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Phylogenetic Relationships among Turbellarian Orders Inferred from 18S rDNA Sequences

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ABSTRACT—The turbellarian flatworm is a key group to understand the origin and the early evolution of triploblastic, bilaterally symmetrical animals, but phylogenetic relationships among turbellarian orders have been a subject of debates for decades, especially on the position of the acoel turbellarians. Some workers have considered the acoel representing the most primitive turbellarian order but others have regarded them as regressive. We determined almost the entire lengths of the nucleotide sequences of 18S ribosomal RNA gene (rDNA) in 17 species from 9 turbellarian orders (the Acoela, Catenulida, Macrostomida, Lecithoepitheliata, Rhabdocoela, Prolecithophora, Proseriata, Tricladida, and Polycladida). After adding the sequences of a cestode, two trematodes and some diploblastic animals obtained from databases, we reconstructed phylogenetic trees using the neighbor-joining, maximum-likelihood and maximum-parsimony methods. All trees significantly indicated that the Acoela is the earliest divergent group among the turbellarian orders. The trees also suggested that the Tricladida evolved in the separate lineage from that of a cluster of the Catenulida, Macrostomida, Lecithoepitheliata, Rhabdocoela, Polycladida, Trematoda and Cestoda after the divergence of the Acoela.

INTRODUCTION

In the traditional taxonomy, the phylum Platyhelminthes (flatworms) comprises three classes, the Turbellaria, the Trematoda (flukes) and the Cestoda (tapeworms) (Hyman, 1951). The turbellarians are primarily free-living aquatic animals of small sizes. Their body consists of a ciliated epidermal covering, a sack-like gut without an anus, and a solid mesenchymal cell mass filling between the epidermis and the gut. Many zoologists have agreed that the turbellarians are the most primitive not only among the platyhelminth classes but among all the bilateral (triploblastic) animal groups (Brusca and Brusca, 1990; Nielsen, 1995). Nevertheless, the origin and the evolution of the turbellarians and their relationships to other animal groups have been the themes of debates for several decades. The body of the acoel turbellarians is a virtually solid cell mass with a mouth on the midventral side and a central syncytial area bearing a digestive functions, lacking any sort of digestive cavity. The order Acoela has traditionally been regarded as the most primitive type of turbellarians on the hypothesis that an acoelomate condition first derived from an acoel-type ancestor, and in the next step of sophistication the gut became more elaborate and the division into distinct germ layers more pronounced as seen in the orders Catenulida and Nemertodermatida (Hyman, 1951; Hadži, 1963; Hanson, 1977; Salvini-Plawen, 1978). A considerable number of zoologists, however, have an opinion that the Acoela is a secondarily reduced group and seemingly primitive features are regressive (see Ax, 1963). Ehlers (1985a,b) and Ax (1987) have recently established a taxonomic system of the phylum Platyhelminthes on the basis of the methodology of cladistic systematics using many light and electron microscopic data. In their cladograms, the order Catenulida is the earliest divergent turbellarian group.

In recent years, molecular phylogenetic methods have been applied to phylogenetic analyses of platyhelminth groups. A large scaled phylogeny of the Metazoa based on complete sequences of 18S ribosomal RNA gene (18S rDNA) has suggested the earliest divergence of the Platyhelminthes among triploblastic phyla (Philippe *et al.*, 1994). However, interrelationships among turbellarian groups have not fully been clarified. The molecular phylogeny of platyhelminth groups so far published were based on partial sequences of 18S rDNA (or 18S rRNA) (Baverstock *et al.*, 1991; Riutort *et al.*, 1992, 1993; Katayama *et al.*, 1993; Rohde *et al.*,1993, 1994, 1995; Kuznedelov and Timoshkin, 1995), and the results are sometimes mutually contradictory and inconclusive. Ambiguousness of the results is partly attributable to the shortage of information available to resolve significantly the relationships among platyhelminth orders. Our previous report on the phylogenetic inference of primitive metazoans using almost the entire lengths of 18S rDNA sequences has suggested that an acoel turbellarian, *Convoluta naikaiensis* diverged early during the triploblastic evolution (Katayama *et al.*, 1995). We have now sequenced almost the entire lengths of 18S rDNA in 17 species from 9 turbellarian orders. The results of the phylogenetic analysis using these sequences together with sequences obtained from databases significantly indicated that not the Catenulida but the Acoela is the most primitive order of the Platyhelminthes.

MATERIALS AND METHODS

Animals

Seventeen turbellarian species from 9 out of 11 turbellarian orders (Cannon, 1986) were used. The names of the used species and their collection sites are summarized in Table 1. The specimens were starved for 10 days, frozen quickly in liquid nitrogen and kept at -80°C until use. Since the adults of *Convoluta naikaiensis* contain symbiotic alga, embryos before algal invasion were used to avoid contamination of algal DNA.

DNA isolation

The frozen samples were powdered and lysed in TE buffer (10 mM Tris-HCl, 0.1 M EDTA, pH 8.0) containing 0.5% sodium dodecyl sulfate. After digestion of samples with proteinase K (100 μg/ml) at 50°C for 3 hr, DNA was extracted with phenol, and precipitated in ethanol and an equal volume of 5.0 M ammonium acetate. Samples resuspended in TE buffer were further purified by RNase A digestion (20 μg/ml) at 37°C for 1 hr, followed by ethanol precipitation.

Amplification of 18S rDNA

The 18S rDNA was amplified by the polymerase chain reaction (PCR) (Saiki *et al.*, 1988) in an Air Thermo-cycler 1645 (Idaho Technology). Almost the entire length of 18S rDNA was amplified using synthetic oligonucleotides, 5'-CTGGTTGATCCTGCCAG-3' (primer 0) and 5'-CCTTGTTACGACTT-3' (primer 10) as the terminal primers. Amplifications were performed in 50 μl of 50 mM Tris-HCl (pH 8.5), 250 μg/ml BSA, 2 mM Mg^{2+} with 0.2 mM each dNTP, 50 pM primers, template DNA (5–10 ng), and 2U Taq DNA polymerase (TOYOBO). The temperature regimen for 35 cycles was 20 sec at 94°C, 30 sec at 50°C, and 90 sec at 74°C.

Determination of DNA sequences

After purification of the amplified DNA by electrophoresis in a 0.8% agarose gel, the nucleotide sequence was directly determined by dideoxy chain-termination (Sanger *et al.*, 1977) using Sequenase version 2.0 (USB) and DNA sequencing high cycle kit (TOYOBO). All DNA samples were sequenced in both directions and from several separate amplifications with terminal primers -0 and -10 and internal primers, the 5' ends of which were labeled with biotin. The internal primers used were primer-1 (5'-CCGGAGAGGGAGCCTGA-3'), primer-2 (antisense of primer-1). primer-3 (5'-CAGCAGCCGCGGTAATT-3'), primer-4 (antisense of primer-3), primer-5 (5'-GCGAAAGCATTTGCCAA-3'), primer-6 (antisense of primer 5), primer-7 (5'-GAAACT(TC)AAAGGAAT-3'), primer-8 (antisense of primer-7), and primer-9 (5'-ACGGGCGGTGTGT(AG)C-3'). The positions corresponding to these primers in the 18S rDNA sequence are shown in Fig. 1. The continuity of the DNA fragments was confirmed by overlapping of the sequences.

Alignment of DNA sequences

In addition to the originally determined 18S rDNA sequences in 17 turbellarian species, we obtained 18S rDNA sequences in 4 diploblastic animals, two trematodes, a cestode, and a yeast from databases. The species and the accession numbers for their 18S rDNA sequences are as follow: *Sycon calcaravis* (Porifera), D15068; *Beroe cucumis* (Ctenophora), D15068; *Trichoplax adhaerens* (Placozoa), L10828; *Anemonia sulcata* (Cnidaria), Z26942; *Gyrodactylus salaris* (Trematoda), Z19562; *Schistosoma mansoni* (Trematoda), X53047; *Echinococcus granulosus* (Cestoda), U27015; *Saccharomyces cerevisiae* (yeast), J01353. Sequences were aligned manually on the basis of maximum nucleotide similarity. Alignment gaps were inserted to account for putative length differences between sequences. We could not unequivocally determine the optimal alignment for the regions containing deletions, insertions, or highly variable sequences. In order to use for phylogenetic inference only regions that we could definitely align, we excluded such regions from analysis as well as the sites where a gap was present for any taxa.

Reconstruction of phylogenetic trees

The phylogenetic trees were reconstructed using programs in the PHYLIP package version 3.57 (Felsenstein, 1989) and fastDNAml (Olsen *et al.*, 1993). Tree-building procedures used were the neighbor-joining (NJ) (Saitou and Nei, 1987), the maximum-likelihood (ML) (Felsenstein, 1981), and the maximum-parsimony (MP) (Fitch, 1971) methods. For the NJ analysis, evolutionary distance values were calculated by the formula of Jukes and Cantor (1969). The degree of support for internal branches of the trees in the NJ and the MP trees was assessed by bootstrap levels of support (Felsenstein, 1985) determined by 500 bootstrap repetitions.

Table 1. Turbellarian species used in the present study and their collection sites

Order	Suborder	Species	Habitat	Collection site
Acoela		*Convoluta naikaiensis* Yamasu	marine	Desaki, Okayama
		Amphiscolops sp.	marine	Zanpa, Okinawa
Catenulida		*Stenostomum leucops* (Dugès)	freshwater	Ushimado, Okayama
Macrostomida		*Macrostomum tuba* Graff	freshwater	Ushimado, Okayama
		Microstomum lineare Müller	freshwater	Ushimado, Okayama
Lecithoepitheliata		*Geocentrophora sphyrocephala* de Man	freshwater	Ushimado, Okayama
Rhabdocoela		*Bothromesostoma* sp.	freshwater	Okayama City
Prolecithophora		*Vorticeros ijimai* Tozawa	marine	Ushimado, Okayama
Proseriata		*Otoplana* sp.	marine	Desaki, Okayama
		Nematoplana sp.	marine	Desaki, Okayama
Tricladida	Maricola	*Ectoplana limuli* (Ijima et Kaburaki)	marine, parasitic*1	Kasaoka City*2
	Paludicola	*Dugesia japonica* Ichikawa et Kawakatsu	freshwater	Kyoto City
		Dendrocoelopsis lactea Ichikawa et Okugawa	freshwater	Hirosaki City*3
	Terricola	*Bipalium* sp.	terrestrial	Ushimado, Okayama
Polycladida	Acotylea	*Notoplana koreana* Kato	marine	Ushimado, Okayama
		Planocera multitentaculata Kato	marine	Ushimado, Okayama
	Cotylea	*Thysanozoon brocchii* (Risso)	marine	Ushimado, Okayama

*1 Living on the ventral surface of the horse-shoe crab, *Tachypleus tridentatus*.
*2 Provided by Dr. N. Soji, Horse Shoe Crab Museum.
*3 Provided by Dr. S. Ishida, Hirosaki University.

RESULTS

Almost the entire lengths of 18S rDNA from 17 turbellarian species were amplified by PCR from the genomic DNA and the sequences (1500-1700 bp) were determined directly from PCR products. The sequences determined have been deposited in databases (DDBJ, EMBL and GenBank) under the following accession numbers: *Convoluta naikaiensis*, D83381; *Dugesia japonica*, D83382; *Planocera multitentaculata*, D83383; *Bipalium* sp., D85086; *Dendrocoelopsis lactea*, D85087; *Ectoplana limuli*, D85088; *Geocentrophora sphyrocephala*, D85089; *Otoplana*.sp, D85090; *Macrostomum tuba*, D85091; *Microstomum lineare*, D85092; *Nematoplana* sp., D85093; *Vorticeros ijimai*, D85094; *Stenostomum leucops*, D85095; *Thysanozoon brocchii*, D85096; *Notoplana koreana*, D85097; *Bothromesostoma* sp., D85098; *Amphiscolops* sp., D85099. The sequence data for *Convoluta naikaiensis*, *Dugesia japonica*, and *Planocera multitentaculata* have already been used in the previous study (Katayama *et al.*, 1995).

The nucleotide sequences were aligned together with those of 4 diploblastic animals, two trematodes, a cestode and *Saccharomyces cerevisiae* (yeast) obtained from databases. Figure 1 shows a sample of the alignment for 7 out of 25 species included in the present analysis. The alignment reveals that the sequences are highly conserved in some regions but highly variable in others. After exclusion of the regions of ambiguous homology, 1121 sites remained for phylogenetic inference. We estimated the average G + C composition of the 1121 nucleotides in the 20 platyhelminth species. Except for 50.0% for *Echinococcus granulosus*, the average G + C contents for other species were within the range of 43.6-47.0% (mean ± SE=45.9 ± 1.59, n=20).

We reconstructed phylogenetic trees by the neighbor-joining (NJ) method based on the pair wise distances (Jukes and Cantor, 1969) (Fig. 2a), the maximum-likelihood (ML) method (Fig. 2b), and the maximum-parsimony (MP) method (Fig. 2c). In those trees, *Saccharomyces cerevisiae* was used as an outgroup. The NJ tree reconstructed on the basis of the two-parameter equation of Kimura (1980) gave the same topology as shown in Fig. 2a. Among the phylogenetic trees reconstructed by the three methods, the topologies were largely congruent with one another, though branching with low bootstrap support showed somewhat conflicting arrangements. The three trees commonly showed that the Acoela was the sister group to the assemblage of all other platyhelminth groups included in the present analysis. This sister-group relationship was supported by a high bootstrapping value (91%) in the NJ tree (Fig. 2a) and by a moderately high value (77%) in the MP tree (Fig. 2c) (because of an enormous computation time required, bootstrapping was not performed in the ML analysis). The other sets of grouping that were commonly seen in the three trees and were supported by high bootstrapping values at least in either the NJ or the MP tree were as follow: The 4 species of the Tricladida formed a monophyletic group, within which *Bipalium* sp. (the Terricola) diverged earlier than the Maricola and the Paludicola. The three species of the Polycladida formed a monophyletic group. The Polycladida and the Macrostomida formed a single cluster. The Trematoda and the Cestoda also formed a single cluster. Though the bootstrap values were not high, *Vorticeros ijimai* (the Prolecithophora) formed a cluster with the Tricladida in the three trees. However, the two species of the Proseriata, *Otoplana* sp. and *Nematoplana* sp. always appeared separately in different clusters, varying in position from tree to tree. We found a common tendency in the topology of the three trees; the assemblage from which the Acoela had branched off divided into two clusters, the one including the Tricladida and the Prolecithophora, and the other consisting of many turbellarian orders, the Trematoda and the Cestoda.

DISCUSSION

The Platyhelminthes comprising the mainly free-living Turbellaria and the parasitic Trematoda and Cestoda is a distinctly delimited group and the monophyly of these groups is widely accepted by traditional (Hyman, 1951) as well as cladistic (Ehlers, 1985a,b; Ax, 1987) researchers. It has traditionally been considered that a monophyletic group consisting of the Trematoda and the Cestoda (the Neodermata) arose from a rhabdocoel turbellarian-like ancestor latest in the platyhelminth evolution (Hyman, 1951). Recent cladistic phylogeny agrees with this traditional view on the position of the Neodermata in the Platyhelminthes (Ehlers, 1985a,b; 1986; Ax, 1987), although not all authors agree in this point (Rohde, 1988). The recent molecular phylogenetic inferences based on partial sequences of 18S rRNA have supported the monophyly of the Neodermata (Baverstock *et al.*, 1991; Rohde *et al.*, 1993). The present analysis on the basis of almost the entire 18S rDNA sequences also supports the idea that the Trematoda and the Cestoda diverged last among the platyhelminth groups as a monophyletic unit closely related with the Rhabdocoela.

There have been two opposing views on the position of the acoel turbellarians in the platyhelminth phylogeny (Willmer, 1990; Rieger *et al.*, 1991a; Nielsen, 1995). The order Acoela is unique in the Turbellaria in having modified cilia, in lacking not only a digestive tract but intercellular matrices (Bedini, 1974; Rieger, 1985; Rieger *et al.*, 1991b; Smith *et al.*, 1986), and in showing a duet type cleavage (Thomas, 1986; Galleni and Gremini, 1989). Many traditional zoologists have felt these characters as primitive and have regarded the Acoela nearest to the ancestor of the turbellarians (Hyman, 1951; Hadži, 1963; Hanson, 1977; Salvini-Plawen,1978), but some workers have regarded the characters as regressive (see Ax, 1963). Recently, based on cladistical analyses, Ehlers (1985a,b, 1992) and Ax (1985, 1987) have claimed that the Catenulida is the earliest divergent group among the Platyhelminthes. Molecular phylogenetic inferences on the basis of partial sequences of 18S rDNA (or rRNA) are not conclusive on this point (Baverstock *et al.*, 1991; Riutort *et al.*, 1992, 1993; Katayama *et al.*, 1993; Rohde *et al.*, 1993, 1994, 1995). The

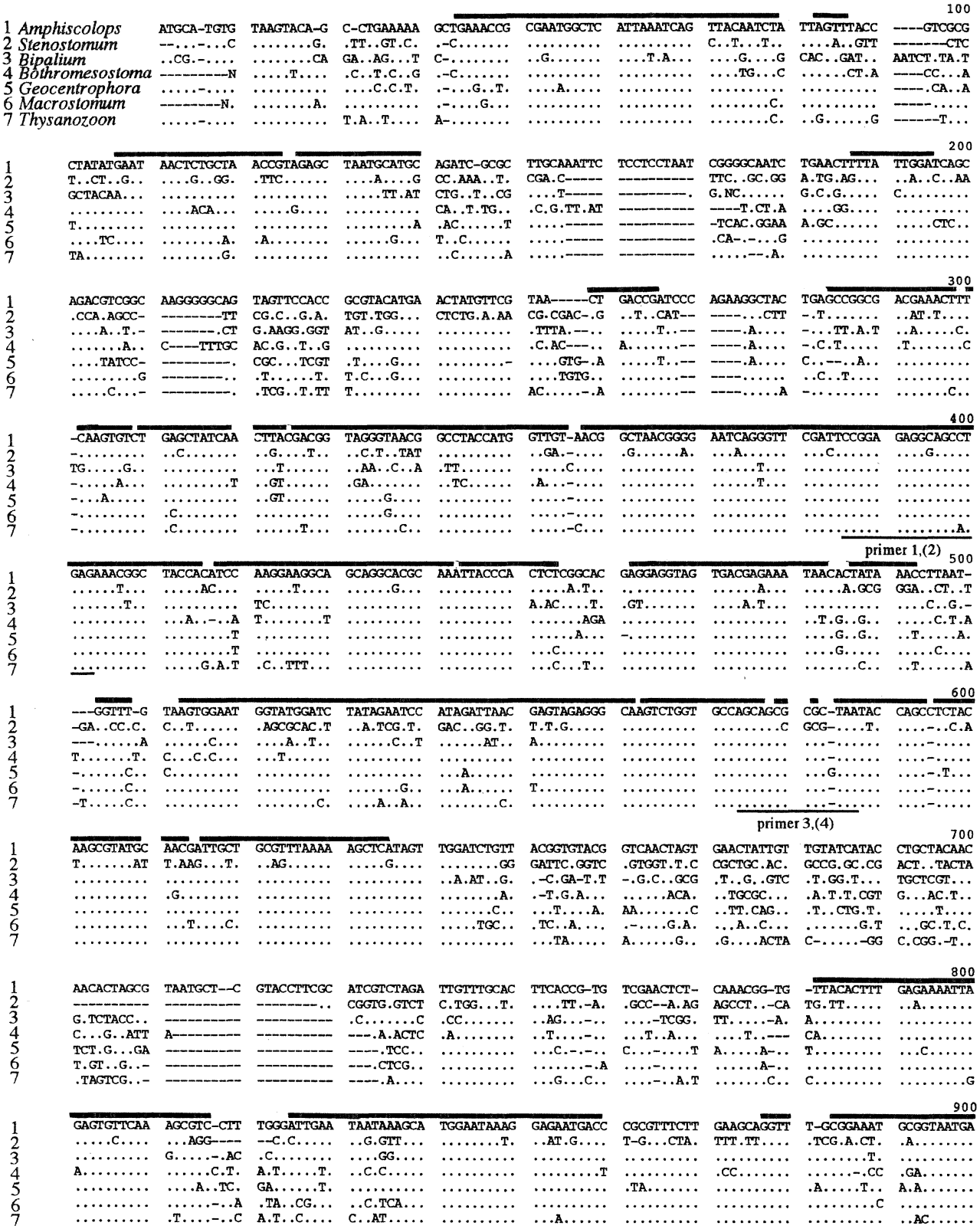
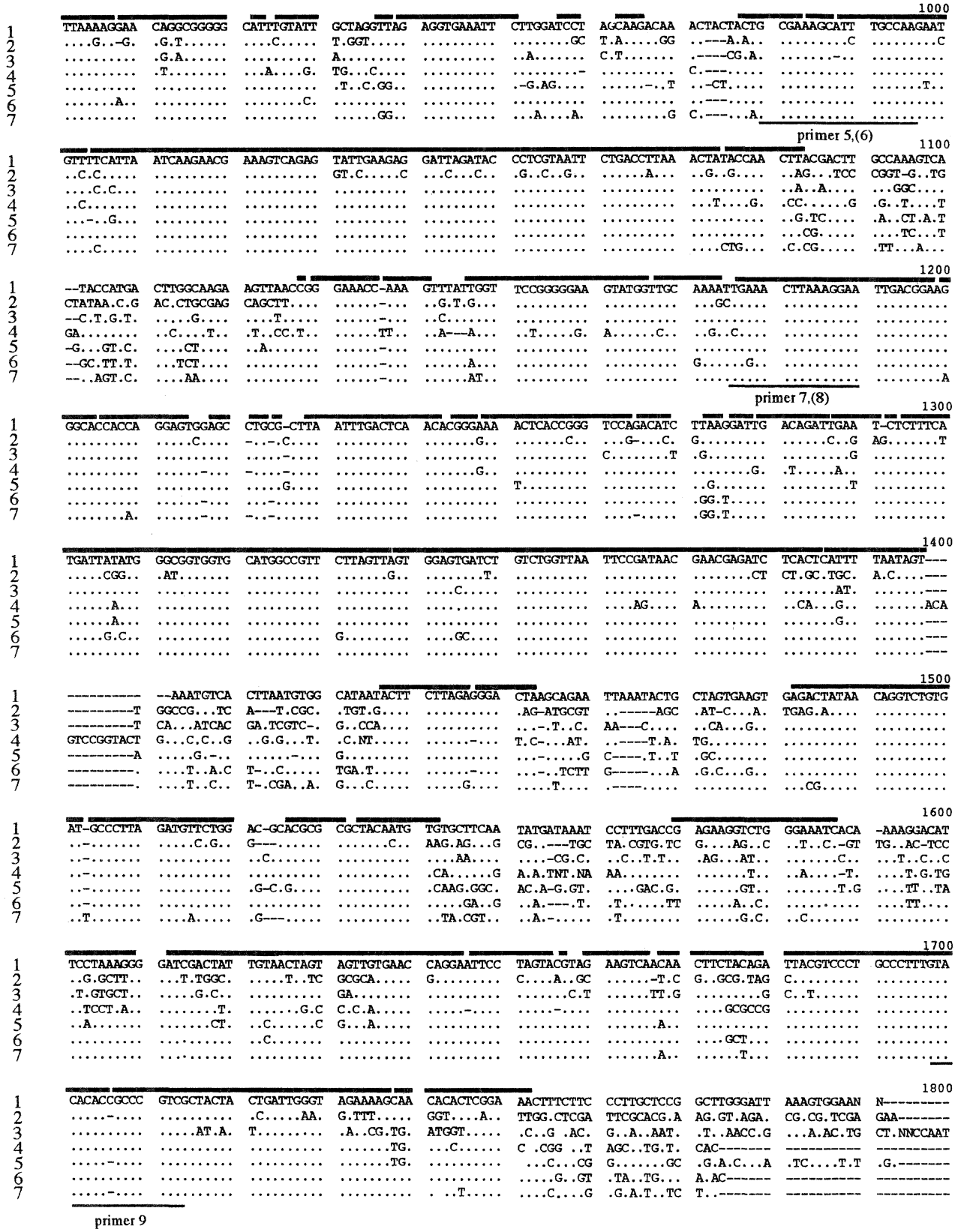
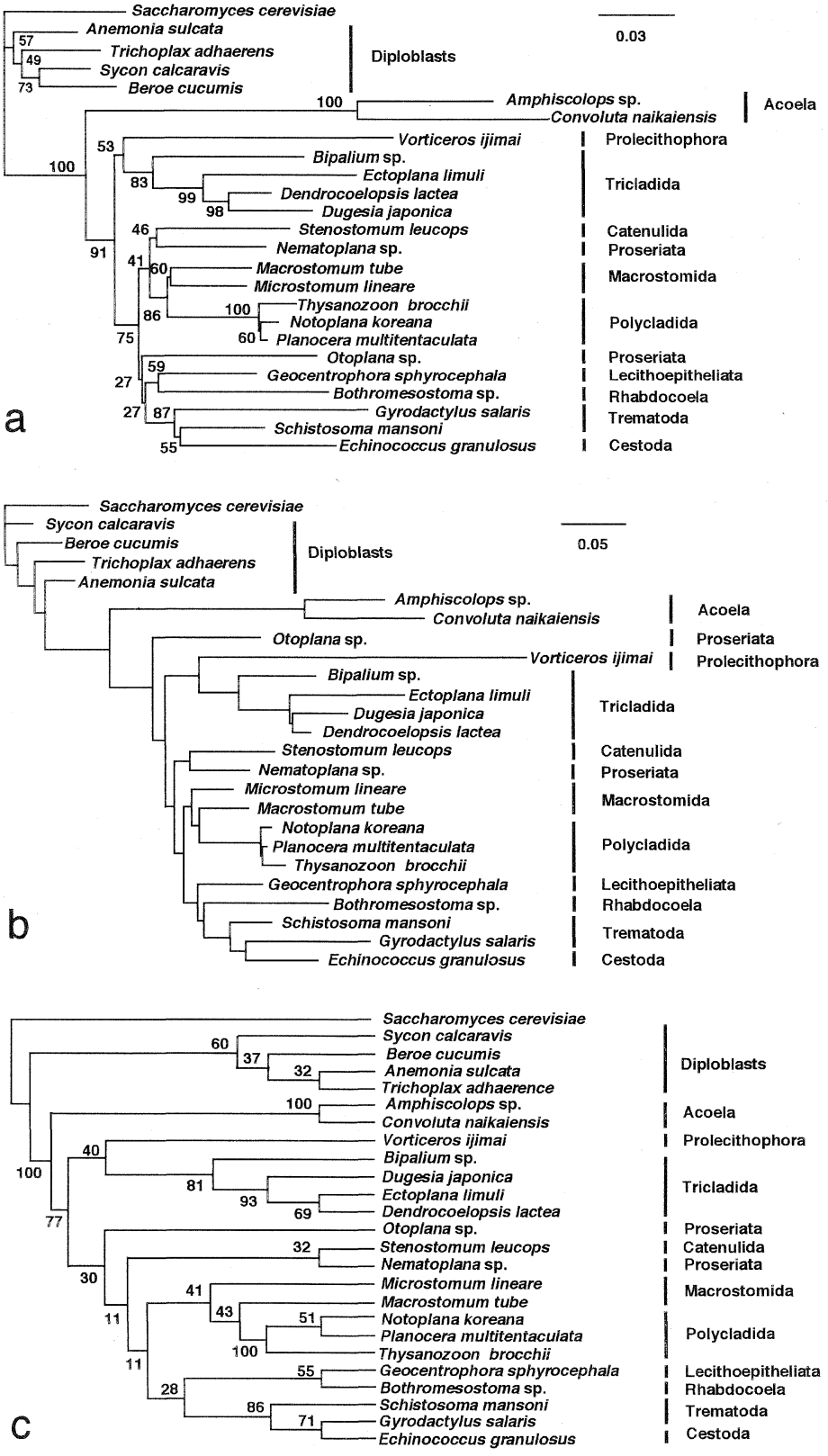


Fig. 1. A sample of the alignment of nucleotide base sequences of 18S rDNA used for the present phylogenetic analysis. Sequences for 7 turbellarian species are picked up from the aligned sequences for 25 species. A period indicates that the base at that position is identical to that in *Amphiscolops* sp., a hyphen indicates a gap, and "N" indicates an undetermined site. The 1121 positions used for phylogenetic inference are shown by thick lines above the alignment. The position corresponding to the internal primers are shown by thin lines below the alignment. The primers whose number is in parentheses are antisense to the sequences shown here. The entire alignment including all 25 sequences is available on the WWW server (http://150.46.174.53/res.htm).



present analysis based on almost the entire sequences of 18S rDNA significantly showed a sister-group relationship of the Acoela to the cluster of all other platyhelminth groups involved. Thus, the present inference is not in agreement with the conclusion of the cladistic systematics but with the traditional view that the Acoela is the most primitive group in the



Platyhelminthes. Molecular data of the Nemertodermatida, which Smith and Tyler (1985) regarded more primitive than the Acoela, as well as the Gnathostomulida, which Ax (1985, 1987) used as the outgoup in his cladistical analysis, will be necessary to determine whether the Acoela is in fact the most primitive platyhelminth group or not.

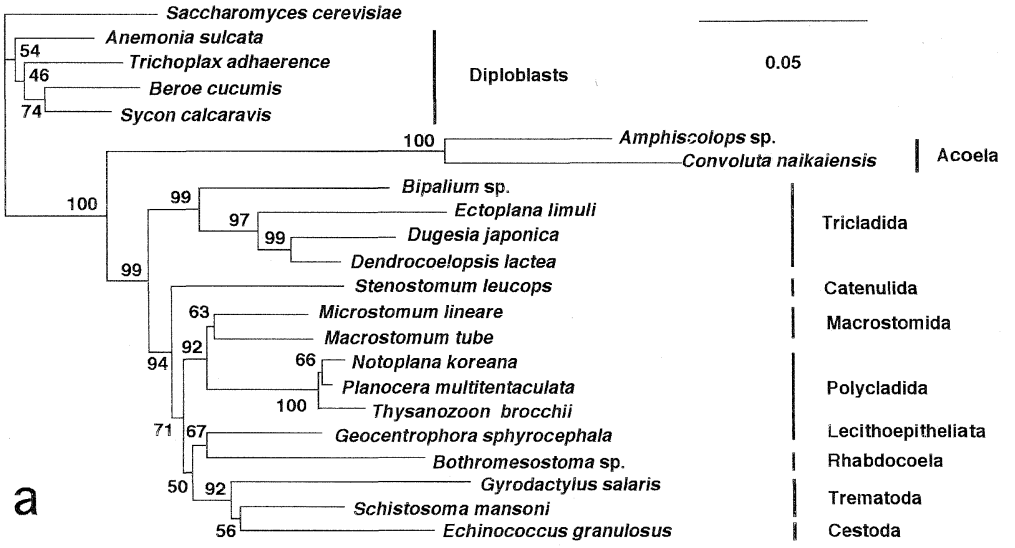
It has been accepted both in the traditional and cladistic systems of phylogeny that the Tricladida is closely related to the Rhabdocoela and this two groups emerged in the late phase of the turbellarian evolution. The present molecular phylogenetic inference seems to be inconsistent with this view. Topology of the phylogenetic trees containing 12 platyhelminth groups (Fig. 2a-c) shows a tendency that the flatworms divided into two clusters after the divergence of the Acoela, where the Tricladida and the Rhabdocoela are separately located. Since a presence of the two proseriate species and a prolecithophoran *Vorticeros ijimai* seemed to blur the conclusion, we tried to reconstruct phylogenetic trees after removing the three species from analysis. The trees deduced by the NJ, ML and MP methods (Fig. 3a-c) showed a basically consistent topology, i.e., the Tricladida formed the sister group to the cluster of the other 7 platyhelminth groups, which was supported by a high bootstrapping value (91%) in the NJ and by a moderately high value (76%) in the MP trees. The three trees also showed that the Catenulida diverged earliest within the assemblage of the 7 platyhelminth groups. Thus, the present results suggest that the Tricladida evolved in the different lineage from the lineage leading to the Rhabdocoela and the Neodermata.

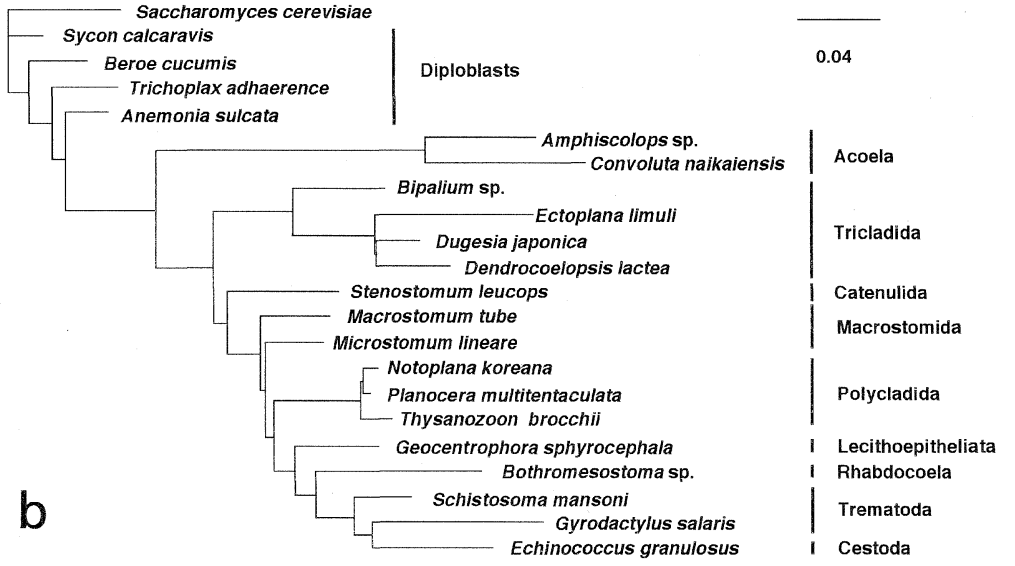
The turbellarians show a variety of patterns in the embryonic development (see Hyman, 1951; Thomas, 1986; Galleni and Gremigni, 1989). In many turbellarian groups, the germ cells contain yolk substance (archoophorans), but in the Tricladida, Prolecithophora Lecithoepitheliata and Rhabdocoela, many yolk cells enclosing the embryo within each egg capsule supply nutrition for embryonic development (neoophorans). There are two modes of cleavage in the archoophorans: duet spiral cleavage of the Acoela and quartet spiral cleavage of the Polycladida and Macrostomida. Cleavage in the neoophoran groups is also classified into two categories: Blastomeres become separated from one another during cleavage in the Tricladida and Prolecithophora, but blastomeres do not become isolated in the Lecithoepitheliata and Rhabdocoela. The present phylogenetic inference suggests that the duet spiral cleavage is the primitive mode from which the quartet spiral cleavage derived. The present results also imply that the neoophoran does not represent a single taxon but yolk cells arose twice convergently in the Tricladida + the Prolecithophora lineage and the Lecithoepitheliata + the Rhabdocoela lineage during the platyhelminth evolution.

We will compare some other detailed points suggested by the present analysis with conclusions of previous workers, though supporting levels of bootstrap for these points were not always high: The order Tricladida contains three subgroups, the Paludicola (fresh water triclads), the Maricola (marine triclads), and the Terricola (terrestrial triclads). According to cladistical analyses on the basis of morphological characters, the Maricola diverged earlier than the Terricola and the Paludicola (Sluys 1989; De Vries and Sluys, 1991). The present results seem to be inconsistent with this view. The phylogenetic trees inferred by three different methods (Fig. 2a-c) commonly suggest that the Terricola diverged first among the three triclad groups. In the traditional (Hyman, 1951) as well as the cladistic phylogeny (Smith *et al.*, 1986; Ax, 1987), the Macrostomida and the Polycladida are regarded as closely related groups. The present inference also suggests a close relationship between the two groups. They form a single cluster in all three trees including 12 platyhelminth groups (Fig. 2a-c). The phylogenetic position of the Prolecithophora could not be determined in the cladgram by Ax (1987) . In the present trees (Fig. 2a-c), a Prolecithophora, *Volticeros ijimai*, always form a cluster with the Tricladida. Kuznedelov and Timoshkin (1995) suggested a close relationship between the two groups. It has long been considered that the Tricladida and the Proseriata are closely related groups (Hofsten,1918). Some workers have proposed that the Tricladida and the Proseriata constitute a monophyletic group, the Seriata (Ax, 1961; Karling, 1974), which is adopted by the cladistic systematics (Ehlers, 1985a,b). In the present phylogenetic trees, however, the two proseriate species are always positioned in separate clusters. In the molecular phylogenetic trees by Rhode *et al.* (1994), two species of the Proseriate are located separately in different clusters. Kuznedelov and Timoshkin (1995) also questioned the close relationship between the Tricladida and the Proseriata on the basis of partial sequencing data of 18S rDNA. In order to obtain a conclusive result on the position of the Proseriata, more proseriate species such as *Bothrioplana* (see Sluys, 1989) must be included in analysis.

Although the present analysis has offered significant inferences on some primary points of turbellarian phylogeny, information obtained from 18S rDNA sequences alone is insufficient to resolve interrelationships among turbellarian orders in detail. Further molecular data are essential for a deeper understanding of turbellarian phylogeny.

Fig. 2. Phylogenetic relationships of turbellarians inferred by (a) neighbor-joining (NJ), (b) maximum-likelihood (ML), and (c) maximum-parsimony (MP) methods. The phylogenetic trees are rooted using *Saccharomyces cerevisiae* as an outgroup. (a) The NJ tree reconstructed using DNADIST and NEIGHBOR program in the PHYLIP package version 3.57 on the basis of the pairwise distance of Jukes and Cantor (1969). (b) The ML tree obtained using fastDNAml algorithm with a transition/transversion ratio of 2.0, which gave the best ML score (ln likelihood =–8621.53972). (c) The consensus MP tree obtained using DNAPARS, SEQBOOT and CONSENSUS program in the PHYLIP package version 3.57. In (a) and (b), branch lengths are proportional to the scale given in substitutions per sequence position. In (a) and (c), the percentage of 500 bootstrap replicates is shown at the node the value is supporting. In (b), bootstrapping was not performed because of the enormous computation time (more than 12 hr per replication).





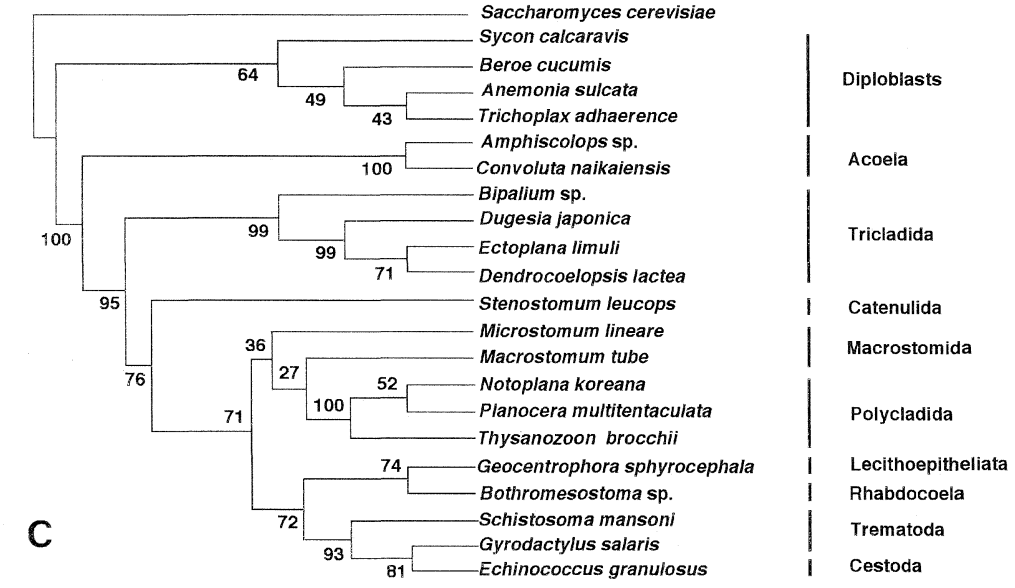


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Fig. 3. Phylogenetic trees reconstructed by (a) NJ, (b) ML, and (c) MP methods after excluding *Vorticeros ijimai*, *Nematoplana* sp., and *Otoplana* sp. from the analyses shown in Fig. 2. The explanation for each tree is the same as shown in Fig. 2 except for "ln likelihood =–8491.68544" in (b).

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