within Rotifera, ((Acanthocephala, Bdelloidea) Monogononta); and (3) possible relationships among the three classes of Acanthocephala examined, ((Palaecanthocephala, Eoacanthocephala) Archiacanthocephala), (Palaecanthocephala (Eoacanthocephala, Archiacanthocephala)), and ((Palaecanthocephala, Archiacanthocephala) Eoacanthocephala).

## Materials and Methods

### Samples

Taxa used in this study are listed in Table 1. Acanthocephala species *Floridosentis mugilis*, *Oligacanthorhynchus tortuosa*, *Oncicola* sp., *Mediorhynchus* sp., *Centrorhynchus microcephalus*, *Filisoma bucerium*, *Arhythmorhynchus brevis*, *Koronacantha pectinaria*, and *Poly-morphus* sp. were collected from their naturally infected vertebrate hosts, washed three times in saline solution, and preserved in absolute ethanol at 4°C. These worms were stained in Mayer's paracarmine, mounted in Canada balsam, and identified using conventional morphological criteria. Two-liter cultures of the rotifers *Asplanchna sieboldi*, *Brachionus platus*, *Lecane bulba*, and *Philodina roseola* were sieved, exhaustively washed in sterile-distilled water, and concentrated to 500  $\mu$ l.

### DNA Manipulations

Genomic DNA was extracted by digestion of specimens with proteinase K (10 mg/ml) in a buffer that contained 200 mM NaOH and 1% SDS and incubated for 2 h at 50°C, followed by standard phenol/chloroform extraction and ethanol precipitation. The near-complete 18S rRNA genes were amplified from all genomic DNA samples by the polymerase chain reaction (PCR) using primers that anneal to conserved regions at both ends of the gene: forward, 5'-AGATTAAGCC-ATGCATGCGT-3'; and reverse, 5'-GCAGGTTACACTACGGAAA-3' (Garey et al. 1996). Each reaction mixture contained 200 ng of genomic DNA, a 200 mM concentration of each dNTP, 100 pmol of each primer, 10 mM Tris-HCl, 50 mM KCl, 3 mM MgCl<sub>2</sub> (final volume, 100  $\mu$ l), and 2 U of Taq polymerase. Thermal cycling used an initial denaturation period of 3 min at 94°C, annealing at 50°C for 3 min, and extension at 72°C for 3 min, followed by another 35 cycles of 1 min at the same denaturation, annealing, and extension temperatures. The PCR products were evaluated in 1% agarose gels, purified following the method described by Vogelstein and Gillespie (1979), and resuspended in 20  $\mu$ l of sterile-distilled water. The PCR products were blunt-end ligated into M13mp19. After transformation of TG1 cells, two positive clones from each taxon containing inserts in opposite orientation were selected for sequencing. The sequencing of inserts in both directions was carried out by the method of chain termination by dideoxynucleotides (Sanger et al. 1977) using M13 primers or primers annealing in conserved internal sequences and <sup>35</sup>S-dATP as radionucleotide.

### Data Analysis

In addition to the 18S ribosomal gene sequences from 9 species of Acanthocephala and 4 species of Rotifera generated for this work, another 12 sequences of Acanthocephala and of 8 outgroup taxa were obtained from GenBank (Table 1). The sequences were initially aligned using the Clustal algorithm (Higgins and Sharp 1988) in DNAMAN (Lynnon BioSoft 1994-97) and then adjusted by eye. The alignment is available from the corresponding author upon request. Phylogenetic relationships were determined by maximum-likelihood analyses (Felsenstein 1981) using the program PAUP\* 4.0d65 (Swofford 1999). The tree searching and model fitting were done in successive steps to increase efficiency. An initial tree was generated by neighbor-joining (Saitou and Nei 1987) with proportional distances and other default settings. A likelihood model was fitted to this initial tree to obtain parameters for the next round of searching. The fitted model included estimates of rates for six nucleotide substitution types, nucleotide frequencies, and the proportion of invariant sites. Rates of substitution at variable sites were assumed to follow a gamma distribution of four rate categories, with the average rate for each category represented by the mean. The shape parameter of the gamma distribution,  $\alpha$ , was estimated from the data. This likelihood model is a general time-reversible model (Yang 1994) with rate heterogeneity. A single heuristic search was conducted using the model parameters estimated from the initial neighbor-joining tree with random sequence addition and tree bisection-reconnection branch swapping to find an initial maximum-likelihood tree. The same model parameters were then reestimated and the parameter values were used in another maximum-likelihood heuristic search as conducted previously, but with five replicates. Likelihood model parameters were then reestimated on the final tree. To compare specific topologies representing specific phylogenetic hypotheses, constraints were defined and searches for maximum-likelihood trees were conducted using the model and heuristic search strategy described above with parameters estimated on the constraint tree. The difference between maximum-likelihood values for trees representing alternative hypotheses were evaluated using the test of Kishino and Hasegawa (1989) implemented in PAUP\*. Other PAUP\* program options, where not specifically mentioned, conform to default settings.

**Table 1.** Taxa examined

Taxon name	Accession No.	Length (bp), G + C (%)	Host	Reference
<b>Acanthocephala</b>				
<i>Arhythmorhynchus brevis</i>	AF064812	1784, 49	<i>Nycticorax nycticorax</i>	This study
<i>Centrorhynchus conspectus</i>	U41399	1750, 49	<i>Strix varia</i>	Garey et al. (1996)
<i>Centrorhynchus microcephalus</i>	AF064813	1735, 49	<i>Crotophaga sulcirostris</i>	This study
<i>Corynosoma enhydry</i>	AF001837	1747, 49	<i>Enhydra lutris</i>	Near et al. (1998)
<i>Echinorhynchus gadi</i>	U88335	1793, 47	<i>Gadus morrhua</i>	Aleshin et al. (1998)
<i>Filisoma bucerium</i>	AF064814	1744, 46	<i>Kyphosus elegans</i>	This study
<i>Floridosentis mugilis</i>	AF064811	1760, 46	<i>Mugil cephalus</i>	This study
<i>Leptorhynchoides thecathus</i>	AF001840	1758, 47	<i>Lepomis cyanellus</i>	Near et al. (1998)
<i>Koronacantha pectinaria</i>	AF092433	1761, 46	<i>Microlepidotus brevipinnis</i>	This study
<i>Macracanthorhynchus ingens</i>	AF001844	1765, 45	<i>Procyon lotor</i>	Near et al. (1998)
<i>Mediorhynchus</i> sp.	AF064816	1758, 45	<i>Casidix mexicanus</i>	This study
<i>Mediorhynchus grandis</i>	AF001843	1769, 45	<i>Sturnella magna</i>	Near et al. (1998)
<i>Moniliformis moniliformis</i>	Z19562	1769, 45	<i>Rattus rattus</i>	Telford and Holland (1993)
<i>Neoechinorhynchus pseudemydis</i>	U41400	1771, 46	<i>Trachemys scripta elegans</i>	Garey et al. (1996)
<i>Neoechinorhynchus crassus</i>	AF001842	1773, 47	<i>Catostomus commersoni</i>	Near et al. (1998)
<i>Oligacanthorhynchus tortuosa</i>	AF064817	1767, 45	<i>Didelphis virginiana</i>	This study
<i>Oncicola</i> sp.	AF064818	1755, 45	<i>Nasua narica</i>	This study
<i>Plagiorhynchus cylindraceus</i>	AF001839	1745, 48	<i>Armadillidium vulgare</i>	Near et al. (1998)
<i>Polymorphus</i> sp.	AF064815	1739, 48	<i>Anas platyrhynchos</i>	This study
<i>Polymorphus altmani</i>	AF001838	1745, 48	<i>Enhydra lutris</i>	Near et al. (1998)
<i>Pomphorhynchus bulbocollis</i>	AF001841	1761, 45	<i>Oncorhynchus mykiss</i>	Near et al. (1998)
<b>Rotifera</b>				
<i>Asplanchna sieboldi</i>	AF092434	1728, 46	Free-living	This study
<i>Brachionus plicatilis</i>	U29235	1736, 47	Free-living	Winnepenninckx et al. (1995)
<i>Brachionus platus</i>	AF154568	1745, 45	Free-living	This study
<i>Lecane bulba</i>	AF154566	1733, 47	Free-living	This study
<i>Philodina acuticornis</i>	U41281	1789, 43	Free-living	Garey et al. (1996)
<i>Philodina roseola</i>	AF154567	1747, 46	Free-living	This study
<b>Gastrotricha</b>				
<i>Lepidodermella squamata</i>	U29198	1742, 50	Free-living	Winnepenninckx et al. (1995)
<i>Chaetonotus</i> sp.	AJ001735	1814, 49	Free-living	Littlewood et al. (1998)
<b>Platyhelminthes</b>				
<i>Opisthorchis viverrini</i>	X55357	1992, 51	<i>Homo sapiens</i>	Korbsrisate et al. (1991)
<b>Annelida</b>				
<i>Lanice conchilega</i>	X79873	1809, 51	Free-living	Winnepenninckx et al. (1995)
<b>Nematoda</b>				
<i>Haemonchus placei</i>	L04154	1758, 47	Adult parasite	Zarlenga et al. (1994)
<i>Nematodirus battus</i>	U01230	1758, 47	Adult parasite	Zarlenga et al. (1994)
<b>Nematomorpha</b>				
<i>Gordius aquaticus</i>	X87985	1797, 47	Free-living	Winnepenninckx et al. (1995)
<b>Priapulida</b>				
<i>Priapulid caudatus</i>	X87984	1811, 49	Free-living	Winnepenninckx et al. (1995)
<b>Kinorhyncha</b>				
<i>Pycnophyes kielensis</i>	U67997	1806, 49	Free-living	Unpublished
<b>Arthropoda</b>				
<i>Artemia salina</i>	X01723	2020, 50	Free-living	Winnepenninckx et al. (1995)

Figures were prepared, in part, using the programs RETREE and DRAWGRAM from PHYLIP (Felsenstein 1999).

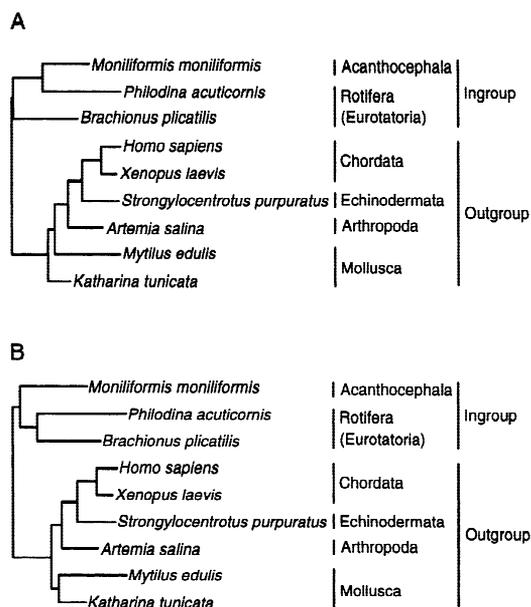
## Results

Near-complete 18S rRNA gene sequences were determined for nine species of Acanthocephala and four species of Rotifera (Table 1). The mean number of nucleotides determined for these sequences was 1756, with a mean proportion G + C of 0.465. Base composition

across all sequences was not significantly heterogeneous ( $\chi^2 = 116.588$ , 108 df,  $p = 0.269$ ).

### *Relationships of Acanthocephala to Rotifera*

The new sequences, together with others determined previously (Table 1), were aligned and analyzed to infer phylogenetic relationships. Initial analysis showed that Acanthocephala was monophyletic and sister to Rotifera, in contrast to recent analysis, where Acanthocephala appears to be part of Rotifera (Garey et al. 1998). To test if



**Fig. 1.** Phylogenetic trees for Acanthocephala, Eurotatoria, and outgroup taxa based on 16S rRNA gene sequence data of Garey et al. (1998). **A** This tree corresponds to the hypothesis that Eurotatoria is paraphyletic and Acanthocephala is sister to Eurotatoria class Bdelloidea, ((Acanthocephala, Bdelloidea) Monogononta). The  $-\ln$  likelihood value for this tree is 4382.478. **B** This tree is the result of a constraint that Eurotatoria is monophyletic, (Acanthocephala (Bdelloidea, Monogononta)). The  $-\ln$  likelihood value for this tree, 4384.405, is not significantly less likely ( $p = 0.642$ ) than the tree shown in A. In all trees the branch lengths are proportional to the inferred number of nucleotide substitutions.

the discrepancy was due to differences in the sequence alignments or in the analysis, four nucleotide sequence data sets/alignments were examined: 16S rRNA gene data from Garey et al. (1998), 18S rRNA gene data from Garey et al. (1996), our expanded 18S rRNA gene data using an alignment based on that of Garey et al. (1996), and our expanded 18S rRNA gene data using our own alignment. Both 16S rRNA and 18S rRNA gene data from Garey et al. (1996, 1998) were downloaded from: <http://chuma.cas.usf.edu/~garey/index.html>. The analytical results associated with each of these data sets are discussed in turn. For the description of results the use of Rotifera is maintained in the text, although, conclusions drawn are restricted to relationships among Acanthocephala and Eurotatoria.

**16S rRNA Gene Data of Garey et al. (1998).** This data set consisted of 9 taxa and 762 aligned nucleotide positions. All five searches resulted in the same maximum-likelihood tree (Fig. 1A), with a topology where Rotifera is paraphyletic, and therefore, Acanthocephala arises from within Rotifera. This tree also has Mollusca paraphyletic. A tree corresponding to the alternative hypothesis, that Rotifera is monophyletic, was generated using searches with a constraint corresponding to this alternative hypothesis. All five searches resulted in the same

**Table 2.** Relationships of Acanthocephala to Rotifera

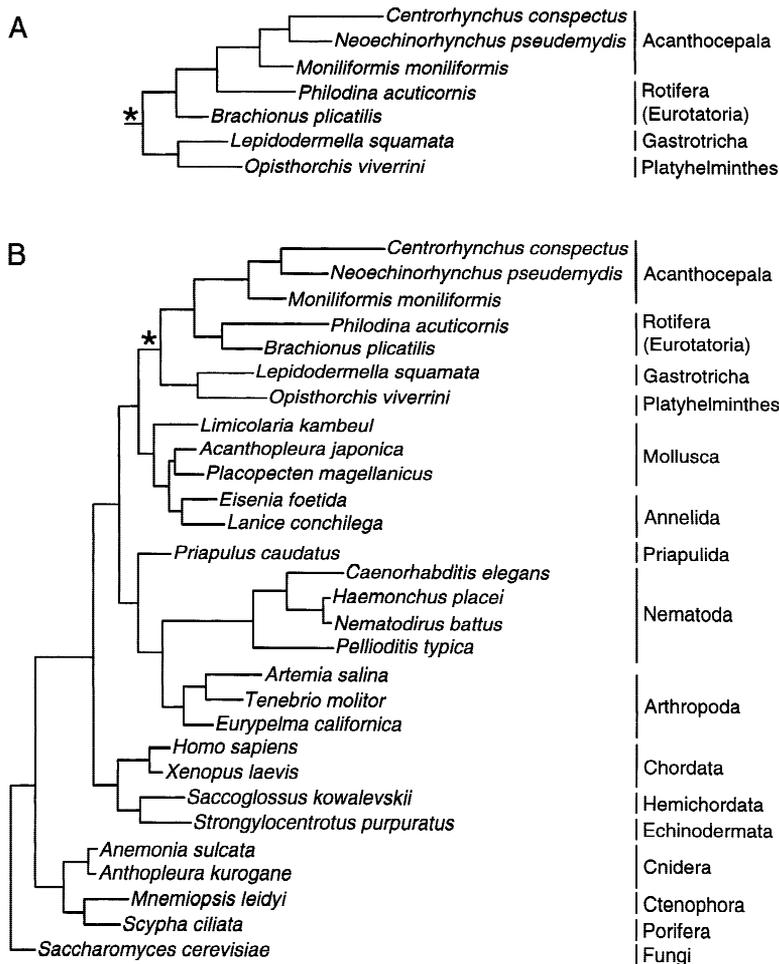
Sequence data sets/alignments	$-\ln$ likelihood <sup>a</sup>	Proportion of invariable sites	Gamma shape parameter
16S rRNA (Garey et al. 1999)			
Fig. 1A	4382.478 <sup>1</sup>	0.141	1.316
Fig. 1B	4384.405 <sup>1</sup>	0.141	1.263
18S rRNA (Garey et al. 1996)			
Fig. 2A	23494.397 <sup>2</sup>	0.071	0.472
Fig. 2B	23499.077 <sup>2</sup>	0.074	0.473
Expanded 18S rRNA, alignment based on Garey et al. (1996)			
Fig. 3	27015.414 <sup>3</sup>	0.063	0.637
Fig. 4	27004.101 <sup>3</sup>	0.060	0.633
Expanded 18S rRNA, our alternative alignment			
Fig. 5	24231.698 <sup>4</sup>	0.083	0.513
Not shown	24248.954 <sup>4</sup>	0.085	0.515

<sup>a</sup> Differences in  $-\ln$  likelihood between the trees representing the two hypothesis, based on the Kishino–Hasegawa test: <sup>1</sup> = 1.927, is not significant (standard deviation of the difference = 4.138,  $t = 0.465$ ,  $p = 0.642$ ); <sup>2</sup> = 4.681, is not significant (standard deviation of the difference = 3.567,  $t = 1.312$ ,  $p = 0.190$ ); <sup>3</sup> = 11.314, is marginally significant (standard deviation of the difference = 5.828,  $t = 1.941$ ,  $p = 0.052$ ); <sup>4</sup> = 17.256, is significant (standard deviation of the difference = 7.599,  $t = 2.271$ ,  $p = 0.023$ ).

maximum-likelihood tree (Fig. 1B). Although not specified in the search constraint, this tree has Mollusca monophyletic. The difference in  $-\ln$  likelihood between the trees representing the two hypotheses is not significant (Table 2).

**18S rRNA Gene Data of Garey et al. (1996).** This data set consisted of 29 taxa and 2520 aligned nucleotide positions. All five searches resulted in the same maximum-likelihood tree (Fig. 2A), with a topology where Rotifera is paraphyletic and Acanthocephala arises from within Rotifera. A tree corresponding to the hypothesis that Rotifera is monophyletic was generated using searches with a constraint corresponding to this alternative hypothesis. All five searches resulted in the same maximum-likelihood tree (Fig. 2B). The difference in  $-\ln$  likelihood between the trees representing the two hypotheses is not significant (Table 2).

**Expanded 18S rRNA Gene Data with Alignment Based on That of Garey et al. (1996).** This data set consisted of 37 taxa and 2527 aligned nucleotide positions. These data include new sequences for nine species of Acanthocephala and four species of Rotifera. With these data we compared two hypotheses. The first, that Acanthocephala is the sister group to Rotifera class Bdelloidea and these two combined form the sister to the rest of Rotifera (Monogononta here), is the hypothesis of Garey et al. (1996, 1998). The second hypothesis is that Acan-



**Fig. 2.** Phylogenetic trees for Acanthocephala, Eurotatoria, and outgroup taxa based on 18S rRNA gene sequence data of Garey et al. (1996). **A** This tree corresponds to the hypothesis that Eurotatoria is paraphyletic and Acanthocephala is sister to Eurotatoria class Bdelloidea, ((Acanthocephala, Bdelloidea) Monogononta). The  $-\ln$  likelihood value for this tree is 23494.397. The asterisk denotes the location of the subtree in A on the full tree in B. **B** This tree is the result of a constraint that Eurotatoria is monophyletic, (Acanthocephala (Bdelloidea, Monogononta)). The  $-\ln$  likelihood value for this tree, 23499.077, is not significantly less likely ( $p = 0.190$ ) than the tree shown in A.

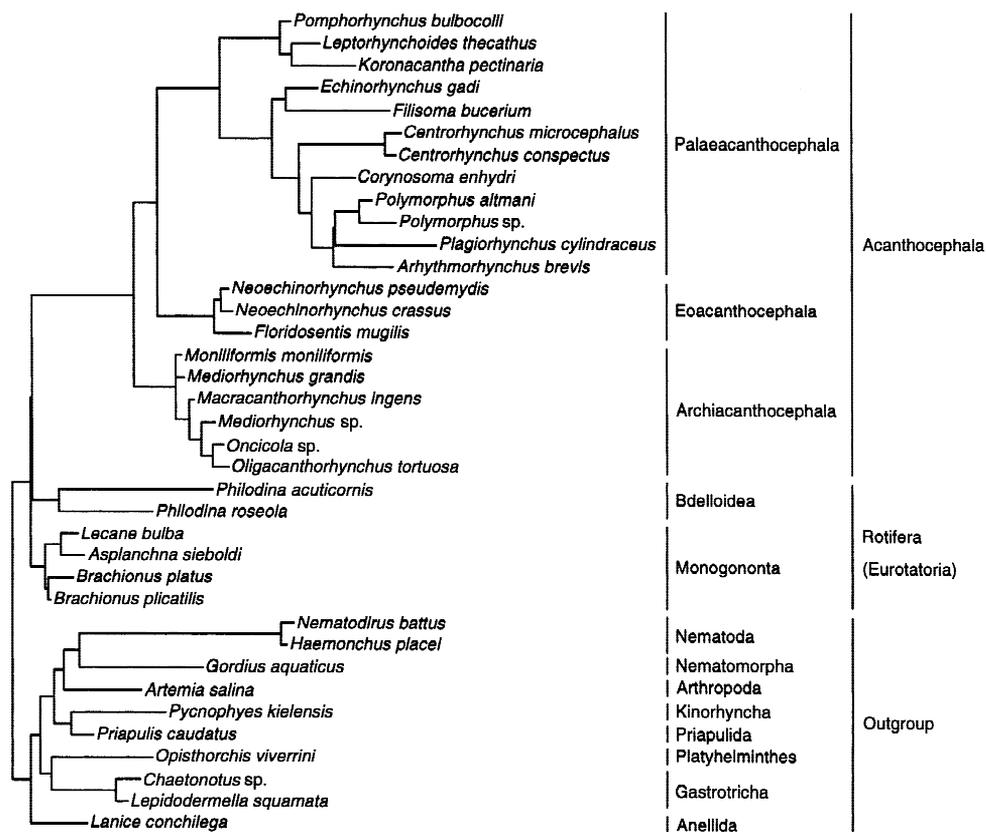
thocephala is the sister group to the whole of Rotifera: this is the hypothesis of Winnepenninckx et al. (1995), Wallace et al. (1996), and Melone et al. (1998). For the constraint searches corresponding to the first hypothesis, ((Acanthocephala, Bdelloidea) Monogononta), all five searches resulted in the same maximum-likelihood tree (Fig. 3). A tree corresponding to the alternative hypothesis, that Acanthocephala is sister to the whole of Rotifera, (Acanthocephala (Bdelloidea, Monogononta)), was generated using searches with a constraint corresponding to this hypothesis. All five searches resulted in the same maximum-likelihood tree with a higher  $-\ln$  likelihood value (Fig. 4). The difference in  $-\ln$  likelihood between the trees representing the two hypotheses is marginally significant, i.e., if rounded to two significant figures (Table 2). A feature of both trees is a paraphyletic *Mediorhynchus*.

*Expanded 18S rRNA Gene Data with Our Alternative Alignment.* This data set consisted of 37 taxa and 2031 aligned nucleotide positions. All five searches resulted in the same maximum-likelihood tree (Fig. 5), with a topology where Rotifera is monophyletic and, as a whole, is the sister group to Acanthocephala, (Acanthocephala (Bdelloidea, Monogononta)). A tree corresponding to the

alternative hypothesis, that Bdelloidea is sister to Acanthocephala, ((Acanthocephala, Bdelloidea) Monogononta), was generated using searches with a constraint corresponding to this hypothesis. All five searches resulted in the same maximum-likelihood tree (not shown). The  $-\ln$  likelihood for this tree is lower and the difference between the trees representing the two hypotheses is significant (Table 2). A monophyletic *Mediorhynchus* is a feature of trees produced with this alignment.

#### *Relationships of Classes Within Acanthocephala*

Where appropriate taxa are present, all trees showed the same relationship among classes within Acanthocephala: Palaeacanthocephala is sister to Eoacanthocephala and these combined form a group that is sister to Archiacanthocephala, ((Palaeacanthocephala, Eoacanthocephala) Archiacanthocephala). We examined the support for this relationship by testing it against other possible relationships of these classes using constraint searches on our expanded data set and alignment. The best tree obtained for one alternative, (Palaeacanthocephala (Eoacanthocephala, Archiacanthocephala)), was found in all five searches and has a  $-\ln$  likelihood of 24253.250. The



**Fig. 3.** Phylogenetic tree for Acanthocephala, Eurotatoria, and outgroup taxa based on our expanded 18S rRNA gene sequence data with an alignment based on that of Garey et al. (1996). This tree corresponds to the hypothesis that Eurotatoria is paraphyletic and Acanthocephala is sister to Eurotatoria class Bdelloidea, ((Acanthocephala, Bdelloidea) Monogononta). The  $-\ln$  likelihood value for this tree is 27015.414.

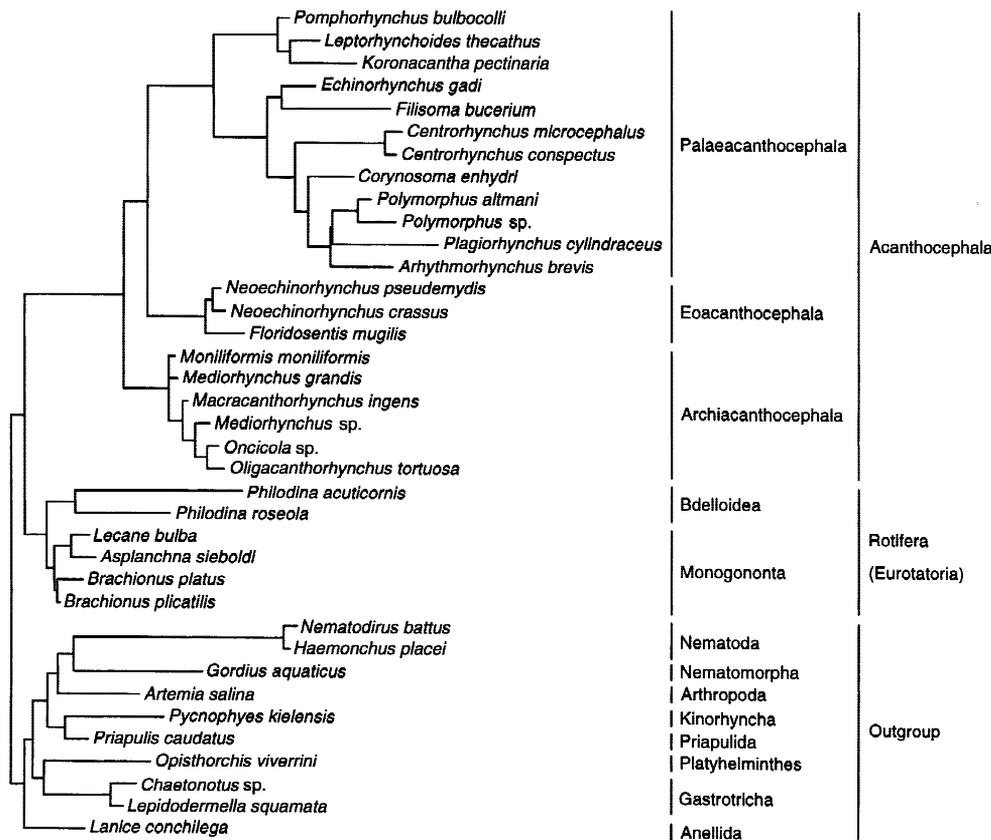
proportion of invariable sites for this tree is 0.089 and the gamma shape parameter is 0.521. The best tree for the other alternative, ((Palaeacanthocephala, Archiacanthocephala) Eoacanthocephala), was found in all five searches and has a  $-\ln$  likelihood of 24253.502. The proportion of invariable sites for this tree is 0.089 and the gamma shape parameter is 0.522. Based on the results of the Kishino–Hasagawa test, both alternative relationships for the classes within Acanthocephala are significantly less likely than that shown in Fig. 5. The difference in  $-\ln$  likelihood between trees for ((Palaeacanthocephala, Eoacanthocephala) Archiacanthocephala) and (Palaeacanthocephala (Eoacanthocephala, Archiacanthocephala)) is 21.552 (SD of the difference = 8.759,  $t = 2.461$ ,  $p = 0.014$ ). The difference in  $-\ln$  likelihood between trees for ((Palaeacanthocephala, Eoacanthocephala) Archiacanthocephala) and ((Palaeacanthocephala, Archiacanthocephala) Eoacanthocephala) is 21.804 (SD of the difference = 8.731,  $t = 2.497$ ,  $p = 0.013$ ).

## Discussion

### Relationships Among Phyla

Recent molecular and morphological data suggest that Acanthocephala is most closely related to Rotifera (Win-

nepenninckx et al. 1995; Wallace et al. 1996). Furthermore, analysis based on morphological characters as well as on 18S ribosomal gene sequences has led to the suggestion that Acanthocephala might be a subtaxon within Rotifera (Lorenzen 1985; Garey et al. 1996, 1998). In our work reported here, the relationships among phyla other than Rotifera and Acanthocephala, including Platyhelminthes, Gastrotricha, Nematoda, Nematomorpha, Priapulida, Kinorhyncha, Arthropoda, and Annelida, as shown in Fig. 5, are highly consistent with other recent reports (e.g., Aleshin et al. 1998). However, hypothesis testing in this study with an expanded data set including six sequences of Rotifera species (two Bdelloidea and four Monogononta), shows that hypotheses other than that of Rotifera monophyly and Rotifera as the sister group to Acanthocephala, (Acanthocephala (Bdelloidea, Monogononta)), are significantly less likely. Therefore, our results are in contrast to those of Garey et al. (1996, 1998), who found that Bdelloidea is the sister group to Acanthocephala, (Acanthocephala, Bdelloidea) Monogononta). However, no conclusion can be reached yet concerning the monophyly of Rotifera as a whole without the inclusion of Seisonidea. The topology of our tree is also supported by a recent analysis using a highly conserved nuclear gene encoding a heat shock protein (hsp 82), where Bdelloidea and Monogononta are monophyletic and form a clade (Eurotatoria) that is the sister



**Fig. 4.** Phylogenetic tree for Acanthocephala, Eurotatoria, and outgroup taxa based on our expanded 18S rRNA gene sequence data with an alignment based on that of Garey et al. (1996). This tree is the result of a constraint that Eurotatoria is monophyletic, (Acanthocephala (Bdelloidea, Monogononta)). The  $-\ln$  likelihood value for this tree, 27004.101, is marginally more likely ( $p = 0.052$ ) than the tree shown in Fig. 5.

group of Acanthocephala (Mark Welch 2000). Our results also support the monophyly of Eurotatoria within Rotifera.

Why do our conclusions differ from those of Garey et al. (1996, 1998)? In general, there are four reasons why results may differ between molecular systematic studies: (1) differences in sequence region(s) sampled, (2) differences in sequence alignment (this point is particularly relevant to rRNA gene sequences for which alignments are substantially subjective), (3) differences in taxa sampled, and (4) differences in phylogenetic inference methods. Three of these reasons are relevant here, the exception being the sequence regions sampled, as we examine the same sequence regions, 16S and 18S ribosomal RNA genes.

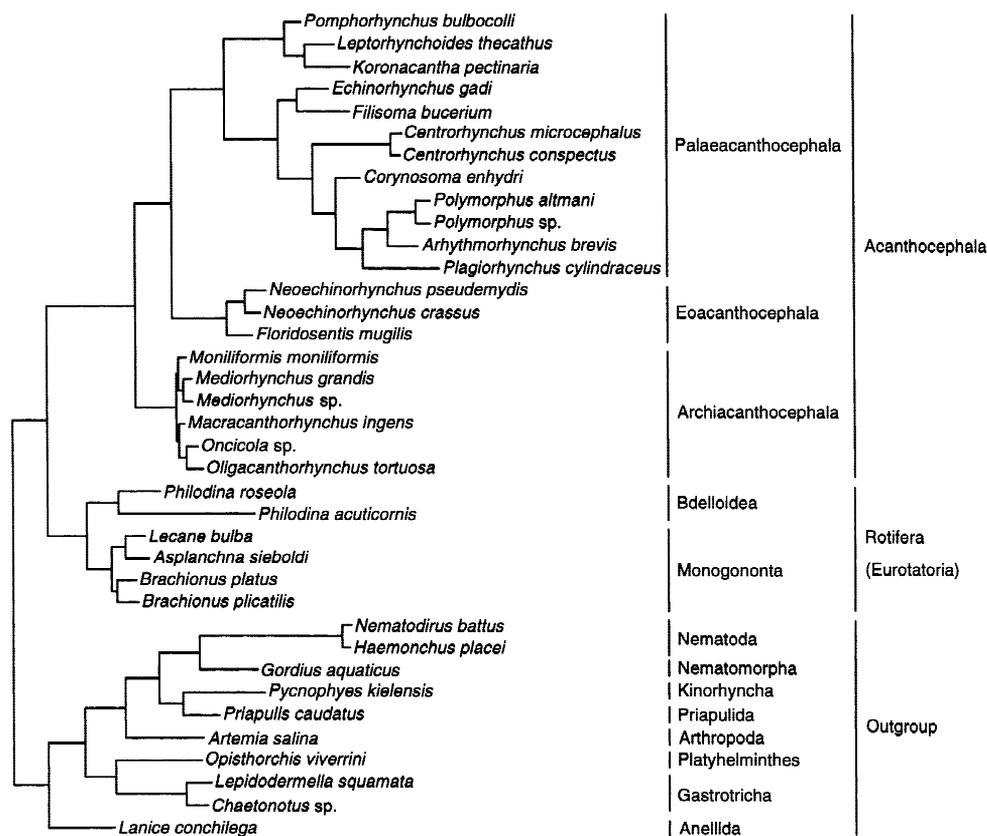
We examined the same sequence alignments used by Garey et al. (1996, 1998) and our expanded data with an alignment based on that of Garey et al. (1996); the trees resulting from analyses of these alignments are presented in Figs. 1–4. However, we prefer the alignment used to produce the tree in Fig. 5, for several reasons. The resulting tree (Fig. 5) demonstrates one reason for our preference, the monophyly of *Mediorhynchus*.

The importance of taxon sampling in the resolution of phylogenetic relationships has been demonstrated (i.e., Graybeal 1998). Several comparisons in our study used

an expanded data set and thus our sampling of taxa is greater than that of Garey et al. (1996, 1998). Additional sampling breadth might be particularly relevant to testing hypotheses concerning the relationships of Acanthocephala and Rotifera, and our study includes sequence data from *Philodina roseola* and thus has two species of Bdelloidea. The branch for *Philodina acuticornis* appears to be relatively long compared to other species of Rotifera.

We used one of the most sophisticated maximum-likelihood models currently available in an attempt to account for frequency of base change types, unequal base frequencies, invariant sites, and rate variation among sites. Furthermore, hypothesis testing in our study was based on the comparison of likelihood values, rather than on comparisons based on minimum evolution and parsimony.

Given the relative short sequences (762 aligned positions for 16S rRNA gene and up to 2527 for 18S rRNA gene), it might not be surprising that the hypotheses cannot always be statistically distinguished. Studies of the sampling properties of DNA sequence data in phylogenetic analysis demonstrate that larger samples of sites than considered here are generally needed to resolve phylogenetic relationships clearly (Cummings et al. 1995, 1999; Otto et al. 1996). Therefore, additional se-



**Fig. 5.** Phylogenetic tree for Acanthocephala, Eurotatoria, and outgroup taxa based on our expanded 18S rRNA gene sequence data and our alignment. This tree is consistent with the hypothesis that Eurotatoria is monophyletic, (Acanthocephala (Bdelloidea, Monogononta)). The  $-\ln$  likelihood value for this tree, 24231.698, is significantly more likely ( $p = 0.023$ ) than the tree for the alternative hypothesis, ((Acanthocephala, Bdelloidea) Mongononta).

quence data would be helpful in producing additional analytical results regarding these hypotheses.

#### Relationships Within Acanthocephala

Our analyses support the hypothesis that Acanthocephala is an independent and monophyletic group that includes three subclasses, each of them representing a class in agreement with current classifications of this parasitic group (Bullock 1969; Amin 1985). The class Polyacanthocephala includes a few species that are parasites of fish and crocodiles (Amin 1987); however, no material from Polyacanthocephala was available for the present study. Determination of Polyacanthocephala relationships with other classes included here, based on sequences data, will be the matter of future study. The phylogenetic relationships within Acanthocephala have been controversial. Based on morphological characters, Archiacanthocephala (Haffner 1950; Golvan 1959), Eoacanthocephala (Van Cleave 1948; Petrotschenko 1956), and Palaeacanthocephala (Lang 1953) have been alternatively proposed as the earliest-emerging class within the phylum. More recent studies based on morphological

and ecological characters do not result in definitive answers either (Conway Morris and Crompton 1982). Our results based on the analysis of the near-complete sequences of 18S ribosomal genes support the hypothesis that Archiacanthocephala is the basal class of the phylum and the sister group of a clade including Eoacanthocephala and Palaeacanthocephala (Fig. 5).

Within Palaeacanthocephala, the species included in our analysis appear separated in the two orders represented by Echinorhynchida (*F. bucerium*, *L. thecathus*, *K. pectinaria*, *P. bulbocolli*, and *E. gadi*) and Polymorphida (*C. microcephalus*, *C. conspectus*, *P. cylindraceus*, *Polymorphus* sp., *P. altmani*, *A. brevis*, and *C. enhydri*). As is apparent in Fig. 5, the branch lengths for Palaeacanthocephala taxa are generally longer than for taxa of Eoacanthocephala and Archiacanthocephala.

The relationships within Archiacanthocephala also are consistent with the existence of the orders: Moniliformida (*M. moliniformis*), Giganthorhynchida (*Mediorhynchus* sp. and *M. grandis*), and Oligacanthorhynchida (*Oncicola* sp., *O. tortuosa*, and *M. ingens*). Similar results concerning the phylogenetic relationships of the higher taxa of Acanthocephala, for a data set of 11 species, have recently been reported (Near et al. 1998).

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