

The Phylogenetic Affinities of the Chaetognaths: A Molecular Analysis¹

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The chaetognaths, or arrowworms, constitute a small and enigmatic phylum of marine invertebrates whose phylogenetic affinities have long been uncertain. A popular hypothesis is that the chaetognaths are the sister group of the major deuterostome phyla: chordates, hemichordates, and echinoderms. Here we attempt to determine the affinities of the chaetognaths by using molecular sequence data. We describe the isolation and nucleotide sequence determination of 18S ribosomal DNA from one species of chaetognath and one acanthocephalan. Extensive phylogenetic analyses employing a suite of phylogenetic reconstruction methods (maximum parsimony, maximum likelihood, evolutionary parsimony, and two distance methods) suggest that the hypothesized relationship between chaetognaths and the deuterostomes is incorrect. In contrast, we propose that the lineage leading to the chaetognaths arose prior to the advent of the coelomate metazoa.

Introduction

Chaetognaths are a small phylum (~100 species) of carnivorous marine invertebrates ranging in size from 2 to 120 mm. Most are planktonic, constituting a significant proportion of the plankton biomass and consuming large quantities of small copepods and fish fry. The affinities of the phylum have long been debated, and present-day workers are far from reaching any consensus of opinion (for review, see Ghirardelli 1968; Bone et al. 1991, chap. 1). In the past 100 or more years, many attempts have been made to ally the chaetognaths to a bewildering variety of taxa. Proposed relatives have included nematodes, mollusks, various arthropods, acanthocephalans, rotifers, and chordates (see Nielsen 1985; van der Land and Norrevang 1985; Bone et al. 1991).

Most recent workers, however, including Hyman (1959, p. 66), Ducret (1978), and Ghirardelli (1981, p. 224), have concluded that the chaetognaths are distant relatives of the three major deuterostome phyla (Hemichordata, Echinodermata, and Chordata), which themselves are convincingly linked by an array of morphological, physiological, embryological, and molecular characters (see Jefferies 1986, chap. 2; Willmer 1990, chap. 12; Holland et al. 1991; present study). This proposed deuterostome relationship is based on several shared, supposedly derived, embryological characters and, indeed, is espoused in current popular textbooks of zoology (e.g., Barnes et al. 1990, chap. 7; Brusca and Brusca 1990, chap. 23) (fig. 1). Even so, perhaps the best way to sum up the current state of affairs is given by Charles Darwin's

1. Key words: chaetognath, acanthocephalan, molecular evolution, ribosomal RNA, PCR. Abbreviations: PCR = polymerase chain reaction; rDNA = ribosomal DNA.

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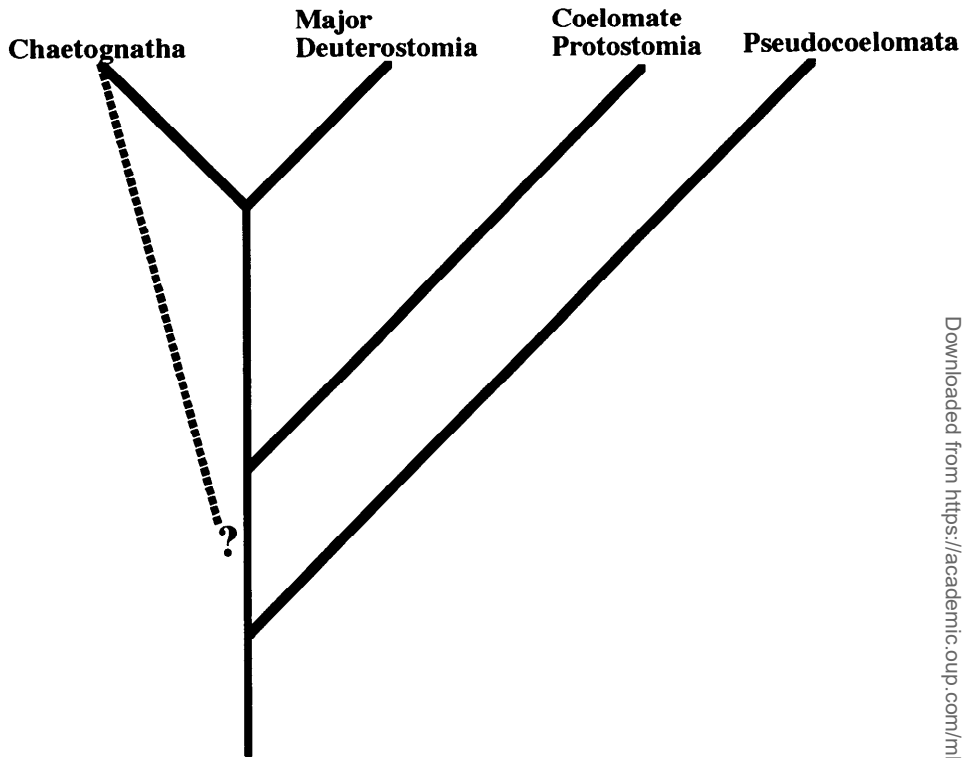


FIG. 1.—Comparison of the traditional view of the phylogenetic position of the chaetognaths as an early branch off the main deuterostome lineage (*unbroken lines*) and the phylogenetic position suggested by the analyses presented here, where the chaetognaths are found not to be the sister group of the deuterostomes (*dotted line*).

(1844) remark when he described the chaetognaths as “remarkable from . . . the obscurity of their affinities.”

Comparative analysis of the primary structure of DNA, RNA, or proteins provides potential additional sources of data to resolve such phylogenetic questions. This paper reports the first molecular sequence data from a member of the phylum Chaetognatha and the first attempt to resolve their affinities by using molecular biological data. To allow evaluation of some of the relationships suggested above, we have used the polymerase chain reaction (PCR; Saiki et al. 1988) to amplify, clone, and sequence the 18S ribosomal DNA (rDNA) from a chaetognath and an acanthocephalan worm. Phylogenetic reconstructions comparing these and other homologous sequences suggest that the chaetognaths are not allied to the three major deuterostome phyla. We also find no support for alternative proposals of close relationships with acanthocephalans or mollusks, but our analyses suggest that the lineage leading to the chaetognaths possibly originated before the advent of the coelomate metazoa.

Material and Methods

Specimen Collection

The chaetognath, *Sagitta elegans*, was collected from coastal waters around Friday Harbor, U.S. The acanthocephalan worm, *Moliniformis moliniformis*, was dissected

from the intestine of a laboratory rat. Genomic DNA extraction was performed as described elsewhere (Holland et al. 1991).

PCR Amplification of Ribosomal DNA

The 18S rDNA positive-strand 5' primer, JM8, described by Holland et al. (1991) was used in conjunction with a negative-strand primer, ITS2, designed to complement the inverse of the 5' end of the 28S rRNA (5'AATCCTGGTTAGTTTCTTTT-CCTCCGCT3'). These primers amplified an ~3-kb fragment containing all but the 5' 38 bp of the 18S rDNA gene, the 5.8S rDNA gene, the two internal transcribed spacers, and the 5' end of the 28S rDNA gene. The PCR was performed on genomic DNA extracted from a single chaetognath or from a portion of the acanthocephalan. The cycling parameters were as follows: 94°C for 3 min, 35 times (94°C for 1 min, 47°C for 1 min 30 s, and 72°C for 4 min), and 72°C for 10 min. The 3-kb fragment was digested at an evolutionarily conserved site 250 bp from the 3' end of the 18S rDNA gene, with *EcoRI*. This yielded two similar-sized fragments from both species, which were subcloned prior to transformation into *Escherichia coli*, strain DH5 α . The chaetognath had an additional *EcoRI* site 560 bp 5' to the conserved site, and the PCR was therefore repeated using a different individual from the same source, and this missing section was cloned. An additional subclone was also obtained, because of the presence of a polymorphic second *EcoRI* site 143 bp 5' to the first. The section from both animals containing the 18S rDNA gene was restriction mapped and subcloned prior to sequence determination using the Sanger method with a kit from Pharmacia and following the manufacturer's instructions. In each case, sequence was determined from the positive strand in one direction and from the negative strand in the opposite direction, with a minimum overlap of 29 bp. In the acanthocephalan, all subclones overlapped by at least this much. There was no overlap at the conserved *EcoRI* site. In the chaetognath all regions except at the conserved *EcoRI* site and at a conserved *PstI* site were covered by overlapping subclones, which overlapped by >29 bp.

Sequence Analysis

Analysis was performed independently on two data sets comprising different species combinations and sequences of different lengths. The first analysis (data set 1) made use of the partial 18S rRNA sequences available from a wide range of taxa. These portions of the 18S rRNA have been frequently used for phylogenetic analysis (e.g., see Field et al. 1988; Turbeville et al. 1991, 1992). The sequences were aligned essentially by following Turbeville et al. (1992), although we decided to omit from our analysis some regions used by these authors, when data were missing or when we could not confidently identify homologous positions. The sequences used originated from the same species as used by Turbeville et al. (1992), with the addition of the acanthocephalan and the chaetognath sequences determined in this study and with the replacement of one protochordate, *Branchiostoma*, with another, *Styela*. In the final analyses, 884 positions were used.

The second analysis (data set 2) used the entire length of the 18S rDNA gene sequenced in this study, together with a number of complete 18S sequences from other metazoa constituting as broad a range of taxa as possible, as well as sequences from three outgroup taxa. These complete 18S rDNA sequences might be expected to give results superior to those discussed above, for two reasons. First, because of the technique used to determine them, there are far fewer ambiguous bases, and, second,

the sequences are more extensive and, hence, theoretically more informative. However, as there are fewer *complete* metazoan 18S sequences, the first data set complements the second by representing a greater variety of taxa. The sequences were initially aligned using the Clustal V multiple alignment program (Higgins et al. 1992) and were fine adjusted by eye, by taking into account rRNA secondary-structure models (Neefs et al. 1991). The rules for inclusion or exclusion of a position from the analysis were as follows:

1. Positions where a base was found in only one species were not used in analyses.
2. In regions of doubtful homology, a position is only used if, by analysis of secondary structure, bases can be shown to be in the same position within a stem region of secondary structure. This is suggested if its predicted pairing partner in the stem varies appropriately.
3. At the start and end of regions that can be confidently aligned and presumed to be homologous, the first and last positions used must not vary between the taxa used. This rule is ignored if rule 2 applies.

The alignment derived and the positions used in analysis are available by e-mail on request from the authors (for address, see Acknowledgments). In our analyses, 1,377 positions were used.

Phylogenetic reconstruction was performed using the maximum-parsimony method of the PAUP 3.0 package (provided by D. Swofford, Illinois Natural History Survey) for identification of the most parsimonious tree and for evolutionary-parsimony analysis (Lake 1987). The maximum-parsimony analysis used a heuristic search in each case and as such was not guaranteed to find the most parsimonious tree; however, under the random-addition option 100 replicates were performed to increase the likelihood of identifying the most parsimonious tree. The evolutionary-parsimony analysis was applied to all positions. The sequences were divided into four groups (for examples, see figs. 3 and 5), and the three possible topologies for all quartets composed of a single sequence from each group were evaluated, and the results were combined. As much as possible, groups were chosen such that one group of unknown position was compared with three groups composed of members of known relationship, such as the outgroup phyla (plant, cnidarian, and fungus), deuterostomes (vertebrates and urochordate), etc. The χ^2 values for combined trees were calculated as described by Lake (1987, appendix), with negative-correlation-values correction as described by Turbeville et al. (1991).

Phylip 3.4 (provided by Dr. J. Felsenstein, University of Washington, Seattle) was used for analyses using the distance matrix programs FITCH (Fitch-Margoliash method; Fitch and Margoliash 1967) and NEIGHBOR (neighbor-joining method; Saitou and Nei 1987). The matrix was calculated using DNADIST with the Kimura correction and with the transition:transversion ratio set to 2. In FITCH, the global search option was used, and negative branch lengths were not allowed. Ten "jumbled" replicates were performed, randomizing the input order; no trees superior to the previous best were found. Bootstrap resampling (500 replicates in each case) was performed using FITCH and NEIGHBOR to gauge support for different branches. Also, the maximum-likelihood program DNAML ("Frequencies" and "Global" options selected) and DNABOOT for parsimony bootstrap analysis (500 replicates) were used. Further investigation of the robustness of the results obtained was undertaken through Felsenstein's implementation of Kishino and Hasegawa's (1989) test. This was used to compare the maximum-likelihood and maximum-parsimony trees with other po-

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tential topologies and to test whether the latter were significantly worse than the optimal topologies derived.

Results

PCR, Cloning, and Sequencing

PCR amplification from genomic DNA allowed the cloning and sequence determination of chaetognath and acanthocephalan 18S rDNA. Amplified bands were cloned, restriction mapped, and subcloned as described. One subclone from each contained the distal 250 bp from the 18S gene, and this was sequenced from a single clone from both animals. The 18S rDNA was cloned and sequenced from a single individual *Moliniformis*. In *Sagitta*, the distal 1,100 bp and proximal 250 bp were sequenced from one individual. A further region of 700 bp, overlapping the 5' end of the 1,100-bp fragment by 143 bp, was sequenced from another individual from the same population. This overlap region appeared to be very slightly polymorphic (4 bp different over 143 bp) within this population of chaetognaths. The sequence from the original 1,100-bp clone was used in all analyses.

Phylogenetic Affinities of *Sagitta*: Data Set 1

Data set 1 contains a composite of three regions of partial 18S sequence from a variety of metazoans, following the alignment of Turbeville et al. (1991). The partial 18S rDNA sequences from two deuterostomes, [*Styela* (urochordate) and *Asterias* (echinoderm)], from five coelomate protostomes [*Golfingia* (sipunculid), *Cerebratulus* (nemertean), *Cryptochiton* (mollusk) and *Chaetopterus*, and *Lumbricus* (annelid)], from three acoelomates [*Fasciola*, *Bothrosomostoma*, and *Dugesia* (platyhelminths)], and from one coelenterate [*Hydra* (cnidarian)] were phylogenetically analyzed in combination with homologous regions from the acanthocephalan *Moliniformis* and the chaetognath *Sagitta*. After alignment and identification of homologous positions, analyses were performed using the reconstruction methods as described.

Maximum-Parsimony Analysis

Of the 884 sites reliably aligned, 499 sites varied, of which 282 (31.9% of the total) were informative. The minimum-length tree found required 1,048 steps and had a consistency index of 0.652 (see fig. 2A). The general topology of the tree is identical to that found by Turbeville et al. (1992), although the relationships within the coelomate clade differ slightly. In particular, the two annelids are found together with the mollusk, while *Cerebratulus* and *Golfingia* are an outgroup to these. This discrepancy is presumably due to the small differences in alignment. The analysis indicates that both the chaetognath and the acanthocephalan fall outside a coelomate clade. They do not form a monophyletic grouping themselves, with the acanthocephalan being an outgroup to the chaetognath plus coelomates. The platyhelminths and cnidarian are an outgroup to all other taxa. Bootstrap analysis lends limited support to this topology, with the chaetognaths being excluded from the coelomate clade in 41% of bootstrap replicates, and the acanthocephalan is excluded from the chaetognath+coelomate group in 36% of bootstrap replicates.

Application of the Kishino-Hasegawa test shows that positioning of the chaetognath within the coelomate protostomes (the most parsimonious positioning possible within this clade) is significantly worse (95% level) than the most parsimonious tree found. When positioned as an early branch from the deuterostomes (a commonly suggested position for the phylum), this is again significantly worse (90% level) than

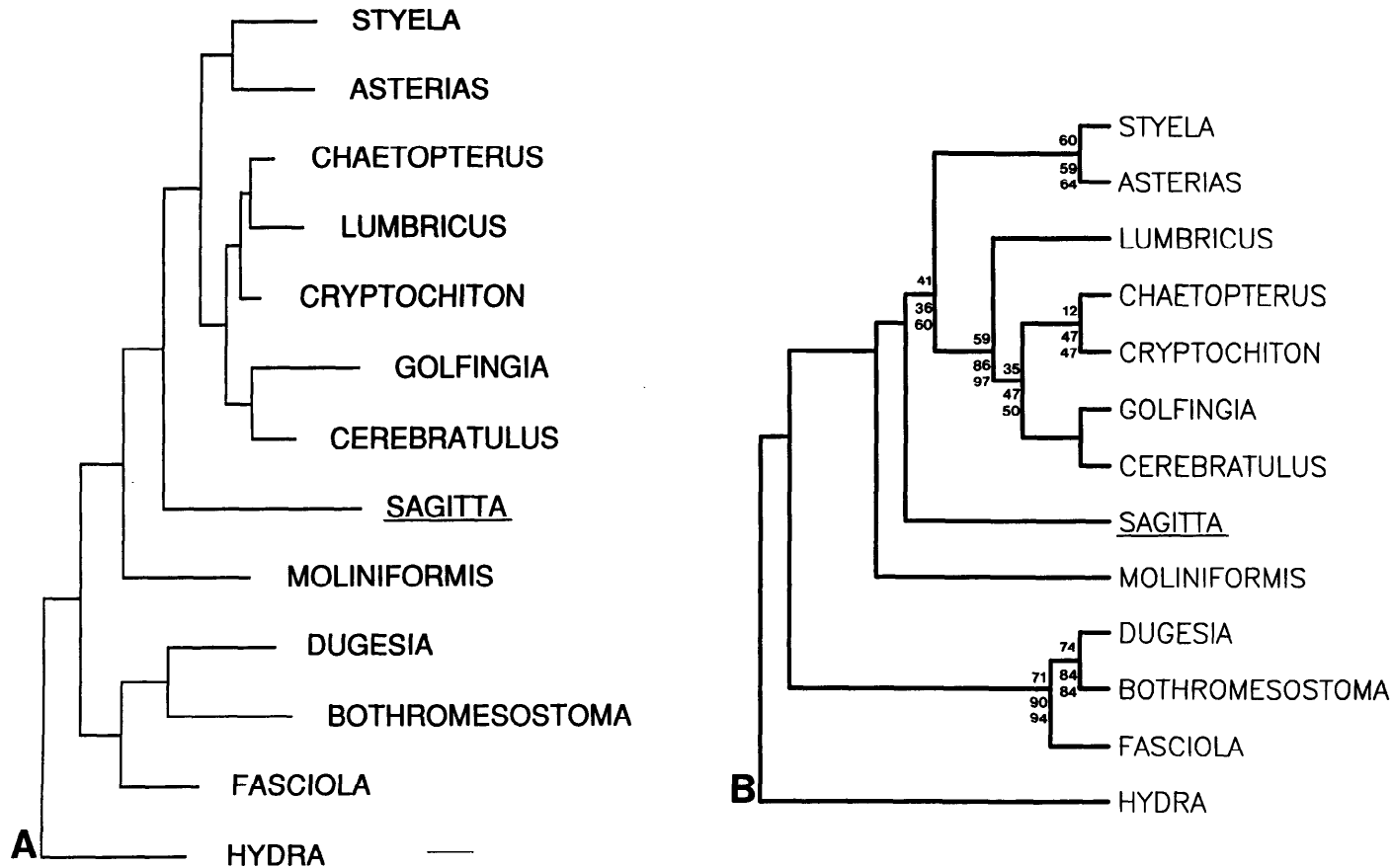


FIG. 2.—Analyses on data set 1. A, Most parsimonious tree found by using the heuristic search option and 100 replicates of the random-addition sequence in PAUP. This tree has 1,048 steps and a consistency index of 0.65. The scale bar represents 30 substitutions. B, Majority-rule consensus tree of the best tree from the following six analyses: maximum parsimony, maximum likelihood, neighbor-joining, Fitch-Margoliash, bootstrapped maximum parsimony, bootstrapped neighbor-joining, and bootstrapped Fitch-Margoliash. Bootstrap percentages are given only for branches that are supported by all methods of bootstrap analysis, and in each case they are derived from 500 bootstrap replicates. The top value (above the branch) is from maximum parsimony; the middle value is from neighbor-joining; and the lower value is from Fitch-Margoliash.

the optimal topology, but, when placed within the deuterostomes as a sister group of *Styela* (the most parsimonious position within the deuterostomes), the tree is not significantly worse.

Evolutionary Parsimony

The first test performed using evolutionary parsimony investigated the relative positions of the chaetognath and acanthocephalan, with reference to the outgroup phyla. The favored topology ($P = 0.0645$) agrees with that suggested by maximum-parsimony analysis: the acanthocephalan is an outgroup to coelomates+chaetognath. The use of different combinations of outgroup confirms this result (data not shown). The second set of analyses tested whether the chaetognath is allied to one or another coelomate group or is the sister group of both (as suggested by maximum-parsimony analysis). When *Hydra* is included in the set of outgroups, the favored topology places *Sagitta* allied to one or other of the coelomate groups [when the outgroup is *Hydra* alone, *Sagitta* is found with the protostomes ($P = 0.048$); when the outgroup is *Hydra*+platyhelminths, *Sagitta* is found with the deuterostomes ($P = 0.0833$); when the outgroup is *Hydra*+platyhelminths+*Moliniformis*, *Sagitta* is found with deuterostomes ($P = 0.0796$)] (see fig. 3A). However, when the outgroup does not include *Hydra*, the favored topology places the chaetognath as an outgroup to the coelomates (deuterostomes and protostomes; $P = 0.0529$), as supported by all other methods of analysis (fig. 3B).

Maximum-Likelihood Analysis

With respect to the position of *Sagitta* and *Moliniformis*, the topology found by maximum-likelihood analysis is identical to that suggested by maximum parsimony, although there is some rearrangement within the coelomate protostomes. A Kishino-Hasegawa test comparing the maximum-likelihood topology with situations in which the chaetognaths are allied to either the deuterostomes or the protostomes failed to show that either of the latter situations was significantly worse than the optimal topology.

Distance Methods

The topology determined above, with the chaetognath and acanthocephalan as outgroups to the coelomates, is also supported by both the Fitch-Margoliash and the neighbor-joining methods of analysis. However, the details of the topologies derived by these distance methods differ, in parts, from that produced by maximum-parsimony analysis. Fitch-Margoliash analysis (sum of squares = 0.28702) reverses the positions of *Sagitta* and *Moliniformis*, placing the chaetognath as outgroup to the coelomates+acanthocephalan (supported by 42% of bootstrap replicates for coelomate/*Moliniformis* monophyly). The neighbor-joining method suggests a slightly different topology. The chaetognath and acanthocephalan are still excluded from the coelomate clade, but the chaetognath is found grouped with the platyhelminths, with the acanthocephalan as an outgroup to this. Bootstrapping lends limited support to this topology (37% support for chaetognath/platyhelminth monophyly).

In both distance analyses the coelomates are consistently grouped to the exclusion of both *Moliniformis* and *Sagitta*: FITCH bootstrap = 60%, and NEIGHBOR bootstrap = 36%. In contrast, resampling support for a clade consisting of *Sagitta* and the coelomate protostomes is lower, as follows: FITCH bootstrap = 4%, and NEIGHBOR bootstrap = 0.2%. Resampling support for a clade consisting of the deuterostomes

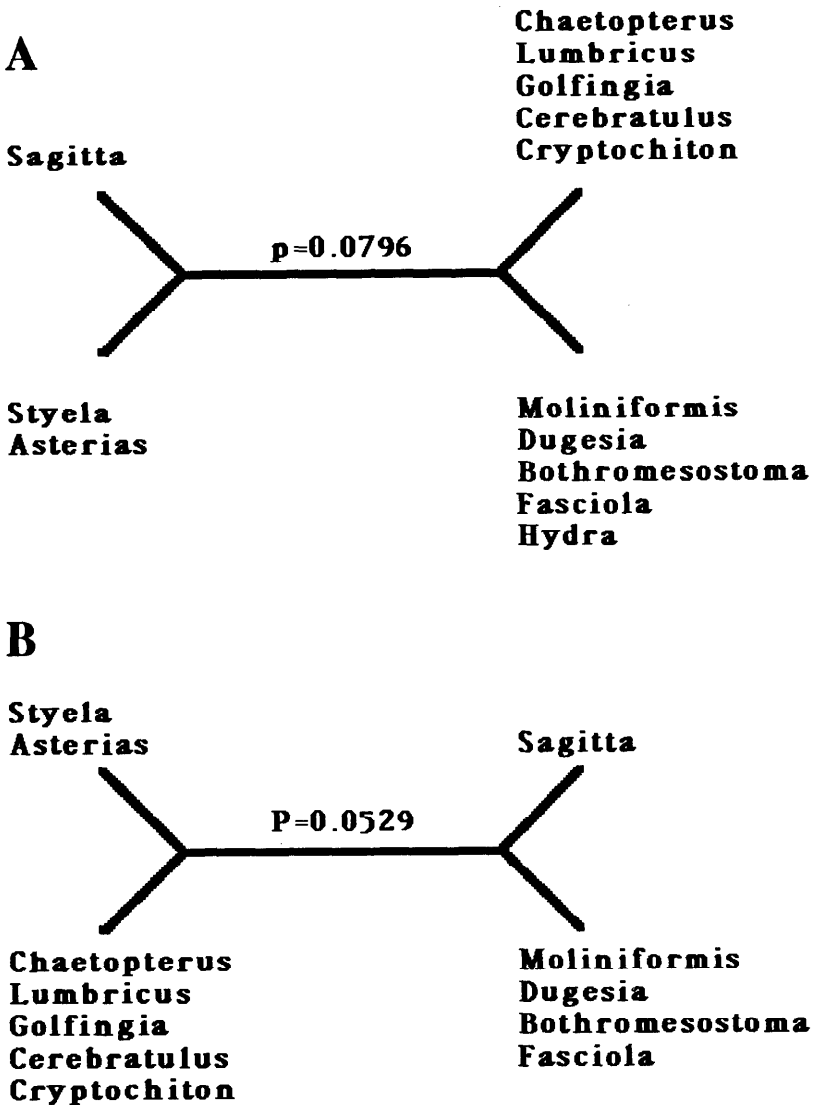


FIG. 3.—Summary of analyses of the position of the chaetognath by using evolutionary parsimony on data set 1. A, Result of using entire set of species. This tree, which positions the chaetognath as sister group of the major deuterostomes, is favored over one in which the chaetognath is found as sister group of the coelomates or as sister group of the coelomate protostomes. B, Result of a repeat of the above analysis, omitting the *Hydra* sequence. The favored topology places the chaetognath as an outgroup to the coelomates. (P is the probability that the data would fit as well as they do if the topology shown were incorrect.)

and *Sagitta* is as follows: FITCH bootstrap = 16%, and NEIGHBOR bootstrap = 28%.

Conclusions from Data Set 1

Ultimately, bootstrapping lends little support to any particular topology. However, the following relationships are found by most or all methods of analysis:

1. The deuterostomes and coelomate protostomes form two separate monophyletic groups.
2. The deuterostomes and coelomate protostomes form a monophyletic group to the exclusion of the chaetognath and acanthocephalan.
3. The platyhelminths form a monophyletic group, which is, in almost all analyses, an outgroup of the coelomates+chaetognaths and acanthocephalan.
4. The chaetognaths and acanthocephalan do not form a monophyletic assemblage: the chaetognaths are closer to the coelomates than is the acanthocephalan.

A majority-rule consensus tree derived from all above methods of analysis, except evolutionary parsimony, is shown in figure 2B.

Phylogenetic Affinities of *Sagitta*: Data Set 2

Data set 2 uses complete 18S rDNA sequences from a range of metazoans and outgroups. These sequences derive from four deuterostomes [*Herdmania* (urochordate), human, mouse, and *Xenopus* (vertebrates)], from four coelomate protostomes [*Artemia*, *Tenebrio* and *Eurypelma* (arthropods), and *Placopecten* (mollusk)], from one platyhelminth (*Opisthorchis*), and from three outgroup taxa [*Glycine* (angiosperm), *Anemonia* (cnidarian), and yeast (fungus)], which were aligned with the complete sequences of the acanthocephalan and chaetognath 18S sequences presented here. The phylogenetic analyses described gave the following results.

Maximum-Parsimony Analysis

Of the 1,377 positions unambiguously aligned, 503 varied, of which 260 (18.9% of the total) were informative. The most parsimonious tree found required 1,010 steps and had a consistency index of 0.672 (see fig. 4A). Contrary to the results gained with data set 1, the chaetognath, acanthocephalan, and platyhelminth are not positioned as sister groups to the coelomate protostomes and deuterostomes. They are found instead within a monophyletic grouping with the coelomate protostomes, the acanthocephalan is positioned within the coelomate protostomes, as sister group of the arthropods (23% support from bootstrapping), and the mollusk is the sister group of the acanthocephalan+arthropods (not supported by bootstrapping). The chaetognath and platyhelminth are grouped together, forming a sister group to this clade. This monophyletic group is supported by 59% of bootstrap replicates. The deuterostomes constitute a monophyletic group, which is supported by 65% of bootstrap replicates.

Analysis of alternative topologies by using the Kishino-Hasegawa test gave the following results: Placement of the chaetognath as sister group of the deuterostomes (its commonly stated phylogenetic position) requires 18 extra steps and is significantly worse (95% level). When placed in its most parsimonious position within the deuterostomes (sister group of the vertebrates), this topology is still significantly worse than the best tree (95% level; 15 extra steps). Placing the chaetognath, acanthocephalan, and platyhelminth *outside* a coelomate clade is not significantly worse.

Evolutionary Parsimony

Various tests were performed using evolutionary parsimony. The results gained were not all congruent with those gained using the other methods. The position of the platyhelminth was tested relative to the positions of the group of deuterostomes, protostomes, and outgroup phyla. This test placed it unexpectedly with the deuterostomes ($P = 0.0447$). *Herdmania* was placed with the vertebrates, rather than with

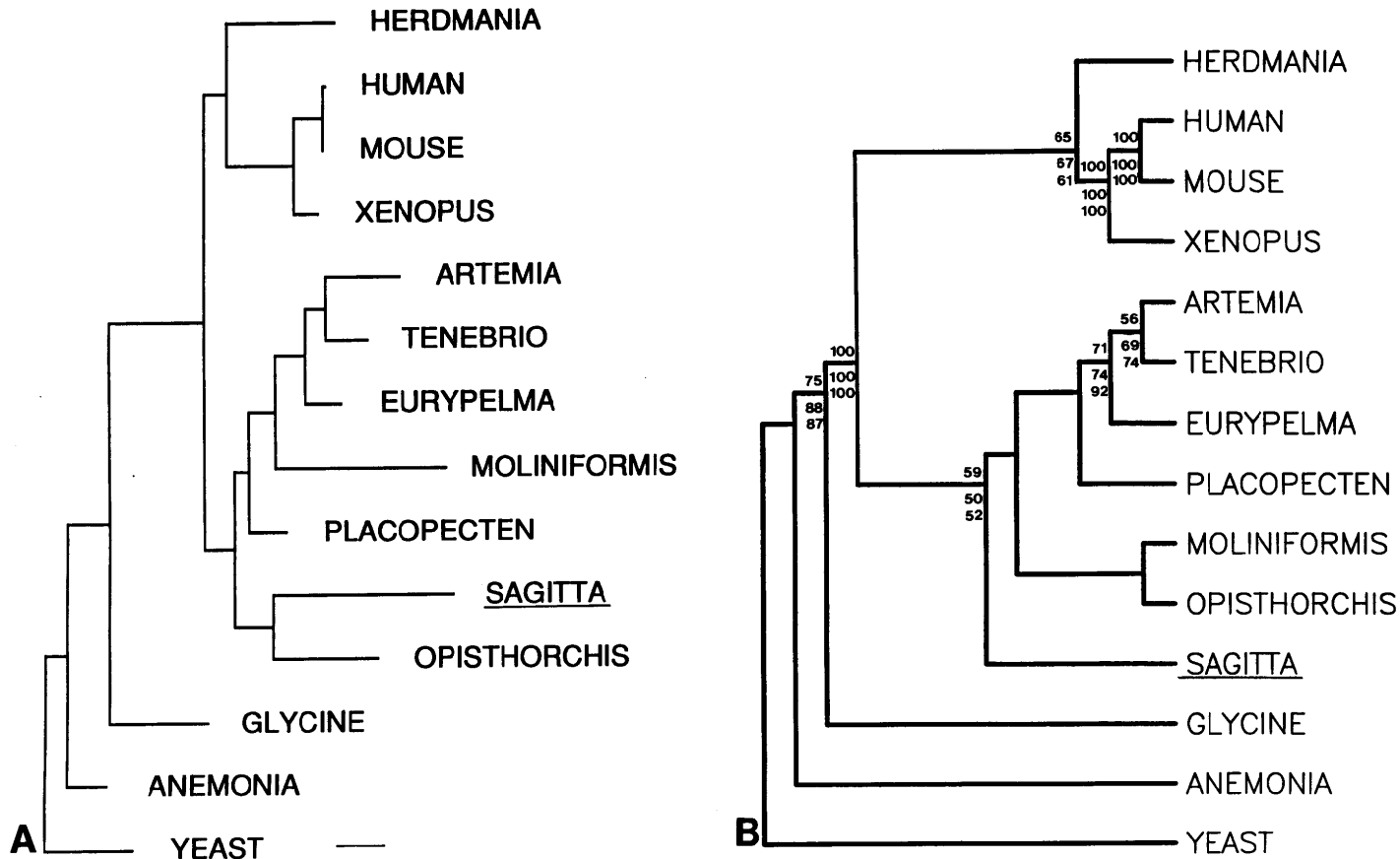


FIG. 4.—Analyses on data set 2. A, Most parsimonious tree found by using the heuristic search option and 100 replicates of the random-addition sequence in PAUP. This tree has 1,010 steps and a consistency index of 0.67. The scale bar represents 30 substitutions. B, Majority-rule consensus tree of the best tree from the following six analyses: maximum parsimony, maximum likelihood, neighbor-joining, Fitch-Margoliash, bootstrapped maximum parsimony, bootstrapped neighbor-joining, and bootstrapped Fitch-Margoliash. Bootstrap percentages are given only for branches that are supported by all methods of bootstrap analysis, and they are derived from 500 bootstrap replicates. The top value (above the branch) is from maximum parsimony; the middle value is from neighbor-joining; and the lower value is from Fitch-Margoliash.

protostomes or outgroup phyla ($P = 0.0784$). *Placopecten* was unexpectedly placed with the deuterostomes ($P = 0.067$), rather than with the arthropods ($P = 0.091$) or outgroup phyla. The difference in P is small, however, and the mollusk/arthropod tree was actually favored *more often* than was the mollusk/deuterostome tree (20 of 36 tests, with 134 counts favoring, and 16 of 36 tests, with 94 counts favoring, respectively). Tests comparing the position of *Moliniformis* relative to the deuterostomes, protostomes, and outgroup phyla placed the acanthocephalan as sister group to the coelomates ($P = 0.12525$), contrary to the results gained from maximum parsimony but similar to the results gained from data set 1.

Tests of the position of *Sagitta* relative to an outgroup, the protostomes, and the major deuterostomes gave varying results, depending on the composition of the outgroup. When all potential outgroup taxa (*Moliniformis*, *Opisthorchis*, *Anemonia*, yeast, and *Glycine*) were used, the corrected P values gave slightly more support to the chaetognath/deuterostome tree (fig. 5A) (chaetognath/deuterostome, $P = 0.0165$; and chaetognath/outgroup, $P = 0.023$). This is despite apparently overwhelming support for a tree in which the chaetognath is placed as outgroup to the coelomate protostomes and deuterostomes. In tests of 80 quartets ($4 \times 4 \times 5 \times 1$) this tree was supported 49 times with 370 counts favoring; chaetognath with deuterostomes, 15.5 times with 169 counts favoring; and chaetognath with protostomes, 15.5 times with 91 counts favoring. The preference for a topology in which the chaetognaths are placed with the deuterostomes seems to be influenced by the coelenterate sequence, as noninclusion of this sequence gives the result that chaetognaths are the sister group of the protostome and deuterostome coelomates ($P = 0.012$) (fig. 5B). Considering the strong support for the topology placing chaetognath as outgroup, we believe that this latter result is the more credible. With regard to the relative positions of chaetognath and acanthocephalan, evolutionary parsimony places the two taxa in a monophyletic group, relative to a coelomate and an outgroup clade ($P = 0.0741$).

Maximum-Likelihood Analysis

The results gained from the maximum-likelihood analysis are similar to those gained with maximum parsimony, except that (a) the mollusk, acanthocephalan, platyhelminth, and chaetognath form a sister group to the arthropods and (b) *Herdmania* is not found within a deuterostome clade but as an outgroup to the bilateria. Kishino-Hasegawa tests show that, if *Herdmania* is constrained in a deuterostome grouping, this tree is not significantly worse than the best tree (difference in ln likelihood = -4.68555 , standard deviation = 8.2645). If this latter tree is compared with one in which the chaetognath is allied to the deuterostomes, this last tree is not significantly worse.

Distance Methods

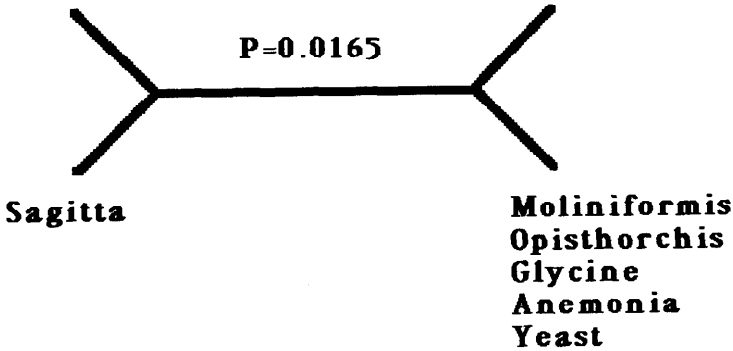
Fitch-Margoliash and neighbor-joining analysis give identical topologies, which are similar to that derived using maximum parsimony (FITCH sum of squares = 0.19943), though there are differences in the arrangement within the clade containing the coelomate protostomes, chaetognath, acanthocephalan, and platyhelminth. Bootstrap resampling supports a monophyletic group consisting of the urochordate and vertebrates (FITCH bootstrap = 61%, and NEIGHBOR bootstrap = 67%) and provides slightly less support for the clade containing the coelomate protostomes and chaetognath, acanthocephalan, and platyhelminth (FITCH bootstrap = 53%, and NEIGHBOR bootstrap = 51%). Bootstrap analysis places the chaetognaths with the deuterostomes in just 13.5% of FITCH bootstrap replicates and in 8.2% of NEIGHBOR

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A

Herdmania
Human
Mouse
Xenopus

Artemia
Tenebrio
Eurypelma
Placopecten

**B**

Herdmania
Human
Mouse
Xenopus

Sagitta

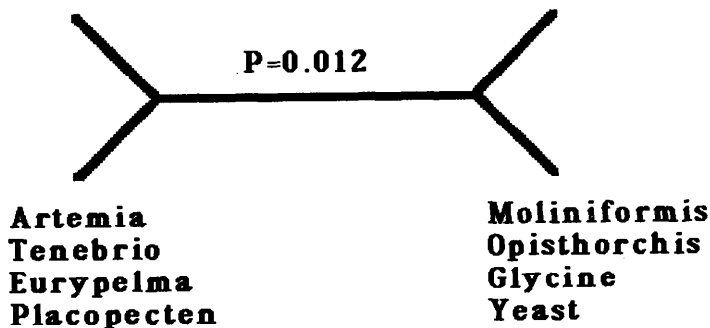


FIG. 5.—Summary of analyses of the position of the chaetognath by using evolutionary parsimony on data set 2. A, Result of using entire set of species. This tree, which positions the chaetognath as sister group of the major deuterostomes, is favored over one in which the chaetognath is found as sister group of the coelomates or as sister group of the coelomate protostomes. B, Result of a repeat of the above analysis, omitting the *Anemonia* sequence. The favored topology places the chaetognath as an outgroup to the coelomates. (P is the probability that the data would fit as well as they do if the topology shown were incorrect.)

bootstrap analyses (the latter is 13.5% when the deuterostomes are considered not to include *Herdmania*).

Conclusions from Data Set 2

Again, from these analyses, certain relationships are repeatedly found.

1. The deuterostomes form a monophyletic group (except in maximum-likelihood analysis, and this grouping was not rejected by Kishino-Hasegawa tests even

here), and there is no evidence to support inclusion of the chaetognaths within this clade.

2. A group consisting of the bilateria minus the major deuterostome phyla is found, except in some evolutionary-parsimony tests.
3. The acelomate, the acanthocephalan, and the chaetognath tend to lie outside the main protostome line, though they form a sister group to the protostomes. (This is not supported by all analyses.)
4. The cnidarian *Anemonia* is consistently placed as an outgroup not included in a metazoan monophyletic grouping, mirroring the results of Christen et al. (1991).

A majority-rule consensus tree for all methods of analysis applied to data set 2 (with the exception of evolutionary parsimony) is shown in figure 4B.

Discussion

Selection of Strategy

The 18S rDNA gene was chosen for this study of the affinities of the chaetognaths and acanthocephalans, for two reasons. First, it is widely recognized as a suitable molecule for phylogenetic analysis of deep branches within the tree of life, such as that attempted here (e.g., see Field et al. 1988; Abele et al. 1989; Holland et al. 1991; Willmer and Holland 1991). Second, it has already been sequenced from a variety of other groups, including many metazoans, thus obviating the need to obtain sequences for comparison from a large number of species. Partial 18S rRNA sequences have often been obtained by direct sequencing from total cellular RNA (e.g., see Field et al. 1988); however, the alternative strategy used here, of cloning 18S rDNA after PCR-mediated amplification from genomic DNA, has several advantages and is becoming the favored technique for this sort of study. Of particular relevance to this study is the sensitivity of PCR, which allowed us to obtain genomic sequences from individual planktonic chaetognaths. Our results show that individuals of minute, even microscopic, species are now readily amenable to molecular analysis.

Complementarity of the Two Data Sets

The need to use two data sets arises from the constraints imposed by the available sequences. Although there are 18S rRNA sequences from a wide range of metazoan groups, many of these are partial sequences derived from direct RNA sequencing. Because of the techniques used to derive these sequences, the latter are shorter and of lower quality (i.e., they have more ambiguous bases) than are sequences derived by conventional cloning. We have complemented the wide variety of phyla represented by these sequences by a second data set, including complete 18S rDNA sequences from a range of taxa comprising fewer phyla. As more complete sequences become available, there will be no need to use two complementary data sets.

Disparity of the Two Data Sets

The major difference between the conclusions reached from analysis of the two data sets lies in the position of the chaetognath, acanthocephalan, and platyhelminth. From data set 1 we find the platyhelminths in the traditionally accepted phylogenetic position as descendant from an early branch prior to the divergence of the pseudocoelomates and coelomates. The acanthocephalan is found as an outgroup to the coelomates, as would be predicted from its pseudocoelomate status (Brusca and Brusca 1990, chap. 12). It is interesting that the chaetognath is also found outside the coelomate

group. Although this result is contrary to the traditional view, we believe that it is quite consistent with more recent ultrastructural analyses. Indeed, chaetognaths have many features in common with the pseudocoelomate "aschelminth" phyla, including, some argue, a pseudocoelom (Willmer 1990, p. 318; Bone et al. 1991, p. 12), though this result is not supported by all authors (Welsch and Storch 1982). The chaetognath is not found grouped with the acanthocephalan, except in the evolutionary-parsimony analysis of data set 2.

The major conclusion of this study—i.e., that the chaetognaths are not related to the major deuterostome phyla—is also supported by the results of our analyses of data set 2. However, the two data sets do differ over the precise location of the chaetognath, acanthocephalan, and platyhelminths; data set 2 places these three groups unexpectedly within or as a sister group of the coelomate protostomes within a coelomate assemblage. We believe that this positioning is artifactual, for several reasons. First, few would disagree that both the platyhelminth and the acanthocephalan are wrongly placed by this particular analysis, which immediately makes one wary of the conclusions drawn. The positioning of the platyhelminth, in the analysis of data set 2, is the only fundamental disparity between this study and previous work using the 18S rRNA (e.g., see Field et al. 1988; Patterson 1989; Lake 1990). Second, confidence tests comparing the "best" topology with one that is more similar to the conclusions of data set 1 cannot reject the latter. Finally, tests using evolutionary parsimony place the acanthocephalan as an outgroup to the coelomates (as seen in data set 1 and as supported by nonmolecular evidence). This is also true of the chaetognath, though this varies according to the analysis performed.

The two data sets do agree in many respects, including the following:

1. The deuterostomes form a monophyletic group, as do the coelomate protostomes.
2. The chaetognaths are not found positioned with the deuterostomes.
3. The chaetognath is usually not found with the acanthocephalan.
4. The chaetognath is usually not found within the coelomate protostome superphylum. Analyses of data set 2 place the chaetognath, along with the platyhelminth, as sister group of the coelomate protostomes. There is no evidence for a link with the mollusks that has been suggested by Casanova (1987).

Phylogenetic Inferences

The hypothesis that the chaetognaths are distant relatives of the major deuterostome phyla (Hemichordata, Echinodermata, and Chordata) (fig. 1) has been advanced by many recent workers (e.g., Hyman 1959, p. 66; Ducret 1978; Ghirardelli 1981, p. 224) and seems to be the generally accepted view on the phylogenetic position of this phylum (Green and Bergquist 1982; Barnes et al. 1990, chap. 7; Brusca and Brusca 1990, chap. 23; Goto et al. 1992). This view is based primarily on certain embryological features claimed to be shared derived characters linking the two groups.

Casanova (1987) discusses the alternative possibility that the chaetognaths are derived from within the mollusks. This conclusion is based primarily on the similarity that circumoral palps found on the chaetognath *Archeterokrohnia palpifera* have to those of certain gymnosome mollusks. Yet another recent suggestion for the affinities of chaetognaths tested here is that of Nielsen (1985) and van der Land and Norrevang (1985), who link the chaetognaths to acanthocephalan worms and rotifers by the presence of an unusual cuticle structure shared by the chaetognath *Eukrohnia hamata* and the other two taxa.

We have tested all three of these proposals by using the molecular data obtained in this study. The results of our analyses suggest that the chaetognaths are *not* allied to the major deuterostome groups. In addition, our analyses give little or no support to the hypotheses, mentioned above, of a molluscan or an acanthocephalan link. *We propose that the most likely position of the chaetognaths is as descendants from an early metazoan branch possibly originating prior to the radiation of the major coelomate groups* (fig. 1).

Implications for Metazoan Evolution

Our result implying that the chaetognaths are not close relatives of the major deuterostome phyla implies that several embryological features said to be derived features shared by the chordates, hemichordates, echinoderms, and chaetognaths (i.e., radial cleavage, deuterostomous mouth formation, and enterocoelous coelom formation) are *not* synapomorphies. Instead, we conclude that these features, if homologous, must be shared ancestral characters (plesiomorphies) or, if not homologous, are shared because of convergence (homoplastic apomorphies). The conclusion that they are not synapomorphies is not unreasonable, because, although cleavage in the chaetognath embryo is radial (Burfield 1927; Kuhl and Kuhl 1965), this character is also found in various nondeuterostomian phyla, such as priapulids (Lang 1953), and (as an apparently modified spiral cleavage after the eight-cell stage) in gastrotrichs (Sacks 1955). Similarly, deuterostomous mouth formation (not from the blastopore) is also found in a variety of other animals, such as the onychophora, various annelids, and some brachiopods. Fiorini (1980) and others argue against the use of this variable character in phylogenetic work. Finally, although chaetognaths do seem to form coeloms during embryogenesis, and although these coeloms are not formed by schizocoely as in protostomes, neither are they formed by a process recognizable as typical deuterostome enterocoely. The embryonic coeloms close later in embryogenesis, and new coeloms form in the adult. The adult cavities may in fact be secondarily derived and, as mentioned above, pseudocoelomic in nature, possessing no peritoneum.

Our analyses suggest that the chaetognaths either lie outside the coelomate assemblage or, possibly, form a sister group to the coelomate protostomes. A more precise placement, however, cannot be inferred, because of the relatively limited range of groups for comparison. Comparison with aschelminth groups may prove valuable in further clarifying the affinities of the chaetognaths, as at least some of these groups have various features in common with chaetognaths. In addition, we urge that all investigations employing molecular data should be complemented by further embryological and ultrastructural studies.

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GenBank, and the DNA Data Bank of Japan; accession numbers are as follows: Z19551 for *Sagitta elegans* and Z19562 for *Moliniformis moliniformis*.

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