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The Phylogenetic Position of the Pterobranch Hemichordates Based on 18S rDNA Sequence Data

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Pterobranchs are a class of deuterostome metazoans that are sessile marine suspension feeders. Although this group has been poorly studied, understanding their phylogenetic affinities is central to understanding early metazoan evolution. Sequence data from the 5′ end of the 18S rDNA gene was collected from a pterobranch, *Rhabdopleura normani*, and combined with other available 18S sequences. Using standard phylogenetic methods, the evolutionary relationships of deuterostome metazoans were reconstructed. The pterobranchs are most closely related to the enteropneust hemichordates. This was confirmed by bootstrap analyses and a topology-dependent cladistic permutation tail probability (T-PTP) test. My analysis agrees with Turbeville *et al.*'s (1994) and Wada and Satoh's (1994) finding that hemichordates are more closely related to echinoderms than to chordates, and it is proposed that Metschnikoff's (1881) name Ambulacraria be adopted for the clade defined by the last common ancestor of the hemichordates and echinoderms. These findings suggest that ciliated gill slits and the dorsal hollow nerve chord are pleisomorphic features of the Deuterostomia.

INTRODUCTION

Pterobranchs are small marine suspension feeding organisms. They are generally only a few millimeters in length and sessile and possess tentaculate arms which account for approximately half the body length (Halanych, 1993). Although pterobranchs are relatively unimportant either economically or in terms of abundance and diversity, understanding their phylogenetic position is pivotal to the study of metazoan evolution because they share several unique characters with both the chordates and the echinoderms.

Although most workers agree that the pterobranchs are deuterostomes (Hyman, 1959; see Willmer, 1990), their position within the Deuterostomia is subject to debate, and two main schools of thought persist. Traditionally, pterobranchs and enteropneusts, or acorn worms, are considered a monophyletic entity called the Hemichordata (Hyman, 1959; Schaeffer, 1987). This hypothesis relies on three criteria of questionable reliability: the presence of a stomal chord, the presence of a collar region, and the presence of a glomerulus. The presence of a stomal chord and a glomerulus are debatable in pterobranchs, and none of the three has been satisfactorily confirmed at the ultrastructural level by microscopy techniques. The continued use of these characters is based largely on the fact that they are included in Hyman's (1959) and Dawydoff's (1948) treatises. Contrary to this hypothesis, some workers (e.g., Jefferies, 1986; Nielsen, 1987) argue that the pterobranchs represent an early divergence event in the deuterostome lineage. These hypotheses usually argue that several of the features found in pterobranchs, especially their feeding apparatus, are primitive structures and postulate that pterobranchs represent an intermediate between the Lophophorata and the Deuterostomia (i.e., pterobranchs are modified tentaculates). Although workers have debated pterobranch affinities, they are arguably one of the most poorly studied taxa of metazoan organisms.

This paper discusses the phylogenetic position of pterobranchs based on sequence data from the 18S nuclear ribosomal gene. This gene was chosen for the analysis because it is conserved in nature making it more applicable to the study of ancient phylogenetic events (Field *et al.*, 1988; Hillis and Dixon, 1991) and because the sequence information is available for several deuterostome metazoans. Furthermore, molecular data are less prone to problems of interpretation and subjective biases than morphological data and provide an independent phylogeny upon which morphological character evolution can be tested.

These analyses demonstrate that the pterobranchs are allied with the enteropneust and are not a basal lineage of deuterostomes. Furthermore, the finding

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that hemichordates are sister taxons to echinoderms and not chordates (Turbeville *et al.*, 1994; Wada and Satoh, 1994) is supported by this analysis. The relevance of these findings for the interpretation of morphological evolution within the deuterostomes is discussed.

MATERIALS AND METHODS

The pterobranch *Rhabdopleura normani* was collected under the Causeway Bridge near Ferry Reach, Bermuda. Total genomic DNA was extracted using a standard proteinase K, phenol–chloroform extraction protocol. The 5′ region of the 18S rDNA gene was amplified via the polymerase chain reaction using the oligonucleotides (5′-CTGGTTGATCCTGCCAGT-3′ and 5′-GAATTACCGCGGCTGCTGGCACC-3′) which correspond to human base positions 3–21 and 632–610, respectively. The PCR product was cloned into a plasmid vector and sequenced (Hillis *et al.*, 1990). The sequence was deposited in GenBank under Accession No. U15664.

The pterobranch sequence was aligned with the corresponding 18S rDNA sequences of other deuterostomes that were extracted from GenBank version 81.0. These are *Saccoglossus cambrensis* (enteropneust 1, GenBank Accession No. X59119), *Balanoglossus carnosus* (enteropneust 2, Accession No. D14359), *Antedon serrata* (crinoid, Accession No. D14357), *Asterias amurensis* (seastar, Accession No. D14358), *Styela plicata* (urochordate, Accession No. M97577), *Lampetra aepyptera* (vertebrate, Accession No. M97573), and *Anemonia sulcata* (cnidarian, Accession No. X53498). The sequences were aligned with the aid of the CLUSTAL V program (Higgins *et al.*, 1992) and proofread by hand. Regions which could not be unambiguously aligned were excluded from subsequent analyses. The alignment can be obtained from the author at internet address "Halanych@utxvms.cc.utexas.edu." For all of the analyses, the cnidarian sequence was designated as the outgroup so that character states could be polarized and the resultant tree(s) rooted. The cnidarian sequence is apparently evolving slow enough to provide more reliable outgroups than other metazoan taxa (e.g., flatworms or nematodes).

The PAUP software package, version 3.1.2d5 (Swofford, 1993), was used for the parsimony analyses, and the PHYLIP software package, version 3.5 (Felsenstein, 1993), was used for neighbor-joining and maximum likelihood analyses. A topology-dependent cladistic permutation tail probability (T-PTP) test examines the significance of monophyly for certain clades by comparing tree lengths of randomized data to tree lengths of real data (Faith, 1991). For the T-PTP analysis conducted herein, data randomization was carried out using a program written by John P. Huelsenbeck.

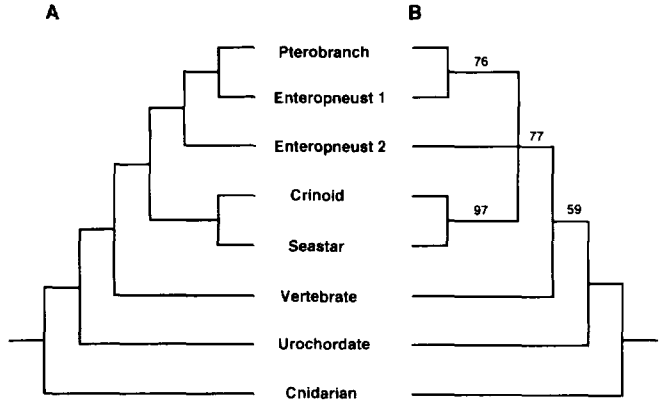

FIG. 1. Parsimony analyses based on equal weighting. (A) A single-most parsimonious tree of 205 steps and a CI = 0.795 was produced by an exhaustive search using the PAUP program. (B) The 50% majority rule consensus tree of 500 bootstrap iterations using the branch and bound algorithm. Branches supported in less than 50% of the iterations have been collapsed.

RESULTS

The alignment of these eight taxa yielded a data matrix of 652 positions of which 528 could be unambiguously aligned. Of those, 133 characters were variable and 57 were phylogenetically informative (i.e, parsimony sites). The g_1 statistic ($g_1 = -0.5587$) indicates that this data set is significantly more structured than random data (Hillis and Huelsenbeck, 1992). The exhaustive search algorithm (with equal character weighting) of the PAUP program found a single-most parsimonious tree of 205 steps and a consistency index of 0.795 (Fig. 1A). This topology was invariant when transversion–transition weights of 1:1 to 3:1 were employed. Two interesting results are apparent in this tree. First, the pterobranch clusters with the enteropneusts, and thus the monophyly of hemichordates is supported. Second, the hemichordates are more closely related to the echinoderms than to chordates. This finding was shown by Turbeville *et al.* (1994) and Wada and Satoh (1994).

Bootstrap analyses were employed to assess the confidence of nodes within the topology. The tree in Fig. 1B represents 500 iterations of a branch and bound parsimony bootstrap analysis. Hillis and Bull (1993) demonstrate that "bootstrap proportions of ≥70% usually correspond to a probability of ≥95% that the corresponding clade is real." Thus, the values for pterobranch–enteropneust 1 node, the echinoderm node, and the hemichordate–echinoderm node (76, 97, and 77%, respectively) are significant.

To determine how robust the findings of the parsimony analyses were, other phylogenetic reconstruction methods were used. A neighbor-joining analysis using the *dnadist* and *neighbor81* programs of the PHYLIP package was conducted. The distances were estima-

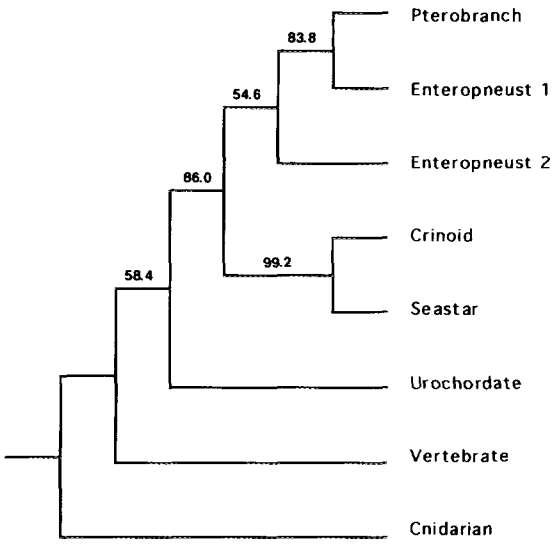

FIG. 2. The result of the neighbor-joining analyses. Using the PHYLIP *dnadist81* program, distances were estimated based on a modified Kimura 2-parameter model with a transversion–transition weighting of 2:1. The topology was then reconstructed with a neighbor-joining algorithm, the *neighbor 81* program. The values along the branches represent the bootstrap percentages based on 500 iterations.

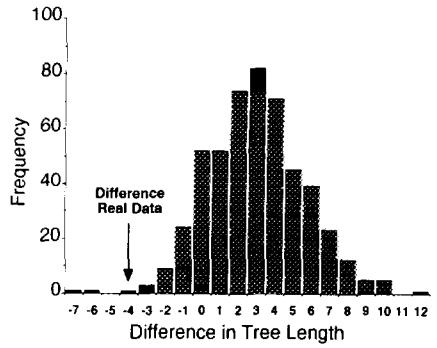

FIG. 3. The results of the T-PTP test, to determine if the monophyly of the pterobranch–enteropneust 1 clade is significantly different from random, are shown. For each randomized data set, the difference between the most parsimonious monophyletic tree and most parsimonious nonmonophyletic tree was determined, and the frequency for each value was plotted. This test supports the monophyly of this clade ($P \leq 0.01$) because the difference for the real data falls outside 99% of the random distribution. The frequency values represent the number of occurrences in 500 randomized data sets.

tions based on a modified Kimura 2-parameter model which used a transversion–transition weighting of 2:1 (Fig. 2). The numbers above the branches represent bootstrap values based on 500 iterations. This topology differs from the parsimony topology in that the relative positions of the urochordate and vertebrate are reversed. A maximum likelihood analysis, using the *dnaml81* program of PHYLIP with a transversion–transition weighting of 2:1, gave the same topology as the parsimony analyses.

Two unexpected results in my analyses were the apparent chordate paraphyly (i.e., urochordate and vertebrate did not form a monophyletic taxon) and enteropneust paraphyly. In all of the analyses, the branch separating the two chordates was weakly supported as the bootstrap values were relatively low. However, the paraphyly of the enteropneusts is well supported by bootstrap values because the pterobranch clusters strongly with enteropneust 1.

To further test the result of the pterobranch and enteropneust 1 being clustered, a T-PTP test was used to determine if the support for monophyly of this branch is statistically different from randomized data (Faith, 1991; Ballard *et al.*, 1992). The T-PTP test utilizes the difference in length of the shortest tree supporting monophyly and the shortest tree not supporting monophyly for the clade in question. The degree of significance is determined by comparing the difference in length of the real data to an expected distribution of the length differences for randomized data. For the real data, the shortest tree not supporting monophyly is 209 steps long, and thus the difference in length between the monophyly tree and the nonmonophyly tree is −4. Figure 3 shows the expected distribution of the length differences for 500 randomized data sets. The difference in tree lengths for the real data falls within the 1% tail of the distribution. Thus, it can be concluded that the data are significantly more structured random ($P \leq 0.01$).

DISCUSSION

Pterobranch Affinities

The results of all of the analyses indicate that the pterobranchs are closely related to the enteropneust and are not a basal lineage of the deuterostomes. The hypothesis that hemichordates are monophyletic is supported, but this analysis did not include the Planctosphaeroidea. Planctosphaeroidea is a rarely collected group of pelagic organisms which have been hypothesized to represent a distinct class of hemichordates, but are typically regarded as a rare larval enteropneust (Hyman, 1959). Using a parsimony criterion, the topology of the 18S rDNA tree suggests that pterobranchs evolved from an organism very similar to modern enteropneusts. The two enteropneusts here (*Balanoglossus* and *Saccoglossus*) share a very similar morphology and their paraphyletic status indicates that several of the enteropneust features are primitive hemichordate characters. This hypothesis is not supported by morphology. The general *Bauplan* of enteropneusts and pterobranchs is radically different prompting several authors to elevate pterobranchs to a distinct phylum (e.g., Dilly, 1975; Nielsen, 1987).

Although the molecular data support an enteropneust–pterobranch clade, three traditional characters (stomal chord, glomerulus, and collar) used to group the them are suspect because ultrastructural evidence for these features is lacking. Pterobranchs were originally considered to have a notochord (Harmer, 1887), but subsequent workers argued that this structure was actually a stomal chord or buccal diverticulum (see Hyman, 1959, for a review). Based on light microscopy and electron microscopy, a stomal chord or a notochord-like structure is absent in pterobranchs (Hyman, 1959; Dilly, 1975; K.M.H. unpublished data). Although buccal diverticula do exist in pterobranchs, their use as a phylogenetically informative character at this level is doubtful. The evolutionary development of diverticula in digestive tracts has occurred numerous times over the course of evolution (e.g., arthropods, mollusks, flatworms). Likewise, convincing ultrastructural evidence which supports the presence of a glomerulus is lacking (K.M.H., unpublished data) and there is nothing in the literature that suggests the collar regions of enteropneusts and pterobranchs share unique characteristics. However, it must be noted that these three characters, as well as other questionable characters (e.g., the putative homology of the larval enteropneust tail and the pterobranch stalk and the putative homology of the enteropneust proboscis and the pterobranch cephalic shield), are congruent with the molecular data.

Another important consideration is that pterobranchs and enteropneusts were first grouped together because of features that have never been present in them or any of their ancestors. This criterion for classification is wholly unacceptable by modern systematic standards which seek to use shared derived characters to group organisms. Some early workers (Bateson, 1885) classified both enteropneusts and pterobranchs as chordates. When the presence of the notochord was refuted (Van der Horst, 1939; Hyman, 1959), they were placed together in their own phylum because they had some but not all of the defining chordate features. Hemichordates possess gill slits and dorsal hollow nerves, but lack a notochord.

Deuterostome Evolution

A finding here not concordant with the traditional metazoan systematics is that the hemichordates are more closely related to the echinoderms than the chordates (Fig. 1A). "The adult echinoderm and the adult hemichordate are so completely different that no one could suspect any relationship between them on the basis of adult anatomy" (Hyman, 1959, p. 197). However, their embryology is similar in many respects, including larval morphology, coelomic development, and the presence of a hydropore. Based on the similarities between the tornaria larvae of enteropneust and the echinoderm larvae (e.g., auricularia), Metschnikoff (1881) proposed that hemichordates and echinoderms be classified together in the Ambulacraria. I propose that the term Ambulacraria be formalized as a node-based name to identify the last common ancestor of the echinoderms and hemichordates and all of the descendants of that ancestor. [de Queiroz and Gautheir (1990) describe the utility of node-based names over other naming procedures.]

This placement of the hemichordates suggests that some of the features of the traditional hemichordate–chordate clade may be more primitive than previously believed. Based on the molecular phylogeny, the presence of ciliated gill slits is a plesiomorphy for the deuterostomes. Therefore, Bather's (1913) assertion, later argued by Jefferies (1986), that primitive echinoderms possess ciliated gill slits is very likely. (However, it is clear that primitive echinoderms are not direct ancestors of the chordates as suggested by Jefferies.) Using a parsimony criterion, gill slits arose once in the basal deuterostome lineage and were subsequently lost twice, once in echinoderms and once in the rhabdopleurid pterobranchs. Of the pterobranch genera, *Rhabdopleura* do not possess gill slits, but *Cephalodiscus* has a single paired gill slit. *Atubaria,* which also has a single pair, is most probably a species of *Cephalodiscus.*

Because a dorsal hollow nerve chord is present in enteropneusts, pterobranchs, and chordates, one would expect that it arose twice (once in hemichordates and once in chordates) or that it was a plesiomorphic feature that was lost in echinoderms. This latter explanation seems more likely for two reasons. First, throughout evolution the loss of complex structure is much more common than two independent gains. Second, the loss of a dorsal hollow nerve chord is conceivable since the nervous system apparently became more simplified with the change from bilateral to pentaradial symmetry (Hyman, 1955, p. 701). Because of the complex metamorphosis in echinoderms, the oral and aboral surfaces topologically represent the right and left sides of the larvae. Thus, the nerve ring found in echinoderms does not correspond to a dorsally derived feature (Hyman, 1955).

Conclusions

Based on 18S rDNA sequence data, the pterobranchs are most closely related to the enteropneusts, or acorn worms. This finding is consistent with the notion of hemichordate monophyly and is inconsistent with hypotheses which posit that pterobranchs are basal deuterostomes. In all of the topologies obtained herein, the hemichordates are more closely related to the echinoderms than to the chordates. The name Ambulacraria is adopted from Metschnikoff to identify the hemichordate–echinoderm clade. The phylogenetic position of the hemichordate also suggests that some features (e.g., ciliated gill slits and the dorsal hollow

nerve chord) evolved much earlier than previously believed.

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