Resultats

“I consider science methodology as the most powerful methodology we have to discover the truth of any mistery” (Michael Ruse)

Capítol I

Acoel flatworms: earliest extant bilaterian metazoans, not members of the Platyhelminthes.

I. Ruiz-Trillo, M. Riutort, D. T. J. Littlewood, E. A. Herniou & J. Baguñà.

Science 283, 1919-1923 (1999)

"Historically -and even today- the Turbellaria dominate much of our phylogenetic thinking on the lower Metazoa; hardly any other group of invertebrates has been accorded a position of comparable importance or been subjected to so many different interpretations" (Peter Ax)

3. P. T. Todorov *et al.*, *Cancer Res.* **58**, 2353 (1998); K. Hirai, H. J. Hussey, M. D. Barber, S. A. Price, M. J. Tisdale, *ibid.*, p. 2359.
4. Protein sequences: Human ZAG [T. Araki *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 679 (1988)]; mouse ZAG [H. Ueyama, H. Naitoh, I. Ohkubo, *J. Biochem.* **116**, 677 (1994)]; rat ZAG [A. Fueyo, J. A. Uría, J. P. Freije, C. López-Otín, *Gene* **145**, 245 (1994)]. Human class I MHC sequences [P. J. Bjorkman and P. Parham, *Annu. Rev. Biochem.* **59**, 253 (1990)]; MHC homolog sequences, SWISS-PROT [A. Bairoch and R. Apweiler, *Nucleic Acids Res.* **26**, 38 (1998)]. Sequence identities: ZAG and HLA-A2, 36%; ZAG and FcRn, 27%; ZAG and mouse CD1d, 23%; ZAG and HFE, 36%; ZAG and MIC-A, 29%.
5. D. R. Madden, *Annu. Rev. Immunol.* **13**, 587 (1995).
6. E. M. Beckman *et al.*, *Nature* **372**, 691 (1994); A. R. Castaño *et al.*, *Science* **269**, 223 (1995).
7. R. P. Junghans, *Immunol. Res.* **16**, 29 (1997); V. Ghetie and E. S. Ward, *Immunol. Today* **18**, 592 (1997); N. E. Simister, E. J. Israel, J. C. Ahouse, C. M. Story, *Biochem. Soc. Trans.* **25**, 481 (1997).
8. J. N. Feder *et al.*, *Nature Genet.* **13**, 399 (1996); J. N. Feder *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 1472 (1998).
9. L. M. Sánchez, C. López-Otín, P. J. Bjorkman, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 4626 (1997). In thermal denaturation studies, ZAG denatures with a transition midpoint of 65°C, compared to 57°C for the peptide-filled class I molecule H-2K^d and 45°C for empty K^d [M. L. Fahnestock, I. Tamir, L. Narhi, P. J. Bjorkman, *Science* **258**, 1658 (1992)].
10. V. Groh *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 12445 (1996). The crystal structure of MIC-A reveals a major rearrangement in domain organization compared to the structures of class I molecules and ZAG. The MIC-A α1-α2 platform is displaced from its α3 domain by 113.5° compared to class I molecules. As a result, the MIC-A α1-α2 platform makes no contact with its α3 domain [P. Li, S. T. Willie, S. Bauer, D. L. Morris, T. Spies, R. K. Strong, personal communication].
11. Protein structures: HLA-A2 [Protein Data Bank (PDB) code 2CLR] [E. J. Collins, D. N. Garboczi, D. C. Wiley, *Nature* **371**, 626 (1994)]; Mouse CD1 (PDB code 1CD1) [Z.-H. Zeng *et al.*, *Science* **277**, 339 (1997)]; Rat FcRn (PDB code 3FRU) [W. P. Burmeister, L. N. Gastinel, N. E. Simister, M. L. Blum, P. J. Bjorkman, *Nature* **372**, 336 (1994)]; Human HFE (PDB code 1A6Z) [J. A. Lebrón *et al.*, *Cell* **93**, 111 (1998)]. Molecular surface areas buried by interaction were calculated with X-PLOR [A. T. Brünger, *X-PLOR. Version 3.1: A System for X-ray and NMR* (Yale Univ. Press, New Haven, CT, 1992)] with a 1.4 Å radius. Identification of pocket residues and calculation of groove surface areas were done based upon earlier analyses of human and mouse class I structures [M. A. Saper, P. J. Bjorkman, D. C. Wiley, *J. Mol. Biol.* **219**, 277 (1991); M. Matsumura, D. H. Fremont, P. A. Peterson, I. A. Wilson, *Science* **257**, 927 (1992)] as described in the CD1 (Z.-H. Zeng *et al.*) and HFE (J. A. Lebrón *et al.*) structure papers. Cut away molecular surfaces of grooves (Fig. 3B) were generated as described in the CD1 structure paper.
12. S. Shibata and K. Miura, *Nephron* **31**, 170 (1982).
13. Structure determination and refinement: HKL [Z. Otwinowski and W. Minor, *Methods Enzymol.* **276**, 307 (1997)]. SHARP [E. De La Fortelle and G. Bricogne, *ibid.*, p. 472]. Solomon [J. P. Abrahams and A. G. W. Leslie, *Acta Crystallogr. D* **52**, 30 (1996)]. CCP4 programs [CCP4: Collaborative Computational Project No. 4, Daresbury, UK, *Acta Crystallogr. D* **50**, 760 (1994)]. O [T. A. Jones and M. Kjeldgaard, *Methods Enzymol.* **277**, 173 (1997)]. R_{free} [A. T. Brünger, *Nature* **355**, 472 (1992)]. The Native II data set (Table 1) was used for refinement. After rigid-body refinement of eight domains in the asymmetric unit (α1-α2 and α3 for each of four ZAG molecules) using CNS [A. T. Brünger *et al.*, *Acta Crystallogr. D* **54**, 905 (1998)], the four molecules were subjected to restrained NCS torsion-angle refinement using the maximum likelihood target function. Tight NCS restraints (300 kcal/mol·Å^2) were applied to all regions except for flexible loops and residues involved in lattice contacts. Intermediate rounds of model building and refinement included the calculation of SIGMAA-weighted [R. J. Read, *Acta Crystallogr. A* **42**, 140 (1986)] simulated annealing omit maps [A. Hodel, S.-H. Kim, A. T. Brünger, *Acta Crystallogr. A* **48**, 851 (1992)]. Final rounds of rebuilding and refinement included tightly restrained individual atomic temperature factor refinement (temperature factor rms deviation for bonded main chain and side chain atoms is 5.7 and 8.8 Å^2, respectively). The model consists of residues 5 through 277 (average *B*: 48 Å^2) with nine carbohydrate residues (average *B*: 61 Å^2) for molecule 1, residues 5 through 278 (average *B*: 56 Å^2) with 11 carbohydrate residues (average *B*: 80 Å^2) for molecule 2, residues 6 through 278 (average *B*: 57 Å^2) with four carbohydrate residues (average *B*: 107 Å^2) for molecule 3, and residues 6 through 249 and 258 through 276 (average *B*: 62 Å^2) with five carbohydrate residues (average *B*: 90 Å^2) for molecule 4 (Wilson $B = 64$ Å^2). Excluding regions that deviate from the NCS, the domains in the NCS-related ZAG monomers are very similar (<0.04 Å rms deviation for Cα atoms). Ramachandran plot statistics (Table 1) are as defined by G. J. Kleywegt and T. A. Jones [*Structure* **4**, 1395 (1996)].
14. Extensive carbohydrate density is found at Asn^{239} (nine ordered carbohydrate residues in molecule 2) and to a much lesser extent at Asn^{89} and Asn^{108} in all four ZAG molecules (Fig. 1) (*13*). Crystal structures of glycoproteins rarely show more than three ordered carbohydrate residues at each glycosylation site [D. E. Vaughn and P. J. Bjorkman, *Structure* **6**, 63 (1998)]. The Asn in the fourth potential N-linked glycosylation site (Asn^{92}) does not show density corresponding to carbohydrate. The bond between Asn^{92} and Gly^{93} can be cleaved by hydroxylamine, confirming that Asn^{92} is not glycosylated (*18*).
15. M. Takagaki *et al.*, *Biochem. Biophys. Res. Commun.* **201**, 1339 (1994); O. Ogikubo *et al.*, *ibid.* **252**, 257 (1998); M. Pfaff, in *Integrin-Ligand Interaction*, J. A. Eble and K. Kühn, Eds. (Chapman & Hall, New York, 1997), pp. 101–121.
16. V. A. Tysoe-Calnon, J. E. Grundy, S. J. Perkins, *Biochem. J.* **277**, 359 (1991); D. Lancet, P. Parham, J. L. Strominger, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3844 (1979); A. Bauer *et al.*, *Eur. J. Immunol.* **27**, 1366 (1997).
17. Structural features that prevent ZAG from binding β_2M include the following residues, which clash with β_2M when it is positioned on the ZAG structure either by interacting with α3 or with α1-α2: Ile^{13}, Thr^{15}, Leu^{30}, Arg^{40}, Gln^{98}, Tyr^{118}, Lys^{122}, Val^{234}, His^{236}, Trp^{245}.
18. L. M. Sánchez and P. J. Bjorkman, unpublished results.
19. G. F. Gao *et al.*, *Nature* **387**, 630 (1997).
20. D. N. Garboczi *et al.*, *ibid.* **384**, 134 (1996); K. C. Garcia *et al.*, *Science* **274**, 209 (1996); Y. H. Ding *et al.*, *Immunity* **8**, 403 (1998); K. C. Garcia *et al.*, *Science* **279**, 1166 (1998).
21. Superpositions based on Cα atoms in the platform β strands reveal that the ZAG platform is more similar to classical class I MHC molecules than to any of the class I homologs [rms deviations for superpositions of platforms: ZAG and HLA-A2, 1.3 Å (147 Cα atoms); ZAG and CD1, 1.1 Å (86 Cα atoms); ZAG and FcRn 1.0 Å (88 Cα atoms); ZAG and HFE 1.0 Å (115 Cα atoms)].
22. L. M. Sánchez, A. J. Chirino, P. J. Bjorkman, G. Hathaway, P. G. Green, K. Faull, unpublished results.
23. Figures 1, 2A (right), 2B, 3A, and 3C were made using MOLSCRIPT [P. J. Kraulis, *J. Appl. Crystallogr.* **24**, 946 (1991)] and RASTER-3D [E. A. Merritt and M. E. P. Murphy, *Acta Crystallogr. D.* **50**, 869 (1994)]. Electrostatic calculations were done and Figs. 2A (left) and 3B were made using GRASP [A. Nicholls, R. Bharadwaj, B. Honig, *Biophys. J.* **64**, A166 (1993)].
24. ZAG, CD1, HFE, and FcRn contain prolines within their α2 domain helices at a position corresponding to Val^{165} in classical class I MHC molecules (*4*). The FcRn and CD1 helices are kinked at a position near their proline residues, whereas the ZAG and HFE helices are similar to the α2 domain helices of class I molecules (*11*). Substitution of Val^{165} for proline in the mouse class I molecule H-2D^d did not interfere with binding and presentation of peptides to T cells, suggesting that no major structural rearrangements occurred [D. Plaksin, K. Polakova, M. G. Mage, D. H. Margulies, *J. Immunol.* **159**, 4408 (1997)].

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Acoel Flatworms: Earliest Extant Bilaterian Metazoans, Not Members of Platyhelminthes

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Because of their simple organization the Acoela have been considered to be either primitive bilaterians or descendants of coelomates through secondary loss of derived features. Sequence data of 18*S* ribosomal DNA genes from non–fast evolving species of acoels and other metazoans reveal that this group does not belong to the Platyhelminthes but represents the extant members of the earliest divergent Bilateria, an interpretation that is supported by recent studies on the embryonic cleavage pattern and nervous system of acoels. This study has implications for understanding the evolution of major body plans, and for perceptions of the Cambrian evolutionary explosion.

"Since the first Metazoa were almost certainly radial animals, the Bilateria must have sprung from a radial ancestor, and there must have been an alteration from radial to bilateral symmetry. This change constitutes a most difficult gap for phylogeneticists to bridge, and various highly speculative conjectures have been made" (*1*, p. 5). So began Libbie Hyman's discussion on the origin of bilaterian Metazoa, and despite a century of morphological studies and a decade of intensive molecular work, the nature of the simplest bilaterian animal remains elusive (*1, 2*). Paleontological and molecular data indicate that most bilaterian phyla appeared and diversified during the Cambrian explosion (*3, 4*). Three main clades emerged—

the Deuterostomia, the Ecdysozoa, and the Lophotrochozoa (*5*), although their branching order is unresolved. The acoel flatworms, traditionally classified as an order of the Platyhelminthes, are perhaps the simplest extant members of the Bilateria and have been viewed as either basal metazoans that evolved from ciliate protozoans ("syncytial or ciliate-acoel theory") (*6*) or a direct link between diploblasts and triploblasts ("planuloid-acoeloid theory") (*1, 7*). However, the lack of complexity has also been interpreted as a loss of derived features of more complex ancestors ("archicoelomate theory") (*8*).

The proposed metazoan phylogenetic trees that include acoels have shown them to branch after the diploblasts, indicating that they are considered primitive triploblastic animals (*9–11*). However, all 18*S* ribosomal DNAs (rDNAs) from acoels that have been sequenced so far show rates of nucleotide substitution that are three to five times the rates of most other Metazoa (*10*), resulting in the long-branch attraction effect in which rapidly evolving taxa cluster and branch together artifactually at the deepest base of the trees (*12*). We examined the relationship of the Acoela to other metazoan taxa by sequencing complete 18*S* rDNAs (*13*) from 18 acoel species (*14*). In addition, we sequenced the 18*S* rDNA of the catenulid *Suomina* sp. and the nemertodermatid *Meara* sp. as additional representatives of basal orders of Platyhelminthes thought to be closely related to acoels. The 18*S* ribosomal gene was chosen because of the large number of sequences available in the molecular data banks (GenBank and European Molecular Biology Laboratory) for representatives of the entire animal kingdom.

To avoid the long-branch effect, we broadly sampled the Acoela to find species that have normal rates of nucleotide substitution (non–fast-clock species). As representatives of most animal phyla a wide range of metazoan species were selected from the data banks (Table 1) and their sequences aligned and compared with those of acoels (*15*). A preliminary phylogenetic analysis (by the neighbor-joining method) showed that all 18 acoels form a very clear monophyletic group that branches at the base of the triploblasts. As expected (*10*), inclusion of the long-branch acoels leads to several inconsistencies in tree topology. Therefore, to select those taxa with uniform rates of change, we first performed a relative rate test (*16*) comparing all the species by pairs with the diploblast species as reference taxa. Because extremely long branches characterize most acoel species, only the four with shortest branches were included in the test. Only one acoel species (*Paratomella rubra*) passed the test, and one other (*Simplicomorpha gigantorhabditis*) came very close (Table 1). Although the latter was not included in subsequent analyses reported here, very similar results were obtained when both species or a single one (*Paratomella rubra*) was used. Of 74 bilaterian species tested (including the four acoels), 57 passed the test. Subsequent analyses were performed with only these 57 species that demonstrated uniform and comparable rates of evolution (representing 21 phyla) plus the four diploblasts representing three phyla. The second step in the analysis was to determine the phylogenetic content of the data resulting from this selection. Two tests were carried out. A plot of the observed (total, transitions or transversions) versus inferred number of substitutions (*4, 17*) showed that, although the curves tend to level off (Fig. 1A), they do not reach a plateau, meaning that the sequences studied are only moderately mutationally saturated. From a likelihood-mapping analysis (*18*) 81.5% of quartets had resolved phylogenies and only 10.7% of all quartet points were in the star-tree region (Fig. 1B), indicating that the rDNA data contain a reasonably high degree of phylogenetic information.

We next built a tree using maximum likelihood (*19*). The best tree that we found is shown in Fig. 2. In this tree, Deuterostomia, Ecdysozoa, and Lophotrochozoa (*5*) form monophyletic groups. Interestingly, the acoelomate and pseudocoelomate groups cluster at the base of the Ecdysozoa and Lophotrochozoa. Most importantly, the tree shows the acoels as the first offshoot after the diploblasts. However, the nemertodermatids, an order of Platyhelminthes usually classified as the sister group of the Acoela and forming the Acoelomorpha (*20, 21*), and here represented by the single species that passed the relative rate test, group within the bulk of the Platyhelminthes rather than with the acoels. On the other hand, both catenulids cluster at the base of the Platyhelminthes. All alternative hypotheses concerning the relationships between acoels and other platyhelminths (*20, 22–24*) and their position within the Bilateria (*10*) were also compared by the Kishino-Hasegawa test and all were significantly poorer

Table 1. List of species included in this study, GenBank accession numbers, and result of the relative rate test (rrt). The names of the 61 species finally selected for analysis are in bold. Ph., phylum; O., order; Cl., class.

Taxa	Species	Acc. number	rrt*	
			5%	1%
Deuterostomia				
Ph. Chordata	***Branchiostoma floridae***	M97571	0	0
	Lampetra aepyptera	M97573	0	0
	Xenopus laevis	X04025	0	0
	Mus musculus	X00686	0	0
Ph. Hemichordata	***Balanoglossus carnosus***	D14359	0	0
	Saccoglossus kowalewskii	L28054	0	0
Ph. Echinodermata	***Antedon serrata***	D14357	0	0
	Ophioplocus japonicus	D14361	0	0
Lophotrochozoa				
Ph. Mollusca	***Acanthopleura japonica***	X70210	0	0
	Lepidochitona corrugata	X91975	0	0
	Argopecten irradians	L11265	0	0
	Chlamys islandica	L11232	0	0
	Nerita albiulla	X91971	0	0
	Limicolaria hambeul	X60374	0	0
Ph. Annelida	***Eisenia foetida***	X79872	0	0
	***Enchytraeus* sp.**	U95948	0	0
	Hirudo medicinalis	Z83752	0	0
	Haemopsis sanguisua	X91401	0	0
	Lanice conchilega	X79873	0	0
	Nereis virens	Z83754	0	0
Ph. Nemertini	***Prostoma eilhardi***	U29494	0	0
	***Lineus* sp.**	X79878	0	0
Ph. Sipuncula	***Phascolosoma granulatum***	X79874	0	0
Ph. Brachiopoda	***Terebratalia transversa***	U12650	0	0
	Lingula lingua	X81631	0	0
Ph. Entoprocta	***Barentsia hildegardae***	AJ001734	0	0
Ph. Bryozoa	***Pedicellina cernua***	U36273	0	0
	Plumatella repens	U12649	0	0
Ph. Phoronida	***Phoronis vancouverensis***	U12648	0	0
Ph. Echiura	***Ochetostoma erythrogrammom***	X79875	0	0
Ph. Pogonophora	***Ridgeia piscesae***	X79877	0	0
	Siboglinum fiordicum	X79876	0	0
Ph. Gastrotricha	***Lepidodermella squammata***	U29198	0	0
	***Chaetonotus* sp.**	AJ001735	0	0

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Table 1. *Continued*

Taxa	Species	Acc. number	rrt*	
			5%	1%
Lophotrochozoa (continued)				
Ph. Platyhelminthes				
O. Acoela†	***Paratomella rubra*‡**	AF102892	0	0
	Simplicomorpha gigantorhabditis‡	AF102894	14	0
	Symsagittifera psammophila‡	AF102893	41	10
	Haplogonaria syltensis‡	AF102900	45	45
O. Tricladida	*Polycelis nigra*	AF013151	9	0
O. Polycladida	***Discocelis tigrina***	U70074	0	0
O. Lecithoepitheliata	***Geocentrophora* sp.**	U70080	0	0
O. Macrostomida	***Macrostomum tuba***	U70081	0	0
	Microstomum lineare	U70083	0	0
O. Proseriata	***Monocelis lineata***	U45961	0	0
O. Nemertodermatida	***Nemertinoides elongatus***	U70084	0	0
	Meara sp.‡	AF051328	32	5
O. Catenulida	***Stenostomum leucops***	U70085	0	0
	***Suomina* sp.**‡	AJ012532	0	0
Cl. Cestoda	*Grillotia erinaceus*	AJ228781	13	0
Cl. Trematoda	***Schistosoma mansoni***	M62652	1	0
	Fasciolopsis bushi	L06668	1	0
Cl. Monogenea	*Neomicrocotyle pacifica*	AJ228787	9	0
Ecdysozoa				
Ph. Tardigrada	***Macrobiotus hufelandi***	X81442	0	0
Ph. Arthropoda	***Odiellus troguloides***	X81441	0	0
	***Aphonopelma* sp.**	X13457	0	0
	Berndtia purpurea	L26511	0	0
	Panulirus argus	U19182	0	0
	Tenebrio molitor	X07801	0	0
	Polistes dominulus	X77785	0	0
	Scolopendra cingulata	U29493	0	0
Ph. Priapulida	***Priapulus caudatus***	X87984	0	0
Ph. Kinorhyncha	***Pycnophyes hielensis***	U67997	0	0
Ph. Nematomorpha	***Gordius aquaticus***	X87985	0	0
Ph. Nematoda	*Trichinella spiralis*	U60231	10	0
	Plectus sp.	U61761	34	8
	Zeldia punctata	U61760	43	14
Other phyla				
Ph. Chaetognatha	*Paraspadella gotoi*	D14362	44	19
Ph. Mesozoa	*Dicyema sp.*	X97157	45	28
	Rhopalura ophiocomae	X97158	29	1
Ph. Gnathostomulida	*Gnathostomula paradoxa*	Z81325	45	32
Ph. Rotifera	*Philodina acuticornis*	U91281	45	44
	Brachionus plicatilis	U49911	0	0
Ph. Acanthocephala	*Moliniformis moliniformis*	Z19562	10	0
	Neoechynorhynchus pseudemydis	U41400	25	0
Diploblasts				
Ph. Placozoa	***Trichoplax adhaerens***	L10828		
Ph. Porifera	***Scypha ciliata***	L10827		
Ph. Cnidaria	***Anemonia sulcata***	X53498		
	Tripedalia cystophora	L10829		

*Figures indicate the number of cases in which the rate of nucleotide substitutions of each species was significantly different (at 5% and at 1% levels) when compared by pairs with a set of 45 species with a uniform rate of substitution. Diploblasts served as reference species. For further details, see (*16*). The complete matrix with all the comparisons is available at ftp://porthos.bio.ub.es/pub/incoming/phylogeny/rrt.xls †A total of 18 species of acoels were sequenced, though only the four earliest branching taxa within that group were used in the metazoan-wide analysis. For the rest of acoel sequences, see (*14*). ‡New sequences reported in this paper.

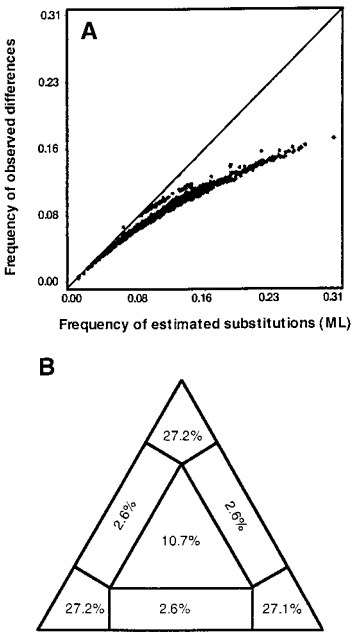

Fig. 1. Phylogenetic content of the data. (**A**) Substitution saturation curve. The *y* axis shows the frequency of observed differences between pairs of species sequences determined with MUST (*4, 17*), and the *x* axis shows the inferred distance between the same two sequences determined by maximum likelihood (ML) with PUZZLE v. 4.0 (*38*). Each dot thus defines the observed compared with the inferred number of substitutions for a given pair of sequences. The resulting curve lies between the diagonal line (no saturation) and a horizontal plateau line (full saturation), which means that the data set is only moderately saturated [for further information see (*4, 17*)]. (**B**) Likelihood mapping analysis (*18*) of the data set, represented as a triangle. Values at the corners indicate the percentages of well-resolved phylogenies for all possible quartets (*18*), and values at the central and lateral regions are percentages of unresolved phylogenies. The cumulatively high percentage (81.5%) from the corner values indicates the data set is phylogenetically informative.

than the phylogeny obtained originally. The robustness of the internal branch separating acoels from the rest of bilaterians was further evaluated by the four-cluster likelihood mapping method (*25*) and resulted in 100% support for this branch.

Because the position of the acoels might be due to the most variable sites of the alignment, we removed them from the whole data set (*26*); the acoels still appeared at the base of the trees, although the phylogenetic signal within the triploblasts almost faded away. Alternatively, the sequence regions that show the highest variation among acoels might represent noisy data that separate them from the rest of the Bilateria. To test this idea, we aligned the 18 acoel sequences, found their most variable positions, and removed the latter from the 61-species alignment (*27*). Again, this resulted in the acoels on a shorter branch at the base of the bilaterian tree (the three trees obtained in both tests are available as supplementary material at www.sciencemag.org/feature/data/986597.shl). Finally, because some important phyla such as Chaetognatha, Acanthocephala, Gnathostomulida, Mesozoa, and Nematoda did not pass the relative rate test and were not included in the maximum-likelihood analyses, the four-cluster likelihood mapping was used again to test the position of these groups against acoels and the rest of the triploblasts. In all cases, acoels and diploblasts cluster together (Table 2). Importantly, of all the phyla tested, some of those previously proposed as "primitive" bilaterians (Mesozoa, Nematoda, and Gnathostomulida), always cluster with the triploblasts.

Our analyses clearly indicate that acoels are not members of the phylum Platyhelminthes, but occupy a key position in the Metazoan tree of life, most likely as the earliest branch within the bilaterian clade that left extant descendants. The monophyly of Platyhelminthes has been criticized (*23, 28, 29*) because of the weakness

Table 2. Four-cluster likelihood mappings to test acoel position against fast-clock phyla. Four-cluster likelihood mappings (*18*) were performed arranging species into three groups: diploblasts (D), acoels (A), triploblasts (T), and a fourth group (X) taken from each of the phyla to be tested. If the phylum under test is more basal than the acoels, it should cluster with high support with the diploblasts. Conversely, if acoels are more basal the phylum under test should cluster with the triploblasts. Results show that acoels cluster more closely to the diploblasts than all other triploblasts.

Fast-clock phyla (X)	Bifurcating tree			Intermediate regions	Star tree
	D A \ T X	D T \ A X	D X \ A T		
Acanthocephala	98.2%	0%	0%	1.8%	0%
Chaetognatha	98.7%	0%	0%	1.3%	0%
Gnathostomulida	75.4%	15.2%	0%	9.4%	0%
Mesozoa	93.1%	0%	0%	5.1%	1.8%
Nematoda	99.7%	0%	0%	0.3%	0%

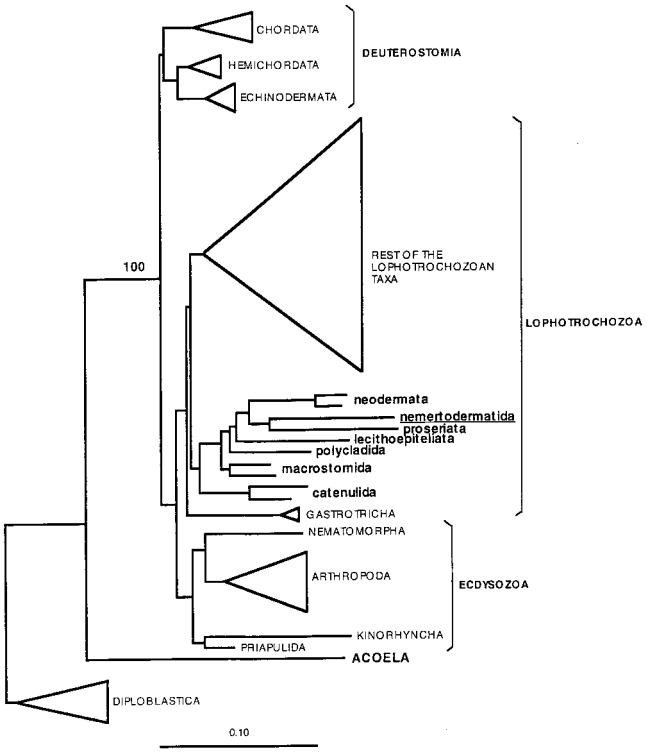

Fig. 2. Diagrammatic representation of the best 18*S* rDNA-based maximum-likelihood tree of 61 metazoan species (bold names are in Table 1) with homogeneous rates of nucleotide substitution. The final matrix included 1181 sites (584 variable and 383 informative under parsimony); log ln = 11,862. The number 100 on the branch separating acoels from the rest of triploblasts represents the percentage of support to that branch obtained by the four-cluster likelihood mapping (*25*). The tree was obtained with fast DNAml (*19*). It illustrates the relationships of the Acoela (bold, upper case) and the rest of the Platyhelminthes (bold, lower case) to the rest of the Metazoa. The general topology of the tree defines three main bilaterian phylogenetic groups: Deuterostomia, Lophotrochozoa (including Platyhelminthes and Gastrotricha as basal phyla), and Ecdysozoa (with Priapulida and Kinorhynchia as basal phyla). The position of the Acoela renders the Platyhelminthes polyphyletic, whereas the Nemertodermatida (underlined) appears buried within the bulk of Platyhelminthes. For taxa and species names, see Table 1, the complete tree with all the species names is available in the supplementary material at www.sciencemag.org/feature/data/986597.shl

of the synapomorphies on which it is based (*22, 24*): multiciliation of epidermal cells, the biciliary condition of the protonephridia, and the lack of mitosis in somatic cells. In contrast, the Acoela have a characteristic set of well-defined apomorphies: a network of ciliary roots of epidermal cells, tips of the cilia with a distinct step, lack of extracellular matrix, absence of protonephridia, and, most importantly, the duet spiral cleavage. The first three features are usually considered to be derived (*22, 24*); the 18*S* molecular data, however, suggest a different interpretation for the other two. The lack of protonephridia in acoels may be a plesiomorphic feature (*29*). The Acoela exhibit duet spiral cleavage, in contrast to the quartet pattern that characterizes the Spiralia and some turbellarian Platyhelminthes. However, acoel cleavage is actually more bilateral than spiral (*30*), suggesting that duet cleavage and typical quartet cleavage are not related. Moreover, all spiralian embryos have both ecto- and endomesoderm and exhibit determinative development, whereas acoel embryos generate only endomesoderm (*30*) and are highly regulative (*31*); the latter two features are considered to be ancestral. Most diploblastic and several triploblastic phyla exhibit a radial cleavage pattern; thus, it is more parsimonious to assume that the first bilaterian also had radial cleavage (*32*). This evidence supports our proposed phylogenetic tree in which the acoels branch before the Cambrian radiation from unknown bilaterian ancestors with radial cleavage and suggests that duet cleavage and quartet spiral cleavage arose independently from an ancestral radial pattern. The structure of the nervous system also indicates that the acoels are not related to the other platyhelminths. Most Platyhelminthes have a bilobed brain with neuropile surrounded by nerve cells and two main longitudinal nerve cords with commisures making an orthogon (*33*). In contrast, the nervous system of acoels comprises a simple brain formed by clusters of nerve cells that lack a neuropile, and a variable number of longitudinal nerve cords that do not make an orthogon (*34*).

The 18*S* rDNA sequences, embryonic cleavage patterns and mesodermal origins (*30*), and nervous system structure data (*34*) support the position of the Acoela as the earliest branching Bilateria (Fig. 2) and the polyphyly of the Platyhelminthes. This argues for an extended period before the Cambrian within which different bilaterian lineages may have originated, with the acoels being the descendants of one of these lineages. This interpretation is supported by recent data on protein sequence divergence (*35*). Direct development, which characterizes all extant acoels, as opposed to the biphasic life cycle with a larval stage and a benthic adult (*36*), probably represents the ancestral bilaterian condition [see (*37*), for a recent discussion]. Our findings suggest that the Acoela (or Acoelomorpha if the Nemertodermatida are shown to remain as their sister group) should be placed in their own phylum.

References and Notes

1. L. H. Hyman, *The Invertebrates. II. Platyhelminthes and Rhynchocoela. The Acoelomate Bilateria* (McGraw-Hill, New York, 1951).
2. R. C. Brusca and G. J. Brusca, *The Invertebrates* (Sinauer, Sunderland, MA, 1990); C. Nielsen, *Animal Evolution, Interrelationships of the Living Phyla* (Oxford Univ. Press, Oxford, 1995).
3. S. Conway-Morris, *Nature* **361**, 219 (1993); R. A. Raff, C. R. Marshall, J. M. Turbeville, *Annu. Rev. Ecol. Syst.* **25**, 351 (1994); S. Conway-Morris, *The Crucible of Creation* (Oxford Univ. Press, Oxford, 1998).
4. H. Philippe, A. Chenuil, A. Adoutte, *Development* **1994** (suppl.), 15 (1994).
5. A. M. A. Aguinaldo *et al*., *Nature* **387**, 489 (1997).
6. J. Hadzi, *The Evolution of the Metazoa* (Macmillan, New York, 1963)
7. L. V. von Salvini-Plawen, *Z. Zool. Syst. Evolutionsforsch.* **16**, 40 (1978).
8. P. Ax, in *The Lower Metazoa*, E. C. Dougherty, Ed. (Univ. of California Press, Berkeley, CA, 1963), pp. 191–224; R. Siewing, *Zool. Jahrb. Anat.* **103**, 439 (1980); R. M. Rieger, in *The Origins and Relationships of Lower Invertebrates*, S. Conway-Morris *et al*., Eds. (Clarendon, Oxford, 1985), pp. 101–122; J. P. S. Smith and S. Tyler, in *ibid.*, pp. 123–142.
9. T. Katayama, M. Yamamoto, H. Wada, N. Satoh, *Zool. Sci.* **10**, 529 (1993); B. Winnepenninckx, T. Backeljau, R. De Wachter, *Mol. Biol. Evol.* **12,** 641 (1995); J. Zrzavy, S. Mihulka, P. Kepka, A. Bezdek, D. Tietz, *Cladistics* **14**, 249 (1998).

10. S. Carranza, J. Baguñà, M. Riutort, *Mol. Biol. Evol.* **14**, 485 (1997).
11. D. T. J. Littlewood, K. Rohde, K. A. Clough, *Biol. J. Linn. Soc.* **66**, 75 (1999).
12. J. Felsenstein, *Syst. Zool.* **27**, 401 (1978); G. J. Olsen, *Cold Spring Harb. Symp. Quant. Biol.* **LII**, 825 (1987).
13. The 18*S* rDNA was amplified and sequenced from high molecular weight genomic DNA as described [(*11*); S. Carranza, G. Giribet, C. Ribera, J. Baguñà, M. Riutort, *Mol. Biol. Evol.* **13**, 824 (1996)].
14. Sequences have been deposited in the GenBank under accession numbers AF102892 to AF102900 and AJ012522 to AJ012530.
15. Sequences were aligned by eye on the basis of the secondary structure model [R. R. Gutell, B. Weibser, C. R. Woese, H. F. Noller, *Prog. Nucleic Acid Res. Mol. Biol.* **32**, 155 (1985)] with GDE v.2.2. Positions that could not be unambiguously aligned were removed from the alignment. The final matrix contained 1181 sites (including 584 variable, 383 informative under parsimony); the full final alignment is deposited under accession number ds37667.dat and is available at ftp://FTP.EBI.AC.UK under directory pub/databases/embl/align.
16. The relative rate test [A. C. Wilson, S. S. Carlson, T. J. White, *Annu. Rev. Biochem* **46**, 573 (1977)] compares genetic distances between each of two species and a reference outgroup. We performed the test according to W. H. Li and M. Tanimura, *Nature* **326**, 93 (1987). A first set of 45 bilaterian species (deuterostomes; ecdysozoans, with the exception of Nematoda; and lophotrochozoans, with the exception of Platyhelminthes) with slow and uniform rates of nucleotide substitution, as reflected in their similarly short branch lengths in most published phylogenetic trees, were selected and compared by pairs with diploblasts as reference outgroups. As expected, all were found to have uniform relative rates of nucleotide substitution. Then, a second set of 29 bilaterian species from phyla known to have rates higher than the first set (Platyhelminthes including acoels, Nematoda, Chaetognata, Mesozoa, Gnathostomulida, Rotifera, and Acanthocephala) were compared with each of the species of the first group (45 species) with diploblasts as reference. The results are expressed in Table 1 as the number of cases in which each species has a rate significantly different from those of the first group at the 1% and at the 5% levels.
17. H. Philippe, *Nucleic Acid Res.* **21**, 5264 (1993). The analysis shows the relation between the number of changes that have occurred (as deduced from the tree), and the number of changes that can be calculated directly from the sequence alignment. When the sequences are saturated, it is expected that although the number of inferred changes still increases (*x* axis), they are no longer detected as observed differences (leveling along the *y* axis), so the line defined by the points levels off from the diagonal in the graph and becomes horizontal.
18. The likelihood mapping analysis [(*38*); K. Strimmer and A. von Haeseler, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 6815 (1997)] is a graphical method to visualize the phylogenetic content of a set of aligned sequences. Likelihoods of all quartet trees for each subset of four species are mapped on a triangle, and the triangle is partitioned in different regions. The central region represents starlike evolution, the three corners represent well-resolved phylogeny, and three intermediate regions between corners represent where it is difficult to distinguish between two of the three trees. The resulting distribution of points indicates whether or not the data are suitable for a phylogenetic reconstruction: the phylogenetic information in the data is higher when the value in the central region is smaller.
19. Tree reconstruction was performed by maximum likelihood with both fastDNAml v.1.1.1.a [G. J. Olsen, H. Matsuda, R. Hangstrom, R. Overbeek, *Comput. Appl. Biosci.* **10**, 41 (1994)] and PUZZLE v. 4.0 (*38*) programs. We built an initial tree with fastDNAML using global rearrangements and jumble options and subjected it to the Kishino-Hasegawa test against alternative topologies using PUZZLE [HKY model of substitution; M. Hasegawa, H. Kishino, K. Yano, *J. Mol. Evol.* **22**, 160 (1985)]. The parameters for rate heterogeneity among sites were inferred from the data set. The best tree found was resubmitted to a global rearrangement search with fastDNAML, taking into account among-site rate variation by using the rate and category parameters calculated by PUZZLE.
20. T. G. Karling, in *Biology of the Turbellaria*, N. W. Riser and M. P. Morse, Eds. (McGraw-Hill, New York, 1974), pp. 1–16.
21. P. Ax, *The Phylogenetic System. The Systematization of Organisms on the Basis of Their Phylogenesis* (Wiley, Chichester, UK, 1987).
22. U. Ehlers, *Das Phylogenetische System der Plathelminthes* (Fischer, Stuttgart, 1985).
23. J. P. S. Smith, S. Tyler, R. M. Rieger, *Hydrobiologia* **132**, 13 (1986).
24. P. Ax, *Multicellular Animals, A New Approach to the Phylogenetic Order in Nature* (Springer-Verlag, Berlin, 1996).
25. To analyze the support for internal branches in a tree without having to compute the overall tree, we performed four-cluster likelihood mapping using PUZZLE 4.0 (*38*). Every internal branch in a completely resolved tree defines up to four clusters of sequences. These four clusters can be defined in the data file, and the program will build a quartet tree for each of all possible combinations of four species, always taking one from each group. The result is represented on a triangle (*18*). The distribution of points within this triangle indicates the level of support for the internal branch under analysis.
26. We used PUZZLE 4.0 (*38*) to find the parameters for among-site rate heterogeneity. Resulting data were divided into eight categories, each with an assigned rate value (from constant to diverse values of variability). In two successive analyses we eliminated from the 61 species alignment those positions that were more variable (category 8) and also the sites with the two most variable categories (7 and 8). From these new data sets trees were reconstructed with fastDNAML (*19*).
27. An alignment including only the 18 acoel sequences was used to find their most variable positions and those sites [category 8, see (*26*)] were removed from the 61 species alignment. From this new data set trees were reconstructed with fastDNAML (*19*).
28. R. M. Rieger, S. Tyler, J. P. S. Smith, G. E. Rieger, in *Microscopic Anatomy of Invertebrates*, vol. 3, *Platyhelminthes and Nemertinea*, F. W. Harrison and B. J. Bogitsh, Eds. (Wiley-Liss, New York, 1991), pp. 7–140.
29. G. Haszprunar, *J. Zool. Syst. Evol. Res.* **34**, 41 (1996).
30. B. C. Boyer and J. Q. Henry, *Am. Zool.* **38**, 621 (1998); J. J. Henry, M. Q. Martindale, B. C. Boyer, personal communication.
31. B. C. Boyer, *J. Exp. Zool.* **176**, 96 (1971).
32. J. W. Valentine, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 8001 (1997).
33. E. Reisinger, *Z. Zool. Syst. Evolutionsforsch.* **10**, 1 (1972).
34. O. I. Raikova, M. Reuter, E. A. Kotikova, M. K. S. Gustafsson, *Zoomorphology* **118**, 69 (1998); M. Reuter, O. I. Raikova, M. K. S. Gustafsson, *Tissue Cell* **30**, 57 (1998).
35. G. A. Wray, J. S. Levinton, L. H. Shapiro, *Science* **274**, 568 (1996); F. J. Ayala, A. Rzhetsky, F. J. Ayala, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 606 (1998); L. Bromham, A. Rambaut, R. Fortey, A. Cooper, D. Penny, *ibid.* **95**, 12386 (1998).
36. G. Jägersten, *Evolution of the Metazoan Life Cycle: A Comprehensive Theory* (Academic Press, London, 1972); R. M. Rieger, *Am. Zool.* **34**, 484 (1994).
37. S. Conway-Morris, *Bioessays* **20**, 676 (1998).
38. K. Strimmer and A. von Haeseler, *Mol. Biol. Evol.* **13**, 964 (1996).
39. We thank M. Martindale, J. Henry, and B. Boyer for their insightful comments and sharing of previous results; S. Carranza for his help in the first stages of this work; A. Adoutte, J. McInerney, G. Olsen, H. Philippe, and K. Strimmer for methodological advice; and R. Bray, L. Colin, M. Dawson, A. Faubel, K. Lundin, L. Martin, O. Raikova, R. Rieger, K. Rohde, J. P. S. Smith, and S. Tyler who provided material for this study. I.R.-T., M.R., and J.B. were supported by CIRIT (Generalitat de Catalunya) grants 1995SGR-00574 and 1997SGR-00057. D.T.J.L. and E.A.H. were funded by a Wellcome Trust Senior Research Fellowship to D.T.J.L. (043965/Z/95/Z). J.B. and D.T.J.L. shared the grant HB1996-0034 from the British-Spanish Joint Research Programme (Acciones Integradas).

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Rapid Dendritic Morphogenesis in CA1 Hippocampal Dendrites Induced by Synaptic Activity

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Activity shapes the structure of neurons and their circuits. Two-photon imaging of CA1 neurons expressing enhanced green fluorescent protein in developing hippocampal slices from rat brains was used to characterize dendritic morphogenesis in response to synaptic activity. High-frequency focal synaptic stimulation induced a period (longer than 30 minutes) of enhanced growth of small filopodia-like protrusions (typically less than 5 micrometers long). Synaptically evoked growth was long-lasting and localized to dendritic regions close (less than 50 micrometers) to the stimulating electrode and was prevented by blockade of *N*-methyl-D-aspartate receptors. Thus, synaptic activation can produce rapid input-specific changes in dendritic structure. Such persistent structural changes could contribute to the development of neural circuitry.

Coordinated patterns of activity help to organize neural circuits throughout the brain (*1*). In particular, activity shapes the structure of sensory maps (*2*) and individual neurons (*3*) through *N*-methyl-D-aspartate (NMDA) receptor–dependent processes, which suggests that synapse-specific associative changes are involved. Relatively little is known about the role of activity in the development of dendritic morphology. A number of studies have addressed whether long-term potentiation (LTP) produces postsynaptic structural changes. Using electron microscopy (EM) analysis of fixed

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Capítol II

The Nemertodermatida are basal bilaterians and not members of the Platyhelminthes.

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Zoologica Scripta, 31: 201-215 (2002)

"Rain will go upwards before acoels and nemertodermatids split apart"
(Reinhard Rieger)

The Nemertodermatida are basal bilaterians and not members of the Platyhelminthes

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Recent hypotheses on metazoan phylogeny have recognized three main clades of bilaterian animals: Deuterostomia, Ecdysozoa and Lophotrochozoa. The acoelomate and 'pseudocoelomate' metazoans, including the Platyhelminthes, long considered basal bilaterians, have been referred to positions within these clades by many authors. However, a recent study based on ribosomal DNA placed the flatworm group Acoela as the sister group of all other extant bilaterian lineages. Unexpectedly, the nemertodermatid flatworms, usually considered the sister group of the Acoela together forming the Acoelomorpha, were grouped separately from the Acoela with the rest of the Platyhelminthes (the Rhabditophora) within the Lophotrochozoa. To re-evaluate and clarify the phylogenetic position of the Nemertodermatida, new sequence data from 18S ribosomal DNA and mitochondrial genes of nemertodermatid and other bilaterian species were analysed with parsimony and maximum likelihood methods. The analyses strongly support a basal position within the Bilateria for the Nemertodermatida as a sister group to all other bilaterian taxa except the Acoela. Despite the basal position of both Nemertodermatida and Acoela, the clade Acoelomorpha was not retrieved. These results imply that the last common ancestor of bilaterian metazoans was a small, benthic, direct developer without segments, coelomic cavities, nephrida or a true brain. The name Nephrozoa is proposed for the ancestor of all bilaterians excluding the Nemertodermatida and the Acoela, and its descendants.

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Introduction

Platyhelminth flatworms have often played a crucial role in discussions of metazoan phylogeny as the sister group or even 'ancestors' of other Bilateria (e.g. Willmer 1990). Although monophyly of the Platyhelminthes has long been assumed, the relationships between the three major platyhelminth clades, Acoelomorpha (Nemertodermatida + Acoela), Catenulida and Rhabditophora are controversial. Difficulties identifying robust morphological synapomorphies uniting Rhabditophora with Catenulida and with the Acoelomorpha led Smith *et al*. (1986) to question the monophyly of the Platyhelminthes, although they did not provide an alternative hypothesis for the sister groups of the three clades. A critical reassessment of morphological evidence within the context of metazoan evolution led Haszprunar (1996) to consider Platyhelminthes a paraphyletic group, with Acoelomorpha being the earliest bilaterian offshoot followed by the Rhabditophora, the Catenulida and the rest of the Bilateria. Another analysis of metazoan phylogeny using a large set of morphological characters in combination with 18S ribosomal DNA (rDNA), also indicated the Platyhelminthes to be a paraphyletic assemblage (Zrzavy *et al*. 1998). However, in most zoology textbooks, flatworms are still featured as a monophyletic group.

Recent analyses of molecular data have challenged the long-held position of Platyhelminthes as ancestral bilaterians as well as flatworm monophyly. 18S rDNA sequence data and Hox gene molecular signatures form the basis for the recognition of three main clades of bilaterian animals: Deuterostomia, Ecdysozoa and Lophotrochozoa (e.g. Aguinaldo *et al*. 1997; Adoutte *et al*. 1999). The acoelomate and 'pseudocoelomate' metazoans, including the bulk of the Platyhelminthes (the Rhabditophora, or probably Rhabditophora + Catenulida), are now referred to positions within these clades by many authors (Balavoine 1997, 1998; Carranza *et al*. 1997; Bayascas *et al*. 1998; Littlewood *et al*. 1999; De Rosa *et al*. 1999; Adoutte *et al*. 2000; Saló *et al*. 2001); but see Giribet *et al*. (2000) for a different view. Thus, Platyhelminthes are now generally considered lophotrochozoans. However, a recent study based on

rDNA placed the flatworm group Acoela as the sister group of all other extant bilaterian lineages (Ruiz-Trillo *et al.* 1999) and separated it from the Rhabditophora which fell within the Lophotrochozoa. Surprisingly, the nemertodermatid flatworms, usually considered the sister group of the Acoela (Ehlers 1985), were grouped inside the rest of the Platyhelminthes (the Rhabditophora) within the Lophotrochozoa, and separately from the acoels. This conflicted with perceived morphological synapomorphies uniting the Acoela and Nemertodermatida into the clade Acoelomorpha (Ehlers 1985; Smith *et al.* 1986) and with apomorphies shared by the Rhabditophora but absent from Nemertodermatida.

The known Nemertodermatida comprise 10–11 species of small marine worms. When the first representative of the group was formally described, Steinböck (1930–31) regarded it as the 'most primitive turbellarian' and classified it within the Acoela. In his description of the second nemertodermatid, Westblad (1937) noted some important differences between the Nemertodermatida and the Acoela. For example, the presence of a permanent intestinal cavity in the former. These features formed the basis for the erection of a separate taxon outside the Acoela, the Nemertodermatida (Karling 1940). The anatomical simplicity of the nemertodermatids was noted by Karling, who considered the Nemertodermatida to be closest to the 'turbellarian archetype' in his phylogenetic analysis of the free-living flatworms (Karling 1974). Later, ultrastructural studies of Nemertodermatida and Acoela revealed similarities in their epidermal cilia interpreted as synapomorphies of the two taxa [Tyler & Rieger 1977; Ehlers 1985; Smith & Tyler 1985; reviewed in Lundin (1997, 1998)], and the taxon Acoelomorpha (Ehlers 1985) was recognized on this basis, with Acoela and Nemertodermatida as sister groups.

The first molecular studies placing the Nemertodermatida within the Rhabditophora, separately from the acoels, were based on one 18S rDNA sequence from a single nemertodermatid species (Carranza *et al.* 1997; Littlewood *et al.* 1999). When the position of the Acoela within the Metazoa was tested using the relative rate test (Wilson *et al.* 1977) to eliminate putatively fast evolving 18S rDNA, nemertodermatids again fell within the Rhabditophora and separate from the Acoela, which branched as the first bilaterians (Ruiz-Trillo *et al.* 1999). This led some workers to question the separation of the Nemertodermatida from the Acoela, suggesting that the early branching of the Acoela was erroneous, and the position of the Nemertodermatida within the Lophotrochozoa was also indicative of acoel relationships (Adoutte *et al.* 2000; Peterson *et al.* 2000; Dewel 2000; Telford 2001). This argument seemed to be reinforced by analyses of elongation factor 1-alpha (EF1-α) from a single species of the Acoela, which placed the group close to the Tricladida, a derived group within the Rhabditophora (Berney *et al.* 2000). However, new EF1-α sequences from additional species of Acoela, Platyhelminthes and other Metazoa do not support a Tricladida–Acoela clade (Littlewood *et al.* 2001). Moreover, a number of morphological synapomorphies present in Tricladida and other Rhabditophora, but missing in the Acoela (e.g. heterocellular female gonads, rhabdites, protonephridia), conflict with a position of the Acoela within the Rhabditophora.

The phylogenetic position of the Nemertodermatida needs to be carefully re-evaluated. Some morphological characters seem to link them to the acoels, but a position among the Rhabditophora within the Lophotrochozoa, as indicated by previous molecular studies (Carranza *et al.* 1997; Littlewood *et al.* 1999), appears unlikely. Therefore, it is important to know whether nemertodermatids join the acoels as the earliest known extant bilaterians separate from the rest of the Platyhelminthes or whether they are members of the Platyhelminthes within the Lophotrochozoa. In this paper, 18S rDNA and mitochondrial nucleotide sequences from three nemertodermatid species were obtained and used to reconstruct the phylogenetic position of the Nemertodermatida. Additional species of acoels and rhabditophorans, as well as a large data set of metazoan sequences, were also included in the analyses.

Materials and methods

Organisms

Three representatives of the Nemertodermatida and one of the Acoela were used to obtain sequences from the 18S rRNA gene. One species of Nemertodermatida and one rhabditophoran were used to obtain sequences from the mitochondrial genes *COI* and *Cytb* (Table 1). The new 18S rDNA sequences were compared with a large set of 18S rDNA sequences from GenBank representing all major metazoan groups (Table 2).

DNA extraction, amplification and sequencing

DNA extraction, polymerase chain reaction amplification and sequencing of 18S rDNA were carried out as described in Norén & Jondelius (1999). For mitochondrial genes, DNA was extracted using the Qiamp DNA Mini Kit (Qiagen Inc.,Valencia, CA, USA). A 700-bp fragment of the *COI* (Cytochrome Oxidase c.: subunit I) gene and 450 bp of the *Cytb* (Cytochrome b) gene were amplified using universal primers. For *Cytb* the primers used were: cytb424–444: 5′-ggW TAY gTW YTW CCW TgR ggW CAR AT-3′ and cytb876–847: 5′-gCR TAW gCR AAW ARR AAR TAY CAY TCW gg-3′ (primer sequences provided by Dr Jeff Boore). The primers used to amplify *COI* were: LCO1490: 5′-ggt caa caa atc ata aag ata ttg g-3′ and HCO2198: 5′-taa act tca ggg tga cca aaa aat c-3′ (Folmer *et al.* 1994).

Sequence alignment and phylogenetic analyses

The 18S rDNA sequences were aligned according to a secondary structure model (Gutell *et al.* 1985). Positions that

Table 1 Taxa sequenced for this study and GenBank Accession nos.

Taxon	Gene	GenBank entry	Collection locality
Nemertodermatida			
Meara stichopi	*18S*	AF327724	Espegrend, Norway
Nemertoderma bathycola	*18S*	AF327725	Kristineberg, Sweden
Nemertoderma westbladi	*18S*	AF327726	Kristineberg, Sweden
Nemertoderma westbladi	*COI*	AF329183	Kristineberg, Sweden
Nemertoderma westbladi	*Cytb*	AF329184	Kristineberg, Sweden
Acoela			
Paraphanostoma cycloposthium	*18S*	AF329178	Kristineberg, Sweden
Paratomella rubra	*COI*	AF329179	Barcelona, Spain
Paratomellla rubra	*Cytb*	AF329180	Barcelona, Spain
Simplicomorpha gigantorhabditis	*Cytb*	GenBank Accession no.	
Rhabditophora			
Microstomum lineare	*COI*	AF329181	Turku, Finland
Microstomum lineare	*Cytb*	AF329182	Turku, Finland

Table 2 Terminals included in the analyses with GenBank Accession nos (sequences reported in this paper are in bold). Terminals marked with * were used only in the parsimony analysis. Terminals marked with § were used only in the maximum likelihood analyses. Unmarked terminals were used in both analyses.

Higher taxon	Terminal	Accession no.	Higher taxon	Terminal	Accession no.
Acoela	*Paratomella rubra*	AF102892		*Lobatostoma manteri**	L16911
	*Symsagittifera psammophila**	AF102893		*Neomicrocotyle pacifica**	AJ228787
	*Simplicomorpha gigantorhabditis**	AF102894	Catenulida	*Stenostomum leucops*	U70085
	*Atriofonta polyvacuola**	AF102895		*Suomina* sp.	AJ012532
	*Paedomecynostomum bruneum**	AF102896	Nemertodermatida	*Nemertinoides elongatus**	U70084
	*Philomecynostomum lapillum**	AF102897		*Meara* sp.*	AF051328
	*Anapaerus tvaerminnensis**	AF102898		***Meara stichopi****	AF327724 **this study**
	*Postmecynostomum pictum**	AF102899		***Nemertoderma bathycola***	AF327725 **this study**
	*Haplogonaria syltensis**	AF102900		***Nemertoderma westbladi***	AF327726 **this study**
	*Actinoposthia beklemischevi**	AJ012522	Deuterostomia		
	Amphiscolops sp.*	AJ012523	Hemichordata	*Balanoglossus carnosus*	D14359
	*Convoluta convoluta**	AJ012524		*Saccoglossus kowalewskii*	L28054
	Acoel sp.3*	AJ012525	Echinodermata	*Antedon serrata*	D14357
	Acoel sp.2*	AJ012526		*Ophioplocus japonicus*	D14361
	*Anaperus biaculeatus**	AJ012527		*Amphipholis squamata**	X97156
	*Aphanostoma virescens**	AJ012528		*Asterias amurensis**	D14358
	*Childia groenlandica**	AJ012529	Chordata	*Branchiostoma floridae*§	M97571
	*Symsagittifera roscoffensis**	AJ012530		*Lampetra aepyptera*§	M97573
	*Convoluta pulchra**	U70086		*Xenopus laevis*§	X04025
	*Praesagittifera naikaiensis**	D83381		*Mus musculus*§	X00686
Rhabditophora	*Polycelis nigra**	AF013151	Lophotrochozoa		
	*Dendrocoelum lacteum**	M58346	Mollusca	*Acanthopleura japonica*	X70210
	*Crenobia alpina**	M583*45*		*Lepidochitona corrugata*	X91975
	Discocelis tigrina	U70074		*Argopecten irradians*	L11265
	*Planocera multitentaculata**	D17562		*Chlamys islandica*	L11232
	Geocentrophora sp.	U70080		*Nerita albicilla*	X91971
	Macrostomum tuba	U70081		*Limicolaria kambeul*	X60374
	Microstomum lineare	U70083		*Antalis vulgaris**	X91980
	Monocelis lineata	U45961		*Liolophura japonica**	X76210
	*Archiloa ribularis**	U70077		*Monodonta labio**	X94271
	*Urastoma cyprinae**	AF167422	Annelida	*Eisenia foetida*	X79872
	*Echinococcus granulosus**	U27015		*Enchytraeus* sp.	U95948
	Schistosoma mansoni	M62652		*Hirudo medicinalis*	Z83752
	Fasciolopsis bushi	L06668		*Haemopis sanguisuga*	X91401
	Gylaunchen sp.*	L06669		*Lanice conchilega*	X79873

Table 2 *continued*

Higher taxon	Terminal	Accession no.	Higher taxon	Terminal	Accession no.
	Nereis virens	Z83754	Kinorhyncha	*Pycnophyes hielensis*	U67997
	*Glycera americana**	U19519	Nematomorpha	*Gordius aquaticus**	X87985
Nemertini	*Lineus* sp.	X79878	Others		
	Prostoma eilhardi§	U29494	Gnathostomulida	*Gnathostomula paradoxa**	Z81325
Sipuncula	*Phascolosoma granulatum*	X79874	Rotifera	*Philodina acuticornis**	U91281
Brachiopoda	*Terebratalia transversa*	U12650		*Brachionus plicatilis*§	U49911
	Lingula lingua	X81631	Acanthocephala	*Moliniformis moliniformis**	Z19562
Entoprocta	*Barentsia hildegardae*	AJ001734		*Neoechynorhynchus pseudemydis**	U41400
Bryozoa	*Pedicellina cernua*	U36273		*Centrorhynchus conspectus**	U41399
	Plumatella repens	U12649	Gastrotricha	*Lepidodermella squammata*	U29198
Phoronida	*Phoronis vancouverensis*	U12648		*Chaetonotus* sp.	AJ001735
Echiura	*Ochetostoma erythrogrammom*	X79875	Diploblasts		
Pogonophora	*Ridgeia piscesae*	X79877	Placozoa	*Trichoplax adhaerens*	L10828
	Siboglinum fiordicum	X79876	Porifera	*Scypha ciliata*	L10827
Ecdysozoa				*Microciona prolifera*	L10825
Tardigrada	*Macrobiotus hufelandi**	X81442		*Tetilla japonica**	D15067
Arthropoda	*Odiellus troguloides*	X81441	Cnidaria	*Anemonia sulcata*	X53498
	Aphonopelma sp.	X13457		*Antipathes galapagensis**	AF100943
	Berndtia purpurea	L26511		*Atolla vanhoeffeni*	AF100942
	Panulirus argus	U19182		*Anthopleura kurogane**	Z21671
	Tenebrio molitor	X07801		*Tripedalia cystophora*	L10829
	Polistes dominulus	X77785	Ctenophora	*Beroe cucumis*§	D15068
	Scolopendra cingulata	U29493		*Mnemiopsis leidyi*§	L10826
Priapulida	*Priapulus caudatus*	X87984	Fungi	*Saccharomyces cerevisiae*§	Z75578

could not be unambiguously aligned were excluded from the analysis, leaving 1655 characters in 104 taxa for the parsimony analysis and 1237 in 66 taxa for the maximum likelihood analysis (with reference to different numbers of taxa used in the analyses, see below). The mitochondrial sequences (*COI* and *Cytb*) were aligned with the software CLUSTALW (Thompson *et al.* 1994), based on the amino acid sequences. The alignments were then edited in the GDE software package (Smith *et al.* 1994). A combined 18S rDNA and mitochondrial data set was compiled from our own data and sequences available from GenBank. Because sequences for all of the genes were not available for some of the species, some terminals are a compilation of sequences from closely related species (see Table 3). In this data set, only the first and second codon positions of the mitochondrial genes were used, giving a total of 2143 characters (750 parsimony informative, 19 taxa).

Long branch attraction (LBA, also known as unequal rate artefacts) may distort the results of phylogeny reconstruction by grouping the longest branches together irrespective of the actual phylogeny (Felsenstein 1978; Hendy & Penny 1989). Although it is difficult to demonstrate LBA with empirical data, it is theoretically possible that the phenomenon may be a concern in phylogenetic analyses that incorporate widely divergent taxa. LBA is a special case of convergence: random similarities on unrelated clades drive them together in a parsimony analysis. It is not known how large the differences in branch length have to be to produce LBA artefacts, but it is obvious that a reduction in homoplasy in the data set will reduce the risk of LBA artefacts. We used a method devised by J. S. Farris to reduce the homoplasy level in our data by recoding the nucleotide sequences into triplets: all occurring triplet combinations of nucleotides were each given their unique code (Farris, prerelease software). Thereby the number of possible character states was increased from four to 64 and the likelihood of chance convergence was reduced [Felsenstein's (1978) initial simulation studies were performed using binary characters]. In an effort to avoid artefacts caused by insufficient taxonomic sampling and to break up any remaining long branches, we included a large selection of terminals from the studied groups: 21 acoel, 17 rhabditophoran and two catenulid terminals were analysed together with the four nemertodermatid sequences available and 60 other metazoans. The recoded data set consisted of 457 characters, all of them parsimony informative. The parsimony analysis (heuristic search with 1000 random additions and the Tree Bisection and Reconnection (TBR) option enabled) was performed with PAUP* (Swofford 2000). Parsimony jackknifing of the combined 18S rDNA and mitochondrial data set (19 terminals, 2143 characters, 750 of which were parsimony informative) was performed with the XAC software (J. S. Farris prerelease software). Five thousand jackknife replicates with nine addition replicates each were performed.

Table 3 Composition of the concatenated 18S ribosomal DNA and mitochondrial data set.

Higher taxon	Species 18S	GenBank 18S	Species mitochondrial	GenBank *COI*	GenBank *Cytb*
Cnidaria	*Metridium* sp.	AF052889	*M. senile*	NC000933	NC000933
Nemertodermatida	*Nemertoderma westbladi*	This study	*N. westbladi*	This study	This study
Acoela	*Paratomella rubra*	AF102892	*P. rubra*	This study	This study
Platyhelminthes1	*Microstomum lineare*	U70082	*M. lineare*	This study	This study
Platyhelminthes2	*Echinococcus granularis*	U27015	*E. multilocularis*	AB018440	AB018440
Annelida1	*Lumbricus terrestris*	AJ272183	*L. terrestris*	U24570	U24570
Annelida2	*Nereis virens*	Z83754	*Platynereis dumerilii*	AF178678	AF178678
Brachiopoda	*Terebratulina retusa*	U08324	*T. retusa*	AJ245743	AJ245743
Mollusca1	*Cepaea nemoralis*	AJ224921	*C. nemoralis*	U23045	U23045
Mollusca2	*Liolophura japonica*	X70210	*Katharina tunicata*	U09810	U09810
Mollusca3	*Helix aspersa*	X91976	*Albinaria coerulea*	X83390	X83390
Nematoda1	*Ascaris suum*	U94367	*A. suum*	X54253	X54253
Nematoda2	*Gnathostoma binucleatum*	Z96946	*Onchocerca volvulus*	AF015193	AF015193
Arthropoda1	*Polistes dominulus*	X77785	*Apis mellifera*	L06178	L06178
Arthropoda2	*Archeta domesticus*	X95741	*Locusta migratoria*	X80245	X80245
Echinodermata1	*Arbacia lixula*	X37514	*A. lixula*	X80396	X80396
Echinodermata2	*Strongylocentrotus purpuratus*	L28056	*S. purpuratus*	X12631	X12631
Chordata1	*Gallus gallus*	AF173612	*G. gallus*	X52392	X52392
Chordata2	*Cyprinus carpio*	AF133089-1st AF021880-2nd	*C. carpio*	X61010	X61010

In the maximum likelihood analyses of the 18S rDNA sequences alone, only the 55 taxa that passed the relative rate test in our previous study (Ruiz-Trillo *et al.* 1999), together with nine outgroup taxa, were used (Table 2, indicated with ?). The three new nemertodermatid sequences were also submitted to the same test. Only two passed and consequently both were added to the data set, giving a total of 66 taxa. The analyses were performed with the FASTDNAML software (Olsen *et al.* 1994) using global rearrangements and jumble options. The categories option was used to take into account the among-site rate variation, introducing the parameters previously calculated with PUZZLE 4.0. Branch support for the ribosomal data set was calculated only for the nodes of interest, through a four-cluster likelihood mapping analysis with PUZZLE 4.0 (Strimmer & von Haeseler 1996) taking into account among-site rate variation. This approach allows an analysis of the support for internal branches in a tree without having to compute the overall tree. Every internal branch in a completely resolved tree defines up to four clusters of sequences. These four clusters can be defined in the data file and the program will build a quartet tree for each of all possible combinations of four species, always taking one from each group. The result is represented on a triangle in which each corner represents one of the three possible unrooted trees uniting the four clusters of species. The distribution of points within this triangle indicates the level of support for the internal branch under analysis. In the analysis to determine the support for the branch separating the Acoela from the rest of the Bilateria, the four clusters were: (A) fungus + diploblasts; (B) Acoela; (C) Deuterostomia; and (D) Protostomia. To determine the support for the branch separating the Nemertodermatida from the rest of the Bilateria (except the Acoela), the groups were: (A) fungus + diploblasts + Acoela; (B) Nemertodermatida; (C) Deuterostomia; and (D) Protostomia.

A further analysis was carried out in which a maximum likelihood tree was built without including any outgroup species, or the Acoela. In this analysis, the long branch separating diploblastic from triploblastic animals is eliminated. Consequently, one would expect the Nemertodermatida to branch close to the Platyhelminthes if their basal position was an artefact (Giribet *et al.* 2000) due to LBA caused by diploblasts. Conversely, if nemertodermatids, together with acoels, are basal triploblasts and the remaining platyhelminths (the Rhabditophora) are derived spiralians, nemertodermatids should not group with the Rhabditophora.

The maximum likelihood analyses of the combined mitochondrial and ribosomal data set were performed as explained above. An additional maximum likelihood analysis was carried out without the most variable positions (category 8 of the gamma distribution). Branch support for this data set was calculated through quartet puzzling (50 000 replicates) with PUZZLE 4.0, taking into account among-site rate variation.

Results

Parsimony analysis

The strict consensus tree from 52 most-parsimonious trees calculated from the triplet-coded 18S rDNA nucleotide data is shown in Fig. 1 (retention index = 0.56). The cladogram shows the Acoela as the sister group of the remaining Bilateria

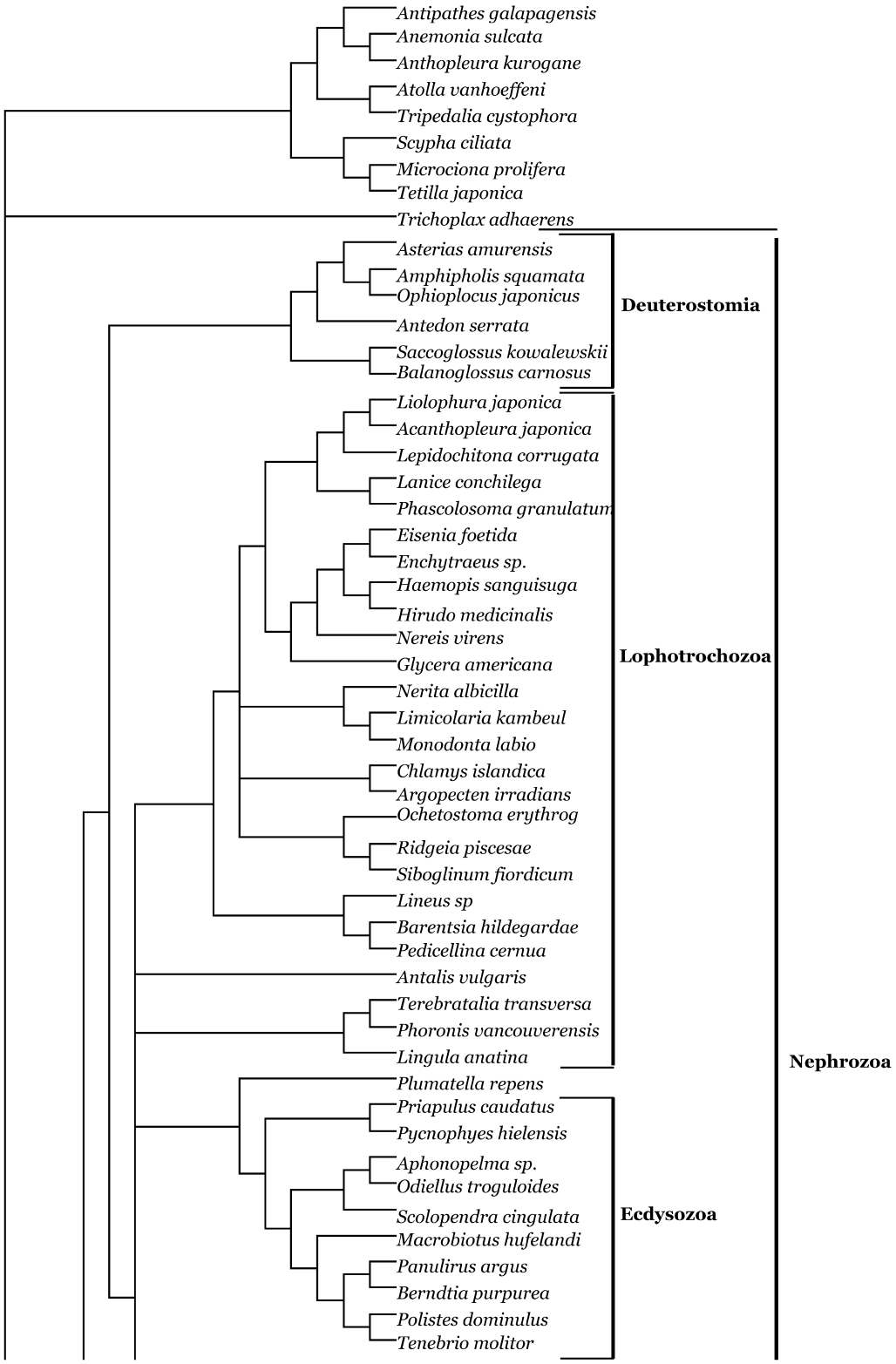


Fig. 1 Strict consensus tree of 52 most-parsimonious trees resulting from a parsimony analysis of the triplet-coded 18S ribosomal DNA data set (PAUP* 1000 random additions/TBR equal weights 457 characters, all of them parsimony informative).

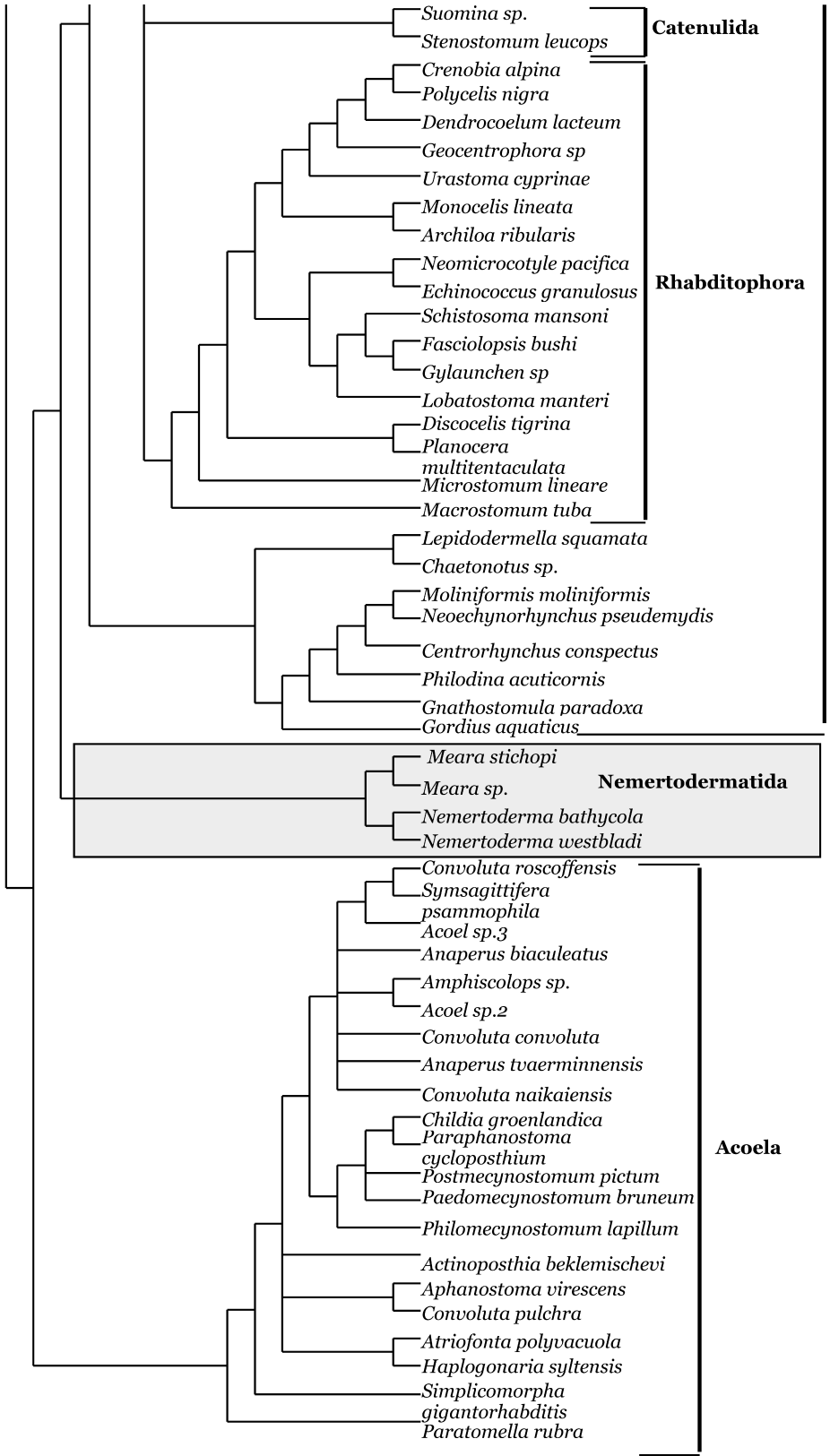


Fig. 1 *continued*

followed by the Nemertodermatida. During our initial analyses, the sequence identified as oriIginating from *Nemertinoides elongatus* (GenBank Accession no. U70084), which was used as a representative of the Nemertodermatida in previous analyses (Carranza *et al*. 1997; Zrzavy *et al*. 1998; Littlewood *et al*. 1999), did not group with the three nemertodermatid species sequenced by us, but with rhabditophoran Platyhelminthes. Therefore it was excluded from further analyses. There is no explicit support for the monophyly of the Lophotrochozoa in the most-parsimonious tree. The cladogram does not conflict with the Lophotrochozoa hypothesis, but the group is not retrieved as a monophylum. A group consisting of the representatives of Nematomorpha, Gastrotricha, Rotifera, Gnathostomulida and Acanthocephala branches basally (between the nemertodermatids and the rest of the Bilateria) in the most-parsimonious tree. The position of the Gastrotricha, Gnathostomulida and Rotifera has been difficult to determine in other studies based on 18S rDNA (Giribet *et al*. 2000) and their basal position here should be viewed with caution.

Maximum likelihood analyses

The tree resulting from the maximum likelihood analysis of the 18S rDNA data set is shown in Fig. 2. The branching order of the Acoela and the Nemertodermatida, highly supported by four-cluster likelihood mapping (98 and 82%, respectively), is identical to that of the parsimony analysis. The deuterostome clade is the sister group of the Ecdysozoa and Lophotrochozoa, which are recovered as monophyletic groups. In this tree, the Nematomorpha, Rotifera (only the single species which passed the relative rate test was included) and Gastrotricha branch within the Ecdysozoa or the Lophotrochozoa. The Gnathostomulida and the Acantocephala did not pass the relative rate test and were not included in this analysis. The resolution within the protostome clade, which includes the flatworm groups Rhabditophora and Catenulida, is higher than in the parsimony analyses.

The analysis of the 18S data without the outgroup gave a tree in which the Nemertodermatida emerge from the branch leading to the deuterostomes, whereas ecdysozoans and lophotrochozoans are grouped together (Fig. 3). We also tested three alternative topologies (using the comparison allowed by the user tree option in FASTDNAML) by forcing the nemertodermatids at three different positions: (1) at the base of the Ecdysozoa, (2) at the base of the Lophotrocozoa, (3) at the base of the Platyhelminthes within the Lophotrochozoa. In the three cases, the likelihood score of the tree was significantly worse than the score of the tree shown in Fig. 2 (difference in LnL: –13.56, standard deviation = 5.39 for trees 1 and 2; –20.61, standard deviation = 6.69 for tree 3).

Combined 18S rDNA and mitochondrial gene analyses

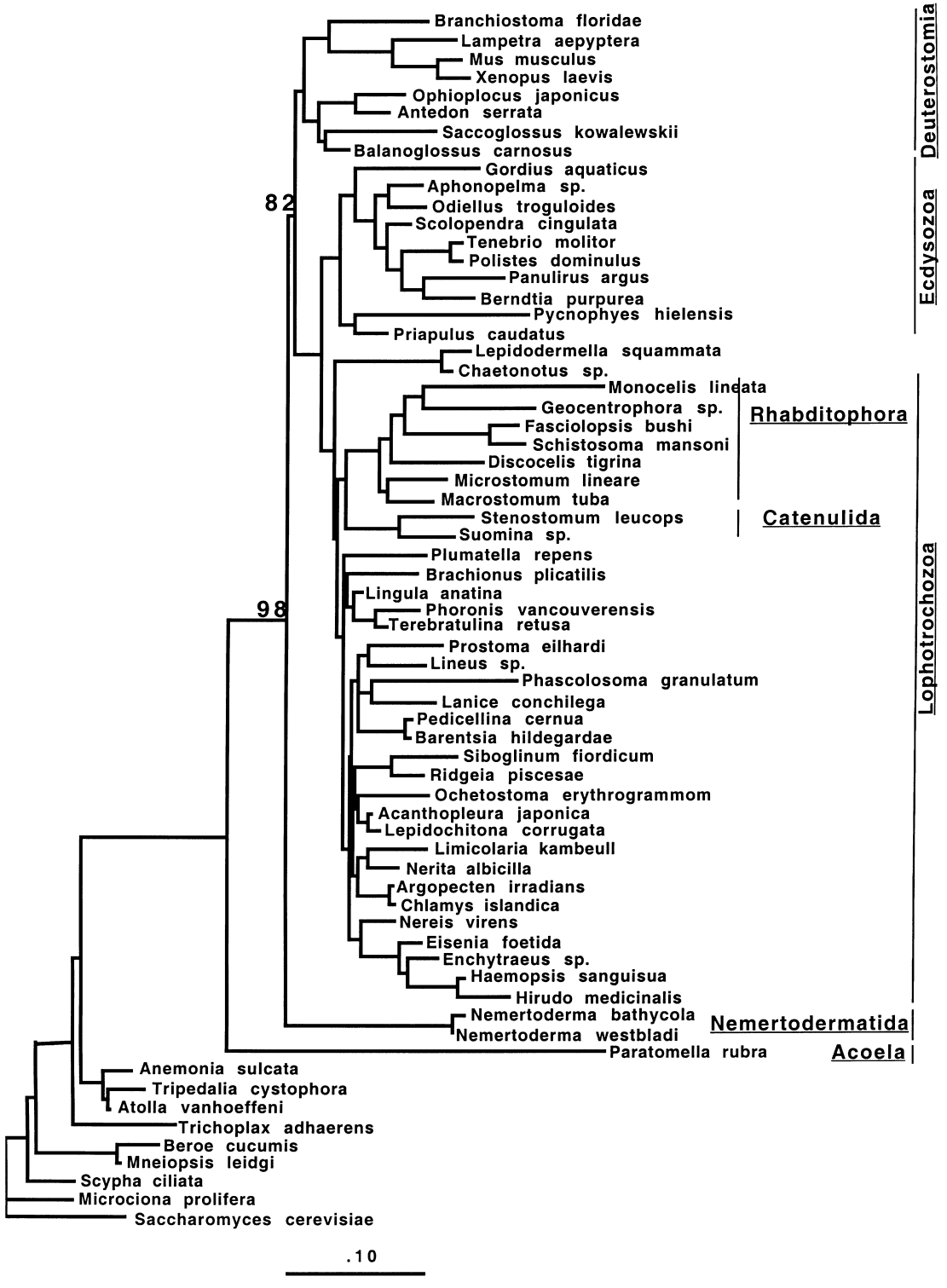
Parsimony jackknifing (Farris *et al*. 1996) of the concatenated 18S rDNA and mitochondrial nucleotide sequences supported a clade consisting of the Nemertodermatida and all other Bilateria, but excluding the Acoela (not shown). The maximum likelihood analysis (Fig. 4) retrieved a tree similar to the 18S rDNA maximum likelihood tree (Fig. 2), except that there is no support for the Ecdysozoa (Nematoda and Arthropoda do not form a monophylum). The branch support for the Acoela and the Nemertodermatida as basal clades of the Bilateria was high (80 and 88%, respectively). Removal of the most variable positions in the maximum likelihood analyses still resulted in strong support for the basal position of acoels and nemertodermatida (92 and 82%).

Moreover, we found our sequences of Nemertodermatida and Acoela to have the standard invertebrate mitochondrial genetic code, not the modification of the mitochondrial genetic code previously reported from the Rhabditophora (Telford *et al*. 2000). The rhabditophoran *Microstomum lineare* did share the modifications of other Rhabditophora.

Discussion

The results presented here strongly support the hypothesis that Nemertodermatida is a clade of basal bilaterians, separate from the Acoela, and not closely related to the Rhabditophora. In addition, the basal position of acoels is further reinforced. Altogether, this renders the Platyhelminthes a non-monophyletic assemblage which, awaiting the precise position of the Catenulida, must be split into three separate monophyletic clades: Acoela, Nemertodermatida and Rhabditophora + Catenulida. The finding that the sequence of *Nemertinoides elongatus* used in all previous analyses did not group with the three nemertodermatid species sequenced here, strongly suggests that it is a sequencing artefact or originated from an undisclosed rhabditophoran flatworm. The position of Nemertodermatida and Acoela separate from Rhabditophora is further supported by the recent finding of a new molecular synapomorphy also corroborated in this work: acoels and nemertodermatids together with catenulids have the standard invertebrate mitochondrial genetic code and do not share the previously reported modifications of the mitochondrial genetic code in the Rhabditophora (Telford *et al*. 2000).

Fig. 2 Tree resulting from the maximum likelihood analysis of the 18S ribosomal DNA data set (FASTDNAML, jumble and global options). Branch support for the Acoela and the Nemertodermatida are indicated at the corresponding nodes (calculated with PUZZLE 4.0). The tree illustrates the position of the Nemertodermatida as the sister group to the rest of the Bilateria except the Acoela. The Rabditophora + Catenulida clade branches within the Lophotrochozoa. For species names and taxonomical assignment see Table 2. Scale bar indicates the number of substitutions per position.


Branchiostoma floridae
Lampetra aepyptera
Mus musculus
Xenopus laevis
Ophioplocus japonicus
Antedon serrata
Saccoglossus kowalewskii
Balanoglossus carnosus
Deuterostomia
Gordius aquaticus
Aphonopelma sp.
Odiellus troguloides
Scolopendra cingulata
Tenebrio molitor
Polistes dominulus
Panulirus argus
Berndtia purpurea
Pycnophyes hielensis
Priapulus caudatus
Ecdysozoa
82
Lepidodermella squammata
Chaetonotus sp.
Monocelis lineata
Geocentrophora sp.
Fasciolopsis bushi
Schistosoma mansoni
Rhabditophora
Discocelis tigrina
Microstomum lineare
Macrostomum tuba
Stenostomum leucops
Suomina sp.
Catenulida
Plumatella repens
Brachionus plicatilis
Lingula anatina
98
Phoronis vancouverensis
Terebratulina retusa
Prostoma eilhardi
Lineus sp.
Phascolosoma granulatum
Lanice conchilega
Pedicellina cernua
Barentsia hildegardae
Siboglinum fiordicum
Ridgeia piscesae
Ochetostoma erythrogrammom
Acanthopleura japonica
Lepidochitona corrugata
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Argopecten irradians
Chlamys islandica
Nereis virens
Eisenia foetida
Enchytraeus sp.
Haemopsis sanguisua
Hirudo medicinalis
Lophotrochozoa
Nemertoderma bathycola
Nemertoderma westbladi
Nemertodermatida
Paratomella rubra
Acoela
Anemonia sulcata
Tripedalia cystophora
Atolla vanhoeffeni
Trichoplax adhaerens
Beroe cucumis
Mneiopsis leidgi
Scypha ciliata
Microciona prolifera
Saccharomyces cerevisiae
.10

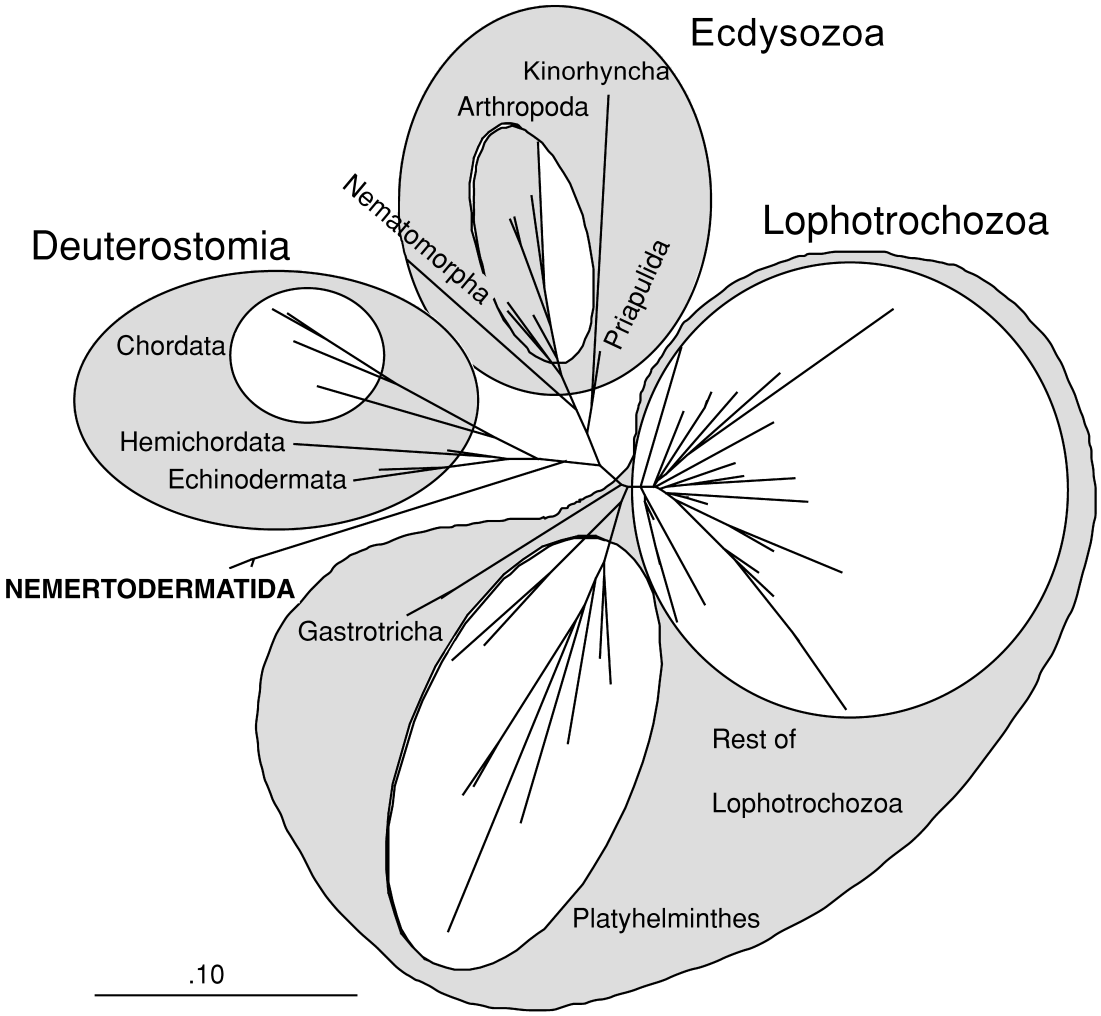

Fig. 3 Unrooted tree resulting from the analysis of 18S ribosomal DNA data of bilaterian phyla not including the Acoela (FASTDNAML, jumble and global options). The three main bilaterian clades are highlighted. The Nemertodermatida emerge at the base of the deuterostomian branch (for more details, see text). For clarity, the names of the species are not included. Scale bar indicates the number of substitutions per position.

To avoid theoretically possible 'long branch' problems, four different strategies were followed. In the parsimony analyses we (1) recoded the nucleotide data into triplets to reduce branch length and homoplasy; and (2) included a large number of terminals (104 taxa) to break up branches and avoid sampling artefacts. In the maximum likelihood analyses we (3) used species that passed the relative rate test in previous analyses (Ruiz-Trillo *et al.* 1999) together with two nemertodermatid species reported here, and (4) performed one analysis with diploblasts and acoels removed. These widely different approaches produced consistent results with respect to the position of the Acoela and the Nemertodermatida. Parsimony analysis of the triplet-coded data set yielded a tree where ecdysozoans and lophotrochozoans form a polytomy. The low resolution within the protostomes might have been anticipated because recoding nucleotides into triplets reduces the number of characters and, therefore, the resolution. Some 'pseudocoelomate' clades (see Fig. 1) occupy positions basal to the Deuterostomia and Protostomia in the most-parsimonious tree. This position must be regarded as highly tentative. While there is no generally accepted position for the Gastrotricha, Gnathostomulida, Rotifera and Acanthocephala, morphological synapomorphies uniting the latter three taxa are known (Rieger & Tyler 1995; Sorensen *et al.* 2000). Most of the 'pseudocoelomate' representatives did not pass the relative rate test (see Table 2) and thus their position could not be reconstructed in the maximum likelihood analysis. The position of the single nematomorph *Gordius aquaticus* in the most-parsimonious cladogram is clearly at odds with the current mainstream view of the Nematomorpha as part of the Ecdysozoa. Inclusion of

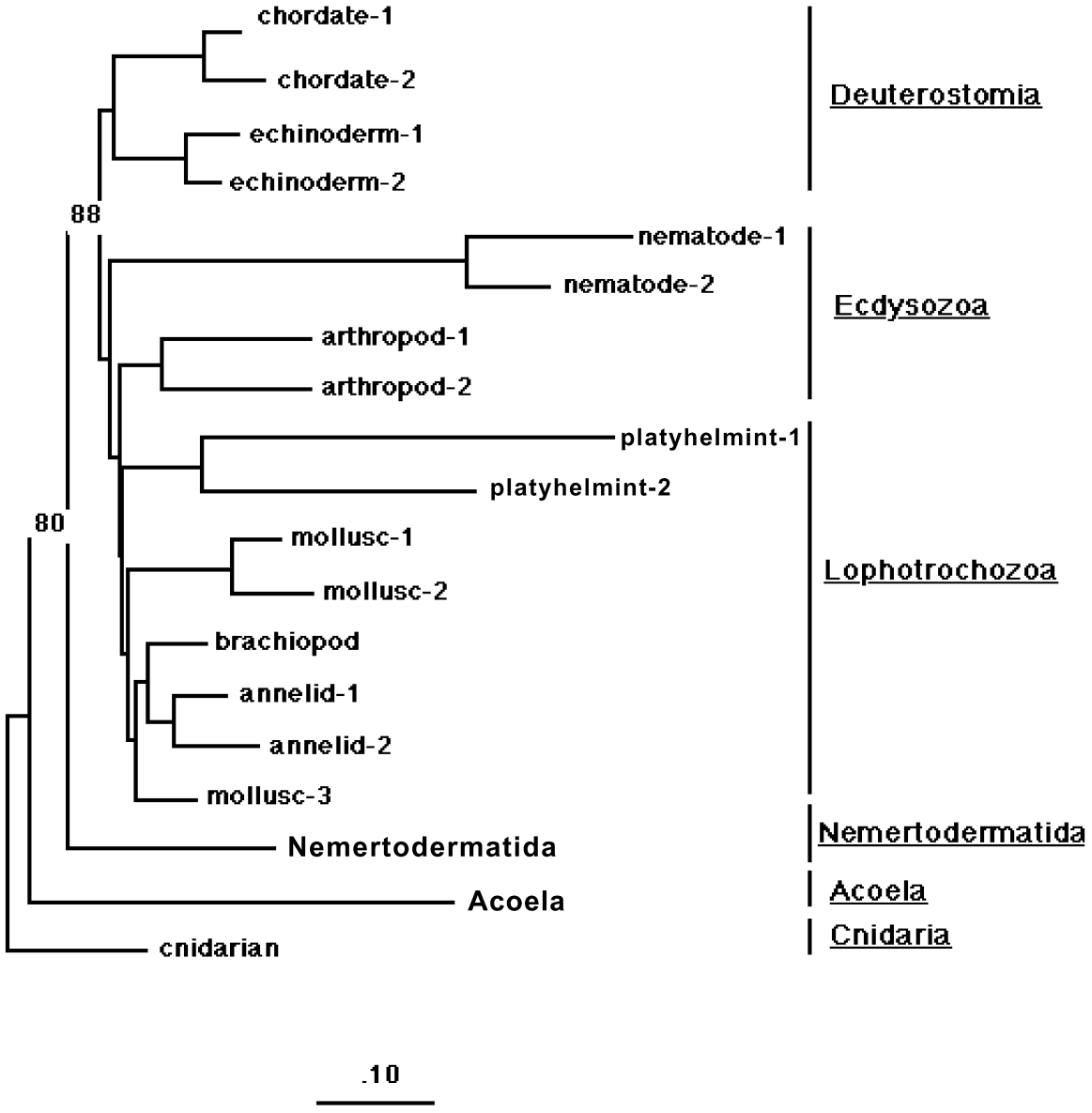

Fig. 4 Tree resulting from the maximum likelihood analysis of a combined 18S ribosomal DNA and mitochondrial data set (FASTDNAML, jumble and global options). Branch support for the Acoela and the Nemertodermatida is shown at the corresponding nodes. The Acoela and the Nemertodermatida are, as in most-parsimonious and maximum likelihood trees based on 18S ribosomal DNA data alone, the two basalmost branches of the Bilateria. For species names see Table 3. Scale bar indicates the number of substitutions per position.

a larger taxonomic sample of Nematomorpha and Nematoda might have produced a different result.

The Deuterostomia, Ecdysozoa and Lophotrochozoa are retrieved in the maximum likelihood trees based on taxa that passed the relative rate test. In these maximum likelihood trees (Fig. 2), Nemertodermatida, together with the Acoela, branch as the most basal groups. Furthermore, removing diploblasts and acoels still resulted in a basal position for the nemertodermatids (Fig. 3), which never grouped with the Rhabditophora. In addition, nemertodermatids do not branch within any of the three bilaterian clades: Deuterostomia, Ecdysozoa and Lophotrochozoa. This is again an indication of their basal bilaterian position. Adding mitochondrial nucleotide sequences to the 18S rDNA sequences and analysing the concatenated sequences by parsimony jackknifing and maximum likelihood (Fig. 4) did not increase the resolution. The resulting maximum likelihood tree is similar to the maximum likelihood tree based on 18S rDNA sequences

alone (Fig. 2), but the Ecdysozoa are not retrieved. Compared with the bootstrap values for the 18S rDNA maximum likelihood tree in Fig. 2, the value for the nemertodermatids increases (82 to 88%) while it decreases for the acoels (98 to 80%). This seems to indicate that mitochondrial sequences add some useful phylogenetic information for nemertodermatids but not for acoels. However, these changes in bootstrap values might also be due to the more restricted species sampling of these analyses (19 species compared with 104 in most-parsimonious and 66 in maximum likelihood analyses). We performed a maximum likelihood analysis of the mitochondrial data alone (*COI* and *Cytb* first and second positions) which supported the basal position of the Acoela separate from the Rhabditophora (77% support in PUZZLE), but the position of the Nemertodermatida was not supported.

Altogether, the most-parsimonious and maximum likelihood analyses agree with regards the position of the Nemertodermatida. Both support a basal position for nemertodermatids, with very high support values in maximum likelihood trees. It is worth noting that the position of the Nemertodermatida and the Acoela as the two basalmost branches in the Bilateria is also retrieved when the data are analysed in nucleotide sequence form with parsimony analysis, i.e. without triplet coding. Thus, it appears that there were no LBA artefacts affecting their positions to begin with.

The basal phylogenetic position for Acoela and Nemertodermatida separate from Rhabditophora is also supported from another set of data. First, acoel embryonic cleavage and cell lineage (Henry *et al*. 2000) show them to exhibit duet spiral cleavage and endomesoderm as the sole source of mesoderm compared with the canonical quartet spiral cleavage and ecto- and endomesoderm in the Rhabditophora and most spiralian lophotrochozoans. An endodermal origin of mesoderm is considered ancestral. Moreover, duet cleavage is actually more bilateral than spiral cleavage (Henry *et al*. 2000), strongly suggesting that duet cleavage and typical quartet cleavage are not related. Finally, acoels and nemertodermatids do not possess protonephridia. In Ehler's (1985) hypothesis, the lack of protonephridia in Nemertodermatida and Acoela is regarded as an apomorphy, deviating from the ground plan of the Platyhelminthes. However, under our phylogenetic hypothesis, the absence of protonephridia is a plesiomorphic state. The presence of protonephridia or homologues, on the other hand, is a synapomorphy uniting all Bilateria except acoels and nemertodermatids.

Despite the basal positioning of both acoels and nemertodermatids, the clade Acoelomorpha (Acoela + Nemertodermatida; Ehlers 1985) was never retrieved in our analyses. Similarities in the structure of the epidermal cilia (interconnecting ciliary rootlets and capped cilia) and the lack of protonephridia are synapomorphies proposed for the Acoelomorpha. The phylogeny of the Nemertodermatida was reconstructed by Lundin (2000) using morphological characters. His study was performed under the assumption of platyhelminth monophyly, and thus is not a critical test of our hypothesis: only taxa belonging to the Nemertodermatida, Acoela, Rhabditophora and the marine worm *Xenoturbella* were included. Although Lundin (2000) seems to prefer the Acoelomorpha hypothesis, his results are entirely compatible with the results presented here after adjusting for our much larger taxonomic sample. Under our hypothesis, the ciliary structures are interpreted as plesiomorphic characters present in the last common ancestor of the Bilateria or independently evolved. It should be noted that knowledge of detailed ciliary morphology of the Bilateria is scant; these features may be more common than assumed when the Acoelomorpha hypothesis was conceived. Recent studies of neuroanatomy also support separate phylogenetic positions for Acoela, Nemertodermatida and Rhabditophora and other bilaterians. A true brain with neuropile is absent in the former two groups (Raikova *et al*. 1998; Reuter *et al*. 1998). The nemertodermatids *Nemertoderma westbladi* and *Meara stichopi* have a very simple nervous system with weak or no centralization. The patterns of neurotransmitters (5-hydroxytryptamine (serotonin) and FMRfamide) in the two species are different from the two dissimilar patterns known from the Acoela and the Rhabditophora (Raikova *et al*. 2000). Finally, the Acoelomorpha hypothesis receives no support from sperm morphology. The Nemertodermatida have uniflagellate spermatozoa with the common 9 + 2 axonemal pattern, which probably evolved from sperm of Franzén's 'primitive type' (Lundin & Hendelberg 1998). Acoel spermatozoa bear two flagella with reverse orientation and sometimes a modified axoneme structure (Raikova *et al*. 2001).

Although the Bilateria are widely recognized as a monophyletic group, the characterization of the bilaterian ground pattern is actually far from simple. This is because the reconstruction of characters of the last common bilaterian ancestor requires knowledge of early bilaterian phylogeny. If a small and structurally simple acoel-like ancestor is hypothesized, the rest of the bilaterian phyla evolved by stages of increasing size and complexity. This widely held notion has recently been substituted, arguing that acoelomate and pseudocoelomate animals are secondarily simplified. Instead, based on a basal split of the Bilateria into two coelomate clades, a rather complex organism (the so-called 'Urbilateria') has been proposed as the ancestral bilaterian. Under this new view the bilaterian ancestor was large and possessed a mouth and anus, coelom, segments and, very likely, some sort of appendages (Rieger 1986; Kimmel 1996; De Robertis 1997; Holland *et al*. 1997; Adoutte *et al*. 1999, 2000). However, as pointed out by Jenner (2000), the provided phylogenies are heavily pruned, leaving out several 'minor' phyla, namely basal ecdysozoans and lophotrochozoans, and the proposed expression pattern

homologies are unreliable and exhibit a strong taxon selection. A second scenario suggests a small ciliated primary larva with a population of set-aside cells [reviewed in Peterson *et al.* (2000)] as the ancestral bilaterian. This scenario hinges on the assumption that 'maximal indirect development' is primitive for bilaterians, direct development being derived (Cameron *et al.* 1998; Peterson *et al.* 2000). This is, at the most, an untested assumption. Moreover, the difficulties of homologizing the so-called primary larvae in different groups suggest that set-aside cells are not homologous but convergent. In addition, the phylogenies provided are again highly pruned and biased towards coelomate, indirectly developing bilaterians. A final, radically different, scenario has suggested a complex, tailed and segmented ancestor, bearing a dorsal brain, serially repeated kidneys, gonocoels and a contractile blood vessel, thought to have evolved from colonial cnidarian-grade organisms (Dewel 2000). Although novel in conception, the evidence to back it up is scant and unconvincing.

The new data summarized in our phylogenetic hypothesis necessitate completely different parsimony-based inferences about the lifecycle and other characteristics present in the last common ancestor of the extant Bilateria. The Acoela and the Nemertodermatida do not possess a true brain or protonephridia. If our hypothesis is accepted, these characters are morphological synapomorphies of the higher Bilateria [see also Haszprunar (1996)]. We propose the name Nephrozoa for Bilateria excluding the Nemertodermatida and the Acoela. Nemertodermatids and acoels are minute, unsegmented bottom dwelling worms with internal fertilization and direct development. Our results therefore imply that the last common bilaterian ancestor was small, benthic, without segments and coelomic cavities, and lacked a planktonic larval stage.

The position of acoels and nemertodermatids at the base of bilaterians has two further implications at different phylogenetic levels. First, if they arose from a cnidarian-like diploblast ancestor, the planula-like features of the Acoela and Nemertodermatida suggest they may have arisen by progenesis (attainment of sexual maturity in larval forms) from a planula-like larva. This is at variance with the traditional planuloid–acoeloid hypothesis, according to which the gastrula-like planuloid ancestor was a fully mature adult, from which both cnidarians and all bilaterians, via an acoel-type organism, arose [reviewed in Willmer (1990)]. Second, if acoels and nemertodermatids are corroborated as basal to all other bilaterians, they could be instrumental in determining the character states of the ancestral Bilateria and so greatly further our understanding of the evolution of the higher bilaterians. Among such characters, the evolution and further deployment of the mesoderm and the nervous system, the embryological origin of the excretory system, and the transition from a single body axis to two orthogonal body axes, are worthy of exploration.

We regard the hypothesis presented here as a current best estimate of the phylogenetic position of the Nemertodermatida and Acoela. It will be tested through the acquisition and analysis of new molecular or morphological data on a Metazoa-wide basis.

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References

Adoutte, A., Balavoine, G., Lartillot, N. & de Rosa, R. (1999). Animal evolution: the end of the intermediate taxa? *Trends in Genetics*, *15*, 104–108.

Adoutte, A., Balavoine, G., Lartillot, N., Lespinet, O., Prud'homme, B. & de Rosa, R. (2000). The new animal phylogeny: reliability and implications. *Proceedings of the National Academy of Sciences USA*, *97*, 4453–4456.

Aguinaldo, A. M. A., Turbeville, J. M., Lindford, L. S., Rivera, M. C., Garey, J. R., Raff, R. A. & Lake, J. A. (1997). Evidence for a clade of nematodes, arthropods and other moulting animals. *Nature*, *387*, 489–493.

Balavoine, G. (1997). The early emergence of platyhelminths is contradicted by the agreement between 18S rRNA and Hox genes data. *Comptes Rendus de l'Academie des Sciences Serie III Sciences de la Vie*, *320*, 83–94.

Balavoine, G. (1998). Are Platyhelminthes coelomates without a coelom? An argument based on the evolution of Hox genes. *American Zoologist*, *38*, 843–858.

Bayascas, J. R., Castillo, E. & Saló, E. (1998). Platyhelminthes have a Hox code differentially activated during regeneration, with genes closely related to those of spiralian protostomes. *Development, Genes and Evolution*, *208*, 467–473.

Berney, C., Pawlowski, J. & Zaninetti, L. (2000). Elongation factor 1-alpha sequences do not support an early divergence of the Acoela. *Molecular Biology and Evolution*, *17*, 1032–1039.

Cameron, R. A., Peterson, K. J. & Davidson, E. H. (1998). Developmental gene regulation and the evolution of large animal body plans. *American Zoologist*, *38*, 609–620.

Carranza, S., Baguna, J. & Riutort, M. (1997). Are the Platyhelminthes a monophyletic primitive group? An assessment using 18S rDNA sequences. *Molecular Biology and Evolution*, *14*, 485–497.

De Robertis, E. M. (1997). The ancestry of segmentation. *Nature*, *387*, 25–26.

De Rosa, R., Grenier, J. K., Andreeva, T., Cook, C., Adoutte, A., Akam, M., Carroll, S. B. & Balavoine, G. (1999). *Hox* genes in

Brachipods and Priapulids: implications for Protostome evolution. *Nature*, *399*, 772–776.

Dewel, R. A. (2000). Colonial origin for Eumetazoa: major morphological transitions and the origin of bilaterian complexity. *Journal of Morphology*, *243*, 35–74.

Ehlers, U. (1985). *Das Phylogenetische System der Platyhelminthes*. Stuttgart: Gustav Fischer.

Farris, J. S., Albert, V. A., Källersjö, M., Lipscomb, D. & Kluge, A. G. (1996). Parsinnony jackknifing outperforms neighbor–joining. *Cladistics 12*, 99–124.

Felsenstein, J. (1978). Cases in which parsimony or compatibility methods will be positively misleading. *Systematic Zoology*, *27*, 401–410.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, *3*, 294–299.

Giribet, G., Distel, D. L., Polz, M., Sterrer, W. & Wheeler, W. C. (2000). Triploblastic relationships with emphasis on the Acoelomates and the position of Gnathostomulida, Cycliophora, Platyhelminthe, and Chaetognatha: a combined approach of 18S rDNA sequences and morphology. *Systematic Biology*, *49*, 539–562.

Gutell, R. R., Weibser, B., Woese, C. R. & Noller, H. F. (1985). Comparative anatomy of 16S-like ribosomal RNA. *Progress in Nucleic Acid Research and Molecular Biology*, *32*, 155–216.

Haszprunar, G. (1996). Platyhelminthes and Platyhelminthomorpha — paraphyletic taxa. *Journal of Zoological Systematics and Evolutionary Research*, *34*, 41–48.

Hendy, M. D. & Penny, D. (1989). A framework for the quantitative study of evolutionary trees. *Systematic Zoology*, *38*, 297–309.

Henry, J. Q., Martindale, M. Q. & Boyer, B. C. (2000). The unique developmental program of the acoel flatworm, *Neochildia fusca*. *Developmental Biology*, *220*, 285–295.

Holland, P. H. W., Kene, M., Williams, N. A. & Holland, N. D. (1997). Sequence and embryonic expression of the amphioxus engrailed gene (*AmphiEn*): the metameric pattern of transcription resembles that of its segment polarity homolog in *Drosophila*. *Development*, *124*, 1723–1732.

Jenner, R. A. (2000). Evolution of animal body plans: the role of metazoan phylogeny at the interface between pattern and process. *Evolution and Development*, *2*, 208–221.

Karling, T. G. (1940). Zur Morphologie und Systematik der Alloeocoela Cumulata and Rhabdocoela Lecitophora (Turbellaria). *Acta Zoologica Fennica*, *26*, 1–260.

Karling, T. G. (1974). On the anatomy and affinities of the turbellarian orders. In N. W. Riser & M. P. Morse (Eds) *Biology of the Turbellaria* (pp. 1–16). New York: McGraw-Hill.

Kimmel, C. B. (1996). Was *Urbilateria* segmented? *Trends in Genetics*, *12*, 329–331.

Littlewood, D. T. J., Olson, P. D., Telford, M. J., Herniou, E. A. & Riutort, M. (2001). Elongation factor 1-alpha sequences alone do not assist in resolving the position of the Acoela within the Metazoa. *Molecular Biology and Evolution*, *18*, 437–442.

Littlewood, D. T. J., Rohde, K. & Clough, K. A. (1999). The interrelationships of all major groups of Platyhelminthes: phylogenetic evidence from morphology and molecules. *Biological Journal of the Linnean Society*, *66*, 75–114.

Lundin, K. (1997). Comparative ultrastructure of the epidermal ciliary rootlets and associated structures in species of the Nemertodermatida and Acoela (Platyhelminthes). *Zoomorphology*, *117*, 81–92.

Lundin, K. (1998). The epidermal ciliary rootlets of *Xenoturbella bocki* (Xenoturbellida) revisited: new support for a kinship with the Acoelomorpha (Platyhelminthes). *Zoologica Scripta*, *27*, 263–270.

Lundin, K. (2000). Phylogeny of the Nemertodermatida (Acoelomorpha, Platyhelminthes). A cladistic analysis. *Zoologica Scripta*, *29*, 65–74.

Lundin, K. & Hendelberg, J. (1998). Is the sperm type of the Nemertodermatida close to that of the ancestral Platyhelminthes? *Hydrobiologia*, *383*, 197–205.

Lundin, K. & Sterrer, W. (2001). The Nemertodermatida. In D. T. J. Littlewood & R. A. Bray (Eds) *Interrelationships of the Platyhelminthes* (pp. 24–27). London: Taylor & Francis.

Norén, M. & Jondelius, U. (1999). Phylogeny of the Prolecithophora (Platyhelminthes) inferred from 18S rDNA sequences. *Cladistics*, *15*, 103–112.

Olsen, G. J., Matsuda, H., Hagstrom, R. & Overbeek, R. (1994). fastDNAml: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *Computer Applied Biosciences*, *10*, 41–48.

Peterson, K. J., Cameron, R. A. & Davidson, E. H. (2000). Bilaterian origins: significance of new experimental observations. *Developmental Biology*, *219*, 1–17.

Raikova, O. I., Reuter, M., Jondelius, U. & Gustafsson, M. K. S. (2000). The brain of the Nemertodermatida (Platyhelminthes) as revealed by anti-5HT and anti-FMRFamide immunostainings. *Tissue and Cell*, *32*, 358–365.

Raikova, O. I., Reuter, M. & Justine, J. L. (2001). Contributions to the phylogeny and systematics of the Acoelomorpha. In D. T. J. Littlewood & R. A. Bray (Eds) *Interrelationships of the Platyhelminthes* (pp. 13–23). London: Taylor & Francis.

Raikova, O. I., Reuter, M., Kotikova, E. A. & Gustafsson, M. K. S. (1998). A commisural brain! The pattern of 5-HT immunoreactivity in Acoela (Platyhelminthes). *Zoomorphology*, *118*, 69–77.

Reuter, M., Mäntylä, K. & Gustafsson, M. K. S. (1998). Organization of the orthogon — main and minor nerve cords. *Hydrobiologia*, *383*, 175–182.

Rieger, R. M. (1986). Über dem Ursprung der Blateria: die Bedeutung der Ultrastrukturforschung für eines neues Verstehen der Metazoenevolution. *Verhandlungen Deutsche Zoological Gesellschatt*, *79*, 31–50.

Rieger, R. M. & Tyler, S. (1995). Sister-group relationship of Gnathostomulida and Rotifera-Acanthocephala. *Invertebrate Biology*, *114*, 186–188.

Ruiz-Trillo, I., Riutort, M., Littlewood, D. T. J., Herniou, E. A. & Baguna, J. (1999). Acoel flatworms: earliest extant Bilaterian Metazoans, not members of Platyhelminthes. *Science*, *283*, 1919–1923.

Saló, E., Tauler, J., Jiménez, E., Bayascas, J. R., Gonzalez-Linares, J., García-Fernández, J. & Baguñà, J. (2001). Hox and ParaHox genes in flatworms. Characterization and expression. *American Zoologist*, *41*, 652–663.

Smith, J. P. S. III & Tyler, S. (1985). The acoel turbellarians: kingpins of metazoan evolution or a specialized offshoot? In S. Conway-Morris, J. D. George, R. Gibson & H. M. Platt (Eds) *The Origins and Relationships of Lower Invertebrates* (pp. 123–142). Oxford: Clarendon Press.

Smith, J. P. S. III, Tyler, S. & Rieger, R. M. (1986). Is the Turbellaria polyphyletic? *Hydrobiologia*, *132*, 71–78.

Smith, S. W., Overbeek, R., Woese, C. R., Gilbert, W. & Gillevet, P. M. (1994). The genetic data environment: an expandable GUI for multiple sequence-analysis. *Computer Applied Biosciences*, *10*, 671–675.

Sorensen, M. V., Funch, P., Willerslev, E., Hansen, A. J. & Olesen, J. (2000). On the phylogeny of the Metazoa in the light of Cycliophora and Microganthozoa. *Zoologischer Anzeiger*, *239*, 297–318.

Steinböck, O. (1930–31). Ergebnisse einer von E. Reisenger & O. Steinböck mit Hilfe der Rask-Orsted Fonds durchgefürten Reise in Grönland 1926. 2. *Nemertoderma bathycola* nov. General nov. spec., eine eigenartige Turbellarie aus der Tiefe der Diskobay; nebst einem beitrag zur Kenntnis des Nemertinenepithels. *Videnskablig Meddelende Fra Dansk Naturhistorisk Forening*, *90*, 47–84.

Sterrer, W. (1998). New and known Nemertodermatida (Platyhelminthes, Acoelomorpha), a revision. *Belgian Journal of Zoology*, *128*, 55–92.

Strimmer, K. & von Haeseler, A. (1996). Quartet puzzling: a quartet maximum likelihood method for reconstructing tree topologies. *Molecular Biology and Evolution*, *13*, 964–969.

Swofford, D. L. (2000). *PAUP: Phylogenetic Analysis Using Parsimony, and other Methods*, *Version 4.0b4*. Sunderland, MA: Sinauer Associates.

Telford, M. J. (2001). Embryology and developmental genes as clues to flatworm relationships. In D. T. J. Littlewood & R. A. Bray (Eds) *Interrelationships of the Platyhelminthes* (pp. 257–261). London: Taylor & Francis.

Telford, M. J., Herniou, E. A., Russell, R. B. & Littlewood, D. T. J. (2000). Changes in mitochondrial genetic codes as phylogenetic characters: two examples from the flatworms. *Proceedings of the National Academy of Sciences USA*, *97*, 11359–11364.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL w: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, *22*, 4673–4680.

Tyler, S. & Rieger, R. M. (1977). Ultrastructural evidence for the systematic position of the Nemertodermatida (Turbellaria). *Acta Zoologica Fennica*, *54*, 193–207.

Westblad, E. (1937). Die Turbellarien-Gattung *Nemertodema* Steinböck. *Acta Societatia Pro Fauna et Flora Fennica*, *60*, 45–89.

Willmer, P. (1990). *Invertebrate Relationship Patterns in Animal Evolution*. Cambridge: Cambridge University Press.

Wilson, A. C., Carlson, S. S. & White, T. J. (1977). Biochemical evolution. *Annual Review of Biochemistry*, *46*, 573–639.

Zrzavy, J., Milhulka, S., Kepka, P., Bezdek, A. & Tietz, D. (1998). Phylogeny of the Metazoa based on morphological and 18S ribosomal DNA evidence. *Cladistics*, *14*, 249–285.

Capítol III

A phylogenetic analysis of Myosin Heavy Chain type II sequences corroborates that Acoela and Nemertodermatida are basal bilaterians.

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"Eppur si muove" (Galileo Galilei)

A phylogenetic analysis of myosin heavy chain type II sequences corroborates that Acoela and Nemertodermatida are basal bilaterians

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Bilateria are currently subdivided into three superclades: Deuterostomia, Ecdysozoa, and Lophotrochozoa. Within this new taxonomic frame, acoelomate Platyhelminthes, for a long time held to be basal bilaterians, are now considered spiralian lophotrochozoans. However, recent 18S rDNA [small subunit (SSU)] analyses have shown Platyhelminthes to be polyphyletic with two of its orders, the Acoela and the Nemertodermatida, as the earliest extant bilaterians. To corroborate such position and avoid the criticisms of saturation and long-branch effects thrown on the SSU molecule, we have searched for independent molecular data bearing good phylogenetic information at deep evolutionary nodes. Here we report a phylogenetic analysis of DNA sequences from the myosin heavy chain type II (myosin II) gene from a large set of metazoans, including acoels and nemertodermatids. Our study demonstrates, both for the myosin II data set alone and for a combined SSU + myosin II data set, that Platyhelminthes are polyphyletic and that acoels and nemertodermatids are the extant earliest bilaterians. Hence, the common bilaterian ancestor was not, as currently held, large and complex but small, simple, and likely with direct development. This scenario has far-reaching implications for understanding the evolution of major body plans and for perceptions of the Cambrian evolutionary explosion.

The identification of the most basal extant bilaterian clade is central to the understanding of the transition from diploblast radially symmetrical organisms to the prevalent and more advanced triploblast Bilateria. To explain this transition, morphological and embryological characters have, over a century, formed the backbone of two confronting sets of hypotheses. The first one, the so-called planuloid-acoeloid hypothesis (1–3), contemplates a simple radial planula-like organism evolving into a similarly simple bilaterian (the acoeloid), which later gave rise step by step to more complex bilaterians. Under this scenario, acoelomate organisms (namely the Platyhelminthes) and/or some pseudocoelomate group represented the extant basal bilaterians. The second set of hypotheses, which stems from Haeckel's gastrea and its modern bilaterogastrea (4) and trochaea (5) versions, is best epitomized as the archicoelomate theory (3, 5, 6). This theory posits a large complex (coelomate and segmented) bilaterian ancestor evolved either from planctonic larval gastraea-like or benthic adult cnidarian-like radial forms. An important corollary is that acoelomates and pseudocoelomates are not basal but derived bilaterians.

In the last 15 years, small subunit (SSU) and Hox gene sequences have regrouped bilaterians into three main superclades: the classical Deuterostomia and the new Lophotrochozoa (7) and Ecdysozoa (8) clades, the last two splitting the former Protostomia. Within this new framework, the acoelomate Platyhelminthes and the pseudocoelomate Nematodes are no longer basal but branch within the lophotrochozoans (9, 10) and the ecdysozoans (8), respectively. In addition, this new phylogeny was considered to support a complex coelomate and segmented bilaterian ancestor (11, 12), which, as a consequence, entailed segmentation and coelom formation as homologous characters across all bilaterians. However different, this scheme also suited alternative hypotheses such as the "set-aside cells" theory (13, 14) and the colonial ancestor theory (15).

This new status quo was soon questioned. A SSU-based study using a large set of Platyhelminthes acoels and other metazoans showed acoels to be the extant earliest branching bilaterians (16), turning Platyhelminthes into a polyphyletic group. In addition, Jenner (17) noted that phylogenies put forward to back the new metazoan molecular trees (10–12, 18) were incomplete and heavily pruned. This evidence turned untenable the claimed homologies of coelom, segmentation, and life cycles between groups as different as annelids, arthropods, and chordates, also questioning the "Urbilateria" as a large and complex organism. However, the proposal of acoels as basal bilaterians was in turn also contested. First it was claimed (14, 19) that, despite several tests that were run to avoid long-branch attraction (LBA) effects (20), the branch length of the single acoel species appearing in Ruiz-Trillo *et al.* (16) trees was still long enough to produce those effects. Second, an order of Platyhelminthes, the Nemertodermatida, considered on morphological grounds the sister group of the Acoela forming with them the Acoelomorpha (21, 22), grouped separately from the Acoela with the rest of the Platyhelminthes within the Lophotrochozoa (9, 16). Such an odd position was considered indicative that placement of acoels as basal bilaterians was probably erroneous (14, 23, 24). Finally, a phylogeny based on sequences of the elongation factor-1 α (EF-1α) gene suggested a close relationship between acoels and higher Platyhelminthes (the Order Tricladida) (25). However, Littlewood *et al.* (26) showed Berney *et al.*'s (25) proposal to be an artifact resulting from improper alignment and poor sampling. Recently, new SSU sequences from three Nemertodermatid species have shown them to be not Platyhelminthes but basal bilaterians, branching only second to acoels (27).

To summarize, the claim of acoels and nemertodermatids as basal bilaterians still holds, although it needs to be substantiated on firmer grounds. Large subunit (LSU) rRNA (28) and the order of mitochondrial genes (29, 30) hold promise, although falling short because of insufficient sampling. A more promising way would be increasing the number of sampled genes. Myosins are a large family of mechanochemical proteins whose members are involved in activities as diverse as cytokinesis, muscle contraction, and organelle motility. They are found in animals, plants, and fungi and contain one or two heavy chains and one or more light chains. The heavy chains contain a catalytic head domain, generally N-terminal, followed by a neck domain and a C-terminal tail. The myosin class II is the conventional two-headed filament-forming protein with a coiled-coil tail. Its head

Abbreviations: ML, maximum likelihood; MP, maximum parsimony; QP, quartet puzzling; BI, Bayesian inference; BPP, Bayesian posterior probability; SSU, small subunit; myosin II, myosin heavy chain type II; LBA, long-branch attraction; NJ, neighbor joining.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF486236–AF486264).

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nucleotide sequence, which was previously shown to bear some interphyla phylogenetic information (31), was chosen to test the basal position of Acoelomorpha.

The aim of this study is to further test the position of acoels and nemertodermatids as basal bilaterians and to infer the phylogenetic value of the myosin heavy chain gene (myosin II) as a tool for inferring deep evolutionary relationships. We have amplified and sequenced 750 bp of the myosin II of 29 metazoans (3 cnidarians, 3 acoels, 1 nemertodermatid, and 22 bilaterian species, comprising 10 phyla). They represent, to our knowledge, the first myosin sequences for several metazoan phyla and the first attempt to infer the metazoan phylogeny by using this nuclear coding gene. Phylogenetic analysis includes maximum likelihood (ML) and Bayesian inference (BI).

Materials and Methods

Sample Organisms and Primer Design. A fragment of the coding region of the myosin II gene was obtained from a broad sample of bilaterian species, 14 from GenBank and 29 sequenced for this study (Table 1). GenBank myosin II sequences were aligned by using CLUSTALW Ver. 1.8 (32) and edited by hand in GDE 2.0 (www.tigr.org/~jeisen/) software. Primers (mio3: GGNGTNYTNGAYATHGC, mio4: GGRAANCCYTTNCKRCADAT) were designed to amplify 750 bp of the myosin II head region. This fragment includes a rather conserved region and a relatively variable one, corresponding to nucleotide positions 1588–2343 of the human myosin II (GenBank accession no. D00943). To avoid amplifying problems found in some species, a new pair of more internal and less degenerate primers (mio 6: CCYTCMARYACACCRTTRCA, mio7: TGYATCAAYTWYACYAAYGAG) was designed.

PCR Amplification and Sequencing. All specimens were either directly processed or quickly frozen at −80°C for RNA extraction, except for *Paratomella rubra*, which was amplified from a cDNA library. Total RNA was isolated by using "Total Quick RNA" (Talent) and cDNA obtained by a standard reverse transcription–PCR by using M-MLV Reverse Transcriptase (Promega). Myosin II was amplified by PCR (50 μl, with 1 unit of Dynazyme polymerase of Fynnzimes, 35 cycles of 45″ at 94°C, 45″ at 48°C, and 55″ at 72°C). To purify PCR products, either Microcon PCR or Ultrafree-DA (Millipore) columns were used. Purified products were directly cycle-sequenced from both strands by using ABI Rhodamine or BigDye (Applied Biosystems), precipitated in DyeEx Spin kit (Qiagen, Chatsworth, CA) column, and run on ABI Prism 377 (Applied Biosystems) automated sequencers. Contigs were assembled by using SEQED VER. 1.03 (Applied Biosystems).

Alignment. The sequences obtained were blasted to reassure myosin II identity. They were translated into amino acid, aligned, and edited by using the GDE software package. Amino acid sites of uncertain alignment were excluded from the final matrix, resulting in 175-aa 525-nt positions for 43 taxa. First and second codon positions were used in the analysis (350 bp).

To analyze the effect of combining myosin II with previously published ribosomal sequences, we searched for SSU rRNA sequences of the same taxa and compiled a new data set. Because SSU rRNA sequences for some of the species were not available, some terminals are a compilation of sequences from closely related species (Table 1). Because a complete cephalopodan ribosomal sequence was not available from GenBank, it was excluded from SSU and combined analyses. SSU nucleotide sequences were aligned according to a secondary structure model (33). Positions that could not be unambiguously aligned were excluded from the analysis, leaving 1,179 homologous positions. The combined data set (available at www.bio.ub.es/~martar) raised the number of positions to a total of 1,529.

Table 1. List of species used in this study with GenBank accession nos.

Higher taxon	Species myosin II	Myosin II	SSU
Cnidaria			
Cnidaria-1	*Anemonia sulcata*	AF486236*	X53498
Cnidaria-2	*Bunodactis verrucosa*	AF486237*	AJ133552†
Cnidaria-3	*Podocoryne carnea*	AF486238*	AF358092
Deuterostomia			
Chordata-1	*Mus musculus*	M76598	X00686
Chordata-2	*Rattus norvergicus*	X15938	X01117
Chordata-3	*Homo sapiens*	D00943	X03205
Chordata-4	*Gallus gallus*	J02714	AF173612
Chordata-5	*Cyprinus carpio*	D89990	U87963
Urochordata	*Halocynthia roretzi*	D45163	AB013016
Lophotrochozoa			
Rhabditophora-1	*Schistosoma mansoni*	L01634	U65657
Rhabditophora-2	*Schmidtea mediterranea*	AF14353	U31084
Rhabditophora-3	*Girardia tigrina*	AF486239*	AF013157
Rhabditophora-4	*Discocelis tigrina*	AF486243*	U70078
Rhabditophora-5	*Thysanozoon sp.*	AF486244*	D85096
Mollusca-1	*Argopecten irradians*	X55714	L11265
Mollusca-2	*Pecten maximus*	AF134172	L49053
Mollusca-3	*Loligo pealei*	AF042349	—
Annelida-1	*Nereis sp.*	AF486248*	Z83754
Annelida-2	*Unclassified Oligochaeta*	AF486250*	X79872†
Annelida-3	*Unclassified Erpobdellidae*	AF486251*	AF272842†
Echiura	*Bonellia viridis*	AF486247*	X79875†
Brachiopoda	*Unclassified Brachiopoda*	AF486245*	U12650†
Rotifera	*Brachionus plicatilis*	AF486264*	U49911
Nemertea	*Lineus sp.*	AF486252*	X79878
Sipuncula	*Phascolosoma granulatum*	AF486254*	X79874
Phoronida	*Phoronis hippocrepia*	AF486246*	AF202112
Ecdysozoa			
Nematoda-1	*Caenorhabditis elegans*	X08065	X03680
Nematoda-2	*Onchocerca volvulus*	M74066	AF036638†
Nematoda-3	*Brugia malayi*	M74000	AF227234†
Chelicerata-1	*Opiliones: Phalangiidae sp*	AF486255*	X81441†
Chelicerata-2	*C. citricola*	AF486257*	AF005447†
Chelicerata-3	*Escorpius flavicardius*	AF486258*	AF005442†
Myriapoda-1	*Lithobius sp.*	AF486259*	AF000773
Myriapoda-2	*Scutigera coleoptrata*	AF486260*	AF000772
Hexapoda-1	*Ammophila sp.*	AF486256*	X77785†
Hexapoda-2	*Empusa sp.*	AF486263*	AJ009317†
Hexapoda-3	*Blatta sp.*	AF486261*	AF220573†
Hexapoda-4	*L. saccharina*	AF486262*	X89484
Priapulida	*Priapulus caudatus*	AF486253*	X87984
Acoela			
Acoela-1	*Paratomella rubra*	AF486242*	AF102892
Acoela-2	*C. roscoffensis*	AF486240*	AJ012530
Acoela-3	*C. convoluta*	AF486241*	AJ012524
Nemertodermatida			
Nemertodermatida	*N. westbladi*	AF486249*	AF27726

*Taxa sequenced in this study.
†Indicates those taxa for which SSU and myosin II sequences belong to different species.

Phylogenetic Analysis. Previous to the phylogenetic analysis, several tests were run: (*i*) a relative rate test in RRTREE (34); (*ii*) a homogeneity test; and (*iii*) a ML mapping analysis, both in TREE-PUZZLE 5.0 (35, 36).

MRBAYES (37) was used to estimate the posterior probability of phylogenetic trees with BI. This method has the advantage of being relatively fast and providing probabilistic measures of tree strength that are more directly comparable with traditional statistical measures than those more commonly used in phylogenetic analyses (38, 39). We generated 100,000 phylogenetic trees by using the Monte Carlo Markov chain with four independent simultaneous chains sampling every tenth one. The first 1,000 trees in the sample were removed to avoid including trees sampled before convergence of the Markov chain. We used the

General Time Reversible Model of gene sequence evolution combined with γ rate heterogeneity to estimate the likelihood of each tree. ML trees were inferred by using FASTDNAML (40), with global rearrangements and jumble options and taking into account the γ distribution (previously inferred from the data by TREE-PUZZLE).

Support for the nodes was obtained by several methods: (*i*) Bayesian posterior probability (BPP) performed in MRBAYES; (*ii*) quartet puzzling analysis (QP) performed in TREE-PUZZLE with the γ distribution and 10,000 replicates; (*iii*) maximum parsimony (MP) 1,000 bootstrap replicate by PAUP 4.b8 (41); and (*iv*) neighbor-joining (NJ) bootstrap inferred from Kimura γ distance in MEGA 2.1 (42), 1,000 replicates.

Furthermore, some competing hypotheses based on published reports were evaluated. We performed both a parametric (Kishino–Hasegawa) and a nonparametric (Shimodaira–Hasegawa) test (RELL; 1,000 replicates) under ML assumptions, a parametric (Kishino–Hasegawa), and some nonparametric (Templeton and winning-site) tests under MP assumptions, as implemented in PAUP 4.B8.

Additional ML analyses (FASTDNAML with jumble, global, and γ distribution options with QP analyses for branch support) were performed only for the combined data set: (*i*) without the taxa that did not pass the relative rate test; (*ii*) by removing the most variable positions by excluding positions from categories 8, 7, and 6 of the γ distribution (resulting in 976 positions); (*iii*) without the two fast-evolving acoels (*Convoluta convoluta* and *Convoluta roscoffensis*); (*iv*) with all acoels removed; and (*v*) without the nemertodermatid.

Results

Previous Tests. Three sets of data were used in the analyses: myosin II first and second codon positions, SSU, and a combined SSU + myosin II data set. A relative-rate test was run to determine whether any taxa had a different substitution rate. The myosin II data set gave no significant differences among taxa at the 2% level. The SSU and the combined data sets resulted in several taxa with increased substitution rates (e.g., all nematodes, all rhabditophoran Platyhelminthes, the acoels *C. roscoffensis* and *C. convoluta*, and the arthropods *Lepisma saccharina* and *Cyrthophora citricola*). It is important to note that *P. rubra* (Acoela) and *Nemertoderma westbladi* (Nemertodermatida) both have homogeneous evolutionary rates, compared with the rest of taxa, in myosin II, SSU, or combined data sets. A test was also run to detect any taxa with a nonhomogeneous nucleotide frequency. At a 5% level, all taxa for the three data sets passed the test.

Finally, a ML mapping analysis tested the phylogenetic information of the data. Results showed the three data sets to be highly suitable for phylogenetic reconstruction, increasing from 78.4 or 83.7% of the myosin and SSU alone, respectively, to 90% of completely resolved phylogenies (only 4.5% of quartet trees fell in the completely unresolved star region) in the combined data set.

Phylogenetic Analysis. BI trees are shown in Figs. 1-3 (ML trees are presented in Figs. 4–6, which are published as supporting information on the PNAS web site, www.pnas.org). The three data sets show Bilateria are monophyletic and that Acoelomorpha are sister groups to all of the other bilaterians, being monophyletic in the myosin II and paraphyletic for the SSU and combined data sets. Ecdysozoa, Lophotrochozoa, and Deuterostomia are validated with varied support. Although support for the nodes was calculated by using several methods, our comments for Figs. 1 and 2 will refer only to BPP. However, all supports tend to follow the same pattern, BPP being the highest and QP the lowest (Table 2).

The myosin II BI tree (Fig. 1) supports (61%) the monophyly of the Acoelomorpha and to a higher degree (82%) the monophyly of Bilatera excluding Acoelomorpha. The superclades Ecdysozoa, Deuterostomia, and Lophotrochozoa are also corroborated with varied support. The myosin II ML tree is very similar, the only difference being the more basal positions for Rotifera and Brachiopoda, although these are only weakly supported. The SSU BI tree (Fig. 2) shows, as in ref. 27, a paraphyletic Acoelomorpha, its position being strongly supported (100%). The three bilaterian superclades are also well supported (100% Ecdysozoa, 100% Deuterostomia, 78% Lophotrochozoa). Again, the only difference between the BI and ML trees is the position of Rotifera (sister group to Platyhelminthes in the ML tree; sister group to a clade comprising Annelida, Mollusca, Brachiopoda, Phoronida, Nemertea, and Sipuncula in the BI tree; both positions with low support).

The combined data set BI tree (Fig. 3) presents the highest nodal support. The addition of myosin II remarkably increases the support for the three superclades, compared with both SSU and myosin II trees alone (Table 2). This increase is clearly seen

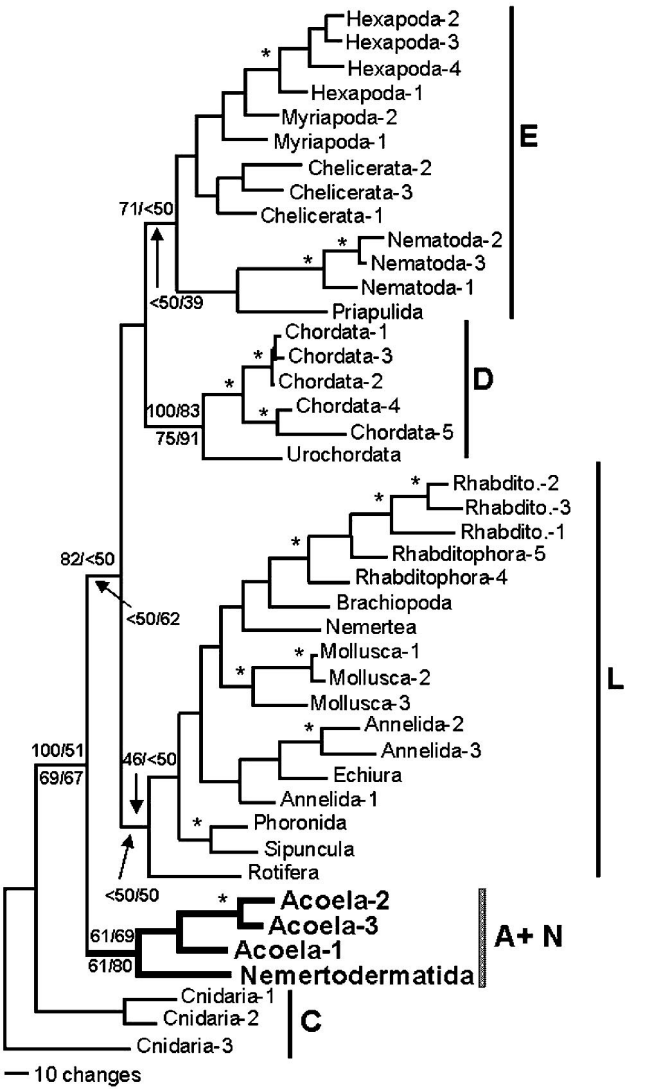

Fig. 1. Phylogeny of bilaterians determined by BI from the myosin II data. Numbers above key nodes refer to the BPP (shown as percentage) and the percentage obtained from a QP 10,000 replicates analysis. Values below branches represent support obtained by MP and NJ 1,000 bootstrap replicates analysis. Asterisks indicate nonkey nodes with more than a 95% BPP. For species names, see Table 1. L, Lophotrochozoa; E, Ecdysozoa; D, Deuterostomia; C, Cnidaria; A, Acoela; N, Nemertodermatida.

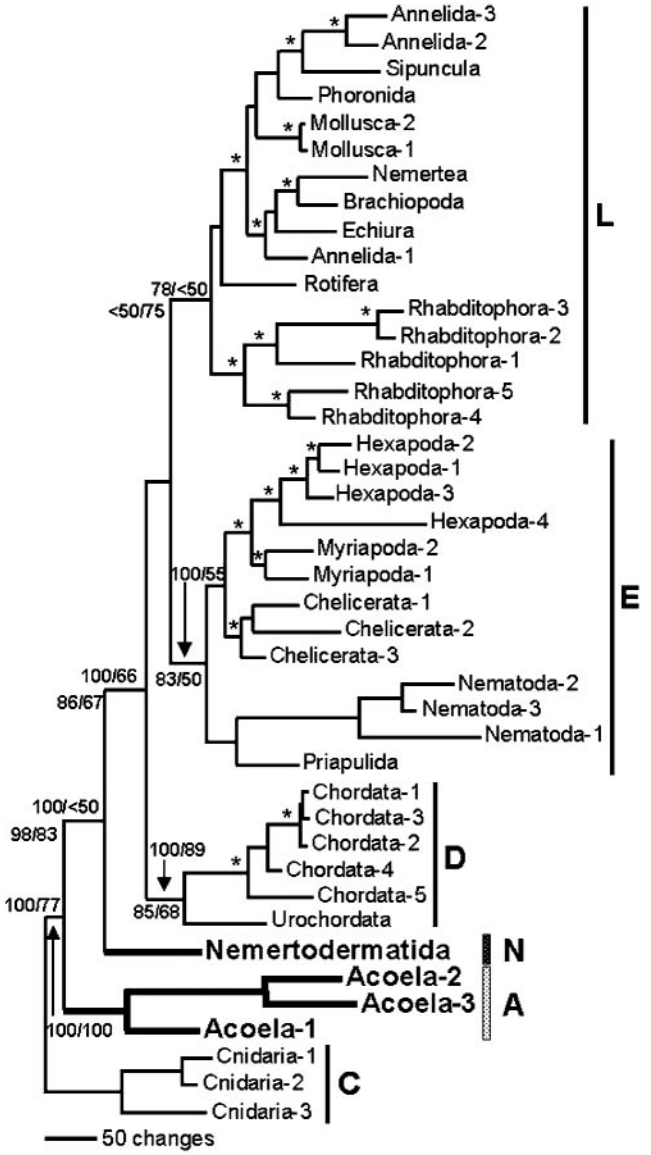

Fig. 2. Phylogeny of bilaterians determined by BI from the SSU data set. For numbers above key nodes, asterisks, abbreviations, and species names, see Fig. 1 legend.

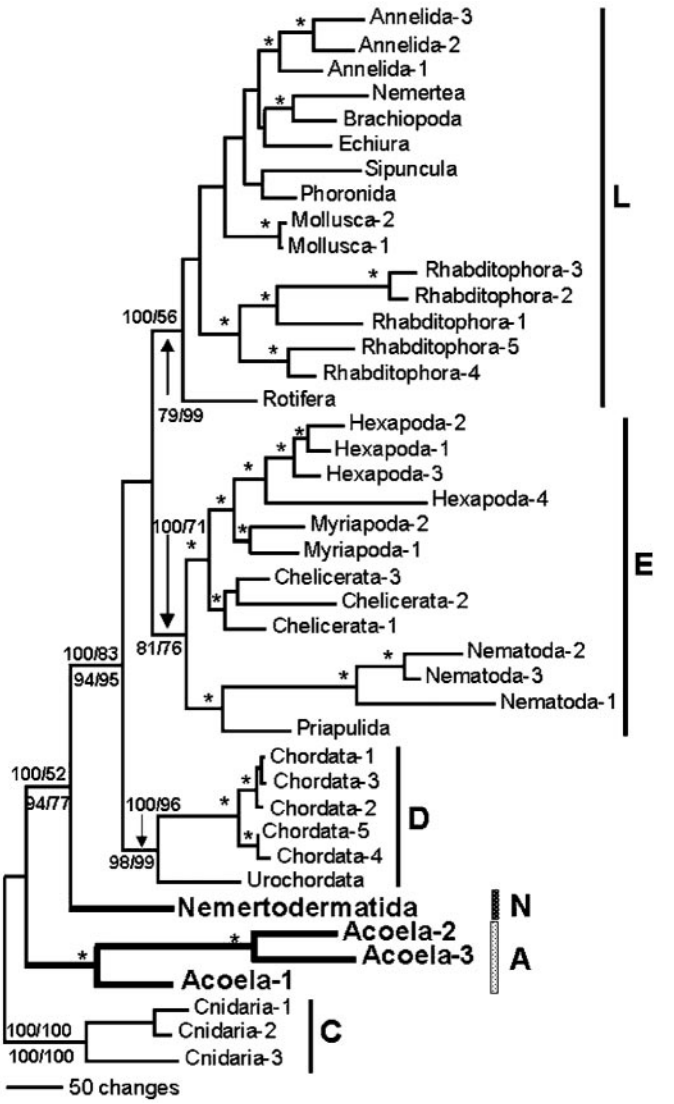

Fig. 3. Phylogeny of bilaterians determined by BI from the combined data set. For numbers above key nodes, asterisks, abbreviations, and species names, see Fig. 1 legend.

with the QP, NJ, and MP methods. QP values supporting the position of Acoelomorpha increase to 83%, Ecdysozoa to 71%, Deuterostomia to 96%, and the Lophotrochozoa to 56%. As in previous data sets, the only differences among the ML and BI trees are within the Lophotrochozoa, Rotifera being either basal lophotorochozoans in the BI tree or sister group to the Platyhelminthes in the ML tree.

To avoid LBA effects, an additional ML analysis was run for the combined data set without fast-clock taxa. The three superclades and the position of Acoelomorpha as sister group to the rest of the Bilateria are recovered with even higher supports, 93% for the position of Acoelomorpha compared with 83% of

Table 2. Comparison of the level of support obtained for the three data sets with different methods

	Myosin II				SSU				Combined			
	BPP	QP	MP	NJ	BPP	QP	MP	NJ	BPP	QP	MP	NJ
Acoelomorpha paraphyletic					100	<50	98	83	100	52	94	77
Acoelomorpha monophyletic	61	69	61	80								
Basal Acoelomorpha	82	<50	<50	62	100	66	86	67	100	83*	94*	95*
Ecdysozoa	71	<50	<50	39	100	55	83	50	100	71*	81	76*
Deuterostomia	100	83	75	91	100	89	85	68	100	96*	98*	99*
Lophotrochozoa	46	<50	<50	50	78	<50	<50	75	100*	56*	79*	99*

Supports obtained from: BPP, QP 10,000 replicate analysis; MP, 1,000 bootstrap replicates, and NJ, 1,000 bootstrap replicates for the three data sets. Asterisks in the combined values indicate those that have increased compared to both SSU and myosin II data sets.

EVOLUTION

Table 3. Results from the ML and MP analyses of competing hypotheses from myosin heavy chain, SSU, and combined data sets

	Myosin II				SSU				Combined			
	ML		Parsimony		ML		Parsimony		ML		Parsimony	
Topologies	KH	SH	KH	W-T	KH	SH	KH	W-T	KH	SH	KH	W-T
Monophyletic Acoelomorpha as basal bilaterians	Best	Best	Best	Best	*	†	*	*	†	†	†	†
Paraphyletic Acoelomorpha as basal bilaterians	†	†	†	†	Best	Best	Best	Best	Best	Best	Best	Best
Acoelomorpha as rhabditophoran triclads	*	*	*	*	*	*	*	*	*	*	*	*
Acoela basal: Nemertodermatida as rhabditophoran triclads	*	*	*	*	*	*	*	*	*	*	*	*
Nemertodermatida basal: Acoela as rhabditophoran triclads	*	*	*	*	*	*	*	*	*	*	*	*
Acoelomorpha as basal Platyhelminthes	*	*	*	*	*	*	*	*	*	*	*	*
Acoela basal: Nemertodermatida as basal Platyhelminthes	*	*	*	*	*	†	*	*	*	*	*	*
Nemertodermatida basal: Acoela as basal Platyhelminthes	*	*	*	*	*	*	*	*	*	*	*	*

Tests include: Kishino–Hasegawa (KH) and Shimodaira–Hasegawa (SH) under ML assumptions and KH and winning site and Templeton (W-T) under MP assumptions. * indicates those hypotheses that are statistically rejected (at a 5% level). † shows those not rejected (at a 5% level).

QP with all taxa included (see Fig. 7, which is published as supporting information on the PNAS web site). Because highly variable positions may, as shown in other groups (43–45), be responsible for an artifactual basal position for the Acoela and Nemertodermatida, the most variable positions were excluded, and this reduced data set was used to infer a ML tree. Although the internal relationships within the Bilateria came up unresolved, Acoela and Nemertodermatida remain sister group to the rest of the bilaterians (data not shown). Further, we tested the effect of eliminating different acoelomorpha taxa. ML trees without the Acoela, leaves Nemertodermatida at the same basal position, with a 93% QP support, and the topology of the rest of the tree unchanged. If Nemertodermatida is removed, the Acoela holds its position, with a QP value of 86%. Finally, if only the two fast-clock acoelomorpha taxa (*C. roscoffensis* and *C. convoluta*) are excluded, the nonfast clock Acoelomorpha, *P. rubra*, and *N. westbladi*, remain basal, with an 87% QP. Importantly, fast-evolving taxa such as Nematoda do not shift to a basal position, still branching within the Ecdysozoa (data not shown).

Finally, all three data sets reject at a 5% level all competing hypotheses both in ML and MP comparisons. The monophyly or paraphyly of Acoelomorpha, with the myosin II and combined data sets, cannot be statistically discerned (Table 3).

Discussion

Our analyses clearly indicate that acoels and nemertodermatids are not members of the phylum Platyhelminthes but most likely represent the earliest branch of the extant bilaterians. These results strongly reinforce the reports based on SSU sequences suggesting acoels (16) and nemertodermatids (27) to be basal bilaterians. In turn, they contradict claims based on SSU and Hox gene sequences (11, 12, 14, 23) and comparative developmental gene expression (13, 18, 46–49), arguing for a large and complex organism (the so-called "Urbilateria") as the bilaterian ancestor.

The Myosin II Gene: A New and Powerful Phylogenetic Tool. In this paper, we present myosin II gene sequences as a new molecular tool for phylogenetic inference. After analyzing a large number of metazoan representatives, we found this new source of data, totally independent of SSU, reproduces the same overall metazoan tree as that previously obtained by SSU and Hox sequences; namely, the monophyly of the three superclades is again corroborated, Lophotrochozoa with the weakest support, and the relationships among them still undecided.

More importantly, when myosin II sequences are added to and analyzed together with SSU sequences, the support values for all deep nodes increase remarkably compared with both SSU and myosin II data sets alone (Table 2). This increase indicates that myosin II sequences bear a strong phylogenetic signal and strengthens the idea that adding more nucleotide data, especially from an independent origin, is a good procedure to increase the resolution of molecular trees. However, candidate molecules have to be carefully checked to avoid unnecessary noise (as a case study, see the EF-1α introduced in ref. 25 and its refutation in ref. 26). This does not seem to be the case for myosin II. In fact, we deem this molecule an ideal candidate to infer robust metazoan phylogenies because of: (*i*) its homogeneous rate of evolution for all species studied; (*ii*) its homogeneity in nucleotide frequency; (*iii*) the considerable amount of phylogenetic information shown by ML mapping analysis; (*iv*) its topological congruence with SSU rRNA data; (*v*) its stability to taxon sampling; and (*vi*) the outstanding increase in support values at the deep nodes when used together with SSU sequences.

The Position of Acoela and Nemertodermatida in the Bilaterian Tree. The myosin II gene data presented here further reinforce Acoela and Nemertodermatida as basal bilaterians, not members of the Platyhelminthes (82% BI; Fig. 1). This position is further strengthened by the fact that a large set of bilaterian clades is included, and that both BI and ML trees have identical topology. Because acoels were first considered basal bilaterians (16), several authors considered this position to be artifactual (11, 12, 14, 24) because of LBA effects (see Introduction). Such effects, however, cannot be pointed out as a putative misleading problem for myosin II sequences. They all show a similar evolutionary rate, and all ML and BI analyses have been performed taking into account, as for SSU sequences, among-site rate variation implemented by a γ distribution. In addition, the topology retrieved for the rest of the tree is similar to those obtained for other molecules [SSU, ref. 16; SSU + large subunit (LSU), ref. 50], evidence that the phylogenetic content of myosin II sequences is coherent along the tree.

Furthemore, competing hypotheses regarding the position of the Acoelomorpha were statistically (parametrically and nonparametrically) rejected under both ML and MP assumptions (Table 3). It is important to note that alternative topologies tested include Acoelomorpha as basal Platyhelminthes (as in ref. 51) or as member of the Rhabditophora (as in refs. 19 and 25). When SSU and myosin II sequences were combined and analyzed together, the basal position of acoels and nemertodermatids is highly reinforced (Fig. 3, Table 2), and the competing topologies also statistically refuted under both MP and ML assumptions (Table 3). However, the combined data set contained some fast-clock organism whose presence could mislead phylogenetic inferences. Additional ML analyses, removing either all fast-clock taxa or the most variable positions or deleting either the acoels or the nemertodermatids, performed to avoid any LBA effect showed that it is not the case. It is also important to note that fast-clock nematodes always grouped, in both SSU and combined data analyses, within the ecdysozoan clade. Were LBA affecting the analyses, they must have

shifted to a basal position. This fact adds credibility to our claim that this data set is not affected by LBA effects.

Despite the evidence listed above supporting the basal positioning of both acoels and nemertodermatids, the clade Acoelomorpha (Acoela + Nemertodermatida; ref. 21) is not always retrieved in our analyses. Whereas myosin II sequences support their monophyly (Fig. 1), SSU and the combined data set show them to be paraphyletic (Figs. 2 and 3). Comparison of the two alternative topologies with the three data sets shows neither topology to be significantly better than the other, except for the SSU data set (Table 3). Therefore, further data are needed to assess whether Acoelomorpha is monophyletic, as several morphological synapomorphies attest (21), or whether it is a paraphyletic assemblage.

Evolutionary Implications. Our results have deep implications for bilaterian phylogeny and taxonomy. First, they imply that the last common ancestor was small, benthic, without segments and coelomic cavities, and likely lacked a planktonic larval stage. This scenario is substantially different from the prevalent view of the bilaterian ancestor (the Urbilateria) as a rather complex organism (see Introduction for main references), calling for critical assessment of the evidence brought forward to back it up. Second, it argues for a period before the Cambrian within which the stem groups of the three bilaterian superclades originated from acoel-like ancestors, present-day acoels and nemertodermatids (albeit modified) being descendants of those ancestors. Third, because acoels and nemertodermatids branch before the rest of the bilaterians, Bilateria could be divided in two inclusive groups: a broad Bilateria including acoels and nemertodermatids and a more derived Bilateria (provisionally named Nephrozoa in ref. 27, or Eubilateria), excluding these two clades. Synapomorphies for all bilaterians would be two orthogonal body axes, anterior nervous system, and endomesoderm, whereas the more derived eubilaterians could be defined by the presence of an excretory system, one way through gut, and further development of the nervous system (e.g., a true brain with neuropil). Finally, the planula-like features of acoels and nemertodermatids again draw attention to the cnidarian planula larva as a model for the precursor of the Bilateria. First proposed as ancestral to all Metazoans (52) or to the Cnidaria and Bilateria (2), the planula larva seems now best suited to give rise to an acoel-like organism by progenesis (attainment of sexual maturity in larval forms). This fact will direct attention to the developmental and genetic events and processes required for such transition and, more specifically, to compare expression of any of the anteroposterior, dorsoventral, mesodermal, and neural marker genes in planula larvae and present-day acoels and nemertodermatids.

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1. Hyman, L. H. (1951) *The Invertebrates* (McGraw–Hill, New York), Vol. II.
2. von Salvini-Plawen, L. V. (1978) *Z. Zool. Syst. Evolutionsforsch.* **16,** 40–88.
3. Willmer, P. (1990) *Invertebrate Relationships* (Cambridge Univ. Press, Cambridge, U.K.).
4. Jägersten, G. (1955) *Zool. Bidr. Uppsala* **30,** 321–354.
5. Nielsen, C. (1995) *Animal Evolution: Interrelationships of the Living Phyla* (Oxford Univ. Press, Oxford).
6. Remane, A. (1963) in *The Lower Metazoa*, ed. Dougherty, E. C. (Univ. of California Press, Berkeley), pp. 247–255.
7. Halanych, K. M., Bacheller, J. D., Aguinaldo, A. M., Liva, S. M., Hillis, D. M. & Lake, J. A. (1995) *Science* **267,** 1641–1643.
8. Aguinaldo, A. M. A., Turbeville, J. M., Lindford, L. S., Rivera, M. C., Garey, J. R., Raff, R. A. & Lake, J. A. (1997) *Nature (London)* **387,** 4453–4456.
9. Carranza, S., Baguñà, J. & Riutort, M. (1997) *Mol. Biol. Evol.* **14,** 485–497.
10. Balavoine, G. (1998) *Am. Zool.* **38,** 843–858.
11. Adoutte, A., Balavoine, G., Lartillot, N & de Rosa, R. (1999) *Trends Genet.* **15,** 104–108.
12. Adoutte, A., Balavoine, G., Lartillot, N., Lespinet, O., Prud'homme, B. & de Rosa, R. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 4453–4456.
13. Davidson, E. H., Peterson, K. J. & Cameron, R. A. (1995) *Science* **270,** 1319–1325.
14. Peterson, K. J. & Eernisse, D. J. (2001) *Evol. Dev.* **3,** 170–205.
15. Dewel, R. A. (2000) *J. Morphol.* **243,** 35–74.
16. Ruiz-Trillo, I., Riutort, M., Littlewood, D. T. J., Herniou, E. A. & Baguñà, J. (1999) *Science* **283,** 1919–1923.
17. Jenner, R. A. (2000) *Evol. Dev.* **2,** 208–221.
18. Cameron, R. A., Peterson, K. J. & Davidson, E. H. (1998) *Am. Zool.* **38,** 609–620.
19. Giribet, G., Distel, D. L., Polz, M., Sterrer, W. & Wheeler, W. (2000) *Syst. Biol.* **49,** 539–562.
20. Felsenstein, J. (1978) *Syst. Zool.* **27,** 401–410.
21. Ehlers, U. (1985) *Das Phylogenetische System der Plathelminthes* (Fischer, Stuttgart).
22. Smith, J. P. S., Tyler, S. & Rieger, R. M. (1986) *Hydrobiologia* **132,** 71–78.
23. Peterson, K. J. & Davidson, E. H. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 4430–4433.
24. Telford M. J. (2001) in *Interrelationships of the Platyhelminthes*, eds. Littlewood, D. T. J. & Bray, R. A. (Taylor and Francis, London), pp. 257–261.
25. Berney, C., Pawlowski, J. & Zaninetti, L. (2000) *Mol. Biol. Evol.* **17,** 1032–1039.
26. Littlewood, D. T. J., Olson, P. D., Telford, M. J., Herniou, E. A. & Riutort, M. (2001) *Mol. Biol. Evol.* **18,** 437–442.
27. Jondelius, U., Ruiz-Trillo, I., Baguñà, J. & Riutort, M. (2002) *Zool. Scripta* **31,** 201–215.
28. Medina, M., Collins, A. G., Silberman, J. D. & Sogin, M. L. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 9707–9712.
29. Boore, J. L. & Brown, W. M. (1998) *Curr. Opin. Genet. Dev.* **8,** 668–674.
30. Boore, J. L. (1999) *Nucleic Acids Res.* **27,** 1767–1780.
31. Korn, E. D. (2000) *Proc. Natl. Acad. Sci. USA.* **97,** 12559–12564.
32. Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680.
33. Gutell, R. R., Weibser, B., Woese, C. R. & Noller, H. F. (1985) *Prog. Nucleic Acid Res. Mol. Biol.* **32,** 155–216.
34. Robinson-Rechavi, M. & Huchon, D. (2000) *Bioinformatics* **16,** 296–297.
35. Strimmer, K. & von Haeseler, A. (1996) *Mol. Biol. Evol.* **13,** 964–969.
36. Strimmer, K. & von Haeseler, A. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 6815–6819.
37. Huelsenbeck, J. P. & Ronquist, F. R. (2002) *Biometrics*, in press.
38. Huelsenbeck, J. P. & Bollback, J. P. (2001) in *Handbook of Statistical Genetics*, ed. Bishop, M. (Wiley, London), pp. 415–439.
39. Huelsenbeck, J. P., Ronquist, R., Nielsen, R. & Bollback, J. P. (2001) *Science* **294,** 2310–2314.
40. Olsen, G. J., Matsuda, H., Hagstrom, R. & Overbeek, R. (1994) *Comput. Appl. Biosci.* **10,** 41–48.
41. Swofford, D. L. (2000) PAUP*, Phylogenetic Analysis Using Parsimony (*and Other Methods) (Sinauer, Sunderland, MA).
42. Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001) *Bioinformatics* **17,** 1244–1245.
43. Brinkmann, H. & Philippe, H. (1999) *Mol. Biol. Evol.* **16,** 817–825.
44. Philippe, H., Germot, A. & Moreira, D. (2000) *Curr. Opin. Genet. Dev.* **10,** 596–601.
45. Philippe, H., Lopez, P., Brinkmann, H., Budin, K., Germot, A., Laurent, J., Moreira, D., Müller, M. & Le Guyader, H. (2000) *Proc. R. Soc. Lond. Ser. B* **267,** 1213–1221.
46. De Robertis, E. M. & Sasai, Y. (1996) *Nature (London)* **380,** 37–40.
47. De Robertis, E. M. (1997) *Nature (London)* **387,** 25–26.
48. Holland, L. Z. (2000) *Curr. Opin. Genet. Dev.* **10,** 434–442.
49. Arendt, D., Technau, U. & Wittbrodt, J. (2001) *Nature (London)* **409,** 81–85.
50. Mallatt, J. & Winchell, C. J. (2002) *Mol. Biol. Evol.* **19,** 289–301.
51. Tyler, S. (2001) in *Interrelationships of the Platyhelminthes*, eds. Littlewood, D. T. J. & Bray, R. A. (Taylor and Francis, London), pp. 3–12.
52. Hyman, L. H. (1940) *The Invertebrates* (McGraw–Hill, New York), Vol. I, pp. 1–726.

Capítol IV

First mitochondrial genome data of an acoel, and *a* nemertodermatid. Gene content and phylogenetic inference.

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In preparation

"In any case, zoologist must persevere with embryological and morphological studies of invertebrates, as these will always be important for the evaluation of relationships" (Libbie Henrietta Hyman)

Mitochondrial genome data of an acoel and a nemertodermatid. Gene content and phylogenetic inference.

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Abstract

The acoelomorphs have been recently shown, based on SSU and myosin molecular data, to be the most basal extant bilaterian clade. We determined 9.6 and 5.4 kb respectively of the mitochondrial genome of the acoel *Paratomella rubra* and the nemertodermatid *Nemertoderma westbladi*. The identified gene arrangements are unique among metazoans. Both taxa, however, share the position of the ribosomal genes and the acoel presents a unique inversion of the conserved portion *cox2-atp8-atp6*. We discuss the data obtained framed within the eubilaterian radiation and the phylogenetic value of the mitochondrial gene rearrangements at deep evolutionary levels.

Introduction

Complete mitochondrial genome sequences are available for over 50 invertebrate animals (Boore 1999; from Genomics Diversity link at http://www.jgi.doe.gov). Nearly all animal mitochondrial DNAs (mtDNAs) are circular molecules, about 16 kb in size, encoding genes for 2 ribosomal subunits RNAs (*srRNA* and *lrRNA*), 22 tRNAs (abbreviated *trnX*, where "X" is the one-letter code for the corresponding amino acid) and 13 proteins subunits (cytochrome oxidase subunits I-III [*cox1-cox3*], cytochrome b [*cob*], NADH dehydrogenase subunits 1-6 and 4L [*nad1-nad6* and *nad4L*], and ATP synthase subunits 6 and 8 [*atp6* and *atp8*]. Among triploblasts animals only the gene a*tp8* has been, independently, lost from this repertoire in some linages. This has been the case in nematodes (Okimoto et al. 1991, 1992; Keddie, Higazi, and Unnasch 1998), parasitic platyhelminthes (Le et al. 2000) and the bivalve mollusk *Mytilus edulis* (Hoffman, Boore and Brown 1992).

Although metazoan mitochondrial sequences are known to evolve rapidly, their gene arrangements often remain unchanged over long periods of evolutionary time. Comparisons of mitochondrial gene arrangements have been very effective for reconstructing some evolutionary relationships (Smith et al. 1993, Boore et al. 1995, Boore and Brown 1998, Boore, Lavrov and Brown, 1998). However, since the rate of rearrangements varies among lineages, being for example quite conserved in vertebrates and quite variable among the mollusks, the gene order characters may provide phylogenetic signal at different taxonomic levels. Only in slow evolving lineages the more ancient signal may be retained. Furthermore, mitochondrial genome data also yield information about genetic code, whose differences have also been used as a phylogenetic character (Telford et al. 2000), tRNAs structure, gene loss, and ribosomal and proteinic sequences. Comparison of mitochondrial protein sequences have previously succesfully been used to infer ancient evolutionary relationships (Boore and Staton 2002).

The acoelomorphs Acoela and Nemertodermatida, previously considered Platyhelminthes, have recently been shown, based on SSU, myosin II, Hox gene and LSU sequences, to be the two most basal extant groups of Bilateria, rendering the Platyhelminthes a polyphyletic assemblage (Ruiz-Trillo et al. 1999, Jondelius et al. 2002, Ruiz-Trillo et al. 2002, Cooke, personal communication, Telford, personal communication). However, while SSU sequence data showed both groups (Acoela and Nemertodermatida) to be paraphyletic, myosin II sequence data showed them as

sister-groups, supporting the clade Acoelomorpha (Ehlers, 1985; Smith et al, 1986). The bulk of the Platyhelminthes, the Rhabditophora, are associated with the lophotrochozoan taxa (Carranza et al. 1997, Adoutte et al. 2000, Ruiz-Trillo et al. 1999 and 2002, von Nickisch-Rosenegk et al. 2001), but, whether they are derived or basal lophotrochozoans is still uncertain. The rest of the bilaterian taxa excluding the acoelomorphs, that is the Eubilateria or Nephrozoa, is divided into three superclades: Deuterostomia, Lophotrochozoa and Ecdysozoa (Jondelius et al. 2002, Ruiz-Trillo et al. 2002).

To date, only a few mitochondrial genomes data from diploblast metazoa has been published. Those of cnidarians (Beaton et al. 1998, Beagley et al. 1998, Pont-Kingdon et al. 2000) have lost all but two mitochondrially encoded tRNAs, and have a prokaryote-like rrnS structure. The mitochondrial genome of the poriferan *Tetilla*, partially sequenced by Watkins & Beckenbach (1999), show some similar features with that of cnidarians, but its genome arrangement, a part of it common in a wide range of bilaterian mitochondrial genomes, and its genetic code are believed to be primitive among the triploblats. However, the characterization of mitochondrial genomes from basal bilaterians, would be valuable in elucidating the process whereby the present form of triploblats mitochondrial genomes were generated.

In this study, we describe the partial mitochondrial genomes of the acoel *Paratomella rubra*, the nemertodermatid *Nemertoderma westbladi*, the two most basal extant bilaterian taxa. They are, to our knowledge, the first representatives of those groups to be examined. The aim of this study is to generate mitochondrial genome data from basal bilaterians in order to 1) better understand the process of metazoan mitochondrial genome evolution, especially the eubilaterian radiation, 2) further test the basal position of acoels and nemertodermatids within the Bilateria, 3) dilucidate whether the clade Acoelomorpha is validated, and 4) test the phylogentic value of mitochondrial gene rearrangements and mitochondrial sequences for deep evolutionary events. Thus, we analyze genomic feature and compare the inferred nucleotide sequences with other animal mitochondrial genomes.

Material and Methods

Molecular analysis

Live specimens of *Paratomella rubra* and *Nemertoderma westbladii* were obtained from Sitges (Spain) and Kristineberg (Sweden) respectively. We isolated

total DNA using Qiagen DNA extraction kit. Initially, we used universal primers in order to amplify short fragments of the genes *cox1* (primers LCO1490 and HCO2198; Folmer et al. 1994), *cox3* (primers COIIIF and COIIIB; Boore and Brown 2000), *cob* (primers Cytb424-444: GGW TAY GTW YTW CCW TGR GGW CAR AT and Cytb876-847: GCR TAW GCR AAW ARR AAR TAY CAY TCW GG) and *rrnL* (primers 16ARL and 16SBRH; Palumbi 1996). We used standard PCR protocol (50 μl, with a 1u of Dynazyme polymerase of Finnzimes, 35 cycles of 20" at 94ºC, 45" at 48ºC and 45" at 72ºC).

The products of those which amplified the expected band (all in *Paratomella rubra*, *cox1* and *rrnL* in *Nemertoderma westbladi*) were purified using Microcon® PCR columns. Purified products were cycle-sequenced directly from both strands using BigDye™ chemistry, precipitated in DyeEx Spin Kit (Qiagen) column, and run on ABI Prism 373 or 377 automated sequencers. Contigs were assembled using SeqEd v1.03. DNA sequences obtained from these fragments were used to design long and species specific oligonucleotides that face out from the fragments. These primers were then employed in "long-PCR" (Barnes 1994) in all possible combinations with Advantage polymerase kit (Clontech). Reactions conditions were as follows:

This generated single fragments of aproximately 3,7 kb., 6 kb. and 9,7 kb. (ranging from *cox1* to *rrnL, rrnL* to *cob,* and *cox1* to *cob* gens, respectively) in *P. rubra*; and approximately 5,3 kb. (ranging from *cox1* and *rrnL* genes) in *N. westbladi*.

Products were purified and cloned using TOPO®XL PCR Cloning kit (Invitrogen). Cloned fragments were purified by a miniprep extraction (Roche), and sequenced as above, with additional primers used to "primer walk" trough both strands of each fragment. Sequences were assembled using Seqman program of the DNAstar software package.

Gene annotation

The protein-encoding genes and the two ribosomal RNA genes were easily identified by Blastx and by comparisons with other published sequences. tRNA were identified either by using tRNAscan-SE (version 1.1, http://www.genetics.wustl.edu/eddy/tRNAscan-SE, Lowe and Eddy 1997) or, where tRNAs were not found using this program, by recognizing potential secondary structures by eye. The 5' ends of protein genes were inferred to be at the first legitimate in-frame start codon (ATN, GTG, TTG, GTT), even if overlapping 3-4 nucleotides with the preceding gene. Protein gene termini were inferred to be at the first in-frame stop

codon unless that codon was located within the sequence of a downstream gene. Otherwise, a truncated stop codon (T) adjacent to the beggining of the next gene was designated as the termination codon and was assumed to be completed by polyadenylation after transcript cleavage (Ojala, Montoya, and Attardi 1981). The 5' and 3' ends of both *rrnL* and *rrnS* genes were assumed to be adjacent to the ends of bordering tRNA genes.

Phylogenetic Analysis

The invertebrate mitochondrial genetic code was used to infer the amino acid sequence of the protein-encoding genes. Amino-acid and nucleotide sequences were aligned first by clustalX as implemented in BioEdit 5.0.6 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), and then revised by eye on GDE2.0 sequence editor (Smith et al. 1994). The genetic code was tested, by eye, on clear conservative positions through all protein-encoding genes.

An alignment of seven protein-encoding genes (*atp6*, *cob*, *cox1*, *cox2*, *cox3, nd1* and *nd6*) was constructed using other published mitochondrial genomes (table 1). Non-clear homologus amino-acid positions were excluded of the alignment, resulting in a final matrix of 1198 amino acid positions. A relative-rate test (RRT) was run in RRTree (M. Robinson-Rechavi and D. Huchon, 2000). A neighbor-joining tree with 1000 bootstrap replicates was constructed using the gamma model as implmented in Mega2.1 software package (Kumar et al. 2001), without the taxa with faster evolutionary rates (except for *Nemertoderma westbadi*).

Results and discussion

Gene content and organization

The 9.795-nt portion of *P. rubra* contains 20 genes: 9 protein-encoding genes (*cox1* and *cob* are incompletes at the 5' and 3' ends, respectively), 9 tRNA genes, and 2 rRNA genes. The 5.243-nt portion of *N. westbladi* contains 14 genes: 4 protein-encoding genes (*cox1* is incomplete at the 3' end), 8 tRNA genes, and 2 rRNA genes (rrnL is incomplete at 5' end). All of the genes identified are found in the same transcriptional orientation, except for *trnL* of *N. westbladi* (figure 1).

Both taxa have a compact mitochondrial genome organization, especially the acoel, in which most genes either abut directly or have some overlapping nucleotides (usually one). In *N. westbladi*, most of the genes are separated by a small number of

noncoding nucleotides (ranging from 2 to 10), usually As and Ts. There are 3 start codons used in the *P. rubra* proteins genes: ATT in 6 cases, GTT in *atp8* and ATA in *cob*. ATG, ATT and GTT are the start codons used in *N. westbladi* protein genes. All protein genes in both taxa, except for *P. rubra atp8*, end on a complete stop codon, either TAA or TAG. *P. rubra atp8* end on an abbreviated stop codon (TA) that is presumably completed by polyadenylation of the mRNA (Ojala et al. 1981).

Paratomella rubra have compact protein coding genes, especially *atp6,* and *nad6*, which are much shorter that the average, both at 5' and 3' ends. However it has, as well as cnidarians, a longer 5' end in the gene *nad1*. Interestingly, its *cox2* sequence shows some similarities with that of the diploblasts. It does not share the longer 5' ends of the diploblasts, but it shares an insertion with cnidarians and fungi (the lofoforates have also that insertion) and a unique Leucine insertion with the poriferan *Tetilla*.

Both taxa bear a gene arrangement unknown in any other metazoan mtDNA (figure 1). Interestingly the arrangement *cox2-atp8-atp6*, considered primitive and quite extensive among metazoans, is found inverted in the acoel, which is unique among metazoans mitochondrial genomes. If we assume the poriferan arrangement (*rrnL-cox2-atp8-atp6*) to be the ancestral diploblast condition, it seems clear acoel lineage suffered an inversion of the conserved *cox2-atp8-atp6* portion (figure 2). That might not be difficult at all, due to the fact that in the octocoralian cnidarians (Beaton et al. 1998), and contrary to the more basal hydrozoans, this portion migrated to the light strand (figure 2). The fact that *atp6* directly follows *atp8* in many mtDNAs has been suggested to be prof that both genes are cotranslated from a single bicistron (Boore & Staton, 2002). However, these genes have also been found separated in several animal lineages, such as Annelida, Mollusca, Platyhelminthes, Nematoda, Tardigrada, Onychofora and Sipuncula, arguing against a constrain about its loss of cotranslation. The two ribosomal genes (*rrnS* and *rrnL*) are also found separated, which is uncommon among metazoan mtDNAs. Interestingly, in both cases, *rrnL* is, separated by a tRNA, at 5' of the gene *atp6* and *rrnS* is, separated by one or more tRNAs, at 5' of the gene *cox3.* This might be a synapomorphy of both taxa, arguing for a sister-group relationship among them, forming the clade Acoelomorpha. Furthermore, the arrangement *rrnS-V-rrnL* is conserved in a variety of animal lineages, such as Vetebrata, Hemichordata, Brachiopoda, Arthropoda and Annelida. Since this arrangament is lacking not only in cnidarian, but also in Acoela and Nemertodermatida, its formation probably occurred after the diversification of the

acoelomorps, and before the eubilaterians diversified into the three extant superclades.

The fact that both taxa, acoels and nemertodermatides, do not share any gene boundaries between them argues for an extended period since both diversificated, or for a rapidly changing lineage, in which there is not a conserved "canonical" gene arrangements. Information of mt genome data from additional acoels and nemertodermatid species might help resolve this issue.

Base composition and codon usage

Overall, both the 9.795 nt and the 5.243 nt determined for *P. rubra* and *N. westbladi* mtDNA respectively are very A+T rich (76.4% and 64.6%), especially the acoel, even for an animal mitochondrial genome (table 2). The nucleotide composition of the reported strand is 28.9% A, 47.5% T, 15.6% G, and 8% C for *P. rubra* and 27.1% A, 37.5% T, 21.6% G and 13.8% C for *Nemertoderma westbladi*. Thus, the reported strand is very rich in T and G, showing that the distribution of the purines versus the pyrimidines between the two strands for each TA and GC pair is highly asymmetric (again especially in the acoel). This asymmetry can be described by skewness (Perna and Kocher, 1995), which measures the relative number of A's to T's by AT-skew ([A-T]/[A+T]) and G's to C's by GC-skew ([G-C]/[G+C]). According to these formulae, skew values are in the range –1 to +1 and compositional asymmetry is greater the closer the skew values (positive or negative), and lower the closer skew values approach zero. AT and GC-skew values are respectively -0.24 and 0.32 for the acoel and –0.16 and 0.22 for the nemertodermatid. As expected, GC-skew values are higher for tRNAs, ribosomal and noncoding nucleotides, in which, in theory, are more free to change and reflect a higher mutational bias, and lower for protein-encoding nucleotides. However AT-skew values are lower than expected for ribosomal, tRNAs and noncoding nucleotides (table 1). A possible explanation is that among ribosomal and tRNAs a similar number of A's and T's is required for stem structure formation, while this is not so obvious on GC-skew due to the lack of appreciable helix-destabilization by GT pairs. Similarly in noncoding regions, the formation of stems might also be crucial in functionally important paired secondary structures, accounting for the relatively low AT-skew.

A higher pyrimidine composition is found in almost all invertebrates mtDNAs and can be assumed to be the ancestral triploblast condition. Vertebrates (Reyes et al. 1998), some ecdysozoans (as *Limulus polyphemus*, Lavrov et al. 2000) and some

lophotrochozoans (as *Terebratulina retusa*, Stechmann & Schlegel, 1999) have convergently gained a richer purine composition, probably due to similar requirements of the mitochondrial replication process (Saconne et al. 2002).

Transfer RNAs

There are 9 sequences in *P. rubra*, 8 in *N. westbladi*, identified with the potential for folding into tRNA-like structures (figure 3 and 4). Each has a seven-member amino-acyl acceptor stem (3 in the acoel with one mistmatch and one in the nemertodermatid with 3 mistmatches) and a five-member anticodon stem (3 in each species with one or two mistmatches). The extra arm have four or five (in two cases) nucleotides. In most of them there are three to five nucleotide pairs in both the DHU and TψC arms. The tRNAs S1 of both species and the L2 of *N. westbladi* lack a paired DHU arm, a condition commonly found in S1 but not in L2 tRNAs. Interestingly, both species share the tRNA S1 structure, without the DHU arm, and the unusually long DHU and TψC arms of the Lysine tRNA.

Genetic code

The standard mitochondrial invertebrate code appear to deviate from the "universal" genetic code with regard to the identities of ATA, TGA and AGR codons (Wolstenholme, 1992). In the standard mitochondrial invertebrate code ATA, TGA and AGR code respectively for M, W and S, instead of I, Stop and R, as in the "universal" genetic code. Diploblasts only deviate in the case of the codon TGA, which codes for W. Platyhelminthes have been inferred to have two further differences, which they share with Echinodermata: AAA encodes N rather than K, and ATA encodes I rather than M (Nakao, M. et al., 2000, Telford et al. 2000).

Our data show that both *P. rubra* and *N. westbladi* share the standard invertebrate mitochondrial genetic code and not the flatworm genetic code, as has been previously inferred for acoels and nemertodermatids (Telford et al. 2000). This means that the ATA and AGR genetic code change is a synapomorphy of the Bilateria.

Phylogenetic reconstruction

The RRT showed that all parasitic platihelmints and the nematodes have much faster evolutionay rates. The acoel and especially the nemertodermatid have also faster evolutionay rates: *P. rubra* just with two of the considered "standard" taxa (the

non-parasitic protosomes and deutetorostomes), but *N. westbladi* with several ones (less that the nematodes or the parasitic platihelmiths). However, we used both taxa to infer their phylogenetic relationships. The results corroborates the Acoelomorpha clade (Acoel + Nemertodermatida) with high boostrap value (89%). As expected, the superclades Ecdysozoa, Lophotrochozoa and Deuterostomia are again validated, and Acoelomorha appear as the most basal bilaterian clade (with low support however). Finally, the phylogenetic signal of mitochondrial protein sequences at such deep evolutionary level seems promising, but needs further analysis with all twelve protein encoding genes to gather their real value.

Conclusions

The results of this study constitute, to our knowledge, the first genomic-level sequence from either acoel or nemertodermatid taxa and the first molecular evolutionary analysis of their mitochondrial genome organization. The comparison of their mitochondrial genome structure and aminoacid sequence analysis corroborate that both taxa constitute a monophyletic clade at the base of the Bilateria.

The position of both ribosomal genes, the tRNA S1 structure and the aminoacid sequence analysis argue for a common ancestor between acoels and nemertodermatids, as recently shown by myosin II sequences (Ruiz-Trillo et al. 2002). However, they migh have diverged long time ago, as shown by their differences in gene order. That is congruent with morphology. They share the cilia structure and the duet-spiral cleavage (Henry et al. 2000; Ulf Jondelius, personal communication). On the other hand they have differences in the pattern of neurotransmitters (Raikova et al. 2000), sperm morphology (Raikova et al. 2001) and statocist structure (Ax, 1996).

The phylogenetic value of mitochondrial gene arrangements at such deep evolutionary level is still a mistery, since additional diploblastic mitochondrial genome data is needed, especially from poriferans, placozoans or ctenophors, in order to be able to infere phylum level metazoan relationships. However, our data show some interesting issues of the basal bilaterian mitochondrial genomes. First, the higher pyrimidine composition is probably ancestral to bilaterians, since both acoelomorphs and diploblasts share it. Second, the "standard" invertebrate code, except for ATG, is a synapomorphy of bilaterians. Third, the formation of the common arrangement *rrnS-V-rrnL* probably ocurred before the eubilaterian radiation and after the acoelomorph diversification. Fourth, acoels have the conserved arrangment *cox-atp8-atp6*, but

inverted respect to *Tetilla sp.*, arguing for a conserved gene arrangement in the 3kb. mitochondrial genome of that poriferan.

Additional mitochonndrial data from acoels and nemertodermatids will surely shed ligh to the bilaterian mitochondrial genome evolutionary history.

References

Ax, P. 1996. *Multicellular animals. A new approach to the phylogenetic order in nature. Vol. I.* Springer-Verlag, Berlin, Germany.

Adoutte, A., Balavoine, G., Lartillot, N., Lespinet, O., Prud'homme, B. & de Rosa, R. 2000. The new animal phylogeny: reliability and implications. *Proc. Natl. Acad. Sci. USA* 97: 4453-4456.

Barnes, W. M. 1994. PCR amplification of up to 35-kb DNA with high fidelity and high yield from bacteriophage templates. *Proc. Natl. Acad. Sci. USA* 91: 2216-2220.

Beagley, C. T., Okimoto, R. & Wolstenholme, D. R. 1998. The mitochondrial genome of the sea anemone Metridium senile (Cnidaria): introns, a paucity of tRNA genes, and a near-standard genetic code. *Genetics* 148: 1091-1108.

Beaton, M. J., Roger, A. J. & Cavalier-Smith, T. 1998. Sequence analysis of the mitochondrial genome of Sarcophyton glaucum: conserved gene order among octocorals. *J. Mol. Evol.* 47: 697-708.

Boore, J. L**.** 1999. Animal mitochondrial genomes. *Nucleic Acids Res*. 27: 1767-1780.

Boore, J. L., Collins, T. M., Stanton, D., Daehler, L. L. & Brown, W. M. 1995. Deducing the pattern of arthropod phylogeny from mitochondrial DNA rearrangements. *Nature*, 376: 163- 165.

Boore, J. L. & Staton, J. L. 2002. The mitochondrial genome of the sipunculid Phascolopsis gouldii supports its association with annelida rather than mollusca. *Mol. Biol. Evol.* 19: 127-137.

Boore, J. L. & Brown, W. M. 1998. Big trees from little genomes: mitochondrial gene order as a phylogenetic tool. *Current Opinion in Genetics & Development* 8: 668-674.

Boore, J. L. & Brown, W. M. 2000. Mitochondrial genomes of Glathealinum, Helobdella, and Platynereis: sequence and gene arrangement comparisons indicate that Pogonophora is not a phylum and Annelida and Arthropoda are not sister taxa. *Mol. Biol. Evol.* 17: 87-106.

Boore, J. L., Lavrov, D. V. & Brown, W. M. 1998. Gene translocation links insects and crustaceans. *Nature* 392: 667-668.

Carranza, S., Baguñà, J. & Riutort, M. 1997. Are the Platyhelminthes a monophyletic primitive group? An assessment using 18S rDNA sequences. *Mol. Biol. Evol.* 14, 485-497.

Ehlers, U. 1985. Phylogenetic relationships within the Platyhelminthes. In *The origin and relationships of lower invertebrates groups*. Morris, S. C., George, J. D., Gibson, R. & Platt, H. M. Pp. 143-158. Oxford University Press, Oxford.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhok, R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotech. 3: 294-299.

Henry, J.Q., Martindale, M.Q., and Boyer, B.C. 2000. The unique developmental program of the acoel flatworm, *Neochildia fusca*. *Dev. Biol.* 220: 285-295.

Hoffmann, R. J., Boore, J. L. & Brown, W. M. 1992. A novel mitochondrial genome organization for the blue mussel, *Mytilus edulis*. *Genetics* 131: 397-412.

Jondelius, U., Ruiz-Trillo, I., Baguñà, J. & Riutort, M. 2002. The Nemertodermatida are basal bilaterians and not members of the Platyhelminthes. *Zoologica Scripta*, 31: 201-215.

Keddie, E. M., Higazi, T. & Unnasch, T. R. 1998. The mitochondrial genome of Onchocerca volvulus: sequence, structure and phylogenetic analysis. Mol. Biochem. Parasitol. 95: 11- 127.

Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. 2001. *MEGA2*: M*olecular Evolutionary Genetics Analysis* software. *Bioinformatics 17, 12*:1244-1245.

Lavrov, D. V., Boore, J. L. & Brown, W. M. 2000. The complete mitochondrial DNA sequence of the horseshoe crab *Limulus polyphemus*. *Mol. Biol. Evol*. 17: 813-824.

Le, T. H., Blair, D. & McManus, D. P. 2002. Mitochondrial genomes of parasitic flatworms. *TRENDS in Parasitology*, 18: 206-213.

Nakao, M. et al. 2000. Mitochondrial genetic code in cestodes. *Mol. Biochem. Parasitol.* 111: 415-424.

Nickisch-Rosenegk, M. Von, Brown, W. M. & Boore, J. L. 2001. Complete sequence of the mitochondrial genome of the tapeworm Hymenolepis diminuta: gene arrangements indicate that platyhelminthes are eutrochozoans. *Mol. Biol. Evol.* 18: 721-730.

Okimoto, R., Chamberlin, H. M., MacFarlane, J. L. & Wolstenholme, D. R. 1991. Repeated sequence sets in mitochondrial DNA molecules of root knot nematodes (Meloidogyne): nucleotide sequences, genome location and potential for host race identification. *Nucleic Acids Res.* 19: 1619-1626.

Ojala, D., Montoya, J. & Attardi, G. 1981. tRNA punctuation model of RNA processing in human mitochondria. *Nature* 290: 470-474.

Okimoto, R., MacFarlane, J. L., Clary, D. O. & Wolstenholme, D. R. 1992.The mitochondrial genomes of two nematodes, Caenorhabditis elegans and Ascaris suum. *Genetics* 130: 471-498.

Palumbi, S. R. 1996. Nucleic acids II: the polymerase chain reaction. Pp. 205-247 in D. M. Hillis, C. Moritz and B. K. Mable, eds. Molecular systematics. Sinauer, Sunderland, Mass.

Perna, N. T. & Kocher, T. D. 1995. Patterns of nucleotide composition at fourfold degenerate sites of animal mitochondrial genomes. *J. Mol. Evol*. 41: 353-358.

Pont-Kingdon, G. A., Vassort, C. G., Warrior, R., Okimoto, R., Beagley, C. T. & Wolstenholme, D. R. 2000. Mitochondrial DNA of *Hydra attenuata* (Cnidaria): A sequence that includes an end of one linear molecule and the genes for l-rRNA, $tRNA^{f\text{-}Met}$, $tRNA^{Trp}$, COII, and ATPase8. *J. Mol. Evol.* 51: 404-415.

Raikova, O. I., Reuter, M., Jondelius, U. & Gustafsson, M. K. S. 2000. The brain of the Nemertodermatida (Platyhelminthes) as revealed by anti-5HT and anti-FMRMamide immunostainings. *Tissue and cell* 32: 358-365.

Raikova, O. I., Reuter, M. & Justine, J. L. 2001. Contributions to the the phylogeny and systematics of the Acoelomorpha. In *Interrelationships of the Platyhelminthes*, pp. 13-23. D. T. J. Littlewood & R. A. Bray eds. London. Taylor & Francis.

Reyes, A., Gissi, C., Pesole, G. & Saccone, C. 1998. Asymmetrical directional mutation pressure in the mitochondrial genome of mammals. *Mol. Biol. Evol*. 15: 957-966.

Robinson-Rechavi M, Huchon D. 2000. RRTree: Relative-rate tests between groups of sequences on a phylogenetic tree. *Bioinformatics*, **16**, 296-297.

Ruiz-Trillo, I., Riutort, M., Littlewood, D. T. J. , Herniou, E. A. & Baguñà, J.1999. Acoel flatworms: earliest extant bilaterian metazoans, not members of the Platyhelminthes. *Science* 283, 1919-1923.

Ruiz-Trillo, I., Paps, J., Loukota, M., Ribera, C., Jondelius, U., Baguñà, J. & Riutort, M. 2002. A phylogenetic analysis of Myosin Heavy Chain type II sequences corroborates that Acoela and Nemertodermatida are basal bilaterians. *Proc. Natl. Acad. Sci. USA*, 99: 11246-11251.

Saccone, C., Gissi, C., Reyes, A., Larizza, A., Sbisà, E. & Pesole, G. Mitochondrial DNA in metazoa: degree of freedom in a frozen event. 2002. *Gene* 286: 3-12.

Smith, J. P. S., Tyler, S. & Rieger, R. R. 1986. Is the Turbellaria polyphyletic?. *Hydrobiologia* 132: 13-21.

Smith, M. J., Arndt, A., Gorski, S. & Fajber, E**.** 1993. The phylogeny of echinoderm classes based on mitochondrial gene arrangements. *J. Mol. Evol.* 36: 545-554.

Smith, S. W., Overbeek, R., Woese, C. R., Gilbert, W. & Gillevet, P. M. 1994. The genetic data environment: an expandable GUI for multiple sequence-analysis. Comp. Appl. Biosci. 10, 671-675.

Stechmann, A. & Schlegel, M. 1999. Analysis of the complete mitochondrial DNA sequence of the brachiopod *Terebratulina retusa* places Brachiopoda within the protostomes. *Proc. R. Soc. Lond. B Biol. Sci.* 266: 2043-2052.

Strimmer, K. & von Haeseler, A. 1996. Quartet puzzling: A quartet maximum likelihood method for reconstructing tree topologies. *Mol. Biol. Evol*. **13**, 964-969.

Telford, M. J., Herniou, E. A., Russell, R. B. & Littlewood, D. T. J. 2000. Changes in mitochondrial genetic code as phylogenetic characters: two examples from the flatworms. *Proc. Natl. Acad. Sci. USA* 97: 11359-11364.

Watkins, R. F. & Beckenbach, A. T. 1999. Partial sequence of a sponge mitochondrial genome reveals sequence similarity to Cnidaria in cytochrome oxidase subunit II and the large ribosomal RNA subunit. J. Mol. Evol. 48: 542-554.

Wolstenholme, D. R. 1992. Genetic novelties in mitochondrial genomes of multicellular animals. *Curr. Opin. Genet. Dev.* 2: 918-925.

Figure legends

Figure 1. Gene map of the sequenced portion of the mt DNA of the acoel *Paratomela rubra* and the nemertodermatid *Nemertoderma westbladi.* All genes are transcribed from left to right, except for tRNA L2. Transfer RNA genes are designated by a single letter for the corresponding amino acid. All other genes are designated by standard annotation and are not to scale. Genes *rrnL*, *atp6*, *rrnS* and *cox3* are depicted to indicate its similar arrangement in both taxa.

Figure 2. Gene map of the published sequenced portion of the mt DNA of the poriferan *Tetilla sp.*, compared to this same portion in the acoel *Paratomela rubra,* the cnidarian *Sarcophyton glaucum* and the standard invertebrate arrangement*.* Arrows show transcription direction. Transfer RNA genes are designated by a single letter for the corresponding amino acid. All other genes are designated by standard annotation and are not to scale. Same genes have the same color along the different taxa. The arrow between *Tetilla sp.* and *P. rubra* indicates the suggested inversion.

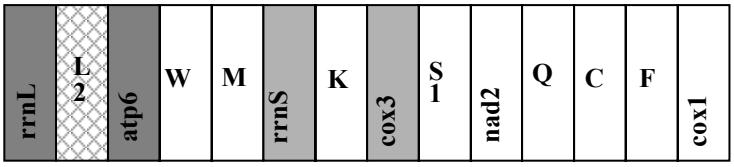
Figure 3. Potential secondary structure of the tRNAS found for *Paratomella rubra*.

Figure 4. Potential secondary structure of the tRNAS found for *Nemertoderma westbladi*.

Figure 5. Neighbor-joining phylogenetic tree based on amino acid sequence alignment of *atp6*, *cob*, *cox1*, *cox2*, *cox3, nd1* and *nd6* protein genes. Numbers above key nodes show 1000 boostrap replicate values.

Paratomella rubra

Nemertoderma westbladi



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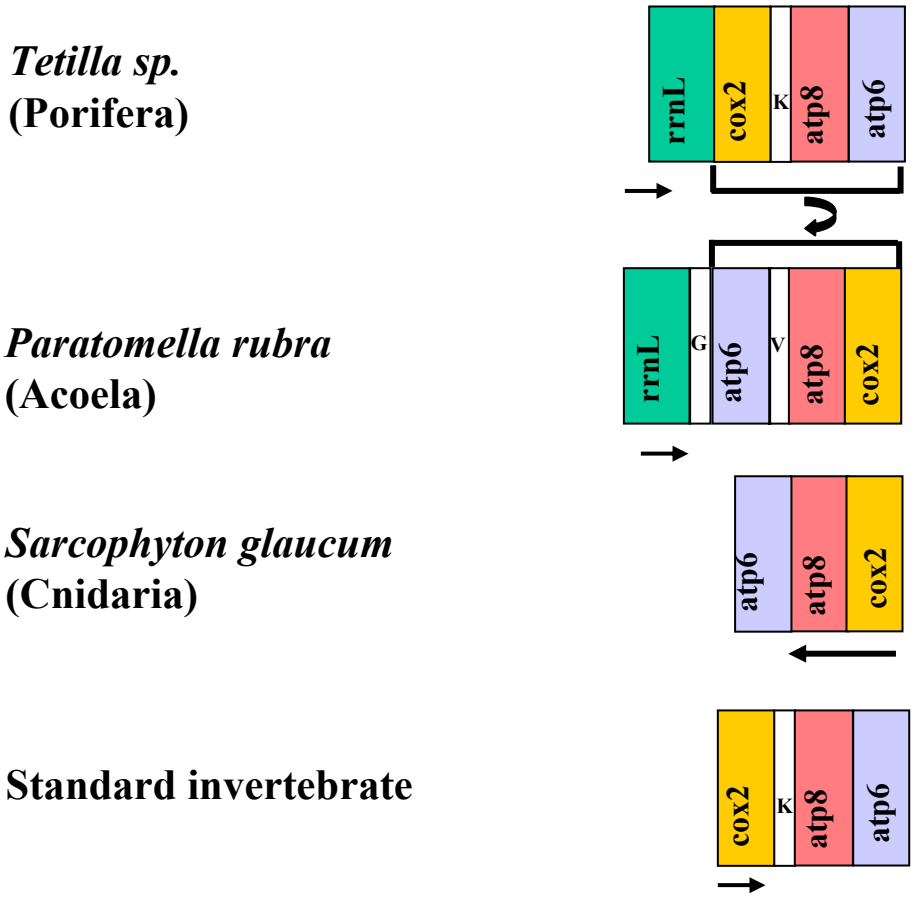

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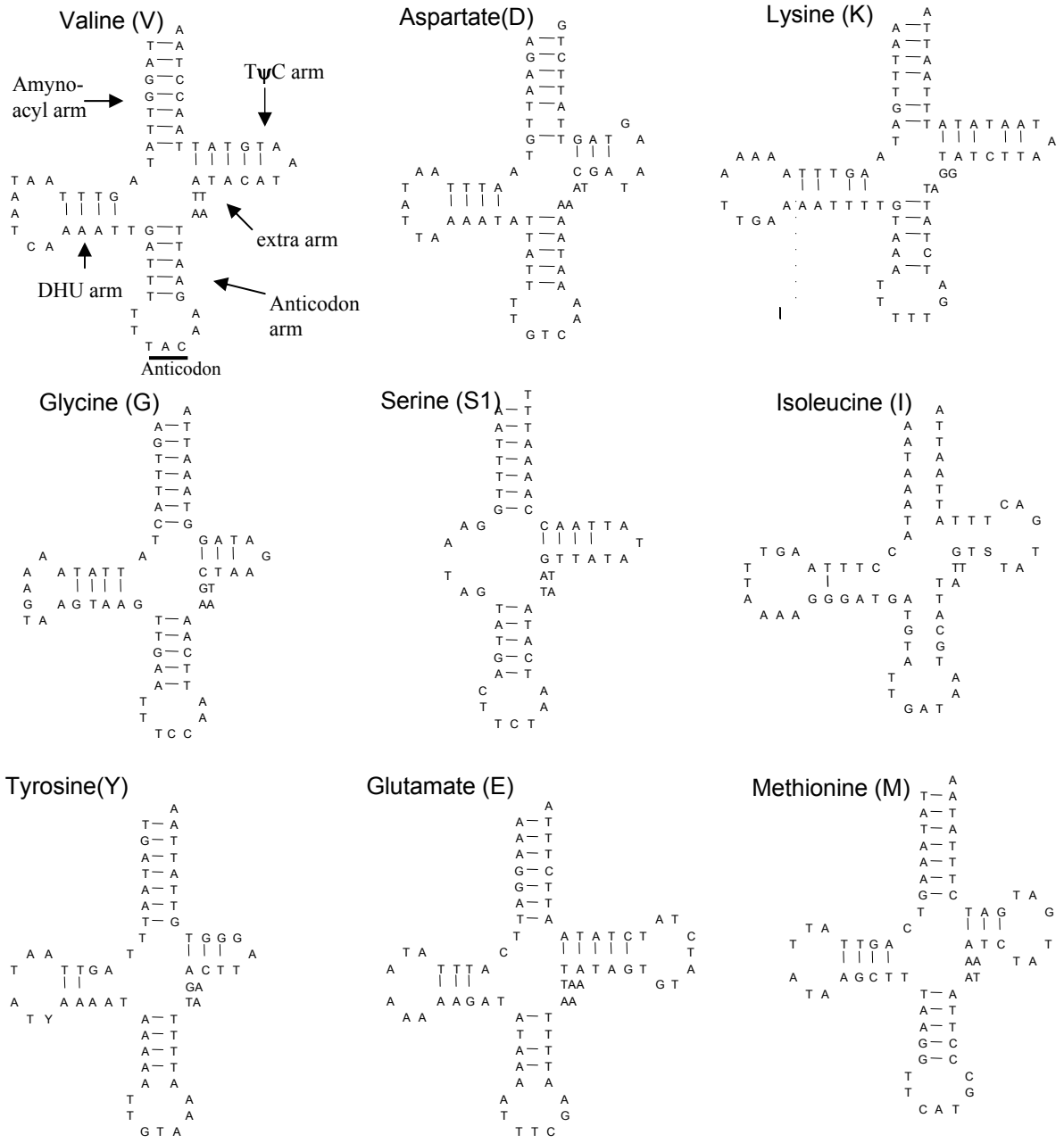

Figure 3. Potential secondary structure of the tRNAS found for *Paratomella rubra*.

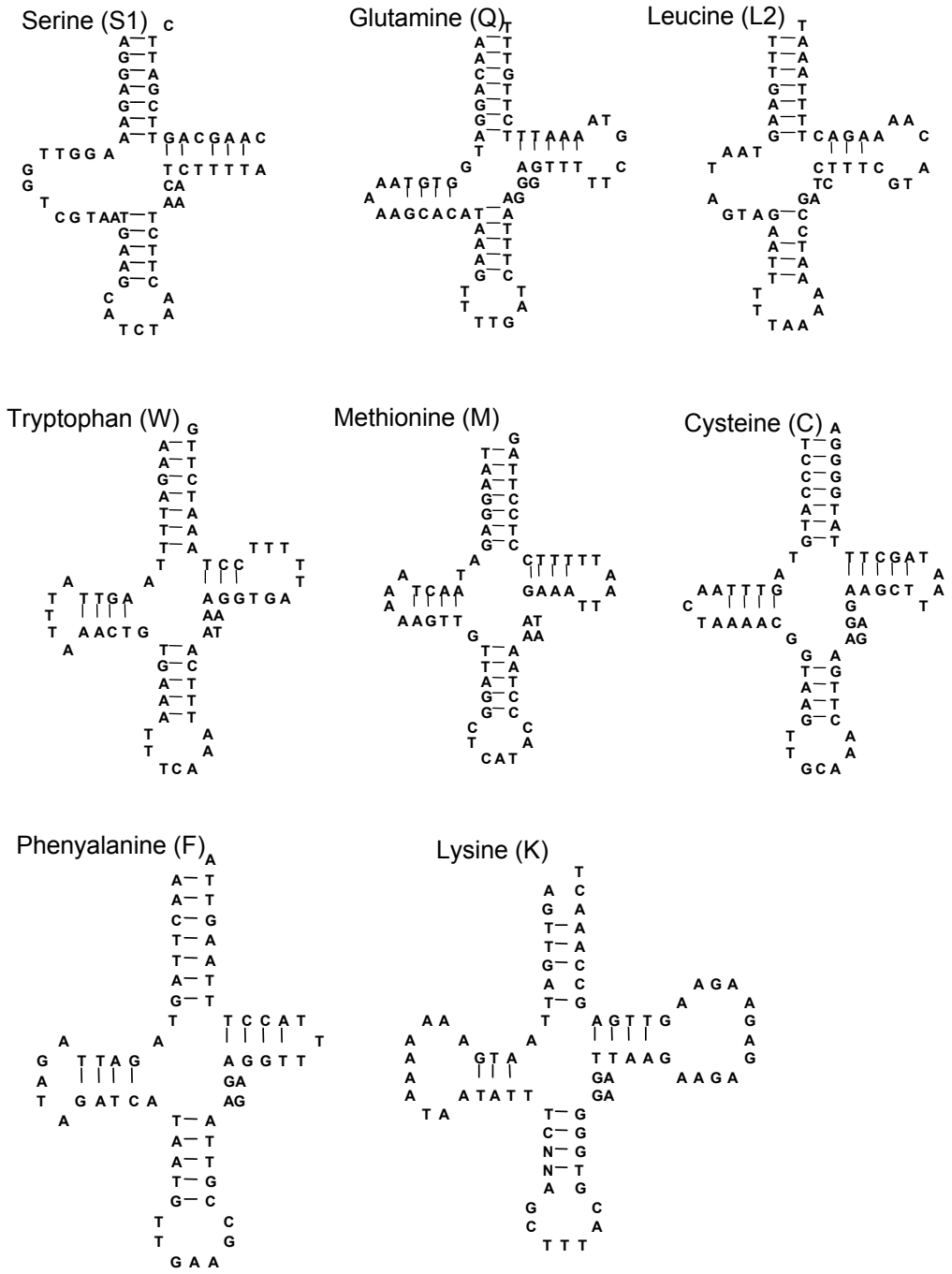

Figure 4. Potential secondary structure of the tRNAS found for *Nemertoderma westbladi*.

Table 1. Nucleotide composition

		No. of nucleotides	T (%)	C (%)	A (%)	G (%)	A+T (%)	G+C (%)	AT-Skew	GC-Skew
Mt DNA	*P.rubra*	9795	47,5	8	28,9	15,6	76.4	23.6	-0.24	0.32
	N.westbladi	5243	37,5	13,8	27,1	21,6	64.6	35.4	-0.16	0.22
Protein genes	*P. rubra*	6771	49,3	9	25,4	16,4	74.7	25.4	-0.31	0.29
	N.westbladi	3388	39,6	14,2	23,6	22,6	63.2	36.8	-0.25	0.22
rRNA genes	*P.rubra*	2204	43	5,9	36,4	14,7	79.4	20.6	-0.08	0.42
	N.westbladi	1351	33,2	13,7	32,9	20,2	66.1	33.9	-0.004	0.19
tRNA genes	*P.rubra*	591	42,2	6,3	40,8	10,7	83	17	-0.01	0.25
	N.westbladi	489	34,4	10,7	34,8	20,2	69.2	30.9	-0.005	0.30
Noncoding	*P.rubra*	242	49,6	5	31,4	14	81	19	-0.22	0.47
	N.westbladi									

Nucleotide composition and AT and GC-Skew values for *P. rubra* and *N. westbladi* mtDNA, protein encoding, ribosomal, tRNAs and non-coding nucleotides. *N. westbladi* lacks information on non-coding regions, since the fragment analysed have only a few non-coding nucleotides.

Table 2. Genetic code for universal, diploblastica, Acoelomorpha, standard invertebrate and Rhabditophora, for the codons TGA, ATA, AGR and AAA

	TGA	ATA	AGR	AAA
Universal	Stop	I	R	K
Diploblastica	W	I	R	K
Acoelomorpha	W	M	S	K
Standard Invertebrate	W	M	S	K
Rhabditophora	W	I	S	N