

Elongation Factor 1-Alpha Sequences Do Not Support an Early Divergence of the Acoela

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The phylogenetic position of the Acoela is a key problem in the understanding of metazoan evolution. Recent studies based on 18S ribosomal DNA (rDNA) sequences have placed the Acoela in an extremely basal position as the sister group to all other extant triploblastic animals, suggesting that the phylum Platyhelminthes is polyphyletic. In order to test the results obtained with 18S rDNA, we sequenced elongation factor 1-alpha (EF1a) for the acoel *Convoluta roscoffensis* and five species of Turbellaria (two polyclads, *Leptoplana tremellaris*, and *Prostheceraeus vittatus*, and three triclads, *Crenobia alpina*, *Schmidtea polychroa*, and *Girardia tigrina*). Phylogenetic analyses of EF1a sequences show that the acoel sequences branch within the Platyhelminthes, in opposition to the 18S rDNA data. Moreover, comparison of the central variable region of EF1a shows similar sequence signatures between *C. roscoffensis* and the three triclad species. Although EF1a sequences fail to prove the monophyly of the phylum Platyhelminthes, they do not confirm the early divergence of the Acoela.

Introduction

Acoel flatworms, ordinarily classified in the phylum Platyhelminthes as an order of the class Turbellaria, have recently been given great attention because of their particularly problematic phylogenetic position. Recent studies based on 18S ribosomal DNA (rDNA) sequences cast doubt on the monophyly of the phylum Platyhelminthes (Carranza, Baguña, and Riutort 1997) and suggested a possible primitive position of acoel flatworms, making them the earliest-diverging Bilateria and the sister group to all other extant triploblastic animals (Ruiz-Trillo et al. 1999).

The 18S rDNA gene is the most commonly used gene in phylogenetic analyses, and its database is the most complete for all living organisms (Hillis and Dixon 1991; Maidak et al. 1999). Like other ribosomal genes, the 18S gene is very easy to amplify because of its large number of copies per genome. Furthermore, the alternation in a single molecule of very conserved and much more variable regions makes 18S rDNA a powerful tool for molecular phylogeny at different taxonomic levels. However, it has been shown that in many phyla, such as Rhombozoa, Dicyemida, Nematoda, Gnathostomulida, and Chaetognatha, 18S rDNA genes do not evolve at a constant rate (Katayama et al. 1995; Winnepenninckx et al. 1995; Pawlowski et al. 1996; Littlewood et al. 1998). As a result, phylogenetic analyses lead to an artifactual basal clustering of the fast-evolving phyla, known as the long-branch attraction (LBA) phenomenon. The Acoela, most of which have been shown to be fast-clock organisms for the 18S rDNA gene (Carranza,

Baguña, and Riutort 1997), represent a particularly good example of the LBA effect.

There are two main ways to overcome the LBA problem. The first one is to broadly sample problematic groups in order to find non-fast-clock species among each of them. This method was successfully used with the phylum Nematoda, shown to be closely related to the Arthropoda (Aguinaldo et al. 1997), and led to a better comprehension of general metazoan phylogeny, with the separation of the triploblastic animals into three main clades, the Lophotrochozoa, the Ecdysozoa, and the Deuterostomia (Halanych et al. 1995; Aguinaldo et al. 1997). Ruiz-Trillo et al. (1999) applied this method to the Acoela and retained the unique non-fast-clock sequence of *Paratomella rubra* out of 18 acoel species. When only taxa with uniform rates of change were included in their analyses, the sequence of *P. rubra* appeared in a very basal position, as the first offshoot of the triploblastic animals. Therefore, the authors concluded that the acoel flatworms do not belong to the phylum Platyhelminthes and are probably the closest living relatives of the first Bilateria.

A second way to avoid the LBA effect is to find another gene for which rates of substitution are more constant between all metazoan phyla. The problem in finding such a gene is that no other database of metazoan sequences is as complete as the one for 18S rDNA. This is the main reason why so few studies of animal phylogeny are based on other genes, despite the general agreement that molecular phylogenetic inference requires congruent results from multiple gene sequences to be really conclusive (Baldauf and Palmer 1993; Regier and Schultz 1997; Abouheif, Zardoya, and Meyer 1998).

Elongation factor 1-alpha (EF1a) is a protein found in all eukaryotic lineages. EF1a is very abundant in the cytosol, where it is involved in the GTP-dependent binding of aminoacyl-tRNAs to the A site of the ribosomes in the second step of translation from mRNAs to proteins. Its universal occurrence and good amino acid sequence conservation makes it an ideal phylogenetic tool with which to determine very ancient relationships, such as the relative branching order of the most primitive

Abbreviations: CI, consistency index; EF1a, elongation factor 1-alpha; LBA, long-branch attraction; NJ, neighbor joining; ML, maximum likelihood; MP, maximum parsimony; QP, quartet puzzling; rDNA, ribosomal DNA; RI, retention index.

Key words: elongation factor 1-alpha, molecular phylogeny, *Convoluta roscoffensis*, Acoela, Platyhelminthes.

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Table 1
List of the Primers Used in this Study

Name	Direction	Nucleotide Sequence (in IUB code)	Amino Acid Sequence	Position ^a
EF2a	Forward	5'-GARGCYCARGARATGGGWAAAGGWTC-3'	EAQEMGKGS	45-53
1ZH	Forward	5'-TCYTTCAARTAYGCNTGGGT-3'	SFKYAWV	53-59
2ZH	Reverse	5'-ATRTGTGCAGTRTGRCARTC-3'	DCHTAHI	362-368
EF9a	Reverse	5'-TCNGCRAAYTTGCARGCAATRTGWGC-3'	AHIACKFAE	366-374

^a Positions correspond to the amino acid residues of human EF1a (GenBank accession number J04617).

Eukaryotes (see, e.g., Hashimoto et al. 1994; Nordnes, Krauss, and Johansen 1994; Baldauf, Palmer, and Doolittle 1996). Furthermore, the nucleotide sequences of EF1a genes were used at a much lower level to study phylogenies of families and genera, using the degenerated third position of codons (see, e.g., Cho et al. 1995; Mitchell et al. 1997). EF1a has also been used for resolving the phylogenetic relationships between animal phyla and classes as a good means to confirm or cast doubt on results based on other genes (Kojima et al. 1993; Regier and Shultz 1997; Kojima 1998).

In this study, we use EF1a in the first attempt to infer the phylogenetic position of the Acoela using a protein-coding gene. A fragment of about 950 nt of EF1a was amplified and sequenced for the acol flatworm *Convoluta roscoffensis*, as well as for three species of Tricladida (*Crenobia alpina*, *Schmidtea polychroa*, and *Girardia tigrina*) and two species of Polycladida (*Prostheceraeus vittatus* and *Leptoplana tremellaris*). We compared it to other metazoan sequences in order to establish the phylogenetic position of the Acoela and test the validity of the results obtained with 18S rDNA sequences.

Materials and Methods

Sample Collection and DNA Extractions

Living specimens of *C. roscoffensis* were collected on the west coast of Cotentin (Normandy, France) and kept alive for several months in the laboratory. Other specimens were collected in Roscoff (Brittany, France) and preserved in 70% alcohol. Two members of the order Polycladida (*L. tremellaris* and *P. vittatus*) were collected near the marine biology station of Luc-sur-Mer (Normandy, France) and also preserved in 70% alcohol. Living specimens of *S. polychroa* and *G. tigrina* (Tricladida, Dugesiiidae) were collected in Geneva, Switzerland, and living specimens of *C. alpina* (Tricladida, Planariidae) were collected above Martigny, Switzerland. They were all kept alive in an aquarium.

DNA from one individual of each species was extracted in guanidine lysis buffer, precipitated with isopropanol, and dissolved in distilled water (Tkach and Pawlowski 1999).

PCR Amplification, Cloning, and Sequencing

PCR amplifications were all performed in a total volume of 50 μ l with an amplification profile consisting of 40 cycles of 30 s at 94 °C, 30 s at 52 °C, and 120 s at 72 °C, followed by 5 min at 72 °C for final extension. To avoid contamination with the endosymbiotic algae of

C. roscoffensis, a set of four metazoan-specific primers was designed for the PCR-amplification of a fragment of about 950 nt of EF1a (see table 1 for the sequences and positions of these primers). These metazoan-specific primers were used to amplify the same fragment of EF1a for the two polyclads and the three triclads. All PCR products were purified using the High Pure PCR purification kit (Roche) and then ligated in the p-GEM-T vector (Promega) and cloned in XL-2 Ultracompetent Cells (Stratagene), all according to the manufacturers' instructions. The clones were then sequenced on an ABI 377 Prism sequencer. The sequences reported in this paper have been deposited in the GenBank database under accession numbers AJ250908–AJ250914.

Phylogenetic Analyses

The sequences of EF1a obtained in this study were manually aligned to other available metazoan and fungal sequences using the Genetic Data Environment software, version 2.2 (Larsen et al. 1993). All species names, taxonomic ranks, and GenBank accession numbers of EF1a sequences used in our analyses are listed in table 2. A relative-rate test was performed using RRTree (Robinson-Rechavi and Huchon 2000).

An evolutionary tree was inferred from the deduced amino acid sequences using the neighbor-joining (NJ) method (Saitou and Nei 1987) applied to Dayhoff's PAM distance matrix (Dayhoff, Schwartz, and Orcutt 1978). The reliability of internal branches was assessed using the bootstrap method (Felsenstein 1988) with 1,000 bootstrap replicates. The Phylo-Win program (Galtier and Gouy 1996) was used for distance computations and NJ building and bootstrapping. All maximum-parsimony (MP) analyses were performed with PAUP* (Swofford 1998). The most parsimonious trees were determined using a heuristic search procedure with 100 random-addition-sequence replicates and tree bisection-reconnection branch swapping. Reliability of internal branches was evaluated with 500 bootstrap replicates. All maximum-likelihood (ML) analyses were carried out with the PUZZLE software, version 4.02 (Strimmer and von Haeseler 1996), except for the exhaustive tree searches using constrained tree topologies, which were performed with the protml program of the MOLPHY package, version 2.3 (Adachi and Hasegawa 1996).

Results

The amplified fragment of EF1a varies in size, from 947 nt (316 amino acids) in the two polyclads to 962 nt

Table 2
List of the Species Used in this Study, Along with
GenBank Accession Numbers

Taxonomic Rank	Accession Number
Ciliophora	
<i>Euplotes crassus</i> ^a	U26260
Apicomplexa	
<i>Plasmodium falciparum</i> ^a	X60488
Euglenophyta	
<i>Euglena gracilis</i> ^a	X16890
Plantae	
<i>Arabidopsis thaliana</i> ^a	X16430
Acrasiomycota	
<i>Dictyostelium discoideum</i> ^a	X55973
Fungi	
Basidiomycota	
<i>Puccinia graminis</i>	X73529
<i>Filobasidiella neoformans</i>	U81804
Ascomycota	
<i>Schizosaccharomyces pombe</i>	D82572
<i>Candida albicans</i>	M29935
<i>Arxula adenivorans</i>	Z47379
Metazoa	
Porifera	
<i>Ephydatia fluviatilis</i>	D49925
Cnidaria	
<i>Eugymnanthea japonica</i>	D49926
<i>Hydra vulgaris</i>	Z68181
Arthropoda	
Chelicerata	
<i>Limulus polyphemus</i>	U90051
<i>Carcinoscorpius rotundicauda</i>	AF063407
<i>Dinothrombium pandorae</i>	U90048
<i>Mastigoproctus giganteus</i>	U90052
<i>Aphonopelma chalcodes</i>	U90045
Myriapoda	
<i>Scutigera coleoptrata</i>	U90057
<i>Polyxenus fasciculatus</i>	U90055
Crustacea	
<i>Limnadia lenticularis</i>	AF063412
<i>Artemia salina</i>	X03349
<i>Speleonectes tulumensis</i>	AF063416
Insecta	
<i>Tomocerus</i> sp.	U90059
<i>Periplaneta americana</i>	U90054
<i>Apis mellifera</i>	AF01526
Platyhelminthes	
Polycladida	
<i>Leptoplana tremellaris</i>	AJ250908 ^b
<i>Prostheceraeus vittatus</i>	AJ250909 ^b
Acoela	
<i>Convoluta roscoffensis</i> 1	AJ250910 ^b
<i>Convoluta roscoffensis</i> 2	AJ250911 ^b
Tricladida	
<i>Crenobia alpina</i>	AJ250912 ^b
<i>Girardia tigrina</i>	AJ250913 ^b
<i>Schmidtea polychroa</i>	AJ250914 ^b
Annelida	
Polychaeta	
Ampharetidae G. sp.	AB003712

Table 2
Continued

Taxonomic Rank	Accession Number
<i>Paralvinella hessleri</i>	AB003711
<i>Eunice yamamotoi</i>	AB003704
<i>Sternaspis scutata</i>	AB003722
<i>Laetmonice</i> sp.	AB003703
<i>Ophelina</i> sp.	AB003708
<i>Maldane cristata</i>	AB003707
Oligochaeta	
<i>Brachiura</i> sp.	AB003715
<i>Allolobophora</i> sp.	AB003714
Hirudinea	
<i>Hirudo medicinalis</i>	U90063
Pogonophora	
<i>Escarpia</i> sp.	AB003718
<i>Lamellibrachia</i> sp.	AB003721
Vertebrata	
<i>Danio rerio</i>	L47669
<i>Xenopus laevis</i>	M25697
<i>Gallus gallus</i>	L00677
<i>Mus musculus</i>	M22432
<i>Homo sapiens</i>	J04617

^a These sequences were not included in the phylogenetic analyses but appear in the partial alignment shown in figure 1.

^b New sequences reported in this paper.

(321 amino acids) in *C. roscoffensis* to 965 nt (322 amino acids) in the three triclads. The mean length of vertebrate EF1a is 1386 nucleotides (462 amino acids). The amplified EF1a of *C. roscoffensis* and the five turbellarians corresponds to amino acid positions 45–374 of human EF1a. The two polyclad sequences share an intron of about 110 nt in the codon corresponding to amino acid 343. The two sequences of *C. roscoffensis* obtained from two different DNA extractions, the first (1) from an individual from Roscoff and the second (2) from an individual from the west coast of Cotentin, differ in five positions, of which four are silent transitions in the third codon positions and one is a transition in the second position which changes glycine to glutamic acid at position 230, as shown in figure 1.

The seven sequences obtained were aligned to diverse eukaryotic sequences (see table 2). The alignment shows that the variation in size between *C. roscoffensis* and the different members of the Turbellaria occurs in the central variable region of EF1a. This region consists of an insertion of about 12 amino acids (positions 214–225) shared only by metazoans and fungi, as shown by Baldauf and Palmer (1993). Whereas the two polyclad sequences are of the same length as those of fungi and most animals, the sequences of *C. roscoffensis* and the three triclads are five and six amino acids longer, respectively. The six additional amino acids of the triclad sequences can be quite readily aligned to the five additional amino acids of the sequences of *C. roscoffensis*, because they share the clear amino acid sequence signature KK(ED)E (fig. 1).

Thirty-eight sequences of diverse metazoan and fungal species were retained for the phylogenetic anal-

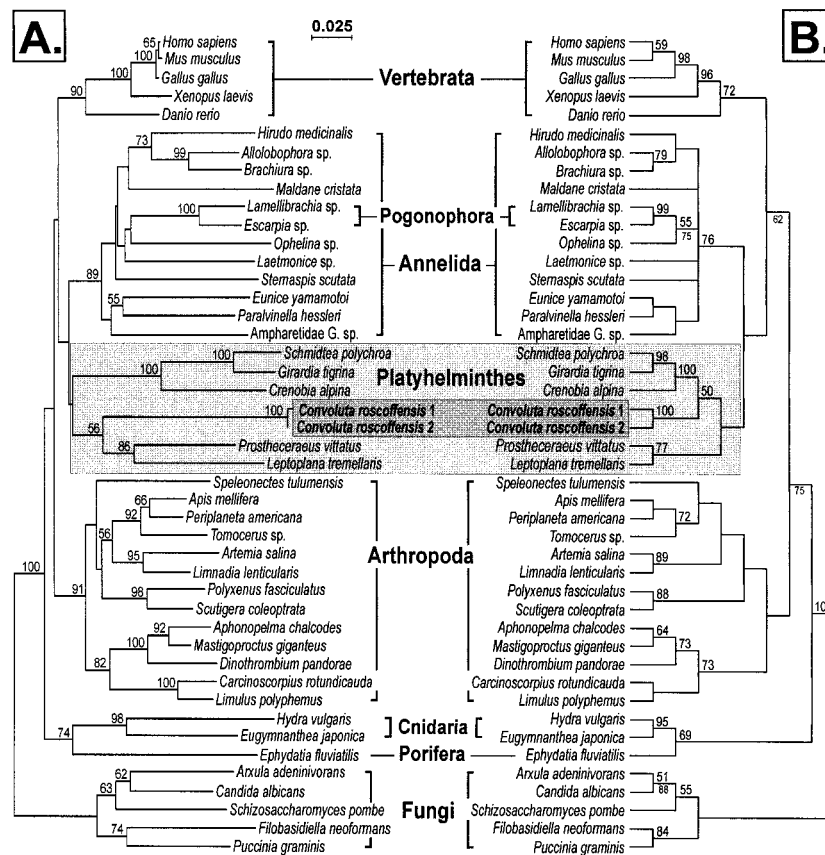


FIG. 3.—Phylogenetic position of the acoel flatworm *Convoluta roscoffensis* inferred from amino acid sequences of EF1a using (A) the neighbor-joining (NJ) method and (B) the maximum-parsimony method. In both trees, the two polyclads, the three triclads, and *Convoluta roscoffensis* appear as a monophyletic unit, a sister group to members of the Lophotrochozoa. The trees are rooted using five fungal sequences as a designated outgroup. A, NJ tree constructed using Dayhoff's PAM distance matrix, with all branch lengths drawn to scale. Numbers at internal nodes indicate percentages of 1,000 bootstrap replicates that support the branch; values less than 50% are omitted. B, Fifty percent majority-rule consensus of the eight most-parsimonious trees (percentiles less than 100% are indicated below the branches) obtained using a heuristic search procedure with 100 random-addition-sequence replicates and tree bisection-reconnection branch swapping. Trees length is 1,025 steps, with a consistency index of 0.447 and a retention index of 0.583. Bootstrap values of 500 replicates are indicated above the nodes; values less than 50% are omitted. The tree differs from the NJ tree mainly in the position of *C. roscoffensis* as the sister group to the three triclad species.

formed by platyhelminths, annelids, and vestimentiferans. Their relative branching order is not resolved. All groups supported by bootstrap values greater than 70% in the NJ tree are also well supported in the MP analysis, except for the arthropod clade, which is supported by only a 47% bootstrap value. As in the NJ tree shown in figure 3A, the Platyhelminthes cluster together, but the main difference between the two trees is that the two sequences from *C. roscoffensis* are placed in the MP tree as the sister group to the three triclads with a 50% bootstrap value, with the two polyclads branching off at the base of the group.

Next, an ML tree was constructed with the quartet puzzling (QP) algorithm (Strimmer and von Haeseler 1996), which automatically assigns estimations of support to each internal branch. Dayhoff's PAM substitution model was used. The frequency of each amino acid was estimated from the data set, and a 5% level chi-square test was performed to confirm that the amino acid composition of each sequence was identical to the average amino acid composition of the whole alignment. The chosen model of rate heterogeneity was a discrete

gamma distribution with eight categories, with all necessary parameters being estimated automatically from the data set. The QP search was conducted with 10,000 puzzling steps. As shown by the likelihood-mapping analysis, 11,592 of the 148,995 quartets analyzed (7.8%) were unresolved, leading to a multifurcating tree with a basal polytomy in the metazoan clade. The tree has a log likelihood value of $-6,507.07$. Six groups of metazoans are defined: the diploblastid clade, the vertebrate clade, the arthropod clade, the clade formed by annelids plus pogonophorans, and two platyhelminth clades. As in the NJ tree, the two sequences of *C. roscoffensis* appear as the sister group to the two polyclads, with a QP reliability of 65%. However, the monophyly of the Platyhelminthes was recovered in only 28% of all intermediate trees generated in the 10,000 puzzling steps, and the three triclad species cluster in a distinct group (data not shown).

Discussion

The results of our study are in opposition to 18S rDNA analyses, which place the acoel flatworms as the

sister group to all other extant triploblastic animals, well apart from other Platyhelminthes (Ruiz-Trillo et al. 1999). Analyses of EF1a provide two independent pieces of evidence favoring a close relationship between the Acoela and the Turbellaria. First, *C. roscoffensis* shares an amino acid sequence signature with the three triclads species, which can be interpreted as a single insertion event in a common ancestor of both lineages (Baldauf and Palmer 1993). Second, in all types of phylogenetic analyses, *Convoluta* always branches as the sister group to members of the Turbellaria. This finding is in agreement with morphological data. The simplicity of acoel morphological features can be interpreted as secondary derived reductions (Ehlers 1985; Smith and Tyler 1985; Smith, Tyler, and Rieger 1986), and there are many rigorous morphological synapomorphies that support a sister group relationship between the Acoela and some members of the Turbellaria, i.e., the Nemertodermatida (Ehlers 1985; Smith and Tyler 1985; Ax 1996). Furthermore, the phylogenetic position of the group formed by *C. roscoffensis* and the Turbellaria in both NJ and MP analyses, placed as the sister group to other members of the Lophotrochozoa (i.e., annelids and pogonophorans), is in agreement with previous studies on the phylogenetic position of the phylum Platyhelminthes (Aguinaldo et al. 1997; Balavoine 1997).

However, the monophyly of the Platyhelminthes was never well supported in our analyses, as shown by very low bootstrap values (8% in the NJ analysis and 21% in the MP analysis). Interestingly, this low support for the platyhelminth clade is apparently mainly due to the important differences between polyclad and triclads sequences. Indeed, if the acoel sequences are not included in the analysis, the five Turbellaria no longer appear monophyletic with the NJ method (data not shown), with the triclads being the sister group to the annelid plus pogonophoran clade. On the other hand, when the three triclads sequences are not included in the analysis, the grouping of the acoel sequences with the two polyclads is strengthened, with a bootstrap support value of 74% (data not shown). Inversely, when the two polyclad sequences are removed from the analysis, the two acoel sequences group with the triclads with a bootstrap support value of 61% (data not shown). Similarly, with the MP method, removing the two polyclad sequences from the analysis strengthens the support value for the acoel plus triclads clade (71%, data not shown). In addition, if the triclads sequences are removed from the analysis, the two sequences of *C. roscoffensis* cluster with the polyclad sequences with a bootstrap support value of 62%. In all cases, the Acoela branch with one of the lineages of Turbellaria, and the platyhelminth clade always remains the sister group to the other lophotrochozoan species. In fact, the two sequences of *C. roscoffensis* share several common amino acid substitutions with the polyclads on the one hand and with the triclads on the other hand, with very few of them being common to the five Turbellaria. This may explain why the monophyly of the platyhelminth clade is never well supported, whereas the monophyly of the acoel plus tri-

clad clade alone and that of the acoel plus polyclad clade alone are supported by quite confident bootstrap values.

Given the weak support of EF1a data for the monophyly of the Platyhelminthes, we were interested in testing their support for the hypothesis of an early branching of the Acoela, suggested by 18S rDNA data. With this aim, we used the exhaustive search mode of the protml program in MOLPHY, version 2.3 (Adachi and Hasegawa 1996), which allows constrained tree topologies, to determine the likelihood values of the totally resolved trees compatible with the six metazoan groups present in the QP tree. We retained only the 20 best tree topologies out of the 945 that were compatible with the QP analysis. Nine of them show the lophotrochozoan clade to be monophyletic, and in four, the Platyhelminthes are monophyletic and a sister group to the annelid plus pogonophoran clade. Moreover, the three diploblastid sequences are the first offshoot of the Metazoa in 18 of the 20 best trees. The Kishino-Hasegawa test (Kishino and Hasegawa 1989) showed at a 5% level that none of the 20 best topologies could be rejected as significantly worse than the other. Next, we performed the same test on two constrained topologies, defined as follows: we used the general topology obtained by Ruiz-Trillo et al. (1999) as a constrained topology, first with the Platyhelminthes monophyletic, then with the two acoel sequences at the base of all other triploblastic species. The first constrained topology was included in the 20 best tree topologies; it has a log likelihood value of $-6,373.49$. However the topology which shows the Acoela as the first offshoot after the diploblasts, which has a log likelihood value of $-6,392.86$, was rejected as significantly worse than the best tree at a 5% level. This test shows that even if EF1a sequences cannot resolve the problem of the monophyly of the phylum Platyhelminthes, they give good evidence in favor of rejecting the hypothesis that acoel flatworms represent the first offshoot of the Bilateria.

A problematic feature of our analyses is the fact that the two sequences of *C. roscoffensis* grouped with the polyclad sequences in both NJ and ML analyses, whereas they grouped with the triclads sequences in the MP analysis. This can be explained by the fact that in both NJ and ML analyses, amino acid substitutions were weighted using Dayhoff's PAM substitution model, whereas in the MP analysis, all amino acid substitutions were equally weighted. As stated above, the two acoel sequences share a few more amino acid substitutions with the three triclads than with the two polyclads, leading to the grouping of *C. roscoffensis* with the triclads in the MP analysis. However, when Dayhoff's PAM substitution model is taken into account, these substitutions are weighted differently, and *C. roscoffensis* groups with the polyclads. Nevertheless, the sequence signature shared by *C. roscoffensis* and the three triclads suggests that the Acoela might be more closely related to the Tricladida than to the Polycladida. Interestingly, Campos et al. (1998) also found a close relationship between Tricladida and Acoela in their molecular phylogeny of the phylum Platyhelminthes, based on partial 18S rDNA sequences. In the analyzed fragment of EF1a, about 15

specific amino acid substitutions define the triclade, whereas only about 10 define the polyclad clade. NJ and ML analyses might thus be misleading because of the numerous amino acid substitutions that define the triclade clade, which tend to group the acel and polyclad sequences.

More sequences from representatives of all turbellarian orders and of members of the Neodermata would perhaps help to clarify this situation. It has been shown (Lecointre et al. 1993; Giribet and Ribera 1998) that increasing the number of taxa in a clade can help to strengthen its monophyly, although it also very rapidly increases the duration of the analyses. However, even more EF1a sequences may not definitively resolve the relationships within the Platyhelminthes. As shown by our analyses, the phylogeny of the phyla for which more EF1a sequences are available is not always well resolved. Whereas the internal topology of the arthropod clade is always well defined in all trees, the one of the annelid plus pogonophoran clade is much vaguer. This shows that the phylogenetic information contained in EF1a sequences is not the same for all metazoan groups. EF1a is thus probably not the perfect gene for inferring good metazoan phylogenies, but neither is 18S rDNA, as shown by Abouheif, Zardoya, and Meyer (1998). Both genes give conflicting results, which prompts us to search for another molecular marker to conclusively resolve the phylogenetic position of Acoela. Our study stresses the need for the use of several different genes to properly determine the phylogenetic position of any group of organisms.

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