

## Evaluating multiple alternative hypotheses for the origin of Bilateria: An analysis of 18S rRNA molecular evidence

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**ABSTRACT** Six alternative hypotheses for the phylogenetic origin of Bilateria are evaluated by using complete 18S rRNA gene sequences for 52 taxa. These data suggest that there is little support for three of these hypotheses. Bilateria is not likely to be the sister group of Radiata or Ctenophora, nor is it likely that Bilateria gave rise to Cnidaria or Ctenophora. Instead, these data reveal a close relationship between bilaterians, placozoans, and cnidarians. From this, several inferences can be drawn. Morphological features that previously have been identified as synapomorphies of Bilateria and Ctenophora, e.g., mesoderm, more likely evolved independently in each clade. The endomesodermal muscles of bilaterians may be homologous to the endodermal muscles of cnidarians, implying that the original bilaterian mesodermal muscles were myoepithelial. Placozoans should have a gastrulation stage during development. Of the three hypotheses that cannot be falsified with the 18S rRNA data, one is most strongly supported. This hypothesis states that Bilateria and Placozoa share a more recent common ancestor than either does to Cnidaria. If true, the simplicity of placozoan body architecture is secondarily derived from a more complex ancestor. This simplification may have occurred in association with a planula-type larva becoming reproductive before metamorphosis. If this simplification took place during the common history that placozoans share with bilaterians, then placozoan genes that contain a homeobox, such as *Trox2*, should be explored, for they may include the gene or genes most closely related to *Hox* genes of bilaterians.

Despite numerous speculations and analyses concerning the evolutionary relationships of the major animal clades, until recently only three distinct hypotheses have been offered concerning the phylogenetic position of Bilateria within the other basal metazoan groups (Fig. 1 A–C). Two of these hypotheses are commonly found in zoology textbooks. One view, an idea that goes back to Haeckel (1) and was championed by Hyman (2, 3), is that Bilateria is the sister group to Radiata (Cnidaria and Ctenophora). A second hypothesis, preferred by Harbison (4) and Wilmer (5), holds that Bilateria and Ctenophora share a more recent common ancestor than either does to Cnidaria. Recent cladistic studies based on morphological characters (6, 7) have supported this second view. A third alternative, which appears to have fallen out of favor, has Bilateria as a nonmonophyletic group, with both cnidarians and ctenophorans derived from flatworm ancestors (8). The strength of these hypotheses is difficult to weigh on morphological evidence alone. The diploblastic groups have disparate body plans with many derived features, only a few of which are potentially informative as to their relative phylogenetic positions. Furthermore, homoplasies are difficult to infer when dealing with such general features as mouths, mesoderm, and symmetry.

Molecular sequence studies provide sets of characters that can be used to both evaluate previous phylogenetic hypotheses and generate new ones. The challenge of testing new hypotheses

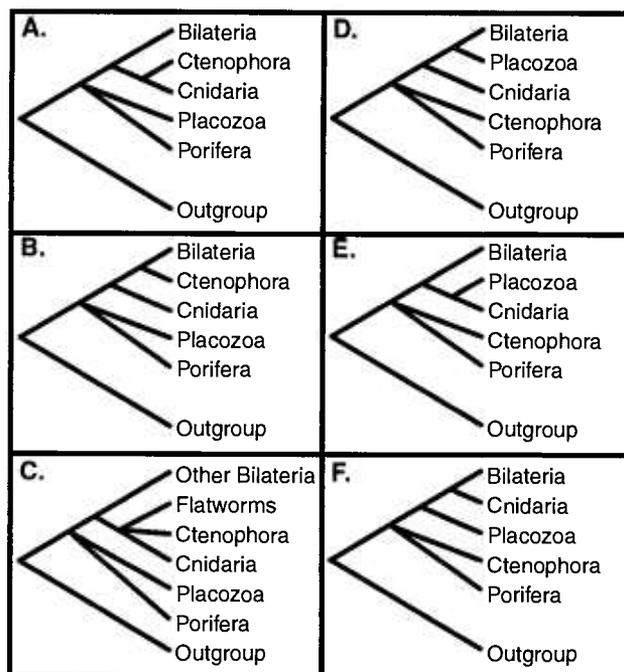


FIG. 1. Six alternative hypotheses for the origin of the Bilateria.

forces us to look at old data in new ways. In the case of bilaterian origins, molecular sequence data for the 18S rRNA gene show a possible relationship of Bilateria to Placozoa and Cnidaria, to the exclusion of Ctenophora and Porifera (9–12). To this point, no assessment of the likelihood of this possibility has been made. More importantly, almost nothing has been written about what this phylogenetic arrangement implies if true. These 18S gene data prompt us to consider three additional hypotheses for the origin of Bilateria (Fig. 1 D–F). Each of the six alternative hypotheses shown in Fig. 1 has different implications for characterizing the origin of Bilateria. Here I evaluate the strengths of the six hypotheses with a data set that includes 10 additional complete 18S gene sequences and clarify inferences based on those hypotheses that appear to be the more robust.

### MATERIALS AND METHODS

All primer sequences, 18S gene sequences, aligned data sets, and PAUP\* data sets are publicly available at the archived data web pages of the University of California Museum of Paleontology ([www.ucmp.berkeley.edu/archdata/Collins98/bilateria.html](http://www.ucmp.berkeley.edu/archdata/Collins98/bilateria.html)) as well as on request.

Abbreviations: Bsi, Bremer support index; 18S, small subunit of rRNA; T-PTP, topology-dependent permutation tail probability. Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF100940–AF100949).

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Table 1. List of species with Linnean classification and GenBank accession numbers

| Species and classification           | GenBank accession number |
|--------------------------------------|--------------------------|
| Sequences generated by the author    |                          |
| Choanoflagellida                     |                          |
| <i>Monosiga brevicolis</i>           | AF100940                 |
| <i>Salpingoeca infusionum</i>        | AF100941                 |
| Cnidaria, Scyphozoa                  |                          |
| <i>Atolla vanhoeffeni</i>            | AF100942                 |
| Cnidaria, Anthozoa                   |                          |
| <i>Antipathes galapagensis</i>       | AF100943                 |
| Ctenophora, Pleurobrachidae          |                          |
| <i>Hormiphora</i> sp.                | AF100944                 |
| Porifera, Calcarea                   |                          |
| <i>Leucosolenia</i> sp.              | AF100945                 |
| Porifera, Demospongiae               |                          |
| <i>Mycale fibrexilis</i>             | AF100946                 |
| <i>Suberites ficus</i>               | AF100947                 |
| <i>Plakortis</i> sp.                 | AF100948                 |
| Porifera, Hexactinellida             |                          |
| <i>Rhabdocalypus dawsoni</i>         | AF100949                 |
| Sequences culled from GenBank        |                          |
| Bilateria, Annelida                  |                          |
| <i>Lanice conchilega</i>             | X79873                   |
| Bilateria, Chordata                  |                          |
| <i>Herdmania momus</i>               | X53538                   |
| <i>Latimeria chalumnae</i>           | L11288                   |
| Bilateria, Echinodermata             |                          |
| <i>Strongylocentrotus purpuratus</i> | L28055                   |
| <i>Amphipholis squamata</i>          | X97156                   |
| Bilateria, Echiura                   |                          |
| <i>Ochetostoma erythrogrammon</i>    | X79875                   |
| Bilateria, Hemichordata              |                          |
| <i>Balanoglossus carnosus</i>        | D14359                   |
| Bilateria, Mollusca                  |                          |
| <i>Tresus nuttali</i>                | L11269                   |
| <i>Limicolaria kambeul</i>           | X66374                   |
| Bilateria, Nematomorpha              |                          |
| <i>Gordius aquaticus</i>             | X87985                   |
| Bilateria, Nemertea                  |                          |
| <i>Lineus</i> sp.                    | X79878                   |
| Bilateria, Platyhelminthes           |                          |
| <i>Stenostomum</i> sp.               | U95947                   |
| <i>Planocera multitentaculata</i>    | D83383                   |
| <i>Schistosoma mansoni</i>           | X53986                   |
| Bilateria, Pogonophora               |                          |
| <i>Siboglinum fiordicum</i>          | X79876                   |
| Bilateria, Vestimentifera            |                          |
| <i>Ridgeia piscesae</i>              | X79877                   |
| Choanoflagellida                     |                          |
| <i>Acanthocephalus unguiculata</i>   | L10823                   |
| <i>Diaphanoeca grandis</i>           | L10824                   |
| Cnidaria, Anthozoa                   |                          |
| <i>Leiopilus fimbriatus</i>          | Z92903                   |
| <i>Anemonia sulcata</i>              | X53498                   |
| <i>Haliplanella lucia</i>            | Z86097                   |
| <i>Bellonella rigida</i>             | Z49195                   |
| <i>Calicogorgia granulosa</i>        | Z92900                   |
| <i>Virgularia gustaviana</i>         | Z86106                   |
| <i>Tubastraea aurea</i>              | Z92906                   |
| <i>Parazoanthus axinellae</i>        | U42453                   |
| Cnidaria, Cubozoa                    |                          |
| <i>Tripedalia cystophora</i>         | L10829                   |
| Cnidaria, Hydrozoa                   |                          |
| <i>Coryne pusilla</i>                | Z86107                   |
| <i>Hydra littoralis</i>              | U32392                   |
| <i>Obelia</i> sp.                    | Z86108                   |
| <i>Selaginopsis cornigera</i>        | Z92899                   |

Table 1. (Continued)

| Species and classification      | GenBank accession number |
|---------------------------------|--------------------------|
| Ctenophora, Lobata              |                          |
| <i>Mnemiopsis leidyi</i>        | L10826                   |
| <i>Incertae setis</i>           |                          |
| <i>Dermocystidium salmonis</i>  | U21337                   |
| <i>Ichthyophonus hoferi</i>     | U25637                   |
| Rosette agent of chinook salmon | L29455                   |
| Placozoa                        |                          |
| <i>Trichoplax adhaerens</i>     | L10828                   |
| <i>Trichoplax</i> sp.           | Z22783                   |
| Porifera, Calcarea              |                          |
| <i>Clathrina cerebrum</i>       | U42452                   |
| <i>Scypha ciliata</i>           | L10827                   |
| Porifera, Demospongiae          |                          |
| <i>Axinella polypoides</i>      | U43190                   |
| <i>Microciona prolifera</i>     | L10825                   |
| <i>Tetilla japonica</i>         | D15067                   |

Genomic DNA was isolated from tissue samples of 10 species (Table 1). The method that most consistently yielded high molecular weight genomic DNA consisted of pulverization of previously frozen ( $-80^{\circ}$ ) tissue in the reagent DNAzol (Molecular Research Center, Cincinnati), followed by centrifugation and ethanol precipitation. The complete sequence for the 18S coding region was amplified from genomic DNA preparations using eukaryotic-specific primers (13) via PCR (30 cycles: 10 s at  $94^{\circ}$ , 60 s at  $37^{\circ}$ , and 180 s at  $72^{\circ}$ ) after an initial 2-min  $94^{\circ}$  denaturation. The PCR products of three species (*Atolla vanhoeffeni*, *Hormiphora* sp., and *Antipathes galapagensis*) were directly sequenced with an Applied Biosystems Prism 377 DNA Sequencer, whereas the remaining PCR products were cloned and pooled (minimum of eight clones) before sequencing with a Li-Cor (Lincoln, NE) model 4000L IR automated DNA sequencer.

Phylogenetic accuracy is increased by adding taxa that break up branches (14–16). Thus, the maximum number of sequences was analyzed given computational limitations imposed by maximum likelihood searches, which were only feasible with data sets up to roughly 50 taxa. Forty-two sequences not generated by me were culled from GenBank to create a 52-taxon data set with reasonably balanced sampling across the basal metazoan groups. Representatives from two nonmetazoan clades were included as outgroups: the single-celled choanoflagellates, and an odd group of fish parasites that include the common aquarium pest Ich. Previous work has established the phylogenetic proximity of these groups to the Metazoa (9, 17, 18). The 52 sequences (Table 1) used in this analysis were aligned by eye with well over 100 published and unpublished metazoan 18S gene sequences by using the Genetic Data Environment sequence editor (written by Steve Smith, Millipore). Characters for which putative homology could not be asserted were excluded to arrive at a final data set of characters for phylogenetic analysis. This data set consists of 1,526 nucleotide characters for the 52 taxa. All subsequent analyses were carried out on this data set by using PAUP\* 4.0 (19).

Three common optimality criteria for evaluating phylogenetic trees were used: parsimony, minimum evolution, and maximum likelihood (see ref. 20 for review). The “best” tree obtained by these methods is the one that optimizes the given criterion. Parsimony seeks to minimize the number of character changes or steps throughout the tree. Seven separate heuristic searches were performed. An initial search with 100 replicates was performed without any topological constraints. A Bremer support analysis to assess branch support (21, 22)



Table 2. Comparison of alternative hypotheses using three methods of phylogenetic reconstruction

| Alternative phylogenetic hypotheses | Number of trees | Cladistic parsimony |               |      | Maximum likelihood |               |      | Minimum evolution |               |          |
|-------------------------------------|-----------------|---------------------|---------------|------|--------------------|---------------|------|-------------------|---------------|----------|
|                                     |                 | Score               | % difference  | Rank | Score              | % difference  | Rank | Score             | % difference  | Rank     |
| A                                   | 8               | 3,517               | 0.486%        | 4    | 18,729             | 0.162%        | 4    | 2,73752           | 0.596%        | 4        |
| B                                   | 2               | 3,521               | 0.600%        | 5    | 18,730             | 0.168%        | 5    | 2,73957           | 0.672%        | 5        |
| C                                   | 6               | 3,590               | 2.571%        | 6    | 18,987             | 1.543%        | 6    | 2,80954           | 3.243%        | 6        |
| D                                   | 5               | <b>3,500</b>        | <b>0.000%</b> | 1    | 18,699             | 0.003%        | 2    | <b>2,72129</b>    | <b>0.000%</b> | <b>1</b> |
| E                                   | 2               | 3,509               | 0.257%        | 3    | 18,700             | 0.006%        | 3    | 2,7236            | 0.085%        | 2        |
| F                                   | 2               | 3,508               | 0.229%        | 2    | <b>18,699</b>      | <b>0.000%</b> | 1    | 2,72532           | 0.148%        | 3        |

Comparison of optimal tree scores under the six alternative hypotheses. Letters A–F refer to Fig. 1. For each methodology of phylogenetic reconstruction, tree scores are ranked and percent difference from the optimal tree score is calculated. Bold type denotes the optimal score for each of the methodologies.

were conducted. The T-PTP was passed at the 95% level or greater for all 40 two-tree comparisons associated with hypothesis A, all 10 with B, and all 30 with C. The T-PTP was not consistently passed for pairs of trees generated under hypotheses E and F. Mean T-PTP values for each of the sets of two-tree comparisons were: A, 0.007; B and C, 0.002; E, 0.075; and F, 0.105. The most parsimonious topology is significantly shorter than the topologies consistent with alternative hypotheses A–C, and it may not be significantly shorter than those consistent with hypotheses E and F.

The unconstrained optimal trees found with the methods of minimum evolution and maximum likelihood are shown in Fig. 3. The minimum evolution tree conforms to hypothesis D, as did the most parsimonious trees, whereas the maximum likelihood tree conforms to hypothesis F. A comparison of the optimal tree scores found with the minimum evolution and maximum likelihood methods under the six alternative hypotheses shows that with both methods of tree reconstruction, topologies constrained to not violate hypotheses A–C are farther from the optimal score than hypotheses D–F (Table 2).

## DISCUSSION

Three phylogenetic methodologies applied to 18S gene sequences suggest that Bilateria, Placozoa, and Cnidaria form a clade to the exclusion of Ctenophora and Porifera. Thus, without considering the philosophical and mathematical debates concerning the most appropriate and accurate algorithms for phylogenetic reconstruction, these data contradict the three hypotheses (A–C) that previously had been proposed for bilaterian origins. The results of the T-PTP tests provide further evidence that undermines hypotheses A–C. However, the T-PTP test is one in a class of techniques, including bootstrapping and jack-knifing, that destroy information by permuting, sampling, and/or deleting characters. The goal in using such strategies is to generate numerous sets of data to which actual data can be compared to make statistical statements. But, actual data used in phylogenetic analyses were generated by evolutionary processes just once. Statistical analyses of such data, with a sample size of one, always will be somewhat questionable. What makes a result from a phylogenetic analysis truly convincing is not high bootstrap values or passing T-PTPs, but a redundancy of results and corroborating evidence.

The three hypotheses that remain (D–F) are difficult to resolve with 18S gene sequence data, although hypothesis D appears to have the most support. Parsimony and minimum evolution analyses both indicate that the sister group to Bilateria is Placozoa. Furthermore, the branch support analysis (Fig. 2) indicates that the node joining Bilateria to Placozoa has a relatively high level of support. On the other hand, the maximum likelihood analysis points to a cnidarian-bilaterian relationship to the exclusion of the placozoans. A strict consensus of hypotheses D–F represents perhaps the best

working hypothesis given the data at hand. Future analyses of additional molecular characters and analyses that combine molecular and morphological characters will be necessary to test this result.

This consensus hypothesis has corollaries that illuminate the early evolution of Bilateria. Two recent phylogenetic studies of Metazoa placed Ctenophora as the sister group to Bilateria based on morphological characters (6, 7). Because these phylogenies were generated by cladistic analyses, they embody specific hypotheses of character evolution. For instance, Schram's analysis (6) suggests that mesoderm, determinate cleavage, and subepidermal muscles are synapomorphies that join Ctenophora and Bilateria. On the other hand, the analysis of Nielsen *et al.* (7) implies that synapses with acetylcholine, multiciliate epithelia, sperm with a single compact acrosome, and mesoderm are characters that were present in the last common ancestor of Ctenophora and Bilateria. The 18S data presented here cast doubt on these proposed homologies. The derived features that members of Ctenophora share with some members of Bilateria either arose independently or were lost in Cnidaria and Placozoa. The former possibility appears to be slightly more likely as it requires one fewer character state changes. If true, this scenario implies that some stem group bilaterians, below the node that joins all living bilaterians, probably did not possess a third tissue layer.

Another inference is that the endomesodermal muscles of bilaterians may be homologous to the endodermal muscles of cnidarians. Two basic types of muscle tissue are found in bilaterian animals (27). In one type, the muscle cells all have the same polarity and form an epithelial sheet, i.e., a myoepithelium. The second type consists of muscle cells without a common polarity embedded in a matrix of connective tissue. Rieger (27) noted that bilaterian muscle tissue originally derived from ectoderm is of the latter type whereas musculature that can be traced to endodermal tissues is usually myoepithelial. Ctenophoran muscles are very specialized (28) and are not epithelial. Cnidarians, on the other hand, possess both types of muscle tissue. The endodermal muscles of anthozoan gastric mesenteries are myoepithelial. Molecular and morphological evidence support a basal position for Anthozoa within Cnidaria (29). It is conceivable that the endomesodermal muscles of the original bilaterian were myoepithelial and derived from the endodermal muscles of a diploblastic ancestor.

The analysis presented here suggests that placozoans branch near the base of Bilateria and may comprise its sister group. Placozoans are composed of a ciliated epithelium that is differentiated dorsally and ventrally, between which is a mesenchymal syncytium (30). Acoel flatworms also possess a central syncytium (31). It is possible that the two syncytia are homologous if acoel flatworms are basal bilaterians. However, there is mounting evidence that at least some flatworms are derived lophotrochozoan protostomes (32), though there remains a possibility that flatworms are polyphyletic and that the

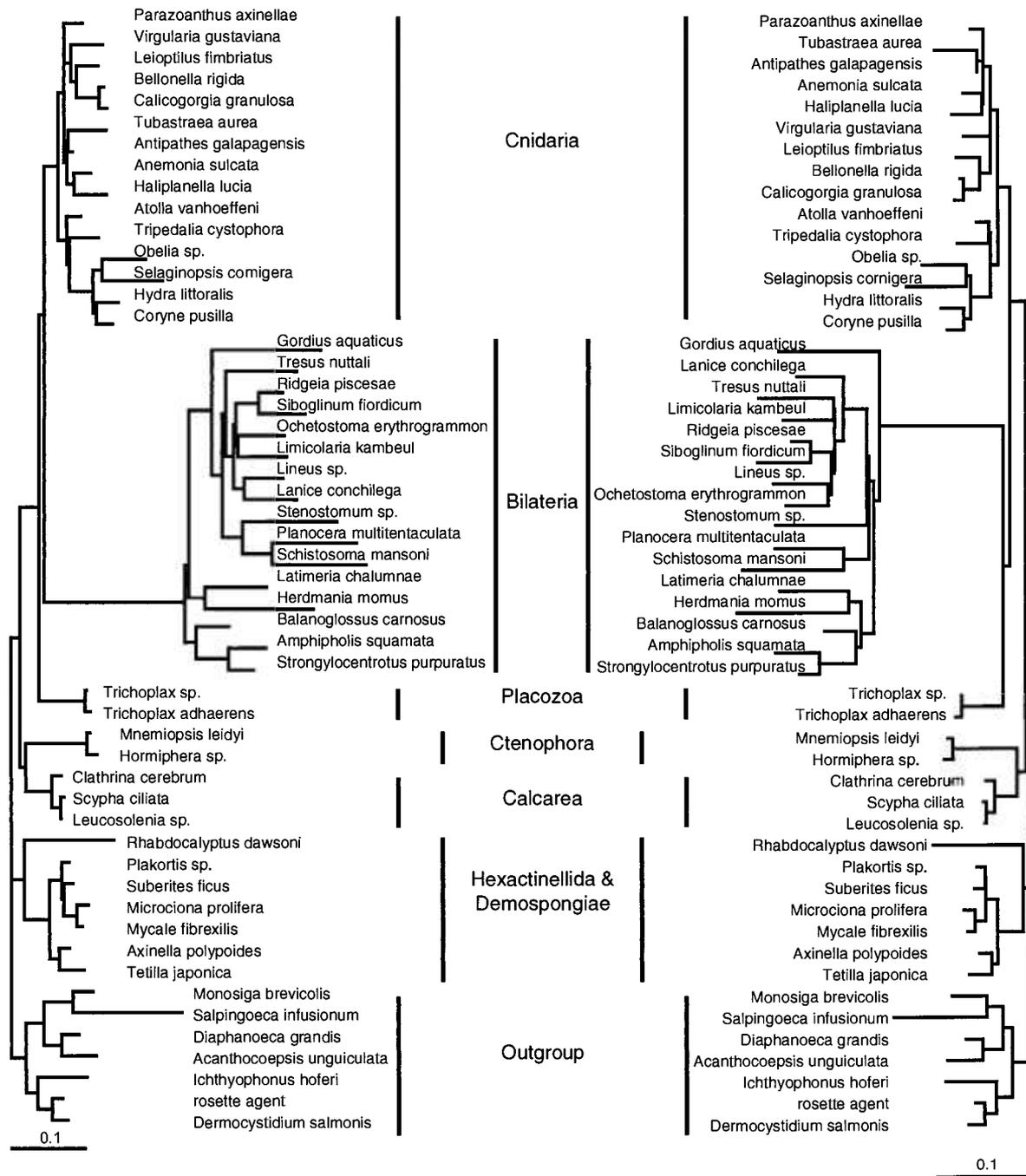


FIG. 3. Optimal trees under the criteria of maximum likelihood (*Left*) and minimum evolution (*Right*). In both analyses the HKY85 model of nucleotide evolution was used with a gamma shape parameter of 0.3365. The maximum likelihood analysis included an assumed transition-to-transversion ratio of 1.643.

acoels are the most basal clade of the Bilateria (33, 34). In any event, it is clear that placozoan development, which has not been observed beyond the 64-cell stage (35), should include gastrulation if Placozoa forms a clade with Bilateria and Cnidaria.

Placozoans are extremely simple animals, with just four distinct somatic cell types (35). In this respect they are simpler than most larvae of cnidarians, ctenophorans, and poriferans. The simplicity of placozoans has been used to argue that they are basal to Cnidaria, Ctenophora, and Bilateria (35, 36). The present phylogenetic analysis contradicts this assertion and instead suggests that placozoans are secondarily simplified. It is not clear whether any such simplification took place during the common history of Bilateria and Placozoa or after Plac-

ozoa diverged from Bilateria. The simplicity of the placozoan body appears to be mirrored by a relatively simple regulatory gene system. A recent study was able to identify just a single placozoan gene resembling *Hox* genes of the Antennapedia class (*Trox2*), whereas similar effort turned up five such genes each in hydrozoan and scyphozoan cnidarians (36). The authors concluded that homology could not be determined for these genes and those known in Bilateria, though others have attempted the difficult task of linking some homeobox genes of diploblasts with *Hox* genes of bilaterians (37). If Placozoa is the sister group to Bilateria, then placozoan genes that contain a homeobox, like *Trox2*, should be explored rather than ignored (37), for they may include the gene or genes most closely related to *Hox* genes of bilaterians.

A long history of discussions concerning bilaterian origins rely on planula-like larvae (see refs. 5 and 38 for reviews). In these scenarios, the ancestral bilaterian is a creeping ciliated larva resembling a planula that began to reproduce before metamorphosis. If placozoans represent an extant lineage stemming from a planula-type organism that also gave rise to Bilateria, then the simplicity of Placozoa would be expected. Simplification usually occurs in the context of dramatic changes in life mode between ancestor and descendant, e.g., parasitism and/or miniaturization. Losses can be of a fundamental nature. For instance, carnivorous sponges have lost the water filtration system that is diagnostic for all other poriferans (39). For placozoans, the dearth of *Hox* genes may be tied to a period of simplification. Regulatory genes are sometimes lost in conjunction with simplifications in animal body plans. For example, barnacles have lost a body region, the abdomen, and also appear to lack a *Hox* gene (*adbA*) that mediates the development of this region in other crustaceans (40). A larva that became able to survive and reproduce would no longer need the cell types or the regulatory genes that were necessary for its adult stage, and they might then be lost. If such an organism did give rise to Bilateria, then this stage in the evolution of Bilateria could be thought of as a phylogenetic bottleneck. The apparent lack of synapomorphies, including homologous *Hox* genes, linking Bilateria to any of the diploblastic groups would be explained.

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