

## Letter to the Editor

### Elongation Factor 1-Alpha Sequences Alone Do Not Assist in Resolving the Position of the Acoela Within the Metazoa

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Following years of debate, and thanks largely to molecular systematic studies, the long-held idea that the phylum Platyhelminthes is the most basal branch of the bilaterian Metazoa has been widely abandoned (e.g., Haszprunar 1996; Carranza, Bagnà, and Riutort 1997; Hausdorf 2000). This new status quo has been upset by a recent paper that has suggested that the Platyhelminthes are polyphyletic and that a single “flatworm” clade, the Acoela, is the most basal extant bilaterian lineage distinct from the other platyhelminths (Ruiz-Trillo et al. 1999). The potential importance of the acoels being confirmed as the outgroup to all other bilaterians is that they would then be instrumental in determining the character states of the ancestral Bilateria through outgroup comparison and thus greatly further our understanding of the evolution of the animals. The currently recognized outgroup—the diploblasts—are generally too evolutionarily and phenotypically distant for this sort of comparison to be informative.

Although recent data support the molecular results (Henry, Martindale, and Boyer 2000), some systematists distrust the findings based on 18S rDNA and have claimed that long-branch attraction (LBA; Felsenstein 1978) affected the final analysis of these data (e.g., Adoutte et al. 2000). Furthermore, according to the same 18S rDNA study (Ruiz-Trillo et al. 1999), the supposed sister group of the acoels, the nemertodermatids (Smith and Tyler 1985; Smith, Tyler, and Rieger 1986; Tyler 2001), do not unite with the acoels at the base of the Bilateria, but instead fall within the turbellarian flatworms. This has suggested that the more controversial position of their sister group, the acoels, was erroneous.

Recently, it has been proposed that the published putative nemertodermatid sequence used by Ruiz-Trillo et al. (1999) (*Nemertinoidea elongatus*, accession number U70083; Carranza, Bagnà, and Riutort 1997) that grouped among the Rhabditophora may have been misidentified (Jondelius et al. 2000).

Berney, Pawloski, and Zaninetti (2000) directly contradict the basal position of the Acoela based on

analyses of elongation factor 1-alpha (EF1a). This gene was chosen with the aim of avoiding LBA. Comparing EF1a sequences derived from a single acoel species, *Convoluta roscoffensis*, with those of other animals and fungi, they forward two lines of evidence suggesting that acoels are not basal metazoans, but rather that they are flatworms, as historically regarded. Their evidence comes not only from a standard phylogenetic comparison of the amino acid and nucleotide sequences of EF1a that places the acoels within the turbellarian flatworms (allied either to the triclads or to the polyclads, depending on the analysis), but also from the presence of a putative insertion of four amino acids in common between the acoels and the triclads that are not found in other EF1a protein sequences compared. Importantly, they must, according to the common possession of the four-amino-acid signature, be derived flatworms allied among the rhabditophorans to the Tricladida.

It is in part the derived position of the acoels within, rather than basal to, the Rhabditophora (the more commonly espoused position according to morphological analyses, e.g., Ehlers 1984; Littlewood, Rohde, and Clough 1999) based on EF1a that has prompted us to examine these claims more closely. In addition to contradicting the results based on 18S rDNA (Ruiz-Trillo et al. 1999), the conclusions of Berney, Pawloski, and Zaninetti (2000) are in conflict with a study (Telford et al. 2000) that has lent support to the idea of a monophyletic Rhabditophora (Ehlers 1984). This analysis of the mitochondrial genetic codes of the flatworms shows that all rhabditophoran flatworms have two differences in their genetic code compared with most other invertebrates: the codons AAA, coding for asparagine as opposed to lysine, and AUA, coding for isoleucine as opposed to methionine (Telford et al. 2000). Acoela, Nemertodermatida, and Catenulida all shared the plesiomorphic condition and were hence excluded from the monophyletic Rhabditophora. Two such convincing synapomorphies are in direct conflict with the interpretation of the EF1a data of Berney, Pawloski, and Zaninetti (2000); clearly, the acoels cannot be both excluded from the Rhabditophora and a sister group of the rhabditophoran triclads or polyclads. Consideration of the morphology casts further doubt on this derived rhabditophoran position of the acoels, as they lack all rhabditophoran or triclad morphological synapomorphies (Tyler 2001). Consequently, we looked carefully at the analyses of Berney, Pawloski, and Zaninetti (2000) to assess their robustness. We provided new EF1a sequenc-

Abbreviations: EF1a, elongation factor 1-alpha; LBA, long-branch attraction; MP, maximum parsimony; NJ, neighbor joining; rDNA, ribosomal DNA.

Key words: elongation factor 1-alpha, Acoela, Platyhelminthes, Metazoa.

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es from three additional species of acoels and seven platyhelminths and combined these with 21 previously published flatworm *Ef1a* sequences to reassess the phylogenetic content of the gene and the homology of the amino acid insertion described. We refute the conclusions of Berney, Pawloski, and Zaninetti (2000).

New sequences were determined from ethanol-preserved flatworms and added to sequences available from GenBank. Berney, Pawloski, and Zaninetti (2000) kindly provided their original alignment files. Genomic DNA was extracted as in Littlewood, Rohde, and Clough (1999). Partial sequences were PCR-amplified using Ready-To-Go (Amersham Pharmacia Biotech) beads and primers EF1a-5' (5'-WCTACMGGWCATCTMATT) and EF1a-3' (5'-AAAGCGACCRAGWGGTGG), which span positions 89–1306 of the EF1a sequence of *Schistosoma mansoni* (accession number Y08487). Cycling conditions were as follows: 3 min at 96°C; 40 cycles of 96°C for 1 min, 54°C for 1 min, and 72°C for 2 min; and 7 min at 72°C. Purified products (Wizard Preps, Promega) were sequenced (following Telford et al. 2000) with the original PCR primers, in addition to internal forward and reverse primers EF-400F (5'-GGTGARTTYGAAGCWGGTAT), EF-710F (5'-AARATYGGYGGYATTGG), and EF-710R (5'-CCA-TACCRCCRCRATYTT). Ten new platyhelminth taxa were characterized (see fig. 1 for details). The alignment of Berney, Pawloski, and Zaninetti (2000), including 45 taxa, was used, but with the addition of 38 sequences from all available new and previously published platyhelminth taxa, an echinoderm, a nematode, two molluscs, and two myzostomids. The inclusion of the new platyhelminth sequences demonstrated additional regions of ambiguity, which were removed prior to phylogenetic analysis. The main exclusion sets affecting all taxa appear in regions 158–161 and 214–224 relative to the *S. mansoni* EF1a sequence (accession number Y08487). These regions, spanning 4 and 21 amino acids, are illustrated in figure 1 (regions A and B, respectively). Regions spanning the introns found in one or more taxa (commonly among the acoel taxa) were removed prior to analysis. New sequences were marginally shorter at the 3' end of the alignment used by Berney, Pawloski, and Zaninetti (2000), and 20 amino acids were omitted from the subsequent analyses, although our alignment provided 10 additional phylogenetically informative positions. The full alignment comprised 250 unambiguously alignable amino acid positions, of which 140 were parsimony-informative; the full alignment may be obtained by anonymous FTP from FTP.EBI.AC.UK under directory pub/databases/embl/align, accession number ds45328).

The partial sequence of *Suomina* (AF288065) was excluded from the analyses, although it provides information on the putative synapomorphy (fig. 1). Phylogenies were estimated with maximum parsimony (MP) and neighbor joining (NJ) using a PAM-weighted amino acid step-matrix (Telford 2001); gaps were treated as missing. Analyses were conducted both including and excluding the echinoderm, mollusc, nematode, and my-

zostomid taxa. Topologies were rooted at the node separating the Fungi from the Metazoa.

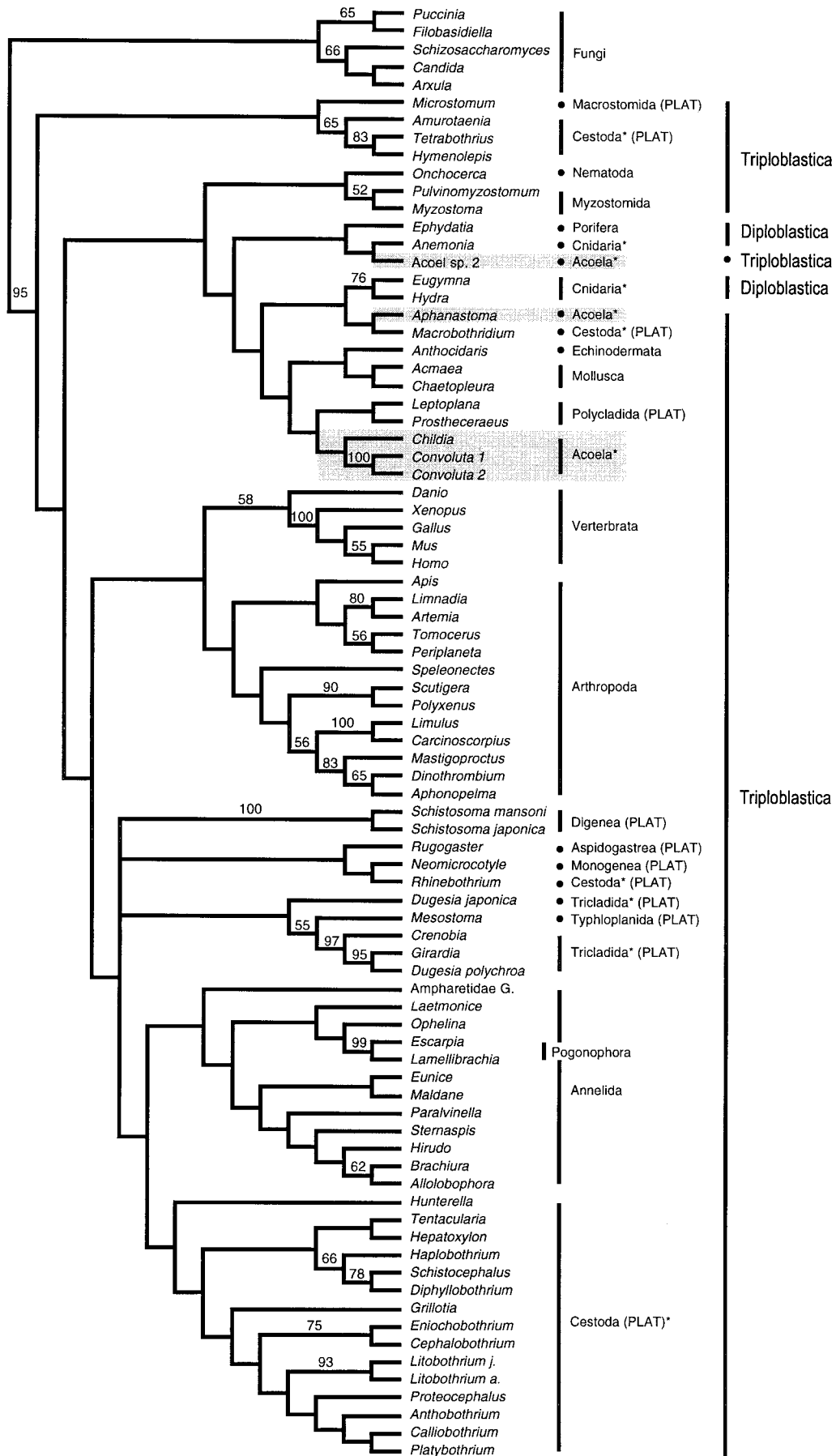
Our alignment of 5 fungi and 78 ingroup species included 38 platyhelminths, of which 4 were acoels. Figure 1 shows a portion of our alignment covering the “12-amino-acid insertion shared by fungi and metazoans” illustrated by Berney, Pawloski, and Zaninetti (2000, p. 1035). Clearly, the insertion shared by the acoel, *Convoluta roscofennis*, and the triclads is not shared by other acoels. Neither *Aphanastoma* nor the undescribed acoel species have any insertion and, although *Childia* has an insertion of 10 amino acids, it is difficult to propose an unambiguous alignment with the KKEE motif in *Convoluta* (see fig. 1). Within the Platyhelminthes, there is little evidence of homology among the amino acid sequences in this region; even within the Cestoda, this region is highly variable. The mollusc *Acmaea testudinalis* (U90061) also shows an insertion of five amino acids (KGNAS), although, again, amino acid positional homology cannot be reliably established. The KK(ED)E motif uniting acoels with triclads or with other members of the Rhabditophora appears unfounded. The KKEE motif shared between *Convoluta* and the triclads either is due to convergence or is an artefact of imposed positional homology in the alignment of Berney, Pawloski, and Zaninetti (2000).

While we recognize the contribution EF1a has made to some phylogenetic studies (e.g., arthropods; Shultz and Regier 2000), we are skeptical as to its utility at a wider metazoan level. We can find no mention in the literature as to its use at this level other than cautionary examples on deeper eukaryote phylogenies (Moreira, Le Guyader, and Philippe 1999; Roger et al. 1999).

At a higher taxonomic level, both MP and NJ yielded biologically unfounded trees, but to illustrate the general problems, we present only the MP results on amino acids of the full set of taxa. MP analysis, employing 20 replicate heuristic searches with the tree bisection-reconnection (TBR) branch-swapping algorithm, yielded two equally parsimonious trees; the strict consensus is illustrated in figure 2. Arthropods, vertebrates, molluscs, myzostomids, and annelids + pogonophorans are each represented as monophyletic groups. Bootstrap support is very weak throughout the tree, except for those nodes uniting relatively closely related taxa. Diploblasts, triploblasts, deuterostomes, and, indeed, Bilateria are each polyphyletic. Even within the polyphyletic “platyhelminths,” acoels, triclads, cestodes, and polyclads are not monophyletic, and the interrelationships among the flatworms bear little resemblance to previously published morphological or molecular estimates (e.g., Littlewood, Rohde, and Clough 1999). NJ trees are comparable in their inability to resolve meaningful relationships within or between metazoan phyla.

Berney, Pawloski, and Zaninetti (2000) omitted nematodes, molluscs, and echinoderms “because of their artifactual branching at the base of the Metazoa”







Rhabditophora and hence unrelated to the other “Turbellaria” (Ehlers 1984; Lundin 2000; Jondelius et al. 2000; Telford et al. 2000). The findings based on EF1a therefore do not contradict Ruiz-Trillo et al. (1999).

Reanalysis of Ef1a sequences shows them to be largely insufficient when considered in isolation. Alone, the gene cannot be used to arbitrate convincingly on the position of the acoels. Morphological studies already seem set to contribute further to our understanding of this problem (Henry, Martindale, and Boyer 2000), but from a molecular perspective there are two obvious alternative approaches, both nicely illustrated by Berney, Pawloski, and Zaninetti (2000). First, it is obviously important to use sequences of genes in addition to 18S rDNA as independent sources of data (Mitchell, Mitter, and Regier 2000). Using multiple genes controls for the possibility of positively misleading, location-dependent processes in sequence evolution (Cummings, Otto, and Wakely 1995), and other genes may help to corroborate or refute the results of Ruiz-Trillo et al. (1999).

The second approach is to look for rare and hopefully nonhomoplasious molecular synapomorphies, characters such as unique insertion/deletion events, mitochondrial gene rearrangements, change in mitochondrial genetic codes, and so on. If the acoels are not basal (presumably deriving from within the lophotrochozoan branch), there should be little difficulty in demonstrating this. Any lophotrochozoan apomorphy shared by the acoels, with the plesiomorphic state being found in both deuterostomes and ecdysozoans, will support a derived position of the acoels (Telford 2000). In contrast, demonstrating the acoels to be basal may be more difficult. To demonstrate this cladistically requires a synapomorphy uniting all Bilateria except acoels. In addition, acoels must have the plesiomorphic condition in common with an outgroup, or their character state might simply be an autapomorphy and, thus, uninformative. This latter condition seems difficult to fulfill due to the evolutionary distance of the closest metazoan outgroups, the diploblasts.

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### LITERATURE CITED

- AOUTTE, A., G. BALAVOINE, N. LARTILLOT, O. LESPINET, B. PRUD'HOMME, and R. DE ROSA. 2000. The new animal phylogeny: reliability and implications. *Proc. Natl. Acad. Sci. USA* **97**:4453–4456.
- BERNEY, C., J. PAWLOSKI, and L. ZANINETTI. 2000. Elongation factor 1-alpha sequences do not support an early divergence of the Acoela. *Mol. Biol. Evol.* **17**:1032–1039.
- CARRANZA, S., J. BAGUÑA, and M. RIUTORT. 1997. Are the Platyhelminthes a monophyletic primitive group? An assessment using 18S rDNA sequences. *Mol. Biol. Evol.* **14**:485–497.
- CUMMINGS, M. P., S. P. OTTO, and J. WAKELY. 1995. Sampling properties of DNA sequence data in phylogenetic analysis. *Mol. Biol. Evol.* **12**:814–822.
- EHLERS, U. 1984. Phylogenetisches System der Plathelminthes. *Verh. Naturwiss. Ver. Hamburg (NF)* **27**:291–294.
- FELSENSTEIN, J. 1978. Cases in which parsimony or compatibility methods will be positively misleading. *Syst. Zool.* **27**:401–410.
- HASZPRUNAR, G. 1996. Plathelminthes and Plathelminthomorpha—paraphyletic taxa. *J. Zool. Syst. Evol. Res.* **34**:41–48.
- HAUSDORF, B. 2000. Early evolution of the Bilateria. *Syst. Biol.* **49**:130–142.
- HENRY, J. Q., M. Q. MARTINDALE, and B. C. BOYER. 2000. The unique developmental program of the acoel flatworm, *Neochildia fusca*. *Dev. Biol.* **220**:285–295.
- JONDELIUS, U., I. RUIZ-TRILLO, J. BAGUÑA, and M. RIUTORT. 2000. Nemertodermatida, a basal bilaterian group. Ninth International Symposium on the Biology of the Turbellaria. *Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain.*
- LITTLEWOOD, D. T. J., K. ROHDE, and K. A. CLOUGH. 1999. The interrelationships of all major groups of Platyhelminthes: phylogenetic evidence from morphology and molecules. *Biol. J. Linn. Soc.* **66**:75–114.
- LUNDIN, K. 2000. Phylogeny of the Nemertodermatida (Acoelomorpha, Platyhelminthes). A cladistic analysis. *Zool. Scripta* **29**:65–74.
- MITCHELL, A., C. MITTER, and J. C. REGIER. 2000. More taxa or more characters revisited: combining data from nuclear protein-encoding genes for phylogenetic analyses of Noctuidae (Insecta: Lepidoptera). *Syst. Biol.* **49**:202–224.
- MOREIRA, D., H. LE GUYADER, and H. PHILIPPE. 1999. Unusually high evolutionary rate of the elongation factor 1 alpha genes from the Ciliophora and its impact on the phylogeny of eukaryotes. *Mol. Biol. Evol.* **16**:234–245.
- ROGER, A. J., O. SANDBLOM, W. F. DOOLITTLE, and H. PHILIPPE. 1999. An evaluation of elongation factor 1 alpha as a phylogenetic marker for eukaryotes. *Mol. Biol. Evol.* **16**:218–233.
- RUIZ-TRILLO, I., M. RIUTORT, D. T. J. LITTLEWOOD, E. A. HERNIOU, and J. BAGUÑA. 1999. Acoel flatworms: earliest extant bilaterian metazoans, not members of Platyhelminthes. *Science* **283**:1919–1923.
- SHULTZ, J. W., and J. C. REGIER. 2000. Phylogenetic analysis of arthropods using two nuclear protein-encoding genes supports a crustacean plus hexapod clade. *Proc. R. Soc. Lond. B Biol. Sci.* **267**:1011–1019.

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FIG. 2.—Results of maximum-parsimony analysis of EF1a sequences from a broadly represented platyhelminth and metazoan taxa set; strict consensus of two trees (see fig. 1 and text). The positions of the acoel taxa are highlighted. All platyhelminths are designated (PLAT), bootstrap values ( $\geq 50\%$ ,  $n = 1,000$ ) are shown above nodes, and poly/paraphyletic clades are designated by asterisks. Tree length = 12,455; consistency index = 0.31; retention index = 0.49; rescaled consistency index = 0.16.

- SMITH, J. P. S., and S. TYLER. 1985. The acoel turbellarians: kingpins of metazoan evolution or a specialized offshoot? Pp. 123–142 in S. CONWAY MORRIS, J. D. GEORGE, R. GIBSON, and H. M. PLATT, eds. *The origins and relationships of the lower invertebrates*. Oxford University Press, Oxford, England.
- SMITH, J. P. S., S. TYLER, and R. M. RIEGER. 1986. Is the Turbellaria polyphyletic? *Hydrobiologia* **132**:13–21.
- TELFORD, M. J. 2000. Evidence for the derivation of the *Drosophila fushi tarazu* gene from a Hox gene orthologous to lophotrochozoan Lox5. *Curr. Biol.* **10**:349–352.
- . 2001. Turning Hox ‘signatures’ into synapomorphies. *Evol. Dev.* (in press).
- TELFORD, M. J., E. A. HERNIOU, R. B. RUSSELL, and D. T. J. LITTLEWOOD. 2000. Changes in mitochondrial genetic codes as phylogenetic characters: two examples from the flatworms. *Proc. Natl. Acad. Sci. USA* **97**:11359–11364.
- TYLER, S. 2001. The early worm—origins and relationships of the lower flatworms. Pp. 3–12 in D. T. J. LITTLEWOOD and R. A. BRAY, eds. *Interrelationships of the Platyhelminthes*. Taylor and Francis, London.

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