Are the Platyhelminthes a Monophyletic Primitive Group? An Assessment Using 18S rDNA Sequences

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In most zoological textbooks, Platyhelminthes are depicted as an early-emerging clade forming the likely sister group of all the other Bilateria. Other phylogenetic proposals see them either as the sister group of most of the Protostomia or as a group derived from protostome coelomate ancestors by progenesis. The main difficulty in their correct phylogenetic placing is the lack of convincing synapomorphies for all Platyhelminthes, which may indicate that they are polyphyletic. Moreover, their internal phylogenetic relationships are still uncertain. To test these hypotheses, new complete 18S rDNA sequences from 13 species of "Turbellaria" have been obtained and compared to published sequences of 2 other "Turbellaria," 3 species of parasitic Platyhelminthes, and several diploblastic and deuterostome triploblastics. Maximum-parsimony, maximum-likelihood, and neighbor-joining methods were used to infer their phylogeny. The results show the order Catenulida to form an independent early-branching clade and emerge as a potential sister group of the rest of the Bilateria, while the rest of the Platyhelminthes (Rhabditophora), which includes the parasites, form a clear monophyletic group closely related to the protostomes. The order Acoela, morphologically considered as candidates to be ancestral, are shown to be fast-clock organisms for the 18S rDNA gene. Hence, long-branching of acoels and insufficient sampling of catenulids and acoels leave their position still unresolved and call for further studies. Within the Rhabditophora, our analyses suggest (1) a close relationship between orders Macrostomida and Polycladida, forming a clear sister group to the rest of orders, (2) that parasitic plathyhelminthes appeared early in the evolution of the group and form a sister group to a still-unresolved clade made by Nemertodermatida, Lecithoepitheliata, Proleumphthora, Proseriata, Tricladiida, and Rhabdocoela; and (3) that Seriata is paraphyletic.

Introduction

The origin of Bilateria (organisms which display bilateral symmetry and clear anteroposterior polarity) can be considered the most important unsolved problem in systematic biology. These animals also share the characteristic of possessing three clearly distinct cell layers (i.e., they have a true mesoderm); hence, they are collectively called triploblastics. Historically, the presence/absence of a true coelom (i.e., a system of cavities within the mesoderm), and, hence, whether the most primitive body form was acoelomate, pseudocoelomate, or coelomate, has been the main issue in discussion of the origin of the Bilateria. The most classical view, adopted in the majority of zoological textbooks, sees the acoelomate Platyhelminthes as an early-emerging clade forming the likely sister group of all the other Bilateria, which themselves would be divided into two coelomate supergroups, protostomes and deuterostomes (Hyman 1951; Salvini-Plawen 1978; fig. 1A). Since egg cleavage in Platyzelminthes is spiral, as in most protostomes, while it is radial in most deuterostomes, this proposal implies several modifications of early embryogenesis in the deuterostome line with respect to the protostome one. To skip that problem, another phylogenetic scheme sees two Bilateria supergroups: the "Spiralia" on the one hand, including the Platyhelminthes, and the "Radialia" on the other (fig. 1B). Under that scheme, the coelom must therefore have originated twice, once in the Spiralia (after the split of one branch from Platyhelminthes) and once in the Radialia (Ax 1987; Brusca and Brusca 1990). A third view is based on the idea that the gastric pouches of coelenterates are homologous with the gastric pouches (enterocoels) that give rise to the coeloms in deuterostomes. Therefore, features of deuterostome development are assumed to be primitive among Bilateria. In this scheme, protostome developmental features are derived, coelom is of early origin, Platyhelminthes are considered derived from a coelomate ancestor by progenesis (Rieger 1985) or by reduction of coelomic cavities in the adult (Reinacle, Storch, and Welsch 1980), and the hypothetical ancestor of the Bilateria would be an "archicoelomate" (Siewing 1980) (fig. 1C).

Knowledge of the actual phylogenetic position of Platyhelminthes is paramount to decide among these alternatives. Platyhelminthes, or flatworms, display a variety of body forms and are successful inhabitants of a wide range of environments. The majority of their 20,000 extant species are parasitic (classes Trematoda, Monogenea and Cestoda). The free-living forms (class Turbellaria) are primarily epifaunal or infaunal inhabitants of the marine and freshwater benthos, but marine and freshwater pelagic and terrestrial forms also occur. The free-living forms range from less than 1 mm to about 50 cm long. Some parasites (tapeworms) may attain lengths of several meters. Major diagnostic features of the phylum (synapomorphies) are disputed. Ehlers (1985, 1986; see also Ax 1987) has proposed as autapomorphies some features of proctenephridia, multiciliation of epidermal cells, and absence of mitosis in epidermal and other somatic cells, but such views have been contested (Smith, Tyler, and Rieger 1986; Rohde 1990). This casts doubt on the monophyly of the Pla-
Fig. 1.—Conflicting traditional phylogenies on the origin of Platyhelminthes and the acoelomate condition. A, evolutionary tree based on the assumption that the acoelomate condition is primitive within the triploblasts. Under this view, Platyhelminthes form the sister group of all the other Bilateria, which themselves would be divided into two coelomate superfamilies, protostomes and deuterostomes (after Hyman, 1951). B, An evolutionary tree based on a very early splitting of Bilateria into two superfamilies, the “Spiralia,” including the Platyhelminthes, and the “Radalia.” In this scheme, the acoelomate condition is primitive within the triploblastic spiraliana, making Platyhelminthes the first descendant group of this lineage but not the sister group of the Bilateria (after Ax 1987; Brusca and Brusca 1990). C, An evolutionary tree based on the proposal that the acoelomate condition arose through neoteny from developmental stages of protostomes prior to the embryonic appearance of coelomic cavities. In this scheme, coelomic cavities (enterocoels) in deuterostomes are homologous to gastric pouches of the diblastic coelenterates, protostome coelomic cavities (schizocoels) are derived, and the hypothetical ancestor of the bilateria would be an “archicoelomate” (after Siewing 1980; Kieger 1985).
mainly concerned parasitic groups (Baverstock et al. 1991; Blair 1993; Rohde et al. 1993, 1995) and a few turbellarian orders (Riutort et al. 1992, 1993; Katayama et al. 1993; Rohde et al. 1993, 1995). The main conclusions are that all the major parasitic groups constitute a monophylum, that class Trematoda is monophyletic whereas Monogenea may be paraphyletic, that there are contradictory views as regards the closest free-living turbellarian to parasitic groups, and that Platyhelminthes may be polyphyletic (with acoels forming a separate clade; Katayama et al. 1995). Most of these data, however, should be taken very cautiously because most studies used only partial 18s sequences and because "lower" platyhelminth orders (Catenulida, Nemertodermatida, Lecithoepitheliata, and Macrostomida) either were not included or were represented by a single species.

We report here the complete 18s rDNA sequences of 13 species of free-living "Turbellaria" belonging to 10 orders. Published complete sequences of two other free-living turbellaria and three parasitic species were also included in some of the analyses. Sequence data were analyzed with maximum-parsimony, distance-matrix, maximum-likelihood, and pattern of resolved nodes (PRN) methods. Our main focus has been to test: (1) the monophyletic or polyphyletic status of the Platyhelminthes, (2) whether Platyhelminthes can be considered the sister group of the rest of bilaterians, (3) the internal relationships among the extant "turbellarian" orders, and (4) which is the closest free-living representative of the parasitic groups.

Materials and Methods

Organisms

Organisms were chosen to provide representatives of the 11 orders of Turbellaria. In addition, published Platyhelminthes sequences were used. Species represented are shown in table 1, and the 13 species for which new sequence data were collected are indicated. Because there is no consensus as to the sister group of Platyhelminthes (see Introduction), we used as outgroup the choanoflagellate Sphaerocca volvox. Since diploblastics have been clearly shown to be the sister group of all Triblastics, the cnidarian Anemonia sulcata, the ctenophore Beroe cucumis, and the placozoan Trichoplax adhaerens were also included in the analysis. Representatives of Deuterostomia were the starfish Asterias amurensis, the cephalochordate Branchiostoma floridae, and the amphiphan Xenopus laevis, while Aphanopelma sp. (Arthropoda, Aracnida), Oediellus trosuloides (Arthropoda, Aracnida), Eseina fetida (Annelida, Oligochaeta), Lanice conchilega (Annelida, Polychaeta), Lioephura japonica (Mollusca, Polyplacophora), Crassotre virgini (Mollusca, Bivalvia), and Prostoma eilhardi (Nemertea) were used as representatives of Protostomia. Because recent analyses indicate that pseudocoelomates, namely the phyla Nematoda and Nematomorpha, may form the sister group of the rest of Bilateria, and since Gastrotricha and Acanthocephala appear as a likely sister groups of Platyhelminthes (Winneppeninx et al. 1995; Doolittle et al. 1996), sequences from the nematomorph Gordius aquaticus, the gastrotrich Lepidodermella squamatar, and the acanthocephalan Moliniformis moliniformis were included in some of the analyses. All nonplatyhelminth species are listed in table 2.

DNA Extraction, Amplification, and Sequencing

High-molecular-weight DNA was purified according to a modification (Garcia-Fernandez, Bagufia, and Sal6 1993) of the guanidine isothiocyanate method initially described for RNA (Chirgwin et al. 1979). The entire length of the 18s rDNA was amplified in two fragments of approximately equal length by the polymerase chain reaction (Saiki et al. 1985) using the primers: 1F, TACCTGTTG ATCCTGCCAG TAG; 5R, CTTGGCAAAAT CTTTCG; 5F, CGCIAAGCAT TTGCAAGA; and 9R, GATCCTTCGG CAAGTTC CACC TAC. The resulting fragments were either blunt cloned and sequenced as described elsewhere (Caranza et al. 1996) or directly sequenced (Nemertinoides,
alignment gaps were inserted to account for putative length differences between the sequences. A secondary-structure model (Gutell et al. 1985) was used in order to optimize alignment of homologous nucleotide positions, resulting in a total of 1,322 positions that could be used in the phylogenetic analyses (675 being variable and 428 being parsimony-informative when the 33 species are compared).

Distance analyses were calculated using the PHYLIP program package v. 3.52 (Felsenstein 1993) and MUST v. 1.0 (Philippe 1993). A distance matrix of the aligned sequences was generated using the program DNADIST and corrected with the two-parameter method of Kimura (1980). The distances were then converted to phylogenetic trees using FITCH (Fitch and Margoliash 1967) and the neighbor-joining (NJ) method of Saitou and Nei (1987) provided by the NEIGHBOR program. Bootstrap resampling (Felsenstein 1985) was accomplished with the use of the programs SEQBOOT (1,000 replicas) and CONSENSE. FASTDNAML v. 1.1.1a (with global rearrangements and reordering of species) was used for maximum-likelihood analyses (Felsenstein 1981; Olsen et al. 1994), and a bootstrap analysis (n = 100) was performed. Maximum-parsimony (MP) analyses (Camin and Sokal 1965) were calculated with the PAUP computer program v. 3.1.1.
Fig. 3.—Neighbor-joining tree including two acel sequences. All branch lengths are drawn to scale. Note long branches leading to and separating the two acel species, which indicates they may be fast-clock organisms. Lack of resolution within the Bilateria clade results from the loss of information due to the difficulty in aligning the acel sequences. Note that Platyhelminthes cannot be considered monophyletic because some Aschelminthes branch between different Platyhelminthes clades, here represented as “Platyhelminthes.” Numbers at nodes are percentages of 1,000 bootstrap replicates that support the branch. Values are shown only if over 50%. This is an unrooted tree, rooted defining the choanoflagellate as the outgroup. For species names, see tables 1 and 2.

(Swofford 1993) using a heuristic search procedure and a branch-swapping algorithm. A bootstrap analysis (n = 100) was also performed. The trees were rooted using the SSU rRNA sequence of the “choanoflagellate” Sphaeroeca volvox. In all analyses, gaps were considered as missing data.

PRN (Lecointre et al. 1994) was performed using the program MUST v. 1.0, to prepare the data, and PRN v. 1 to test the robustness of the nodes obtained. Instead of simply examining the bootstrap proportions (BP) at important nodes as a criterion of robustness of the corresponding nodes, PRN introduces a procedure of BP analysis which involves following the values of BP as a function of increasing number of nucleotides. PRN was used for the same taxa used in the distance analysis, but only the parsimony-informative positions were included, under the following conditions. The alignments of the N species were each submitted to random sampling of a given number of sites through the use of the PRN program running on UNIX platforms. Ten different sequence lengths were chosen (10, 15, 25, 50, 75, 100, 150, 200, 250, 300), and, for each, 200 samples were drawn. Thus a total of 2,000 subsets of sequence alignments were obtained, each including all N species. Each of these subsets was used to construct a neighbor-joining tree, which was submitted to 1,000 bootstrap replicates.

Selection of the nodes was then carried out to keep only those with an ascending tendency and that appeared more frequently. At a given node, one could therefore graphically display the evolution of BP as a function of the number of nucleotides used to generate the tree.

The sequence alignment and the weighting mask which determined the nucleotide positions taken for the analyses are available from the ftp site: por-thos.bio.ub.es/users/ftp/pub/teu/l8Sphylogeny.

Results

A distance tree including all the sequences is represented in figure 3. Some features are consistent with what is known of animal evolution. As expected, the diploblast branched before all triploblast groups. However, the most interesting feature of this tree is the long branches leading to and separating the two acel representatives. In fact, acel sequences diverge so much from the rest of the organisms studied (not only from Platyhelminthes) that alignment of sequences turned out to be very difficult. Nonetheless, at the cost of losing many informative sites, the two acels and all the representatives from the other groups (diploblasts, protostomes, “aschelminthes,” deuterostomes, and platyhelminths) were included. The analysis considers only
those positions that were unambiguously aligned for the two acoels. This reduced the available positions from 1,322 to 1,131 and the variables from 675 to 512. In this tree (rooted with the choanoflagellate *Sphaeroeca volvox*), the acoels constitute an early sister branch to the rest of bilaterians (67% bootstrap). However, the loss of informative sites leaves the internal relationships of the bilaterian group unresolved. The very long branches of the two acoels suggest that we are in front of fast-clock organisms; hence, their position in the tree could be artifactual. To avoid their disturbing influence, they were not included in subsequent analyses.

The distance analyses, Fitch-Margoliash and neighbor-joining methods, not including the acoels, resulted in identical topologies (fig. 4). Two general features of the tree were unexpected. First, Platyhelminthes appear to be a paraphyletic group, as the order Catenulida do not cluster with the rest of Platyhelminthes (=Rhabditophora + Nemertodermatida) constitute a clear monophyletic group which appears as the sister group of a protostome + deuterostome clade. Numbers at nodes are percentages of 1,000 bootstrap replicates that support the branch, only values over 50% being represented, with the exception of the bilaterian sister group to Catenulida (45%). All branch lengths are drawn to scale. For species names, see tables 1 and 2.

The 50% majority-rule consensus tree of 10 maximum-parsimony (MP) trees of equal length is shown in figure 5. It has a length of 1,943 steps, with a consistency index (CI) of 0.495 and a retention index (RI) of 0.504. Platyhelminthes and Deuterostomia are well-supported monophyletic groups (91% and 81%, respectively), while the Protostomia show a sequential branching pattern not well supported by bootstrap (only 27%), similar to that obtained in the distance tree (fig. 4). Although most of the more parsimonious trees (9 of 10) group platyhelminths with deuterostomes, this grouping is not supported by bootstrap (only 24%). This analysis and the bootstrap analyses suggest that the available se-
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Fig. 5.—Fifty percent majority-rule consensus of 10 most parsimonious trees (percentiles indicated below the nodes). Bootstrap values are indicated above the nodes, only those over 50% being shown. Total length of the tree is 1,943 steps; the overall consistency index is 0.495. Branch lengths are unrelated to evolutionary distance. The topology of this tree only differs from the distance tree of figure 4 in that the monophyletic 'Platyhelminthes' (=Rhabditophora + Nemertodermatida; Ehlers 1985) form a clade with the deuterostomes. However, the low bootstrap values for this clade and for the protostome lineage suggests instead a polytomy (see text). For species names, see tables 1 and 2.

Discussion

This paper explores one of the most important unsolved problems in systematic biology, the origin of Bi-
Lateria. Using complete 18S rDNA sequences and different methods of phylogenetic analysis, we have studied the monophyletic, phylogenetic position, and internal phylogeny of one of the most likely candidates for sister group of all the other Bilateria, the Platyhelminthes. The analyses show the Catenulida branching after the diploblasts, whereas the Rhabditophora, which form a monophyletic clade, branch early on in the evolution of the protostomes. In addition, all analyses give a similar internal phylogeny for the Rhabditophora, with the parasites as a monophyletic group branching unexpectedly early within the evolution of the group.

The Acoela: Primitive or Derived Fast-Clock Organisms?

Our 18S analyses show Acoela as the first Bilateria to branch after the diploblasts, although with only moderate support (67% bootstrap) (fig. 3). An early divergence of acoel flatworms in triploblast evolution had previously been reported by Katayama et al. (1993) based on partial 18S rDNA sequences. Later, Katayama et al. (1995) used the acoel Convoluta natkatensis with two dicemids, one mixozoan, the nematode Caenorhabditis elegans, and other diploblast and triploblast organisms to position the mesozoan dicemids within the Metazoa. A clade made by the acoel, dicemids, mixozoa, and C. elegans was found to be the sister group to the rest of Bilateria. All the members of this clade, however, had very long branches, which may explain why species so diverse grouped together.

The NJ tree supporting the early branching of acoels (fig. 3) leads, however, to several inconsistencies. First, protostomes and deuterostomes cluster together but with low bootstrap (less than 50%), and protostomes appear paraphyletic, with arthropods and the single nematomorph included forming a sister group to the deuterostomes. When acoels are not introduced, deuterostomes appear highly supported (94%, 81%, and 84% in NJ, MP, and ML trees, respectively; figs. 4–6), whereas protostomes remain paraphyletic because aschelminthes (NJ trees; fig. 4) or Platyhelminthes (ML trees; fig. 6) appear buried within them. Second, the bulk of Platyhelminthes appears only weakly supported (51% in fig. 3); instead, when acoels are not included, Platyhelminthes are very highly supported (figs. 4–6; see below). Finally, and most importantly, the lines leading to and separating the two acoels are extremely long, which may lead to artifactual grouping. Indeed, another long-branch organism, the acanthocephalan Moliniformis, is attracted close to acoels. Early branching of acoels may be explained in two ways. Either they really are very primi-
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The number of parsimony informative sites (B) was plotted vertically as a function of increased number of nucleotides (abscissa) (for further details, see Materials and Methods). The graphs correspond respectively to the monophyly of (A) Macrostomida (Microstomum and Macrostomum), (B) Polycladida (Planocera and Discocelis) + Microstomum, (C) Polycladida (Planocera and Discocelis) + Macrostomum, and (D) Macrostomida (Microstomum and Macrostomum) + Polycladida (Planocera and Discocelis).

FIG. 7—The patterns of resolved nodes (PRN) for four nodes taken from the phylogenies of figures 4–6 concerning the clustering of Polycladida and Macrostomida. In each graph, the 200 bootstrap proportions (ordinate) obtained for a given number of nucleotides (10, 15, 25, 50, 75, 100, 150, 200, 250, and 300) are plotted vertically as a function of increased number of nucleotides (abscissa) (for further details, see Materials and Methods). The graphs correspond respectively to the monophyly of (A) Macrostomida (Microstomum and Macrostomum), (B) Polycladida (Planocera and Discocelis) + Microstomum, (C) Polycladida (Planocera and Discocelis) + Macrostomum, and (D) Macrostomida (Microstomum and Macrostomum) + Polycladida (Planocera and Discocelis).

In acoels are scant, although they are known to have generation times of 3–4 weeks in optimal conditions (Faubel, personal communication). This is considerably shorter than most generation times reported for other Turbellaria (Heitkamp 1988) and gives support for such an effect in acoels. Whether their long branches stem from shorter generation times as predicted by the generation time effect hypothesis or whether they are due to increased rates of change in their lineage is an open question. Acoels have been proposed in the past to be the most primitive Platyhelminthes. However, most features considered primitive in acoels are based on negative evidence (absence of characters), though the
"brain" and the longitudinal nerve cords closely resemble the cnidarian and the ctenophore condition (Haz-sprunar 1996). Recent reappraisals, however, place acoels as a rather derived group (Ehlers 1985; Smith, Tyler, and Rieger 1986; Willmer 1990).

Are the Catenulida an Early Bilaterian Group?

In the past, Catenulida have been considered as an aberrant member of Platyhelmintes (Reisinger 1924) or to represent one of the first offshoots in their evolution (Ax 1963; Karling 1974). Some (Sterrer and Rieger 1974) have raised the question of whether this group should even be classified within the Platyhelmintes. This is because the postulated synapomorphies between Catenulida and Rhabditophora: protonephridia, ciliary rootlet system, and mode of epidermal replacement (Ehlers 1985) have not been proved to be homologous with the cnidarian and the ctenophore condition (Haz-sprunar 1985) have not been proved to be homologous. Moreover, the promising PRN node found linking Catenulida to Acoelomorpha are not known. Synapomorphies of the Catenulida are the unpaired excretory system, the special organization of the cyrtocyte, the dorsorostral position of the male copulatory organ, other features are plesiomorphies, common to most Platyhelmintes, and some (e.g., sparsely ciliated epidermis, monoligated epidermal sensory receptors, lack of frontal glands, and lack of rhadites) are common to lower Eumetazoa.

With the exception of trees incorporating acoels (fig. 3), Catenulida is the first group branching after the diploblasts, although with low or moderate bootstrap support (45%, 71%, and 45% for NJ, MP, and ML trees, respectively). Moreover, the promising PRN node found for Catenulida + Diploblasts + choanoflagellates (Protozoa) is a good indicator of the basal position of this group. However, their basal position in all trees could be a consequence of a greater rate of evolutionary change for this group (similar to what happens with Acoela). In trees constructed using algorithms reflecting rate inequalities along branches such as those used here (fig. 4), one essentially carries out a relative-rate test on several species simultaneously, a rate difference being manifested by a difference in branch lengths (Philippe et al. 1994). Clearly at variance to what happened with acoels, the catenulid branch is similar in length to the rest of metazoans (only some platyhelmintes have somewhat longer branches). A relative-rate test (Wilson, Carlson, and White 1977) was carried out, resulting in very similar distances for deuterostomes (21.8%), protostomes (21.4%), "Platyhelmintes" (23.5%), and Catenulida (Stenostomum) (20.7%) when calculated using the choanoflagellates as an outgroup. Similar results are obtained when the diploblasts are used as outgroup (18% for deuterostomes, 17.9% for protostomes, 19.2% for "Platyhelmintes" and 17.6% for the Catenula with respect to the ctenophore). In all cases, only the "Platyhelmintes" seem to have slightly higher rates.

When "lower" groups, reportedly considered basal to the triploblastic Bilateria (Winnepenninckx et al. 1995), such as phylum Gastrotricha, phylum Acanthocephala, and phylum Nematomorpha (classically classified as phylum Aschelminthes) were included in the analysis, they fell within the protostomates (NJ tree, fig. 4) or formed the sister group of Rhabditophora (ML tree, fig. 6, for Gastrotricha and Acanthocephalans). In both cases, Catenulida branched earlier than any other Bilateria, including these presumptive lower groups. However, when acoels are introduced (NJ trees, fig. 3), acanthocephalans and gastrotricha branch sequentially after them, although this probably results from attraction among long branch groups. Gastrotricha and Acanthocephala were reported by Winnepenninckx et al. (1995) to form a weakly supported clade with the Platyhelmintes. Our analyses, using more species, support the relationship of rhabditophoran platyhelmintes with these aschelminth groups, calling for further studies.

Altogether, and despite the fact that only a single representative has been sequenced so far, our data support the hypothesis that Catenulida constitute an independent clade that branched off early in the evolution of Bilateria and, hence, that Platyhelmintes probably are paraphyletic.

The Bulk of "Platyhelmintes": the Monophyletic Rhabditophora

The bulk of "Platyhelmintes," the so-called Rhabditophora sensu Ehlers (1985), appear in all analyses as a clear monophyletic group. Rhabditophora, which includes the parasitic classes (Neodermata), has a main autapomorphy, the presence of lamellated rhabdies, and several synapomorphies such as the presence of duo-gland adhesive system and duo-cell weir and multiciliated terminal cell in the protonephridia. There is ample consensus in considering the Rhabditophora as monophyletic (Karling 1974; Ehlers 1985; Smith, Tyler, and Rieger 1986; Rohde 1990). This is supported here by very high or moderate bootstraps (99% and 91% for NJ and MP trees, respectively, and 65% for ML trees) and agrees with the morphological character-based phylogenetic schemes of Karling and Ehlers (see fig. 2). 18S sequence data, however, show a major difference with them: the clustering of Nemertodermatida within Rhabditophora and not with Acoela forming the Acoelomorpha sensu Ehlers (1985). Because only one species (Nemertinoides elongatus) has been analyzed here, the position of nemertodermatids should be left open.

Internally, NJ and ML trees reproduce two main monophyletic clades with higher bootstrap values: Macrostomida + Polycladida and the rest of Rhabditophora plus Nemertodermatida. Macrostomida and Polycladida appear as close groups in Karling's (1974) and Ehlers' (1985) phylogenetic proposals (fig. 2) but do not cluster together. Smith, Tyler, and Rieger (1986) see Polycladida within a clade with the bulk of Rhabditophora, whereas Macrostomida form a sister group to the enigmatic Haplophyryngida. Finally, according to Rohde (1990), Macrostomida form a clade with Prolecithophora this being the sister group of Prosiriata and Neodermata. This state of flux as regards Macrostomida may reflect the uncertainties of linking Macrostomidae and Microstomidae in a single order (Rieger, personal communication), indicating the need
to include more species in future analyses. Despite these
difficulties, the high bootstraps found in NJ trees, the
promising PRN nodes for Microstomidae + Macrostom-
idae (fig. 7A) and Polycladida + Macrostomidae (fig.
7D), and the presence of homochorial gonads and en-
dolecithal eggs in both groups are good indicators of
close affinities between Polycladida and Macrostomida.

The sister group to Polycladida + Macrostomida
reproduces the Neophora (Westblad 1948; Ehlers 1985)
with high or moderate bootstraps (78%, 86%, and 57%
for NJ, MP, and ML trees, respectively). The main syn-
apomorphies of this group are the presence of hetero-
cellular female gonads and the ectolecithal eggs. The
internal phylogeny of Neophora, however, is not well
resolved. Rohde (1990) has noted that apomorphies for
the Lecithoepitheliata, Prolecithophora, Proseriata, and
Tricladida are insufficient to determine their position
within the Platyhelminthes. The low bootstraps found
here for most neoophoran branchings indicate that at the
present level of knowledge, 18S rDNA sequences are
unable to settle the issue. Even so, all trees reproduce
some interesting regularities. First, leaving Nemertoder-
matida aside, parasites appear as a clear monophyletic
group basal to the rest of neophorans (see below). Sec-
ond, Tricladida often clusters with Rhabdocoela with
low or moderate bootstraps (although PRN nodes are
promising; data not shown), forming a highly derived
group. And finally, Proseriata always show a rather basal
position close to the parasites (Neodermata) and far
from their presumed sister group, the Tricladida. These
results indicate that Seriata are a paraphyletic group and
that Neodermata are a monophyletic basal group.

Parasitic platyhelminthes (Neodermata; Ehlers
1985) are most often considered either the sister group
of the Dalycylioida, rhabdocoels with doliform pharynx
(Ehlers 1985; Brooks 1989), or an early branch of rhab-
docoels which retained the less specialized roseulate
pharynx (Kotikova and Joffe 1988). Based on the sim-
ilarity between ultrastructure of the flame bulbs of Neo-
dermatida and those of most "primitive" proseriates, Roh-
de (1990) considers them not related to rhabdocoels but
as the sister group of the Proseriata. The NJ, MP, and
ML trees built here support the monophyly of Neoder-
matida and its basal position within the evolution of neoo-
phorans. Cestodes begin their life cycle as parasites of
their presumed host, giving the typical life cycle with two larval stages
and three host species (Stunkard 1975). This plausible
scenario perfectly fits the early branching of parasitic
groups from primitive "turbellarians" found here and
argues against proposals on parasitism as a late develop-
ment from advanced turbellarian rhabdocoels (Ehlers
1985; Brooks 1989).

Taxonomic and Phylogenetic Considerations

The general topology of the trees here obtained
agrees to a large extent with previous proposed phylog-
ies of the animal kingdom (Field et al. 1988; see the
reanalysis of their data by Patterson 1989 and Lake
1990), followed by those of Christen et al. (1991),
Adoutte and Philippe (1993), Chenuil (1993), and Wain-
right et al. (1993). All show two main features: first, a
clear split between diploblasts and triploblasts; second,
a poor resolution for the major coelomate phyla, es-
specially for protozoans often giving big multifurca-
tion that include annelids, molluscs, arthropods, nemertines,
and other minor phyla. In addition, the positions of
pseudocoelomates and acelomates remain uncertain.
Philipe, Chenuil, and Adoutte (1994) have provided
convincing evidence that unresolved bushy multifurca-
tions arise because the resolving power of the presently
available complete 18S rDNA database is only about 40
Myr. In other words, multifurcations derived
from molecular data have provided molecular corrobo-
ration for the "Cambrian explosion." In this context, the
uncertain position of the Rhabditophora as regards pro-
tostomes and deuterostomes (see figs. 3–6) may
indicate that the bulk of "Platyhelminthes" appeared and
evolved during this fast Cambrian radiation. In contrast,
the early branching of Catenulidida supports an acelo-
mate grade of organization for the first bilaterian, al-
though the small number of species studied leaves the
issue unresolved and calls for a deeper study.

As regards the hypothesis put forward on the origin
of Bilateria (fig. 1A–C) the phylogenetic derivations of
our results could also be of interest. First, Platyhelmin-
thes as a whole cannot be considered the sister group of
the rest of Bilateria as they are paraphyletic. Second, the
bulk of "Platyhelminthes," represented by the mono-
phylectic Rhabditophora, are more related, as could be
expected, to protostomes than to deuterostomes. This
suggests that Rhabditophora are either protostomes or
most likely, an early branch on the lineage leading to
the bulk of protostomes (fig. 1B). This rules out the
hypothesis that sees "Platyhelminthes" (Rhabditophora)
as the most primitive Bilateria (fig. 1A) or as a derived
group from coelomate ancestors (fig. 1C). Third, all
analyses and trees, with the exception of those incor-
porating acelous, show the catenulid Stenostomum
branching after the diploblasts and earlier than any tri-
ploblast here studied, including potential primitive
groups such as gastrotrichs, acanthocephalans, and ne-
matormorphs (Winnepeinnickx et al. 1995).

To summarize, we have shown that Platyhelminthes
as a whole do not seem to be primitive. Although our
results do not contradict the idea of Platyhelminthes
being monophyletic, the persistence of the node, albeit
with a low bootstrap, locating the Catenulida as the sister group to the rest of the Bilateria, casts some doubts on its monophyly. Long-branching of acoels and the paucity of sampling in both acoels and catenulids calls for a large sampling of these primitive groups which hold promise to be the key to the evolution of the Bilateria.

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