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Triploblastic Relationships with Emphasis on the Acoelomates and the Position of Gnathostomulida, Cycliophora, Plathelminthes, and Chaetognatha: A Combined Approach of 18S rDNA Sequences and Morphology

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Abstract.—Triploblastic relationships were examined in the light of molecular and morphological evidence. Representatives for all triploblastic "phyla" (except Loricifera) were represented by both sources of phylogenetic data. The 18S ribosomal (rDNA) sequence data for 145 terminal taxa and 276 morphological characters coded for 36 supraspecific taxa were combined in a total evidence regime to determine the most consistent picture of triploblastic relationships for these data. Only triploblastic taxa are used to avoid rooting with distant outgroups, which seems to happen because of the extreme distance that separates diploblastic from triploblastic taxa according to the 18S rDNA data. Multiple phylogenetic analyses performed with variable analysis parameters yield largely inconsistent results for certain groups such as Chaetognatha, Acoela, and Nemertodermatida. A normalized incongruence length metric is used to assay the relative merit of the multiple analyses. The combined analysis having the least character incongruence yields the following scheme of relationships of four main clades: (1) Deuterostomia [((Echinodermata + Enteropneusta) (Cephalochordata (Urochordata + Vertebrata)))]; (2) Ecdysozoa [(((Priapulida + Kinorhyncha) (Nematoda + Nematomorpha)) ((Onychophora + Tardigrada) Arthropoda))]; (3) Trochozoa [((Phoronida + Brachiopoda) (Entoprocta (Nemertea (Sipuncula (Mollusca (Pogonophora (Echiura + Annelida)))))))]; and (4) Platyzoa [((Gnathostomulida (Cycliophora + Syndermata)) (Gastrotricha + Plathelminthes))]. Chaetognatha, Nemertodermatida, and Bryozoa cannot be assigned to any one of these four groups. For the first time, a data analysis recognizes a clade of acoelomates, the Platyzoa (sensu Cavalier-Smith, Biol. Rev. 73:203–266, 1998). Other relationships that corroborate some morphological analyses are the existence of a clade that groups Gnathostomulida + Syndermata (= Gnathifera), which is expanded to include the enigmatic phylum Cycliophora, as sister group to Syndermata. [Ecdysozoa; Metazoa; morphology; phylogeny; Platyzoa; 18S rRNA; Triploblastica.]

Metazoan and, in particular, triploblastic relationships are becoming clearer as phylogenetic techniques are applied to morphological and molecular characters, although the first high-level analysis combining both morphology and molecules (total evidence) was not published until 1998 (Zrzavý et al., 1998). That study constituted the most comprehensive analysis published to date for three reasons: (1) all metazoan phyla and problematic groups were coded for morphology; (2) the most complete molecular sampling (in terms of number of phyla) was included; and (3) this was the first time that metazoan relationships were examined on the basis of the total evidence. As a result of this analysis Zrzavý et al. (1998) elevated a considerable number of groups to phylum status, and merged others.

Almost simultaneously with Zrzavý et al., Cavalier-Smith (1998) proposed a "new system of life" based on six kingdoms, with major changes in metazoan classification. His new system tried to synthesize many of the new relationships that molecular systematists (in particular "18S systematists") had proposed in recent years, although it was not based on a character-based phylogenetic analysis. One of these groups, Ecdysozoa, might seem already to be a well-recognized metazoan group, although it was just proposed in 1997 (Aguinaldo et al., 1997). Another new group was Platyzoa, which includes a series of acoelomate and pseudocoelomate (nonecdysozoan) taxa. Also many

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new phyla, as well as other supra and infraphylum categories, were erected and new names introduced.

The ultimate responsibility for the enlarged number of phylogenetic hypotheses of relationships among animal phyla lies with the exponential growth of molecular data and the refinement of morphological and anatomical information through electron microscopy. The goal of this paper is to evaluate these hypotheses of relationship by using new molecular data in combination with the morphological data set published by Zrzavý et al. (1998).

BACKGROUND

Cavalier-Smith (1998), in his six-kingdom system of life, proposed dividing triploblastic animals into four infrakingdoms of protostome animals (Lophozoa, Chaetognathi, Ecdysozoa, and Platyzoa) and into two infrakingdoms of deuterostomes (Coelomopora and Chordonia). Lophozoa (previously referred to as Eutrochozoa [Ghiselin, 1988; Eernisse et al., 1992] or Lophotrochozoa [Halanych et al., 1995]) included the classical protostome coelomates (Annelida, Echiura, Sipuncula, Pogonophora, Mollusca, Nemertea), Entoprocta, and Cycliophora plus the "lophophorates" (Bryozoa, Phoronida, and Brachiopoda). Zrzavý et al. (1998) used the name Trochozoa for all Lophozoa except Brachiopoda and Phoronida. Ecdysozoa (Aguinaldo et al., 1997) included the molting animals (Nematoda, Nematomorpha, Kinorhyncha, Priapulida, Loricifera, Onychophora, Tardigrada, and Arthropoda) (see also Giribet and Ribera, 1998; Zrzavý et al., 1998). Chaetognathi include the enigmatic phylum Chaetognatha. Perhaps the most surprising taxon in this new system of classification is the so-called Platyzoa. The components of this group were defined as ciliated nonsegmented acoelomates or pseudocoelomates that lack a vascular system and have a straight gut (when present), with or without anus. Platyzoa thus include Rotifera, Acanthocephala, Gastrotricha, Gnathostomulida, and Plathelminthes (Cavalier-Smith, 1998).

This system relocates many taxa far from their previous positions in the classifications. For example, Platyzoa were divided into two phyla, Acanthognatha (= Rotifera, Acanthocephala, Gastrotricha, and Gnathostomulida), and a monophyletic Plathelminthes. However, Gastrotricha and Gnathostomulida are considered independent phyla by most authors, whereas Plathelminthes have been found to be nonmonophyletic in several morphological and molecular analyses.

What Is the Evidence for the Monophyly of the Platyzoa?

Winnepenninckx et al. (1995) presented an "aschelminth" phylogeny based on 18S rDNA sequences, including platyzoan sequences of Gastrotricha, Rotifera, Acanthocephala, and Rhabditophora, which turned out to constitute a monophyletic clade. Carranza et al. (1997) also used 18S rDNA sequences of several platyzoan groups (Gastrotricha, Acanthocephala, Acoela, Nemertodermatida, Catenulida, and Rhabditophora) to address the question of Plathelminthes monophyly. The data suggested monophyly of Rhabditophora (including the Nemertodermatida), and nonmonophyly of Plathelminthes (to the exclusion of Catenulida and Acoela).

In general, molecular phylogenies have proposed monophyly of Plathelminthes + Syndermata (e.g., Eernisse, 1998; Littlewood et al., 1998); Plathelminthes + Gastrotricha + Syndermata (e.g., Winnepenninckx et al., 1995; Carranza et al., 1997); Gnathostomulida + Gastrotricha (Zrzavý et al., 1998); and Syndermata + Cycliophora (Winnepenninckx et al., 1998). Together all these groups, except Cycliophora, constitute the Platyzoa sensu Cavalier-Smith (1998).

Different relationships among the platyzoan taxa have been proposed by several authors on the basis of morphological data (e.g., Lorenzen, 1985; Wallace et al., 1995, 1996; Neuhaus et al., 1996; Ahlrichs, 1995, 1997; Nielsen, 1995; Nielsen et al., 1996; Haszprunar, 1996); a summary of these hypotheses is shown in Figure 1. Meglitsch and Schram (1991) (see also Schram, 1991) made the first attempt to analyze metazoan phylogeny using parsimony algorithms. Schram and Ellis (1994) reanalyzed an amended data set by using more-standard parsimony procedures. Their consensus cladogram contained a basal clade of triploblastic animals consisting of Gastrotricha, Rotifera, Acanthocephala, Chaetognatha, Nematoda, Nematomorpha, Kinorhyncha, Priapulida, and Loricifera (= "Aschelminthes"). This clade was followed by a clade containing

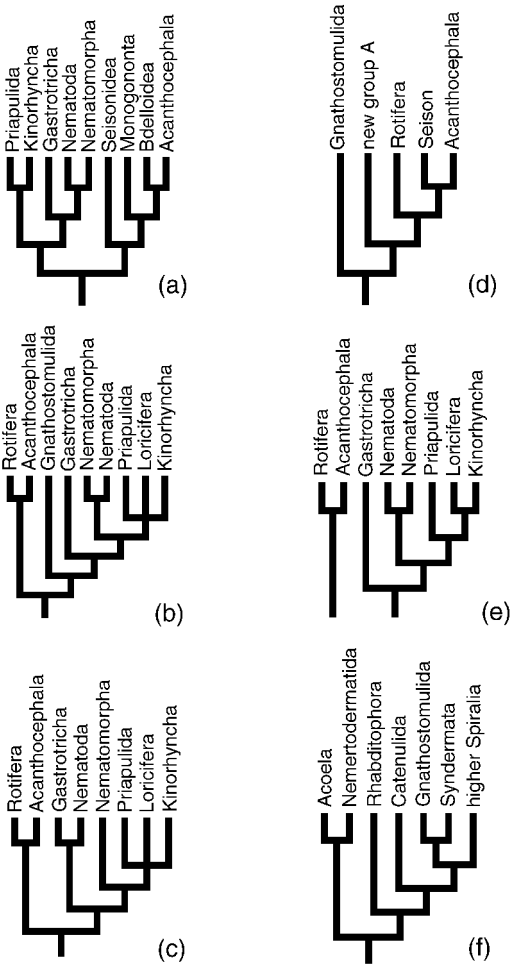

FIGURE 1. Phylogenetic hypotheses for different platyzoan taxa. (a) Lorenzen (1985). (b) Wallace et al. (1995, 1996). (c) Neuhaus et al. (1996); (d) Ahlrichs (1995, 1997); (e) Nielsen (1995); Nielsen et al. (1996). (f) Haszprunar (1996).

Gnathostomulida + Plathelminthes (= Plathelminthomorpha), as sister group to coelomates. Eernisse et al. (1992) recoded Schram's 1991 data matrix and created a new morphological data matrix for 26 selected taxa. Plathelminthomorpha were monophyletic, but Gastrotricha, Rotifera, Acanthocephala, and some minor phyla were not included in the analyses. Backeljau et al. (1993) performed another analysis recoding some of Schram's 1991 characters. Their results showed little resolution at the base of the triploblastic animals. The position of Gastrotricha, Chaetognatha, and Syndermata, among others, was unresolved in the consensus tree. Plathelminthomorpha were monophyletic within a clade of spiralian worms plus panarthropods.

Wallace et al. (1995, 1996) analyzed relationships among pseudocoelomate taxa, and found the following pattern: (Syndermata (Gnathostomulida (Gastrotricha + Introverta))) (Fig. 1b). In this case, only Plathelminthes and Polychaeta were used to test "pseudocoelomate" monophyly. The parsimony analysis of Nielsen et al. (1996) recognized three independent groups that involve platyzoan taxa: Cycloneuralia (including the Gastrotricha), Parenchymia (including the Plathelminthes), and Syndermata. With respect to Gnathostomulida, Nielsen (1995) considered them to be a modified annelid and thus did not include this taxon in his analysis. More recently, the morphological parsimony analysis of Zrzavý et al. (1998) supported monophyly of Gastrotricha + Syndermata, as well as monophyly of Plathelminthes (Catenulida + Acoela + Nemertodermatida + Rhabditophora), and Plathelminthomorpha (Gnathostomulida + Plathelminthes). Plathelminthomorpha was the sister group to Trochozoa (including Cycliophora and excluding the "lophophorates"), and Gastrotricha + Syndermata were sister group to Plathelminthomorpha + Trochozoa. Cycliophora appeared as the sister group to Entoprocta.

GNATHOSTOMULIDA: ENIGMATIC AS EVER?

The marine worm group Gnathostomulida was originally described by Ax (1956) from two species, *Gnathostomula paradoxa* from the North and Baltic Seas and *Gnathostomaria lutheri* from the Mediterranean, although he considered them an order of the phylum Plathelminthes. Later, Ax (1960) separated them from the Turbellaria and created a new class. Riedl (1969) elevated the Gnathostomulida to the rank of phylum, and Sterrer (1972) divided the Gnathostomulida into the orders Filospermoidea (two families, including *Haplognathia*), and Bursovaginoidea (nine families, including *Gnathostomula*). More recently, Ax (e.g., 1984, 1985, 1996) considered Gnathostomulida as the sister group to Plathelminthes, the two of them constituting the supraphyletic category Plathelminthomorpha (Ax, 1984). Sterrer et al. (1985) questioned the putative sister group relationship between

Gnathostomulida and Plathelminthes, and postulated alternative hypotheses of relationships for Gnathostomulida. One of these hypotheses supported a relationship of Gnathostomulida + Gastrotricha based on the monociliary epithelium and the structure of protonephridia (see also Rieger and Mainitz, 1977). The other hypothesis postulated a grouping of Gnathostomulida + Rotifera based on tubular reinforcement of the inner jaw lamella (see also Rieger and Rieger, 1977, 1980; Rieger and Tyler, 1995).

The cladistic analyses of Meglitsch and Schram (1991), Schram (1991), Eernisse et al. (1992), Backeljau et al. (1993), and Schram and Ellis (1994) suggested monophyly of Plathelminthomorpha. However, a study on the epidermis of several members of the Platyzoa (Ahlrichs, 1995, 1997) indicated a putative clade Gnathifera (= Gnathostomulida + Syndermata) as opposed to Plathelminthomorpha (Fig. 1d). This group has also been proposed by Kristensen (1995), based on the jaw apparatus of a new freshwater taxon from Greenland (New group A); by Haszprunar (1996), based on four morphological characters (Fig. 1f); and by Herlyn and Ehlers (1997), based on an anatomical study of the pharynx of one gnathostomulid.

Analyses of molecular data from 18S rDNA sequences published to date are ambiguous with respect to the position of gnathostomulids. Littlewood et al. (1998) used a sequence of *Gnathostomula paradoxa* that came out as a member of Ecdysozoa. However, using the same gnathostomulid sequence, Zrzavý et al. (1998) found *Gnathostomula* to be a sister taxon to the two gastrotrich sequences included in their analyses.

In summary, morphology has suggested that gnathostomulids are either a member of the Plathelminthes, the Annelida, or are sister group to Plathelminthes, Gastrotricha, Rotifera, or Syndermata. The most recent morphological analyses seem to favor a sister group relationship between Gnathostomulida and Syndermata. Additionally, a relationship with Chaetognatha and with Gastrotricha has been proposed by molecular data.

Cycliophora

The phylum Cycliophora was described by Funch and Kristensen (1995) on the basis of a single species, *Symbion pandora*, living on the mouthparts of the Norway lobster *Nephrops norvegicus*. Originally a morphologic resemblance of cycliophorans with rotifers had been postulated by Wingstrand (unpublished observations cited by Funch and Kristensen, 1997), but later, *Symbion pandora* was thought to be related to Entoprocta and Ectoprocta (Funch and Kristensen, 1995), or just to Entoprocta (Funch and Kristensen, 1997). The first cladistic morphological analysis including the phylum Cycliophora was that of Zrzavý et al. (1998), which placed *Symbion* as sister group to Entoprocta. This putative relationship of (*Symbion* + Entoprocta) was also postulated in the classification of the metazoan kingdom by Cavalier-Smith (1998), who considered them to be members of the phylum Kamptozoa. The first molecular analysis of *Symbion pandora* used 18S rDNA sequences to place it as the sister group to Syndermata (Winnepenninckx et al., 1998), as predicted by Wingstrand, but other taxa—postulated to be related to Syndermata (Gnathostomulida, Plathelminthes, and Gastrotricha)—were not included in the molecular analysis.

Plathelminthes

Modern classifications of Plathelminthes recognize three groups: Acoelomorpha (= Acoela + Nemertodermatida), Catenulida, and a third group, Rhabditophora, that contains the remaining "turbellarian" orders plus the parasitic classes (e.g., Karling, 1974; Ehlers, 1985, 1986, 1995; Ax, 1984, 1996). The position of the parasitic groups (= Neodermata) within the Rhabditophora has been disputed (e.g., Ehlers, 1985; Ax, 1984).

Molecular analyses of Plathelminthes are numerous in the literature, but only a few have investigated higher-level relationships within the group (Katayama et al., 1993, 1995, 1996; Rohde et al., 1993, 1995; Katayama and Yamamoto, 1994; Carranza et al., 1997; Giribet and Ribera, 1998; Zrzavý et al., 1998; Littlewood et al., 1999; Ruiz-Trillo et al., 1999). The common conclusions of these studies are (1) all the major parasitic groups constitute a single lineage; (2) Rhabditophora is monophyletic, including the parasitic forms; (3) Plathelminthes may be polyphyletic with acoels forming a separate clade; (4) nemertodermatids are either included within Rhabditophora or form an independent clade; and (5) catenulids

either form a separate clade or are sister to Rhabditophora.

Catenulida has been considered to be one of the earliest diverged groups of Plathelminthes (Karling, 1974; Ax, 1963, 1996; Ehlers, 1985, 1986) because of the presence of several morphological traits considered plesiomorphic for the Plathelminthes: two cilia in the terminal cell of the protonephridium, and epidermal cells weakly multiciliated, usually with two cilia per cell. Other authors consider the putative synapomorphies uniting Catenulida and Rhabditophora—structure of protonephridia, structure of cilia, epidermal movement (Ehlers, 1985, 1986)—to be convergent (Sterrer and Rieger, 1974; Smith et al., 1986). Furthermore, the absence of synapomorphies for Catenulida + Acoelomorpha has led some authors to consider that catenulids are not plathelminths (e.g., Haszprunar, 1996). The morphological parsimony analysis of Zrzavý et al. (1998) suggested a polytomy among Catenulida, Acoelomorpha, and Rhabditophora, whereas the morphological analysis of Littlewood et al. (1999) showed the Catenulida as sister group of the Rhabditophora. Phylogenetic studies using 18S rDNA data considered Catenulida either as sister group to Rhabditophora (Rohde et al., 1993; Carranza, 1997; Giribet and Ribera, 1998), included within Rhabditophora (Katayama et al., 1996), or originating independently of Rhabditophora (Carranza et al., 1997; Zrzavý et al., 1998). The combined (morphological + 18S rDNA) parsimony analysis of Zrzavý et al. (1998) concluded that the origin of Catenulida was independent of that of other Plathelminthes and elevated Catenulida to the phylum rank; the combined analysis of Littlewood et al. (1999), however, placed Catenulida and Rhabditophora as sister taxa.

Acoelomorpha (Acoela + Nemertodermatida) have been considered monophyletic (Karling, 1974; Tyler and Rieger, 1977; Ehlers, 1985, 1986; Ax, 1984, 1996). Three synapomorphies were proposed by Ax (1996): network formed by interconnecting rootlets of epidermal cilia; shaft region in epidermal cilia; and absence of protonephridia. Other authors have considered the rootlet system (Lundin, 1997, 1998) and the presence of degenerating epidermal (pulsatile) bodies (Lundin and Hendelberg, 1996) as the synapomorphies for the group. Embryology of Acoela is autopomorphic (Boyer, 1971), and that of Nemertodermatida is unknown. Reuter et al. (1998) have also suggested that the brain-like structure of Acoela is not homologous with the brains of other Plathelminthes, which may indicate an independent origin of Acoela. Nemertodermatida constitute an enigmatic group with only eight known species (Sterrer, 1998), defined by the presence of a statocyst with two statoliths and several parietal cells (Ax, 1996). Steinböck (1930) described the first species of the group, *Nemertoderma bathycola*, which was dredged from a muddy bottom 300–400 m deep off Greenland and at the time was considered to be the most primitive bilaterian.

Whether the Acoelomorpha or the Catenulida represents the first branching event of Plathelminthes has been disputed. Karling (1974), and Zrzavý et al. (1998; morphological tree) did not resolve the position of Catenulida and Acoelomorpha. The morphological analysis of Zrzavý et al. (1998) and Littlewood et al. (1999) supported monophyly of Acoelomorpha, but the molecular and the total evidence analyses did not. Acoela and Nemertodermatida were each elevated to the phylum rank by Zrzavý et al. (1998). Other molecular analyses of 18S rDNA sequences have suggested that Acoela have a different origin from the remaining Plathelminthes (Katayama et al., 1995, 1996; Carranza et al., 1997; Zrzavý et al., 1998; Littlewood et al., 1999; Ruiz-Trillo et al., 1999), whereas Nemertodermatida have been found to belong within Rhabditophora (Carranza et al., 1997; Zrzavý et al., 1998; Littlewood et al., 1999).

Chaetognatha

Chaetognaths still constitute one of the most enigmatic animal phyla in terms of their phylogenetic relationships. Darwin (1844) mentioned the obscurity of their affinities, and later Ghirardelli (1968) argued that chaetognaths were not closely related to any extant metazoan phylum. These ideas reflect the lack of unambiguous synapomorphies to unite chaetognaths with other phyla. Often placed within the Deuterostomia because of their method of coelom formation (Hyman, 1959), Meglitsch and Schram (1991) questioned the enterocoelic nature of their body cavities. Both development and morphology of the adult nervous system are typically protostomian, the early determination of the

germ cells is typical of many "aschelminths," and the toothed oral membrane with its very high content of chitin resembles the mastax of rotifers (Nielsen, 1995).

The first cladistic analysis of the metazoan phyla (Meglitsch and Schram, 1991) placed chaetognaths within a clade of "aschelminths." Nielsen (1995) also placed Chaetognaths in a clade of "aschelminths" that included Gastrotricha, Nematoda, Nematomorpha, Priapulida, Kinorhyncha, Loricifera, Rotifera, and Acanthocephala; in his cladistic analysis, however, Chaetognatha constituted the sister group to Syndermata but did not group with the remaining aschelminths. The morphological analysis of Zrzavý et al. (1998) placed Chaetognatha as the sister group of a clade containing all other protostome phyla except lophophorates.

Chaetognath affinities have been proposed on the basis of molecular analyses using 18S rDNA sequence data (Telford and Holland, 1993; Wada and Satoh, 1994; Halanych, 1996). Telford and Holland (1993) and Wada and Satoh (1994) concluded that chaetognaths were not deuterostomes, although the taxonomic sampling used did not allow their phylogenetic position to be established more accurately. Halanych (1996) concluded that chaetognaths were sister group to nematodes, postulating an evolutionary scenario for the origin of chaetognaths from a vermiform benthic organism. The clade containing (Chaetognatha + Nematoda) was sister group to Plathelminthes. Other analyses (Eernisse, 1998) placed chaetognaths either as sister group to Nematomorpha, within the Ecdysozoa, with the Nematoda at the base of the tree, or in a clade containing (Nematomorpha + Nematoda + Chaetognatha) within Ecdysozoa. Littlewood et al. (1998) placed Chaetognatha as sister group to Gnathostomulida with both as sister group to Nematoda, within Ecdysozoa. Chaetognaths have extremely divergent 18S rDNA sequences in comparison with other metazoans and in all the analyses published so far have tended to group with other divergent sequences, such as those of nematodes or gnathostomulids.

Metazoan Phylogeny—Is There a Rooting Problem for the Bilateria?

Recent analyses of metazoan taxa based on large 18S rDNA data sets (Eernisse, 1998; Giribet and Ribera, 1998; Littlewood et al., 1998; Zrzavý et al., 1998; Giribet and Wheeler, 1999) basically agreed in the monophyly of the triploblastic animals (= Bilateria) and in the presence of four main groups of triploblastic animals: Deuterostomia, Ecdysozoa, Platyzoa, and Trochozoa. However, the relationships among these four groups, some of which appear to be paraphyletic, have been problematic. It also has not been possible to resolve the internal relationships among these four main clades consistently, especially with regard to whether the first dichotomy within the Bilateria is Deuterostomia versus Protostomia, or Platyzoa versus coelomates (Zrzavý et al., 1998). For this reason, it has been suggested that there might be a rooting problem for the Bilateria, because the branch separating them from the diploblastic animals is too long and thus may have accumulated too many changes (Giribet and Wheeler, 1999:Fig. 2). As Wheeler (1990) pointed out, distant outgroups may lead to spurious relationships based on random similarity, a phenomenon that may apply also to the phylogenetic reconstruction of the Bilateria on the basis of 18S rDNA sequences.

THE NEW ANALYSIS

In an attempt to resolve the myriad of hypotheses proposed for the interrelationships among triploblastic phyla, especially for the interrelationships among the "aschelminth" and platyzoan phyla, we have analyzed an enlarged 18S rDNA data set combined with a morphological data matrix.

1. Taxonomic sampling was improved within each phylum, with 23 unpublished 18S rDNA sequences, including two new Gnathostomulida (*Gnathostomula* sp. and *Haplognathia* sp.), one "archiannelid" (*Dinophilus gyrociliatus*), one new Nemertodermatida (*Meara stichopi*), and new sequences of other several phyla: Mollusca, Sipuncula, Echiura, Nemertea, Brachiopoda, Phoronida, Bryozoa, Priapulida, Onychophora, Arthropoda, and Enteropneusta (see Appendix 1).
2. Only triploblastic taxa were analyzed, to avoid rooting with distant outgroups. This strategy may be useful to test several of the hypotheses formulated here, and to avoid problems with putative sequence heterogeneity.
3. Data were analyzed by using the direct optimization method (Wheeler, 1996), which avoids intermediate alignment steps.

4. A sensitivity analysis using seven parameter sets for three data sets (18S complete; 18S without five variable regions; 18S without variable regions + morphology) were conducted to avoid formulating nonrobust hypotheses.
5. Character congruence (ILD of Mickevich and Farris, 1981) was used as an external criterion to choose the parameter set that minimizes incongruence among molecules and morphology.

In total, we used complete 18S rDNA sequences of 145 species of Bilateria (~1,800–2,300 bp), and 276 morphological characters for 36 morphological terminals. All major triploblastic groups or putative phyla (except Pterobranchia and Loricifera) were represented. The morphological data set was extracted from Zrzavý et al. (1998) and adjusted for the taxa represented by use of molecular data. We tested five main hypotheses:

1. Are the members of the Platyzoa (sensu Cavalier-Smith, 1998) directly related to each other, or are certain platyzoans more closely related to the introvertan pseudocoelomates?
2. Is the phylum Gnathostomulida related to other acoelomate taxa (Platyzoa) or to the introvertan pseudocoelomates included within Ecdysozoa?
3. Are the Plathelminthes monophyletic?
4. Is *Symbion pandora* (Cycliophora) more closely related to Entoprocta or to Syndermata?
5. Is the phylum Chaetognatha a member of the Ecdysozoa?

Materials and Methods

Taxon Sampling

Multiple sampling within each triploblastic phylum was attempted, and additional taxa were added to phyla for which only one sequence was available. This was not possible for Kinorhyncha, Cephalochordata, or Cycliophora. Sequence data from the phylum Loricifera are not existent, and for the hemichordate class Pterobranchia only a small fragment of the 18S rDNA gene is available in GenBank and it was not included in our analyses. Complete 18S rDNA sequences of the following groups (and no. of species sampled) were included in this investigation: Polychaeta (9), Clitellata (4), Mollusca (10), Sipuncula (2), Echiura (2), Pogonophora (2), Nemertea (3), Brachiopoda (4), Phoronida (2), Bryozoa (4), Entoprocta (2), Cycliophora (1), Rotifera (2), Acanthocephala (4), Gastrotricha (2), Gnathostomulida (3), Acoela (2), Nemertodermatida (2), Catenulida (2), Rhabditophora (15), Priapulida (2), Kinorhyncha (1), Nematomorpha (2), Nematoda (8), Onychophora (2), Tardigrada (2), Arthropoda (22), Enteropneusta (2), Echinodermata (10), Urochordata (3), Cephalochordata (1), Craniata (11), and Chaetognatha (2). Among the 145 18S rDNA sequences used in this investigation, 37 have been obtained by the present authors (25%). Higher taxonomic ranks used in the paper are summarized in Tables 1 and 2. Taxonomy of the terminal taxa is given in Appendix 1.

DNA Sequences

Genomic DNA samples were obtained from fresh, frozen, or ethanol-preserved tissues in a solution of guanidinium thiocyanate homogenization buffer following a modified protocol for RNA extraction (Giribet et al., 1999). The 18S rDNA loci were amplified by polymerase chain reaction in two or three overlapping fragments of ~950, 900, and 850 bp each, with primer pairs 1F–5R, 3F–18Sbi, and 5F–9R, respectively, and were sequenced by using standard cycle-sequencing protocols (see primers and detailed protocols in Giribet et al., 1999). All the new sequences have been deposited in GenBank (see accession codes in Appendix 1).

Morphological Data

The morphological data set was extracted from the data matrix presented by Zrzavý et al. (1998). Taxa representing diploblastic animals, as well as certain triploblastic taxa for which no molecular data were available (Xenoturbellida, Pterobranchia, Loricifera, *Buddenbrockia*, Lobatocerebromorpha, and Myzostomida), were excluded from the morphological data set. In total, 36 terminal higher-taxa and 276 characters were used for the morphological analysis. All characters were treated as unordered, and no differential weighting was applied. The coding strategy combines an exemplar approach of the molecular taxa (real taxa) with a groundplan approach for the morphological terminals. This approach has been adopted because of

TABLE 1. Triploblastic phyla recognized by three different authors.

Cavallier-Smith (1998)	Zrzavý et al. (1998)	Brusca and Brusca (1990)
Bryozoa	Bryozoa	Ectoprocta
Kamptozoa	Entoprocta Cycliophora	Entoprocta ?
Mollusca	Mollusca	Mollusca
Brachiozoa	Phoronozoa	Phoronida Brachiopoda
Annelida	Annelida Pogonophora Echiura	Annelida Pogonophora Vestimentifera Echiura
Sipuncula Nemertina Chaetognatha	Sipuncula Nemertea Chaetognatha	Sipuncula Nemertea Chaetognatha
Arthropoda	Arthropoda	Arthropoda Pentastomida
Lobopoda	Onychophora Tardigrada	Onychophora Tardigrada
Nemathelminthes	Nematoda Nematomorpha Cephalorhyncha	Nematoda Nematomorpha Priapula Kinorhyncha Loricifera
Acanthognatha	Syndermata Gastrotricha Gnathostomulida	Rotifera Acanthocephala Gastrotricha Gnathostomulida
Platyhelminthes	Acoela Nemertodermatida Rhabditophora Catenulida	Platyhelminthes
Hemichordata Echinodermata	Hemichordata Echinodermata	Hemichordata Echinodermata
Urochordata Chordata	Chordata	Urochordata Cephalochordata Chordata
?	Xenoturbellida	?

the difficulties of coding all morphological traits for the exact same species as are used in the molecular approach; further coding of the morphological data for exemplar species will be presented in the future, because it seems to us to be a more defensible strategy.

Phylogenetic Analyses

Homology concept in sequence data.—Although most molecular analyses use strict base-to-base correspondences (a fixed alignment) as their primary homology statement, this introduces ambiguity and does not allow the accommodation of sequences of substantially unequal length (see Wheeler, 1996). In contrast, our first hypothesis of homology corresponds to secondary structure features (see below), followed by a dynamic base-to-base correspondence, as described by the "direct optimization" method (Wheeler, 1996). To do this, the sequences are divided into the smallest possible unambiguously recognizable homologous regions. For the first split we used primer regions, and then we identified secondary structure features. In total, the 18S rDNA molecule was divided into 47 regions (excluding the external primers 1F and 9R) for each terminal taxon. The 47 input files contained the unaligned sequences of all terminal taxa. All 47 sequence files, parameter files, and batch files are available from the anonymous ftp site ftp.science.amnh.org pub/molecular/data/gnathostomulida.

Sequence data analysis: direct optimization.—Sequence data were analyzed by using the

TABLE 2. Some proposed nomenclature involving acoelomate groups. Names in bold type are accepted in the present work.

Acanthognatha (Cavalier-Smith, 1998)
Rotifera + Acanthocephala + Gastrotricha + Gnathostomulida
Cephalorhyncha (Malakhov, 1980; Nielsen, 1995) (= Scalidophora [Lemburg, 1995])
Priapulida + Kinorhyncha + Loricifera
Cycloneuralia (Nielsen, 1995) (= Nemathelminthes s.s. [Neuhaus, 1994; Schmidt-Rhaesa, 1997])
Gastrotricha + Nematoda + Nematomorpha + Priapulida + Kinorhyncha + Loricifera
Ecdysozoa (Aguinaldo et al., 1997)
Nematoda + Nematomorpha + Priapulida + Kinorhyncha + Loricifera + Onychophora + Tardigrada + Arthropoda
Gnathifera (Ahlrichs, 1995, 1997)
Gnathostomulida + Rotifera + Acanthocephala
Introverta (Nielsen, 1995) (= Cycloneuralia [sensu Ahlrichs, 1995])
Nematoda + Nematomorpha + Priapulida + Kinorhyncha + Loricifera
Monokonta (Cavalier-Smith, 1998) (= Neotrichozoa [Zrzavý et al., 1998])
Gastrotricha + Gnathostomulida
Nematoida (Schmidt-Rhaesa, 1996)
Nematoda + Nematomorpha
Nematozoa (Zrzavý et al., 1998)
Nematoda + Nematomorpha + Onychophora + Tardigrada + Arthropoda
Parenchymia (Nielsen, 1995)
Nemertini + Plathelminthes
Plathelminthomorpha (Ax, 1984)
Gnathostomulida + Plathelminthes
Platyzoa (Cavalier-Smith, 1998)
Rotifera + Acanthocephala + Gastrotricha + Gnathostomulida + Plathelminthes
Syndermata (Ahlrichs, 1995, 1997; Zrzavý et al., 1998) (= Trochata [Cavalier-Smith, 1998])
Rotifera + *Seison* + Acanthocephala

direct optimization method described by Wheeler (1996) and implemented in the computer program POY (Gladstein and Wheeler, 1997). The method assesses directly the number of DNA sequence transformations (evolutionary events) required by a phylogenetic topology without the use of multiple sequence alignment. This is accomplished through a generalization of existing character optimization procedures to include insertion and deletion events (indels) in addition to base substitutions. The crux of the procedure is the treatment of indels as processes rather than as patterns implied by multiple sequence alignment. That is, the necessary indel events that occurred during reconstructing the ancestor of two given sequences are counted but are not reflected in a "fixed alignment." The result of this procedure is directly compatible with parsimony-based tree lengths and appears to generate more efficient (simpler) explanations of sequence variation than does multiple alignment (Wheeler, 1996). Moreover, the method is much less demanding than parsimony-based multiple sequence alignment algorithms and yields more congruent results than multiple sequence alignments when congruence among partitions is used as a criterion (Wheeler and Hayashi, 1998).

Sensitivity analysis.—Character transformations were weighted differentially to see how they affect phylogenetic conclusions (sensitivity analysis sensu Wheeler, 1995). A parameter space of two analytical variables was examined: insertion–deletion ratio, and transversion–transition ratio (tv:ts) (as in Wheeler, 1995). When the tv:ts was set as $\neq 1$, the insertion–deletion cost was set according to the cost of transversions; that is, gap:change = 2, tv:ts = 2: gaps cost twice as much as transversions, and transversions cost twice as much as transitions. In total, seven combinations of parameters were used in the analysis: gap:tv:ts = 111, 121, 141, 211, 221, 241, and 411. We considered this an effective way to explore the data and to discern between well-supported relationships (those supported throughout a wide range of parameters) and poorly supported relationships (those that appear only with very particular parameter sets). The molecular analyses were performed for the complete sequences (all 47 fragments) and for a reduced data set that excluded 5 of the 47 regions (E10-1, E10-2, E21-1-2, 41, and 47) showing large variations in sequence length among the sampled taxa.

Morphological data analysis.—A parsimony analysis of the morphological data set was

TABLE 3. Tree length for the individual partitions (18S = 18S rDNA data set; Morph = morphological data set weighted as the highest molecular cost [molecC]) and for the combined data set (Total). The congruence among partitions is measured by using the ILD metrics (Mickevich and Farris, 1981). IndelC = insertion–deletion cost ratio; Tv:Ti = transversion-transition ratio; molecC = highest molecular cost (is calculated multiplying IndelC x Tv:Ti).

IndelC	Tv:Ti	molecC	18S	Morph	Total	ILD
1	1	1	11,336	2,185	13,628	0.00785
1	2	2	16,892	4,370	21,563	0.01396
1	4	4	27,848	8,740	36,952	0.00985
2	1	2	12,799	4,370	17,329	0.00923
2	2	4	19,691	8,740	28,769	0.01175
2	4	8	33,013	17,480	51,312	0.01596
4	1	4	15,213	8,740	24,373	0.01723

performed with the computer program NONA v. 1.9 (Goloboff, 1998). The tree search strategy adopted involved a heuristics algorithm with random addition-sequence and TBR branch-swapping. No further, more-sophisticated strategies were required, because all of the 100 replications performed yielded the same result. Branch support (Bremer, 1988) up to five extra steps was calculated by using a heuristic procedure and holding a maximum of 10,000 trees with NONA (Goloboff, 1998).

Combined analysis.—Morphological and molecular data were combined directly and analyzed using the "direct optimization" method (Wheeler, 1996). For the combined analyses, the reduced molecular data set (the one excluding the five hypervariable regions) was used. The morphological transformations were weighted as equal to the greatest of the molecular costs (= indels). Branch support (Bremer, 1988) was calculated by using a heuristic procedure implemented in POY.

Character congruence.—Congruence among partitions (morphological and molecular) was measured by the ILD metrics (Mickevich and Farris, 1981) (see Table 3). This value is calculated by dividing the difference between overall tree length and the sum of its data components: ILD = $(\text{length}_{\text{combined}} - \Sigma\ \text{length}_{\text{individual sets}})/\text{length}_{\text{combined}}$. Character congruence is thus used as the criterion to choose our best (most congruent) tree, the tree that minimizes character conflict among all the data. This is understood as an extension of parsimony (or any other minimizing criteria); in the same sense that parsimony tries to minimize the number of overall steps in a tree, the "character congruence analysis" tries to find the model that minimizes incongruence for all the data sources.

In the present case, seven parameter sets were analyzed (a total of 21 analyses) that took ~4 months of computing time on a cluster of 10 PC processors (200 MHz) working in parallel. Other phylogenetic studies have explored wider ranges of parameter sets, as well as different relative weights between morphology and molecules (i.e., Wheeler and Hayashi, 1998), but the currently available computer technology did not allow us to undertake the same approach in a reasonable amount of time.

RESULTS

The trees presented here have been rooted arbitrarily in the branch that separates deuterostomes from protostomes, as has been found in several morphological analyses (e.g., Nielsen et al., 1996; Zrzavý et al., 1998). However, other possibilities have been also proposed. Thus, the term monophyly does not strictly apply to the unrooted topologies presented.

Molecular Analysis

Figure 2 shows summary trees (Deuterostomia collapsed) of the results for parameter set 111 (gap cost = tv = ts = 1; the parameter set that minimizes incongruence [see below]) for the complete data set (all 47 fragments included; Fig. 2a) and for the reduced data set (42 fragments included; Fig. 2b). When the complete data set is used, the platyzoan taxa (in red) appear in four independent clades: (1) grouping the acoels (*Amphiscolops* and *Convoluta*) plus the two gnathostomulid species of the genus *Gnathostomula* within the Ecdysozoa (in green); (2) a clade containing the cycliophoran (*Symbion*), the two gastrotrics (*Chaetonotus* and *Lepidomermella*), the other gnathostomulid (*Haplognathia*), and

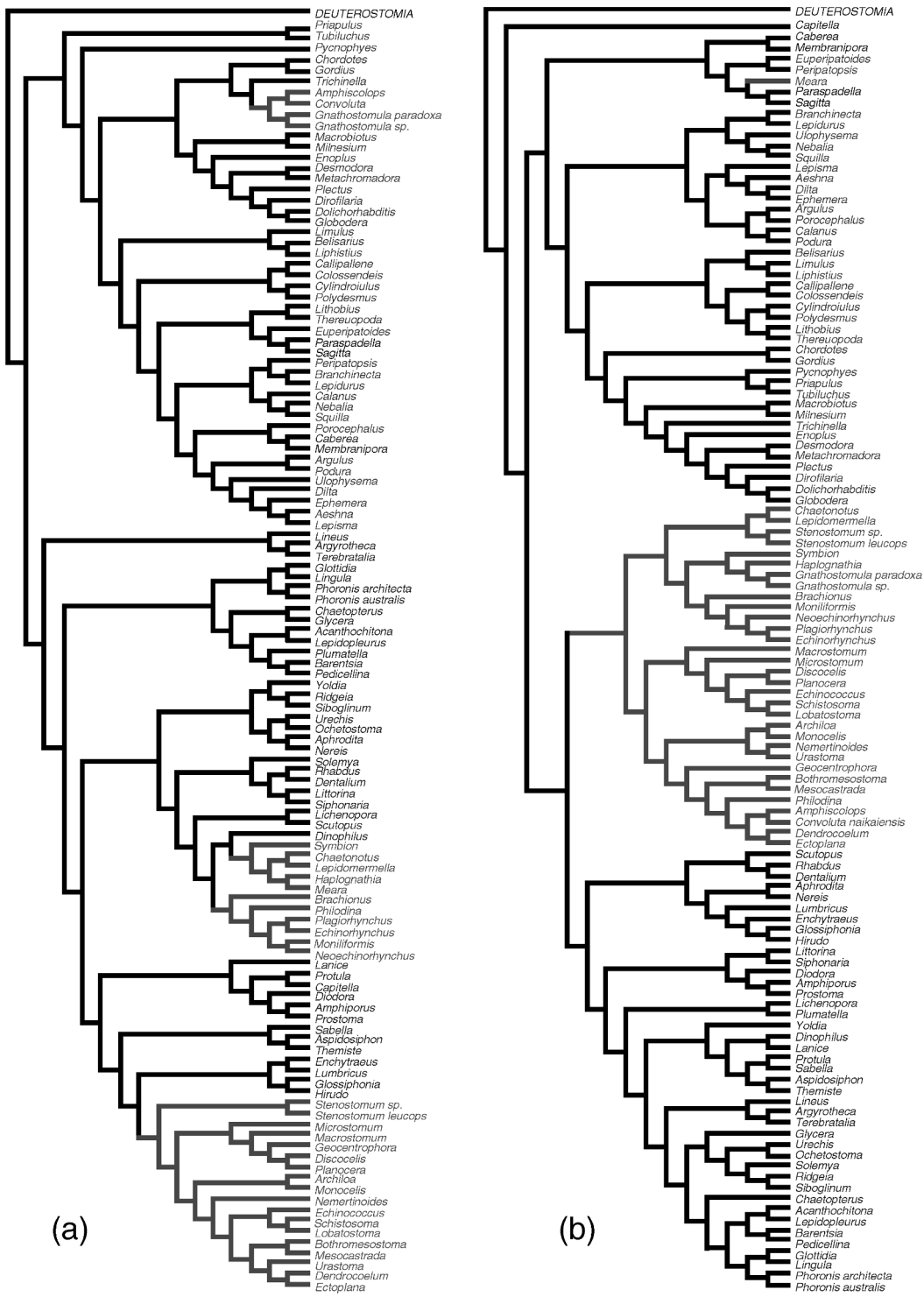

FIGURE 2. 18S rDNA trees for parameter set 111 (gap = change; tv = ts), when the complete gene sequence is used (a), or when the most heterogeneous regions are removed (b). The deuterostomes have been collapsed. Colors represent major protostome groups: Ecdysozoa (green), Platyzoa (red), and Trochozoa (blue). Branches of the platyzoan taxa are represented in red.

one nemertodermatid (*Meara*); (3) a clade containing the Syndermata; and (4) a clade containing the remaining plathelminths (including the nemertodermatid *Nemertinoides*) (Fig. 2a). The last three clades appear scattered among the trochozoan taxa (in blue).

The same parameter set for the data set that excludes the five hypervariable regions (Fig. 2b) yields platyzoan monophyly (in red) except for *Meara* (one of the two nemertodermatid sequences), which groups with the chaetognaths. Platyzoa is the sister group to a clade of trochozoans (excluding *Capitella*, *Caberea*, and *Membranipora*—a polychaete and two gymnolaematan bryozoans, respectively). Platyzoa + Trochozoa constitute the Spiralia. In this tree, four main groups (deuterostomes, ecdysozoans, trochozoans, and platyzoans) are recognized, except for the taxa previously mentioned, plus onychophorans and chaetognaths. This structure is unstable to parameter variation.

Morphological Analysis

The parsimony analysis of the morphological data matrix resulted in 16 trees of 455 steps (consistency index = 0.466; retention index = 0.701). The strict consensus of these 16 trees is represented in Figure 3. In the consensus tree (arbitrarily rooted between deuterostomes and protostomes), Deuterostomia, Trochozoa, Ecdysozoa, and Platyzoa are recognized. The "lophophorates" appear between deuterostomes and protostomes (their relationship depends on the point where the tree is rooted). Other relevant results (that conflict with the molecular analyses) are that Syndermata branches outside the Platyzoa; Chaetognatha appears as sister group to Ecdysozoa; and the cyclophoran is placed within the Trochozoa.

Branch support = 1 for almost all supraphyletic nodes, except for the following: all the chordate phyla (Bremer support [bs] = 3); the Trochozoa (excluding Entoprocta) (bs = 3) and some other groupings within this clade; Syndermata (bs = 5); Nematoida (bs = 3); (Priapulida (Kinorhyncha (Onychophora, Tardigrada, Arthropoda))) (bs = 2, 2, >5; for each respective node); and Plathelminthomorpha (bs = 2). All the clades with branch support >2 are also found in the total evidence tree, except for Acoelomorpha (Acoela + Nemertodermatida), which has a morphological branch support of 4 but is not found in the combined analysis tree.

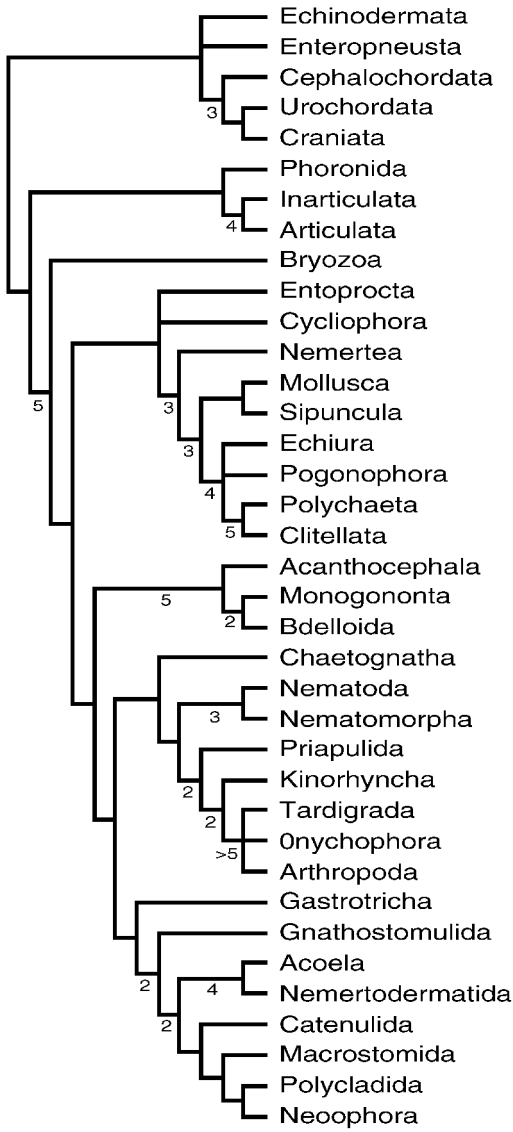

FIGURE 3. Strict consensus of 16 trees of 455 steps (consistency index = 0.466; retention index = 0.701) based on the morphological data of Zrzavý et al. (1998). Bremer support values >1 are indicated.

Combined Analysis

The total evidence analyses achieved a minimum of incongruence at equal weights (indel = tv = ts = morphology; parameter set 111) (Table 3). Thus, this tree (Fig. 4) represents our best hypothesis of relationships, being the one that minimizes incongruence among the different sources of data. A summary tree with the taxonomic categories as coded for the morphological analysis is represented in Figure 5.

Our best tree presented four main convex (nonpolyphyletic) groupings: the Deuterostomia and three main groups of protostome animals—Ecdysozoa, Platyzoa, and Trochozoa. Three major taxa (Chaetognatha, Nemertodermatida, and Bryozoa) could not be assigned to any of the four main groups.

Deuterostomia (bs = 17) contained two main clades, one of nonchordate animals (Echinodermata + Enteropneusta) and one of chordate animals (Cephalochordata (Urochordata + Craniata)) (Coelomopora and Chordonia, respectively, sensu Cavalier-Smith, 1998). Ecdysozoa (bs = 11) could also be divided into two main clades, one including the introvertan pseudocoelomate phyla ((Priapulida + Kinorhyncha) (Nematoda + Nematomorpha)) (= Introverta, sensu Nielsen, 1995), and another including arthropods and related phyla ((Onychophora + Tardigrada) Arthropoda). Trochozoa (bs = 3) includes the following groups: ((Phoronida + Brachiopoda) (Entoprocta (Nemertea (Sipuncula (Mollusca (Pogonophora (Echiura + Annelida)))))), being the grouping with the lowest branch support.

Platyzoa (bs = 5) constitute a clade that includes all the classical acoelomate phyla (except Nemertodermatida). Two main subgroups were recognized: one containing (Gnathostomulida (Cycliophora (Monogononta (Bdelloidea + Acanthocephala)))) and another containing (Gastrotricha + Plathelminthes). The internal structure of Plathelminthes was as follows: (Catenulida (*Macrostomum* ((*Microstomum* + Polycladida) ("Neoophora" + Acoela)))). Catenulids were sister group to the remaining Plathelminthes; Macrostomids were nonmonophyletic, being *Microstomum* sister to Polyclads; Neoophora contained the remaining orders, including the Acoela as sister group to Tricladida.

Discussion

Platyzoa

Platyzoan taxa, to the exclusion of Nemertodermatida, were convex in our favored tree (monophyletic if the rooting was correct, or paraphyletic if the root was placed within the Platyzoa). This result is consistent with some molecular analyses (e.g., Winnepenninckx et al., 1995). However, putative "long-branch" problems with certain groups of Plathelminthes and other acoelomates may have been influential in the failure by other authors to obtain this clade by molecular data analyses (see below).

Most morphological analyses have considered platyzoans to be polyphyletic or paraphyletic, although many times this hypothesis has been assumed without testing. Thus, Syndermata and Gastrotricha (and sometimes Gnathostomulida) have been often related to the introvertan ecdysozoans (i.e., Lorenzen, 1985; Wallace et al., 1995, 1996; Neuhaus et al., 1996; [shown in Figs. 1a–c, this paper]). Other authors considered Syndermata as an independent clade (i.e., Fig. 1e). Haszprunar (1996; shown in Fig. 1f here) proposed a paraphyletic grade of platyzoans, that led to the "higher spiralians."

The absence of coelom (as defined histologically) is the only morphological synapomorphy that might define Platyzoa, although its optimization is ambiguous (this character state is also present in Entoprocta, Nemertodermatida, Kinorhyncha, Nematoda, and Nematomorpha; it is coded as unknown for Syndermata). This clade is thus mainly defined by molecular characters.

Gnathifera: Gnathostomulida, Cycliophora, and Syndermata

The two classes of the phylum Gnathostomulida (Bursovaginoidea and Filispermoidea) appeared to be monophyletic under most parameter sets in the molecular analyses, although not in those with high gap costs. The monophyly of the phylum was not tested in the morphological analysis, for which the three species were coded identically. Our combined tree also supported gnathostomulid monophyly, not surprisingly, given that the three species have been coded identically for the morphology.

Gnathostomulids appeared as sister taxa to a clade containing (*Symbion* + Syndermata). The close relationship of *Symbion* (Cycliophora) with Syndermata agrees with the molecular analysis of Winnepenninckx et al. (1998) but contradicts the original hypothesis of a close relationship to Entoprocta (Funch and Kristensen, 1995, 1997). That the Gnathostomulida constitute the sister taxon of (Syndermata + Cycliophora) could be considered an extension of the Gnathifera hypothesis (Ahlrichs, 1995, 1997; Haszprunar, 1996; Herlyn and Ehlers, 1997; Kristensen, 1995; Rieger and Rieger, 1977, 1980; Rieger and Tyler, 1995) (see Figs. 1d, 1f), which did not include the phylum Cycliophora.

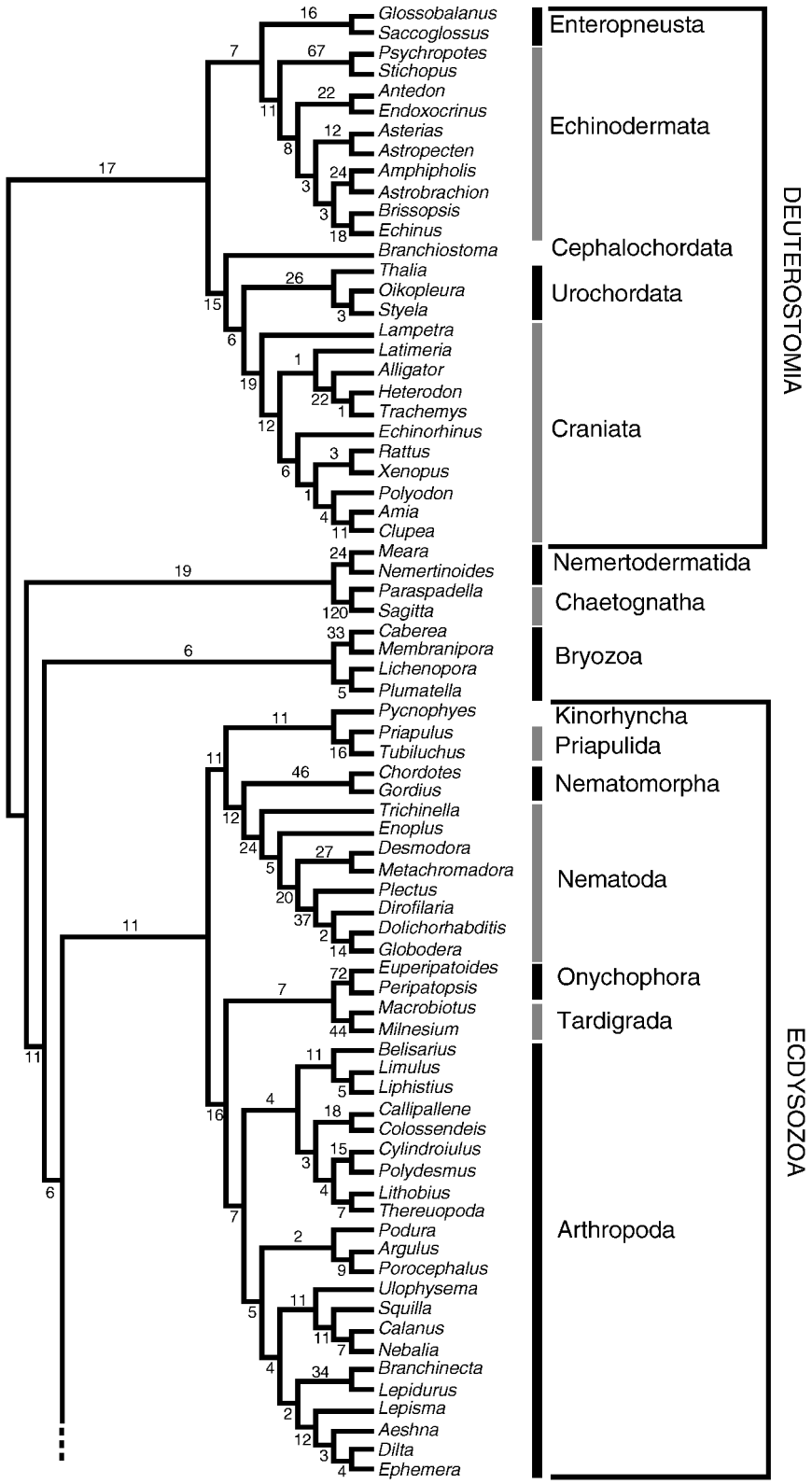

FIGURE 4. Total evidence tree (molecular data with the five most heterogeneous regions removed) for parameter set 111. Numbers on branches indicate Bremer support values.

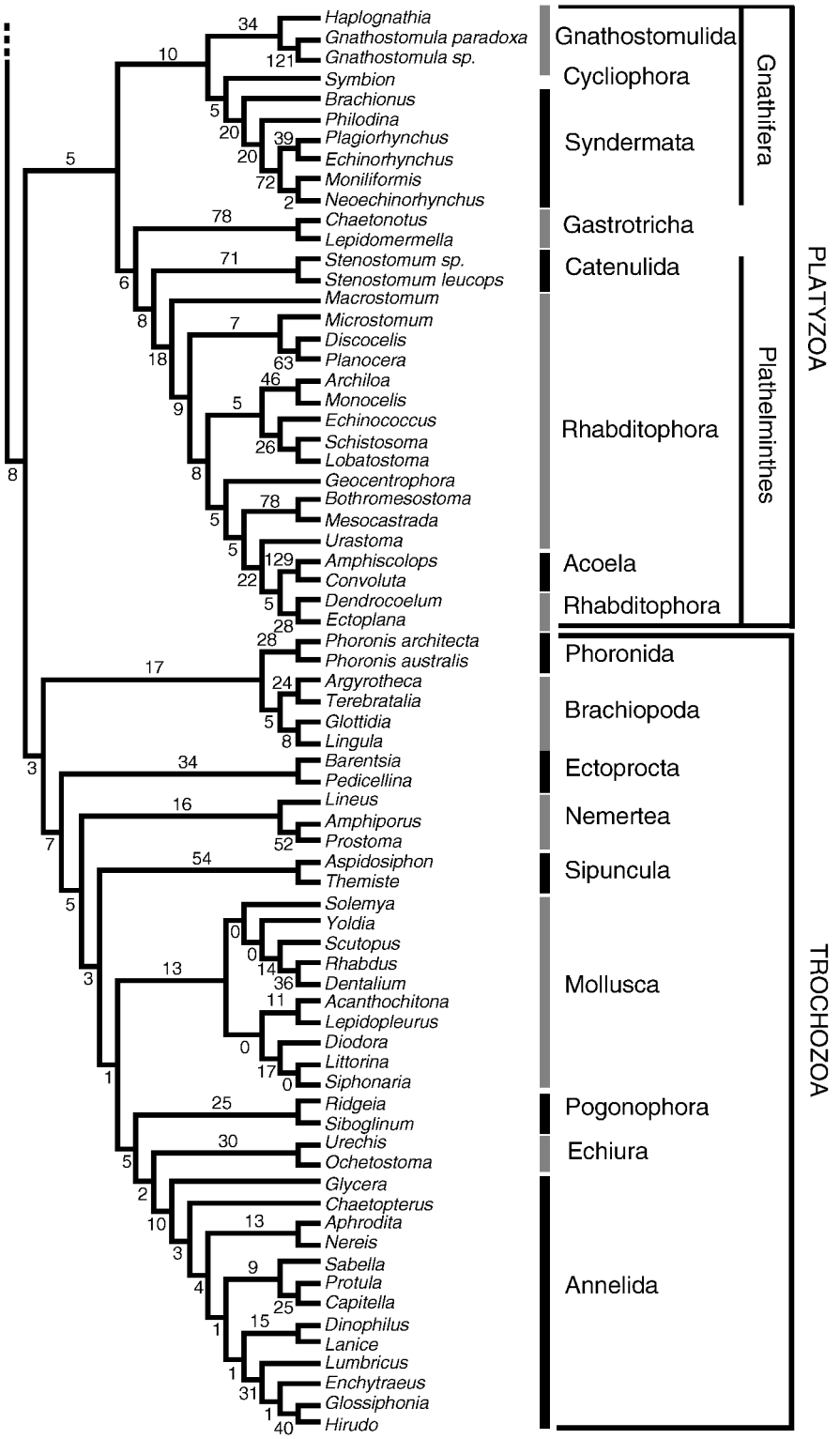

FIGURE 4. Continued.

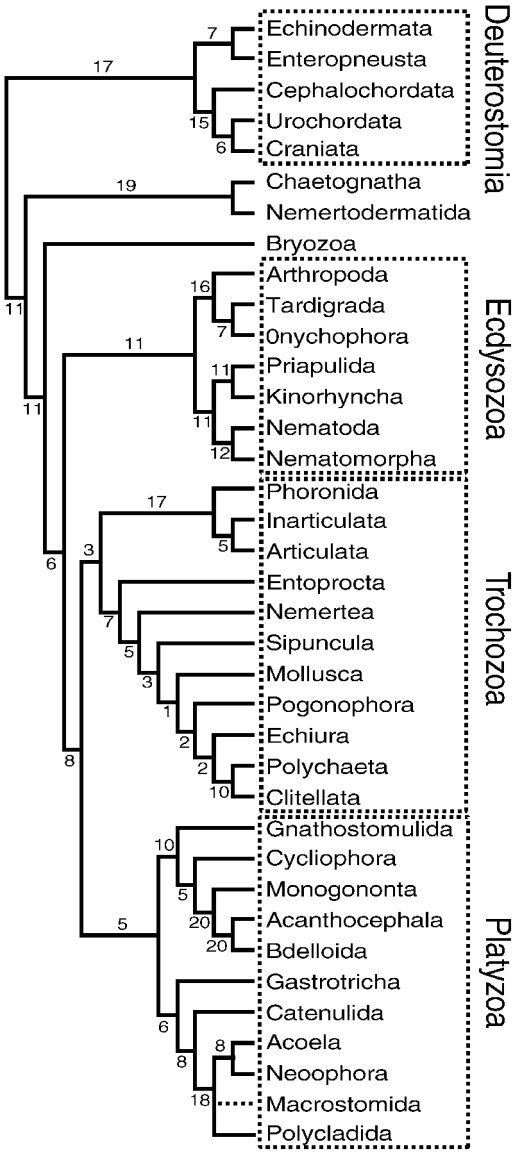

FIGURE 5. Summary tree of Figure 4 with the terminal taxa as coded for the morphology. The dashed line in Macrostomida indicates nonmonophyly. Numbers on branches indicate Bremer support values.

In consequence, the Plathelminthomorpha hypothesis (Ax, 1984; Meglitsch and Schram, 1991; Schram, 1991; Eernisse et al., 1992; Backeljau et al., 1993; Schram and Ellis, 1994; Zrzavý et al., 1998), which postulated a sister group relationship of Gnathostomulida with Plathelminthes, was rejected. Other hypotheses, such as the relationship of Gnathostomulida with Ecdysozoa (Littlewood et al., 1998), were rejected as well by the current analyses. Under one parameter set (221), however, the reduced molecular data set placed gnathostomulids within ecdysozoans.

The monophyly of Gnathifera is supported by one unambiguous morphological synapomorphy (presence of protonephridia with channel cell completely surrounding lumen), although this state was coded as unknown for Cycliophora. The inclusion of Cycliophora within the Gnathifera was entirely because of the molecular data.

Syndermata was proposed by Zrzavý et al. (1998) as a new phylum combining Rotifera and Acanthocephala based on the paraphyletic status of Rotifera. Paraphyly of Rotifera with respect to Acanthocephala had already been proposed by Lorenzen (1985) and subsequent authors (Ahlrichs, 1995, 1997; Garey et al., 1996; Giribet and Ribera, 1998; Littlewood et al., 1998, 1999; Winnepenninckx et al., 1998). Our results agree with this hypothesis and also suggest a sister group relationship between Cycliophora and Syndermata.

Plathelminthes, Nemertodermatida, Acoela, and Putative "Long Branches"

Nemertodermatids were represented in our analyses by two sequences of the genus *Meara* and *Nemertinoides*, and morphology was coded identically for both taxa. The molecular data analyses placed *Nemertinoides* together with *Urastoma* in eight of nine parameter sets, related either to other Platyzoa or within Deuterostomia. Only in one case did *Nemertinoides* come out as sister taxon to *Meara* within a clade of platyzoan taxa (parameter set 121), as was expected. However, the position of *Meara* was extremely parameter-dependent.

The 18S rDNA of *Nemertinoides elongatus* was sequenced by S. Carranza, although it did not appear related to *Urastoma* in his analyses (Carranza et al., 1997). The same species was used by Zrzavý et al. (1998), but in their analysis it appeared as sister to the rhabdocoelan *Bothromesostoma* sp. This *Nemertinoides* sequence was also used by Littlewood et al. (1999), but in that case, it grouped with some proseriate sequences. In our analyses, *Bothromesostoma* appeared as sister taxon to *Mesocastrada* (both Rhabdocoela) throughout all the parameters explored. *Meara stichopi* is a new sequence that has not been included in any previous analyses. It seems that to

resolve their definitive position, more sampling might be needed within the Nemertodermatida, even though only eight species are known. The combined analyses "forced" the two nemertodermatids to group together because they had been coded identically for the morphology. In the best tree, they appeared to be sister group to chaetognaths, but in the second best tree (parameter set 121; tree not shown) they appear to be the sister group to all the remaining Plathelminthes, thus making the phylum monophyletic.

According to our analyses, the only morphological "synapomorphy" for Nemertodermatida + Chaetognatha would be the presence of hermaphroditism, which is highly homoplastic and is found in some Priapulida, Kinorhyncha, Gnathostomulida, Gastrotricha, Plathelminthes, Nemertodermatida, Clitellata, and Mollusca.

Plathelminthes (to the exclusion of Nemertodermatida) appeared as a clade in our best tree with a branch support of eight (parameter set 121 also included Nemertodermatida with the remaining Plathelminthes). Catenulida appeared as the sister taxon to the remaining groups, and Acoela was sister group to Tricladida. The phylogenetic position of catenulids has been problematic, as noted above (see, for example, Carranza et al., 1997; Giribet and Ribera, 1998). Zrzavý et al. (1998) elevated the group to the phylum category; however, our total evidence results disagree (see also Rhode et al., 1993; Carranza, 1997; Giribet and Ribera, 1998; Littlewood et al., 1999; Ruiz-Trillo et al., 1999).

The Acoela were also elevated to the phylum category by Zrzavý et al. (1998). The phylogenetic position of acoel plathelminths certainly has been problematic. Morphological synapomorphies with other plathelminths are scarce (densely multiciliated epidermal cells, frontal organ, or frontal glandular complex [Ax, 1996]). Furthermore, because most of the molecular analyses published so far did not place acoels with the remaining plathelminths, many authors have concluded that plathelminths are polyphyletic (Katayama et al., 1995; Carranza et al., 1997; Zrzavý et al., 1998; Littlewood et al., 1999; Ruiz-Trillo et al., 1999), although a molecular analysis (Norén and Jondelius, 1997) showed monophyly of Plathelminthes, including the acoel *Praesagittifera*. Some of these authors have also proposed that acoels might represent a basal triploblastic animal (Zrzavý et al., 1998; Ruiz-Trillo et al., 1999).

The available 18S rDNA sequences of acoels have large phenetic dissimilarities with respect to other metazoan taxa, which result in branches considerably longer than the average branches of the tree (see Carranza et al., 1997:Fig. 3). When such a phenomenon occurs, positioning of these taxa based solely on sequence data is difficult. This phenomenon, often referred to as "long branch attraction" (among other names), has been used, perhaps excessively, to explain many anomalies in phylogenetic trees, and although "long branch" problems may exist, they are difficult to demonstrate empirically.

A putative "long branch" example in the literature is the position of nematodes in metazoan trees based on 18S rDNA sequence data. Nematodes are recognized as members of the Introverta (sensu Nielsen, 1995) in many morphological analyses, but they have also repeatedly been found at basal positions within triploblastic animals (as is the case of Acoela) (Aguinaldo et al., [1997] summarize this problem). Two subsequent strategies were followed that seemed to solve this putative "long branch" problem. The first, adopted by Aguinaldo et al. (1997), was to sequence the 18S rDNA loci of several nematodes and use only the most slowly evolving taxa. This strategy led them to "reposition" nematodes with their morphologically recognized relatives, the other introvertan pseudocoelomates, and to define the clade Ecdysozoa. The second strategy, following the recommendation of Hillis (1996) for "breaking up long branches" by using a larger taxonomic sampling, was followed by Giribet and Ribera (1998), leading to the same conclusions reached by Aguinaldo et al.

Acoels (as well as in nematodes and other putative "long branch" metazoans) tend to "migrate" to the base of the triploblastic taxa in 18S rDNA phylogenetic trees instead of grouping with the bulk of the Plathelminthes. This could be their actual position in the metazoan phylogenetic tree (e.g., Ruiz-Trillo et al., 1999). However, it could also be a problem because of the branch lengths present in the phylogenetic trees separating diploblastic from triploblastic animals. In consequence, the removal of this branch could be a third way of avoiding certain "long branch attraction" problems. If acoels were basal triploblastics, and

the remaining plathelminths were derived spiralians, acoels should not group with the remaining Plathelminthes. On the other hand, if their basal position was an artifact, when eliminating the diploblastic taxa from the analyses, the acoel sequences should be repositioned together with the other plathelminths. And this seems to be the case in the current analysis, although we recognize that the position of this taxon is strongly parameter dependent.

In the analyses of Zrzavý et al. (1998), molecular data were used to suggest that nemertodermatids (*Nemertinoides*) were related to other Plathelminthes, whereas acoels were not. Our molecular tree (without variable regions) using parameter set 111 (Fig. 2b) suggests that *Nemertinoides* and the acoels are derived Rhabditophora, whereas *Meara* (the other nemertodermatid) is not. This result was extremely unstable to parameter variation. For example, parameter set 121 suggested that both nemertodermatids (*Meara* + *Nemertinoides*) were sister to catenulids and that acoels were related to rhabditophorans; parameter 141 suggested that *Nemertinoides* was related to *Urastoma*, and *Meara* was related to the Acoela, both clades unrelated to the remaining platyzoans; and so on. Thus, we can be confident neither of the position of Acoela nor of Nemertodermatida. In the absence of additional evidence, we cannot consider Acoela, Nemertodermatida, or Catenulida as independent animal phyla as suggested by Zrzavý et al. (1998), and we recommend caution against erecting new taxonomic ranks that are based on poorly supported analyses.

Other Protostome Groups

Phoronozoa is another new phylum proposed by Zrzavý et al. (1998) (= Brachiozoa of Cavalier-Smith, 1998). This was based mainly on the paraphyletic status of Brachiopoda with respect to Phoronida in molecular analyses and stands in contrast to the morphological analyses of Carlson (1995). Zrzavý et al. (1998) used sequences of one phoronid (*Phoronis vancouverensis*), two inarticulate brachiopods (*Glottidia pyramidata* and *Lingula lingua*), and one articulate brachiopod (*Terebratalia transversa*). In the present analysis, we used two phoronid sequences (*P. architecta* and *P. australis*) but excluded the *P. vancouverensis* sequence as potentially being of doubtful origin. For brachiopods, we used the same sequences as in Zrzavý et al. (1998), plus a second articulate brachiopod (*Argyrotheca cordata*). Our sampling suggested monophyly of Brachiopoda, monophyly of Phoronida, and monophyly of (Phoronida + Brachiopoda). This result prompts us to consider Brachiopoda and Phoronida as monophyletic groups, and we thus recommend preserving the phylum status for both groups, Phoronida and Brachiopoda (but see Cohen et al., 1998; Cohen, 2000).

Synapomorphies supporting Trochozoa are (1) the presence of a haemal system (also present in Deuterostomes, Panarthropods; reduced in Nemertea); (2) the presence of respiratory pigments (also present in Enteropneusta, Chordonia, Priapulida and Nematoda); and (3) the presence of a primary larva (also present in Enteropneusta, Echinodermata, Bryozoa, Cycliophora, and Polycladida; reduced in Clitellata).

Synapomorphies for Ecdysozoa are (1) an absence of epidermal ciliation (also absent in Chaetognatha and Acanthocephala); (2) the presence of two-layered cuticle (also in Gastrotricha, Entoprocta, Sipunculida, Mollusca, Pogonophora, and Annelida); (3) the presence of cuticular molting (also in Clitellata); (4) the presence of ecdysone (known only in Nematoda, Onychophora, and Arthropoda); and (5) nonciliated intestinal cells (also in Gnathostomulida).

Chaetognatha

The position of the phylum Chaetognatha continues to be one of the most enigmatic issues in metazoan phylogeny. The grouping of Chaetognatha with Nemertodermatida (bs = 19) in our favored tree (Figs. 4, 5) is difficult to justify on the basis of morphological/anatomical characters (see above). Despite the high branch support, this result is not corroborated by any other parameter set. The placement of chaetognaths with nematodes, gnathostomulids, and nematomorphs (Halanych, 1996; Eernisse, 1998; Littlewood et al., 1998) is also unstable. Disregarding their "relationship" with nemertodermatids, chaetognaths appear between the protostomes and the deuterostomes, a conflicting phylogenetic position that has sustained considerable debate for decades (e.g., Hyman [1959] vs. Meglitsch and Schram [1991]).

CONCLUSIONS

Gnathostomulida appear to be a member of Gnathifera, a group that might also include Cycliophora. In our analyses, Gnathifera are more closely related to the other platyzoan acoelomates (Gastrotricha and Plathelminthes) than to the introvertan pseudocoelomates that form part of the Ecdysozoa. Platyzoa thus may be monophyletic (if the root is placed between deuterostome and protostome animals) and sister group to Trochozoa. Platyzoa + Trochozoa would constitute the group named Spiralia. Other possibilities are that Platyzoa is a sister group to the remaining triploblastic animals (acoelomates vs. coelomates), or that Platyzoa constitutes a grade of basal triploblastic animals.

Higher groupings found in our favored tree were Cephalorhyncha (Nielsen, 1995) (= Scalidophora [Lemburg, 1995]); Ecdysozoa (Aguinaldo et al., 1997); Gnathifera (Ahlrichs, 1995, 1997); Introverta (Nielsen, 1995) (= Cycloneuralia [Ahlrichs, 1995]); Nematoida (Schmidt-Rhaesa, 1996); Platyzoa (Cavalier-Smith, 1998); and Syndermata (Ahlrichs, 1995, 1997; Zrzavý et al., 1998) (= Trochata [Cavalier-Smith, 1998]). Higher groups not supported by our best tree are Acanthognatha (Cavalier-Smith, 1998); Cycloneuralia (Nielsen, 1995) (= Nemathelminthes s.s. [Neuhaus, 1994; Schmidt-Rhaesa, 1997]); Monokonta (Cavalier-Smith, 1998) (= Neotrichozoa [Zrzavý et al., 1998]); Nematozoa (Zrzavý et al., 1998); Parenchymia (Nielsen, 1995); and Plathelminthomorpha (Ax, 1984).

Our analysis also disagrees with the phylum status of Catenulida (Zrzavý et al., 1998), Acoela (Zrzavý et al., 1998), and Phoronozoa (Zrzavý et al., 1998) (= Brachiozoa [Cavalier-Smith, 1998]). Relationships of Bryozoa, Nemertodermatida, and Chaetognatha to the four main triploblastic groups (Deuterostomia, Ecdysozoa, Platyzoa, and Trochozoa) were unresolved, and to some extent they depend on the root position.

Commonly, Rotifera, Acanthocephala, Gastrotricha, and Introverta are included in the "Nemathelminthes" (see Lorenzen, 1985; Malakhov, 1980). Neuhaus (1994) excluded Rotifera and Acanthocephala and named Nemathelminthes s. str. to the putative clade containing the remaining taxa. None of those concepts (Nemathelminthes or Nemathelminthes s. str.) is supported by our data.

The results shown here are certainly subject to revision, because sampling within some of the most diverse phyla, and in particular for certain aberrant taxa, must be improved. We also recognize that the addition of the diploblastic animals to the tree could determine the position of certain groups of triploblastics, although the difficulty in doing so based on 18S rDNA sequence data, according to previously published studies, made us investigate the new approach adopted here. Despite the recognition of these problems, our analyses seem novel in discerning among well-corroborated versus unstable hypotheses of relationships. The placement of certain taxa are stable and probably definitely established through the combination of molecular and morphological data, such as the position of the phylum Cycliophora. Others, such as Chaetognatha, Acoela, and Nemertodermatida (among others) are highly parameter-sensitive, and inferences based on the currently available data are, at least, poorly supported.

NOTE ADDED IN PROOF

The sequence of *Nemertinoides elongatus* published in GenBank and used in this study has been demonstrated to be a sequence artifact (Jaume Baguñà, pers. comm.).

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APPENDIX 1

Taxon sampling used in the molecular analyses and GenBank accession codes. Asterisks refer to the taxa sequenced by the authors. Taxonomy based in different sources (e.g., Brusca and Brusca, 1990; Ax, 1996).

	Taxon sampled	GenBank accession no.
Annelida–Polychaeta (9 spp.)		
Order Phyllodocida	*Nereis virens*	Z83754
	Aphrodita aculeata	Z83749
	Glycera americana	U19519
Order Spionida	*Chaetopterus variopedatus*	U67324
Order Capitellida	*Capitella capitata*	U67323
Order Terebellida	*Lanice conchilega*	X79873
Order Sabellida	*Sabella pavonina*	U67144
	Protula sp.	U67142
Order Dinophilida	*Dinophilus gyrociliatus*	AF119074*
Annelida–Clitellata (4 spp.)		
Order Prosothecata	*Enchytraeus* sp.	Z83750
Order Opisthopora	*Lumbricus rubellus*	Z83753
Order Arhynchobdellida	*Hirudo medicinalis*	Z83752
Order Rhynchobdellida	*Glossiphonia* sp.	Z83751
Mollusca (10 spp.)		
Class Caudofoveata	*Scutopus ventrolineatus*	X91977
Class Polyplacophora	*Lepidopleurus cajetanus*	AF120502*
	Acanthochitona crinita	AF120503*
Class Gastropoda	*Diodora graeca*	AF120513*
	Littorina obtusata	X94274
	Siphonaria pectinata	X94274
Class Scaphopoda	*Dentalium pilsbryi*	AF120522*
	Rhabdus rectius	AF120523*
Class Bivalvia	*Solemya velum*	AF120524*
	Yoldia limatula	AF120528*
Sipuncula (2 spp.)		
Class Phascolosomida	*Aspidosiphon misakiensis*	AF119090*
Class Sipunculida	*Themiste alutacea*	AF119075*
Echiura (2 spp.)		
Order Echiuroinea	*Ochetostoma erythrogrammon*	X79875
Order Xenopneusta	*Urechis caupo*	AF119076*
Pogonophora (2 spp.)		
Class Perviata	*Siboglinum fiordicum*	X79876
Class Obturata	*Ridgeia piscesae*	X79877
Nemertea (3 spp.)		
Class Anopla	*Lineus* sp.	X79878
Class Enopla	*Prostoma eilhardi*	U29494*
	Amphiporus sp.	AF119077*

APPENDIX 1. Continued.

	Taxon sampled	GenBank accession no.
Brachiopoda (4 spp.)		
Class Inarticulata	*Glottidia pyramidata*	U12647
	Lingula lingua	X81631
Class Articulata	*Terebratalia transversa*	U12650
	Argyrotheca cordata	AF119078*
Phoronida (2 spp.)	*Phoronis architecta*	U36271
	Phoronis australis	AF119079*
Bryozoa (4 spp.)		
Class Stenolaemata	*Lichenopora* sp.	AF119080*
Class Gymnolaemata	*Membranipora* sp.	AF119081*
	Caberea boryi	AF119082*
Class Phylactolaemata	*Plumatella repens*	U12649
Entoprocta (2 spp.)	*Pedicellina cernua*	U36273
	Barentsia hildegardae	AJ001734
Cycliophora (1 sp.)	*Symbion pandora*	Y14811
Rotifera (2 spp.)		
Class Bdelloidea	*Philodina acuticornis*	U41281
Class Monogononta	*Brachionus plicatilis*	U29235
Acanthocephala (4 spp.)		
Class Palaeoacanthocephala	*Plagiorhynchus cylindraceus*	AF001839
	Echinorhynchus gadi	U88335
Class Archiacanthocephala	*Moniliformis moniliformis*	Z19562
Class Eoacanthocephala	*Neoechinorhynchus pseudemydis*	U41400
Gastrotricha (2 spp.)		
Order Chaetonotida	*Chaetonotus* sp.	AJ001735
	Lepidodermella squammata	U29198
Gnathostomulida (3 spp.)		
Order Filospermoidea	*Haplognathia* sp.	AF119084*
Order Bursovaginoidea	*Gnathostomula paradoxa*	Z81325
	Gnathostomula sp.	AF119083*
Plathelminthes–Nemertodermatida (2 spp.)	*Meara stichopi*	AF119085*
	Nemertinoides elongatus	U70084
Plathelminthes–Catenulida (2 spp.)	*Stenostomum leucops*	U70085
	Stenostomum sp.	U95947
Plathelminthes–Acoela (2 spp.)	*Convoluta naikaiensis*	D83381
	Amphiscolops sp.	D85099
Plathelminthes–Rhabditophora (15 spp.)		
Order Macrostomida	*Macrostomum tuba*	U70081
	Microstomum lineare	U70083
Order Polycladida	*Discocelis tigrina*	U70079
	Planocera multitentaculata	D17562
Order Lecithoepitheliata	*Geocentrophora* sp.	U70080
Order Proseriata	*Archiloa rivularis*	U70077
	Monocelis lineata	U45961
Order Rhabdocoela	*Bothromesostoma* sp.	D85098
	Mesocastrada sp.	U70082
Order Prolecitophora	*Urastoma* sp.	U70086
Order Tricladida	*Dendrocoelum lacteum*	M58346
	Ectoplana limuli	D85088
Cestoda	*Echinococcus granulosus*	U27015
Trematoda	*Schistosoma mansoni*	M62652
	Lobatostoma manteri	L16911
Priapulida (2 spp.)		
	Priapulus caudatus	D85088
	Tubiluchus corallicola	AF119086*
Kinorhyncha (1 sp.)		
Order Homalorhagida	*Pycnophyes kielensis*	U67997
Nematomorpha (2 spp.)		
Class Gordioida	*Chordotes morgani*	AF036639
	Gordius aquaticus	X80233
Nematoda (8 spp.)		
Order Araeolaimida	*Plectus aquatilis*	AF036602
Order Desmodorida	*Desmodora ovigera*	Y16913
Order Chromadorida	*Metachromadora* sp.	AF036595
Order Enoplida	*Enoplus brevis*	U88336

APPENDIX 1. Continued.

	Taxon sampled	GenBank accession no.
Order Trichocephalida	*Trichinella spiralis*	U60231
Order Rhabditida	*Dolichorhabditis* sp.	AF036591
Order Tylenchida	*Globodera pallida*	AF036592
Order Spirurida	*Dirofilaria immitis*	AF036638
Onycophora (2 spp.)	*Peripatopsis capensis*	AF119087*
	Euperipatoides leukarti	U49910
Tardigrada (2 spp.)		
Class Eutardigrada	*Macrobiotus hufelandi*	X81442*
	Milnesium tardigradum	U49909
Arthropoda (22 spp.)		
Class Pycnogonida	*Colossendeis* sp.	AF005440*
	Callipallene sp.	AF005439*
Class Chelicerata	*Limulus polyphemus*	U91490*
	Belisarius xambeui	U91491*
	Liphistius bicoloripes	AF007104*
Class Branchiopoda	*Branchinecta packardi*	L26512
	Lepidurus packardi	L34048
Class Maxillopoda	*Argulus nobilis*	M27187
	Ulophysema oeresundense	L26521
	Calanus pacificus	L81939
	Porocephalus crotali	M29931
Class Malacostraca	*Nebalia* sp.	L81945
	Squilla empusa	L81946
Class Myriapoda	*Thereuopoda clunifera*	AF119088*
	Lithobius variegatus	AF000773*
	Polydesmus coriaceus	AF005449*
	Cylindroiulus punctatus	AF005448*
Class Hexapoda	*Podura aquatica*	AF005452*
	Dilta littoralis	AF005457*
	Lepisma sp.	AF005458*
	Aeschna cyanea	X89481
	Ephemera sp.	X89489
Enteropneusta (2 spp.)	*Glossobalanus minutus*	AF119089*
	Saccoglossus kowalevskii	L28054
Echinodermata (10 spp.)		
Class Crinoidea	*Antedon serrata*	D14357
	Endoxocrinus parrae	Z80951
Class Holothuroidea	*Stichopus japonicus*	D14364
	Psychropotes longicauda	Z80956
Class Echinoidea	*Echinus esculentus*	Z37125
	Brissopsis lyrifera	Z37119
Class Ophiuroidea	*Amphipholis squamata*	X97156
	Astrobrachion constrictum	Z80948
Class Asteroidea	*Asterias amurensis*	D14358
	Astropecten irregularis	Z80949
Urochordata (3 spp.)		
Class Apendicularia	*Oikopleura* sp.	D14360
Class Thaliacea	*Thalia democratica*	D14366
Class Ascidiacea	*Styela plicata*	M97577
Cephalochordata (1 sp.)	*Branchiostoma floridae*	M97571
Craniata (11 spp.)		
Cephalaspidomorphi	*Lampetra aepyptera*	M97573
Chondrichthyes	*Echinorhinus cookei*	M91181
Actinopterygii	*Amia calva*	X98836
	Polyodon spathula	X98838
	Clupea harengus	X98845
Coelacanthiformes	*Latimeria chalumnae*	L11288
Amphibia	*Xenopus laevis*	X04025
Testudines	*Trachemys scripta*	M59398
Lepidosauria	*Heterodon platyrhinos*	M59392
Archosauria	*Alligator mississippiensis*	M59383
Eutheria	*Rattus norvegicus*	X01117
Chaetognatha (2 spp.)		
Order Phragmophora	*Paraspadella gotoi*	D14362
Order Aphragmophora	*Sagitta elegans*	Z19551