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Molecular Evidence for Acanthocephala as a Subtaxon of Rotifera

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Abstract. Rotifers are free-living animals usually smaller than 1 mm that possess a characteristic wheel organ. Acanthocephalans (thorny-headed worms) are larger endoparasitic animals that use vertebrates and arthropods to complete their life cycle. The taxa Acanthocephala and Rotifera are considered separate phyla, often within the taxon Aschelminthes. We have reexamined the relationship between Rotifera and Acanthocephala using 18S rRNA gene sequences. Our results conclusively show that Acanthocephala is the sister group of the rotifer class Bdelloidea. Rotifera was nonmonophyletic in all molecular analyses, which supports the hypothesis that the Acanthocephala represent a taxon within the phylum Rotifera and not a separate phylum. These results agree with a previous cladistic study of morphological characters.

Key words: Rotifers — Acanthocephalans — Bdelloidea—Lemniscea

Introduction

The Acanthocephala are intestinal parasites of vertebrate definitive hosts and are characterized by an eversible proboscis with hooks that serve as a holdfast. The epidermis projects inward at the proboscis base to form a pair of lemnisci, which are thought to be involved in extension of the proboscis. Acanthocephalans have a syncytial epidermis with unique circulatory channels (lacunar system) that promote the direct absorption of nutrients through the body wall. The phylum is divided into three classes (Archiacanthocephala, Palaeacanthocephala, and Eoacanthocephala) based on the location of lacunar canals, the persistence of ligament sacs in females, the number and type of cement glands in males, proboscis hooks, and host taxonomy and ecology (Bullock 1969; Dunnagan and Miller 1991). Obligatory parasitism and lack of obvious free-living sister groups has hampered the study of morphological changes associated with the evolution of parasitism in Acanthocephala and other parasites (Brooks and McClennan 1993).

The phylum Rotifera is composed of three classes: Seisonidea, Monogononta, and Bdelloidea. In addition to the wheel organ, rotifers have a mastax, a foot with pedal (adhesive) glands, and a syncytial epidermis. Many rotifers also display eutely. The Seisonidea, which is considered a basal rotifer group, consists of a single genus (*Seison*) which is epizoic on certain marine crustaceans. In *Seison* the wheel organ is reduced to bristles and gonads are paired. The Monogononta are found mostly in freshwater, and the males are nonfeeding dwarfs possessing a single gonad. The Bdelloidea include freshwater and terrestrial taxa, males are absent, and reproduction is exclusively by parthenogenesis (Hyman 1951; Brusca and Brusca 1990; Dunagan and Miller 1991; Ruppert and Barnes 1994).

Rotifera and Acanthocephala, along with other pseudocoelomate phyla including Nematoda, Gastrotricha, Kinorhyncha, and Priapulida, are usually grouped with the taxon Aschelminthes (Hyman 1951; Marcus 1958;

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Clark 1979; Brusca and Brusca 1990; Ruppert and Barnes 1994). There is both morphological (Lorenzen 1985; Ruppert 1991; Malakhov 1994; Neuhaus 1994) and molecular evidence (Winnepenninckx et al. 1995) that Aschelminthes is not a valid taxon because these phyla do not constitute a monophyletic group. Despite this finding, there is growing evidence (Melone and Ferraguti 1994; Raff et al. 1994; Neuhaus 1994; Rieger and Tyler 1995; Winnepenninckx et al. 1995) that Acanthocephala are closely related to Rotifera. This has been suggested by various authors (Hafner 1950; Remane 1963; Nielsen 1995) and subsequently supported by a cladistic analysis of structural characters by Lorenzen (1985), which indicated that the Acanthocephala share most recent common ancestry with rotifers of the class Bdelloidea. A recent molecular study of the 18S rRNA from a monogonont rotifer and an archiacanthocephalalan also suggested a relationship between Rotifera and Acanthocephala (Winnepenninckx et al. 1995). The results of Lorenzen's cladistic study have been viewed as controversial (Clément 1993; Markevich 1993), and this hypothesis has not been tested rigorously with independent evidence.

Materials and Methods

A culture of the bdelloid rotifer *Philodina acuticornis* was provided by Dr. Terry Snell. The cultures were expanded using a commercial fish food infusion. Rotifer culture was transferred to a beaker and chilled on ice for 15 min, causing the rotifers to adhere to the beaker walls. The vessel was drained and rinsed with ice-cold water three times to remove debris and free-swimming microorganisms. A fresh volume of cold distilled water was added to the vessel and allowed to warm up to room temperature for 3 h to allow clearance of food from the digestive tract of the rotifers. The culture was chilled on ice to cause the reattachment of the rotifers to the vessel wall and two additional washes of ice-cold water were carried out. The drained culture was examined microscopically and found to be free of contaminating organisms and lysed in DNA extraction buffer (Hempstead et al. 1990) and stored frozen. Total DNA was prepared according to Hempstead et al. (1990). The 18S rRNA gene was amplified in two overlapping fragments, cloned into M13, and sequenced fully in both directions as described (Winnepenninckx et al. 1995).

The acanthocephalans *Neoechinorhynchus pseudemydis* and *Centrorhynchus conspectus* were collected from their vertebrate hosts and stored at −80°C. Voucher specimens were fixed in acetic acid–formalin–alcohol and some individuals were stained and mounted for identification following Bullock (1969). DNA was extracted from frozen specimens. The 18S rRNA gene was amplified (94°C 4-min initial denaturing followed by 25 cycles: 94°C 30 s, 60°C 30 s, 72°C 90 s) using primers corresponding to conserved regions at the extreme ends of the 18S rRNA gene (5′-AGATTAAGCCATGCATGCGTAAG-3′ and 5′-TGATCCTTCTGCAGGTTCACCTAC-3′), cloned into pGem-T vector (Promega Corp, Madison, WI) and sequenced completely in both directions with a variety of custom primers using a commercial cycle-sequencing kit (Amersham, Cleveland, OH).

The sequences obtained above were aligned with 18S rRNA gene sequences from 26 additional organisms acquired from Genbank. The following is a listing of the phylum, binomial name, three-letter code used in the figures (first letter of genus, first two letters of species), and Genbank accession number of each sequence used in the analyses. The entries for the new sequences presented here are underlined. Taxa were chosen from complete 18S rRNA sequence entries in Genbank to represent major groups of protostome, deuterostome, and aschelminth phyla. Diploblasts and Fungi were used to root the tree. Chordata: *Homo sapiens,* **Hsa,** M10098; *Xenopus laevis,* **Xla,** X02995; Hemichordata: *Saccoglossus kowalevskii,* **Sko,** L28054; Echinodermata: *Strongylocentrotus purpuratus,* **Spu,** L28056; Arthropoda: *Artemia salina,* **Asa,** X01723; *Tenebrio molitor,* **Tmo,** X07801; *Eurypelma californica,* **Eca,** X13457; Priapulida: *Priapulis caudatus,* **Pca,** X87984; Mollusca: *Limicolaria kambeul,* **Lka,** X66374; *Acanthopleura japonica,* **Aja,** X70210; *Placopecten magellanicus,* **Pma,** X53899; Annelida: *Eisenia fetida,* **Efo,** X79872; *Lanice conchilega,* **Lco,** X79873; Rotifera: *Brachionus plicatilis,* **Bpl,** U29235; *Philodina acuticornis,* **Pac,** U41281; Acanthocephala: *Moniliformis moniliformis,* **Mmo,** Z19562; *Neoechinorhynchus pseudemydis,* **Nps,** U41400; *Centrorhynchus conspectus,* **Cco,** U41399; Gastrotricha: *Lepidodermella squamata,* **Lsq,** U29198; Platyhelminthes: *Opisthorchis viverrini,* **Ovi,** X55357; Nematoda: *Pellioditis typica,* **Pty,** U13933; *Caenorhabditis elegans,* **Cel,** X03680; *Haemonchus placei,* **Hpl,** L04154; *Nematodirus battus,* **Nba,** U01230; Cnidaria: *Anemonia sulcata,* **Asu,** X53498; *Anthopleura kurogane,* **Aku,** Z21671; Ctenophora: *Mnemiopsis leidyi,* **Mle,** L10826; Porifera: *Scypha ciliata,* **Sci,** L10827; Fungi: *Saccharomyces cerevisiae,* **Sce,** M27607.

Sequences were aligned according to a secondary structure model (Van de Peer et al. 1994) using the DCSE editor (De Rijk and De Wachter 1993). The alignment is available by sending email requests to garey@next.duq.edu. Bootstrapped neighbor-joining and maximum parsimony trees were carried out with MEGA (Kumar et al. 1994) and PHYLIP (Felsenstein 1993) or PAUP (Swofford 1993), respectively. Kimura's two-parameter distance model with a correction for unequal rates of substitution at different sites was used as previously described (Winnepenninckx et al. 1995) for the neighbor-joining tree. PHYLIP was used for the maximum likelihood tree. Robustness of clades in the maximum-parsimony trees was assessed by examining 1,000 bootstrap replicates and decay indices, which were calculated as the number of steps that must be added before each clade present in the minimum length trees was no longer supported (Donoghue et al. 1992). Confidence probability values and bootstrap values for the neighbor-joining tree were determined using PHYLTEST (Kumar 1995) and MEGA (Kumar et al. 1994), respectively. Alternate topologies were tested using minimum-evolution criteria with four-cluster analysis (Kumar 1995; Rzhetsky et al. 1995) and with parsimony using Templeton's pairwise parsimony test (Templeton 1983).

Results and Discussion

The 18S rRNA genes from the archiacanthocephalalan *Moniliformis moniliformis* and the monogonont rotifer *Brachionus plicatilis* have been published previously (Telford and Holland 1993; Winnepenninckx et al. 1995). We have sequenced the 18S rRNA gene from the palaeacanthocephalan *Centrorhynchus conspectus,* the eoacanthocephalan *Neoechinorhynchus pseudemydis,* and the bdelloid rotifer *Philodina acuticornis.* Therefore, our dataset includes sequences representing all three acanthocephalan classes and the two major rotifer classes. Analyses of a sequence dataset (excluding sites containing gaps) for 29 taxa by neighbor-joining, maximum parsimony, and maximum likelihood tree inference methods recovered similar topologies (Fig. 1). These three inference methods all recovered the same topology with respect to the Rotifer–Acanthocephalan relationship (Fig. 1), and monophyly of the Acanthocephala is

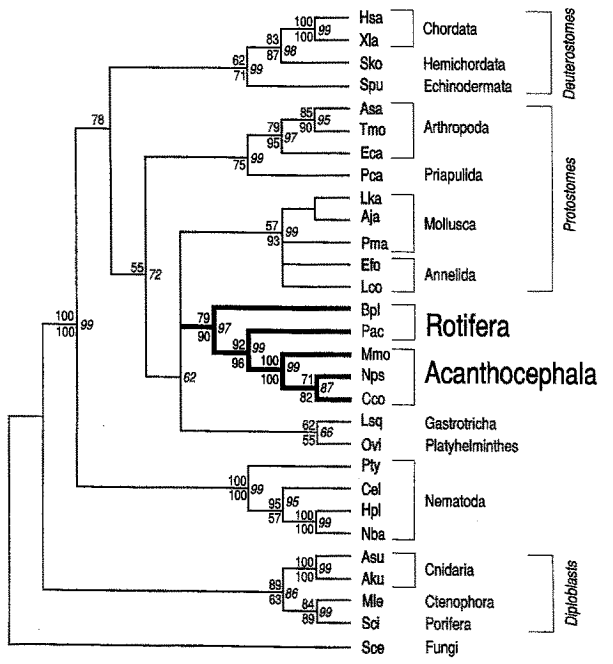

Fig. 1. The tree is a consensus derived from neighbor-joining, maximum parsimony, and maximum likelihood analyses of a secondary structure based alignment of near-complete 18S rRNA genes from 29 taxa. *Numbers above and below* each fork represent percentage of 1,000 bootstrap replicates that support the branch in the maximum parsimony tree and the neighbor-joining tree, respectively, and are shown only where greater than 50%. *Numbers to the right* of each branch are confidence-probability values that the branch length is significantly greater than zero and are shown only when greater than 50. See text for the definitions of the *three-letter codes* at each terminal node.

strongly supported. Maximum parsimony bootstrap values were 79% for Rotifera + Acanthocephala and 92% for bdelloid rotifer + Acanthocephala. Neighbor-joining bootstrap and confidence probability values (Kumar 1995) were 90% and 92% respectively for Acanthocephala + Rotifera and higher for bdelloid rotifer + Acanthocephala. In the maximum likelihood tree, all branches were significantly positive ($P < 0.01$). Alternate topologies using this dataset were explored using four-cluster analysis (Kumar 1995), which indicated with high probability that Rotifera + Acanthocephala was better than either taxon with other groups by minimum evolution criteria (Rzhetsky et al. 1995; see Table 1a). The G + C contents of the acanthocephalan and rotifer sequences were found to be slightly lower than the other nondeuterostome triploblasts included in the analyses (45.7 ± 2.23 and 49.4, ± 1.7% G + C, respectively, error range equals 1 SD). This is much less variation in G + C composition than is known to result in incorrect tree topology (Lockhart et al. 1992; Steel et al. 1993), and therefore compositional bias is not likely to be a factor in the phylogenetic placement of acanthocephalans and rotifers in our analyses. The neighbor-joining tree in Fig. 2 shows branch lengths drawn to scale, and it can be seen that the branches leading to *Philodina acuticornis* and *Centrorhynchus conspectus* are particularly long. It has been demonstrated (Lockhart et al. 1992; Hillis et al. 1994) that long branches can result in incorrect tree topologies. However, the *P. acuticornis* and *C. conspectus* sequences do not cluster with the other long branches leading to the nematodes (Fig. 2). Further, we reanalyzed the data set used in Fig. 2 but omitted either the *C. conspectus* sequences or both *P. acuticornis* and *C. conspectus* sequences with no change in the topology (not shown); therefore, it is doubtful that long branches have resulted in topological errors in our analyses regarding rotifers and acanthocephalans.

An analysis of a subset of 20 taxa by maximum parsimony (PAUP 3.1.1) of all 2,140 sites (sites with gaps included; gaps treated as missing data) recovered a topology (Fig. 3) similar to Fig. 1. This single most parsimonius tree (3,740 steps; CI of 0.452) was obtained using a heuristic search algorithm of PAUP (set to TBR, MULPARS, and steepest descent options). The g1 statistic (Hillis and Huelsenbeck 1992) for this tree (–0.981, 10,000 random trees) was significant ($P < 0.01$). The relationship among rotifers and acanthocephalans was identical to that recovered in the analysis of 29 taxa, and this clade received strong support by bootstrap resampling and analysis of decay indices (Fig. 3). The 20-taxon dataset was also used to test alternative phylogenetic hypotheses (Templeton 1983) for rotifer–acanthocephalan relationships. Alternative hypotheses (Table 1b) were found to be significantly worse than the most parsimonious tree. In the molecular analysis a topology with Acanthocephala and Praipulida as sister taxa required 71 additional substitutions. A topology with Acanthocephala and Platyhelminthes as sister taxa required 49 additional substitutions, and a topology representing Rotifera as a monophyletic group with Acanthocephala as the sister taxon required the addition of 28 more substitutions. The results strongly support the hypothesis that acanthocephalans share an immediate common ancestor with bdelloid rotifers, and are inconsistent with previous proposals concerning which extant group is most closely related to acanthocephalans (VanCleave 1941; Conway Morris and Crompton 1982).

These results are also in agreement with the cladistic study of Lorenzen (1985), which supported (a) monophyly of Rotifera + Acanthocephala as reflected by homology of the cuticle-like structure outside the syncytial epidermis; (b) monophyly of Bdellodea + Acanthocephala as indicated by the presence of lemnisci, which are shared-derived features of acanthocephalans and certain bdelloid rotifers, and that the proboscis of bdelloid rotifers works much like the introvert found in Acanthocephala; and (c) monophyly within Acanthocephala because of the lacunar system, uterine bell, spiny proboscis originating from the epidermal basement membrane, and the presence of acanthella, a juvenile stage of acanthocephalans. Lorenzen's arguments are consistent with our

Table 1. Results of testing alternate topologies

Table 1a. Four-cluster analysis (Kumar 1995; Rzhetsky *et al.* 1995). Comparison of alternate branching orders of metazoan groups. CP_I and CP_{II} are the confidence-probability values supporting the best tree as better than alternate tree I or II, respectively. Relationship of Rotifera (R), Acanthocephala (A), and diploblasts (D) with:

	Best tree	Alternate I	CP_I	Alternate II	CP_{II}
Annelida (An)	([A,R],[Ar,D])	([A,Ar],[R,D])	99	([A,D],[R,Ar])	98
Arthropoda (Ar)	([A,R],[Ar,D])	([A,Ar],[R,D])	99	([A,D],[R,Ar])	96
Deuterostomes (De)	([A,R],[De,D])	([A,De],[R,D])	99	([A,D],[R,De])	99
Gastrotricha (G)	([A,R],[G,D])	([A,G],[R,D])	99	([A,D],[R,G])	99
Mollusca (M)	([A,R],[M,D])	([A,M],[R,D])	99	([A,D],[R,M])	99
Nematoda (N)	([A,R],[N,D])	([A,N],[R,D])	99	([A,D],[R,N])	99
Platyhelminthes (Pl)	([A,R],[Pl,D])	([A,Pl],[R,D])	99	([A,D],[R,Pl])	92
Priapulida (Pr)	([A,R],[Pr,D])	([A,Pr],[(R,D])	99	([A,D],[(R,Pr])	86

Taxa used in each cluster: Annelida (Efo, Lco); Arthropoda (Asa, Tmo, Esa); Deuterostomes (Hsa, Xla, Sko, Spu); Gastrotricha (Lsq); Mollusca (Lka, Aja, Pma); Nematode (Pty, Cel, Hpl, Nba); Platyhelminthes (Ovi); Priapulida (Pca)

Table 1b. Analysis of alternative hypotheses using Templeton's (1983) pairwise parsimony method

	Length of tree	Difference in steps	Standard deviation	Significantly worse?
Most parsimonious tree (Fig. 2)	3,470			
Acanth. + Rotifera (monophyletic)	3,498	28	7.4851	Yes
Acanth. + Annelida	3,554	84	12.0027	Yes
Acanth. + Arthropoda	3,541	71	11.5320	Yes
Acanth. + Deuterostomes	3,540	70	11.1381	Yes
Acanth. + Gastrotricha	3,523	53	11.2720	Yes
Acanth. + Platyhelminthes	3,519	49	11.3604	Yes
Acanth. + Priapulida	3,541	71	11.7925	Yes

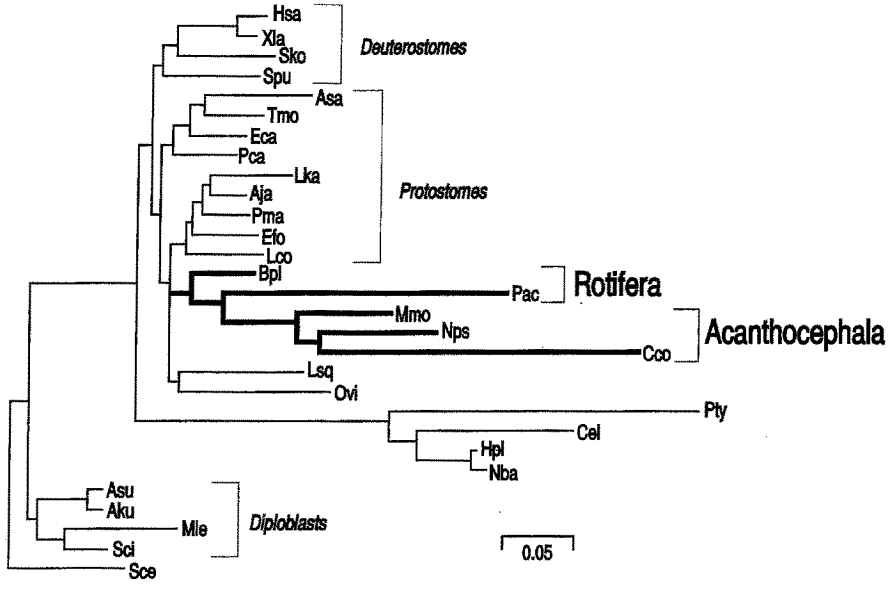

Fig. 2. Neighbor-joining tree from Fig. 1 showing branch lengths drawn to scale (number of substitutions per site). See Fig. 1 for bootstrap and confidence-probability values. Phylum names other than Acanthocephala and Rotifera are omitted for clarity. Removal of sequences representing the taxa Pac or both Pac and Cco from the analysis did not change the topology of the tree.

gene tree and (a, b and c) are indicated in Fig. 3. Based on previous morphological evidence and the new molecular evidence presented here, we propose that Acanthocephala be considered a taxon of Rotifera, united with the class Bdelloidea under the superclass Lemniscea.

Until now a free-living sister group of a major obligate parasitic taxon has not been identified (Brooks and McClennan 1993), hampering comparative studies of character change associated with the evolution of parasitism. Comparisons between acanthocephalans and free-

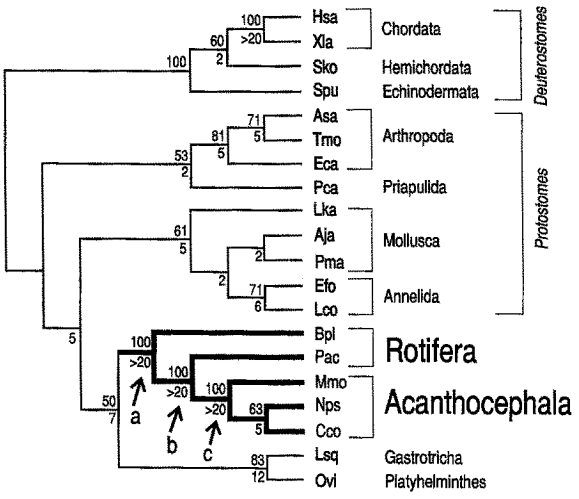

Fig. 3. Maximum-parsimony tree obtained from analysis of all 2,140 sites (679 phylogenetically informative) of 20 taxa. *Numbers above* each fork represent percentages of 1,000 bootstrap replicates that support the branch and are shown only where greater than 50%. *Numbers below* the branch represent a decay index (Donoghue *et al.* 1992). The *three-letter* codes at each terminal node are defined in the text. *The letters a, b, and c* refer to morphological support for the tree described in the text.

living rotifers should prove instrumental in solving long-standing problems such as the relative importance of secondary character loss vs character innovation in the evolution of a parasitic life history (Brooks and McClennan 1993).

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