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

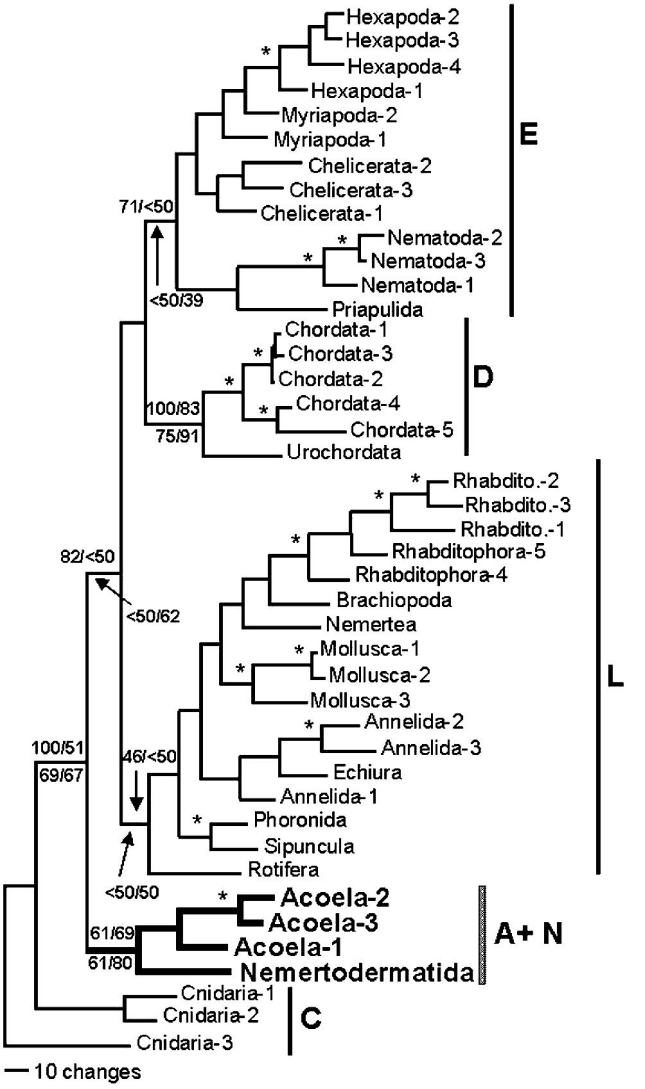

Fig. 1. Phylogeny of bilaterians determined by BI from the myosin II data. Numbers above key nodes refer to the BPP (shown as percentage) and the percentage obtained from a QP 10,000 replicates analysis. Values below branches represent support obtained by MP and NJ 1,000 bootstrap replicates analysis. 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.

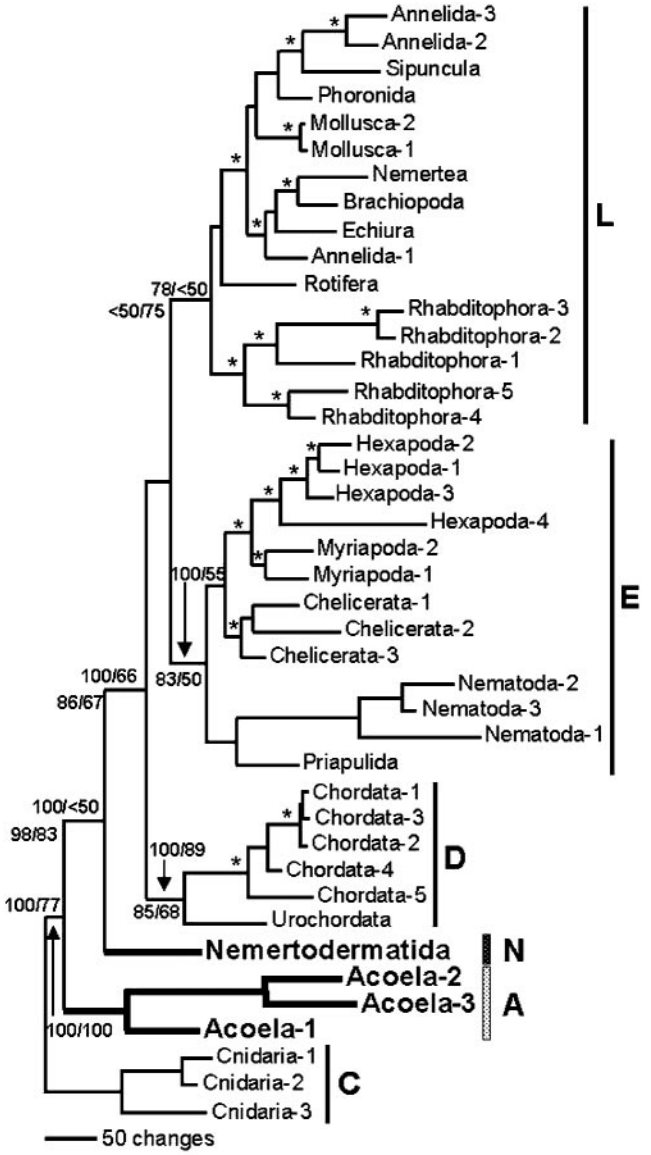

Fig. 2. Phylogeny of bilaterians determined by BI from the SSU data set. For numbers above key nodes, asterisks, abbreviations, and species names, see Fig. 1 legend.

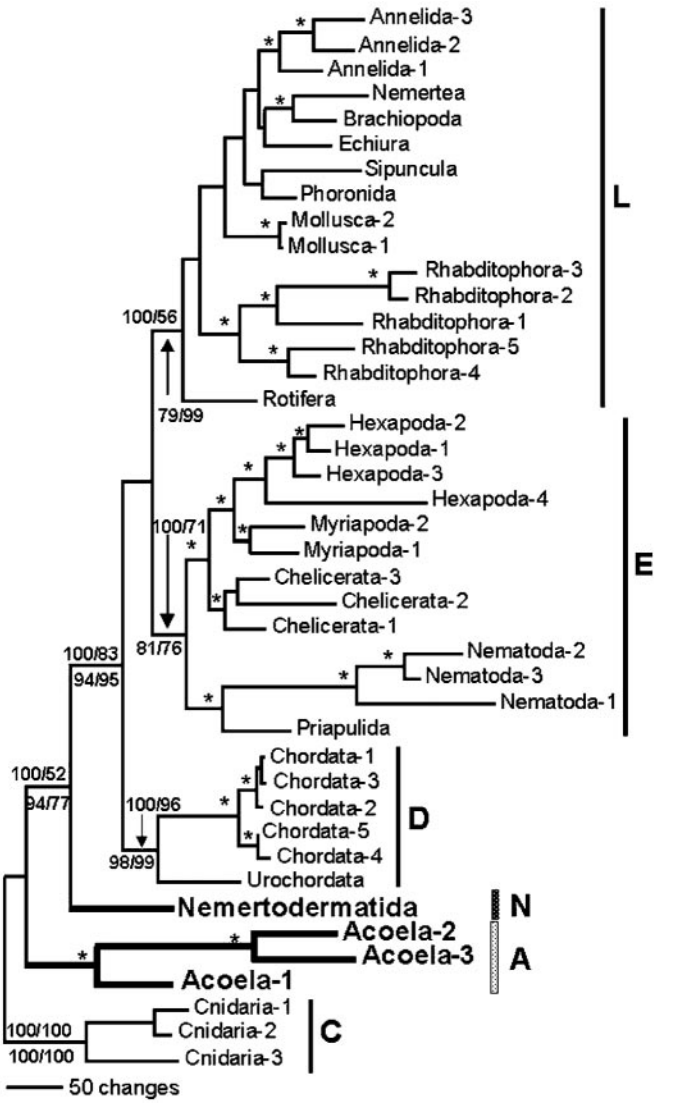

Fig. 3. Phylogeny of bilaterians determined by BI from the combined data set. For numbers above key nodes, asterisks, abbreviations, and species names, see Fig. 1 legend.

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.

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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