

The higher phylogeny of Phylactolaemate bryozoans inferred from 18S ribosomal DNA sequences

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ABSTRACT: Freshwater bryozoans (Phylactolaemata) are organized among five distinct families. Comparative morphology of these families suggests a linear series, from simple to complex, which is generally interpreted as an evolutionary trend. We sought to test this widely accepted hypothesis by analysis of partial 18S rDNA sequences from 8 species representing the five families, rooted with 2 species of cnidarians. Genetic variation among the bryozoan species was small. The two monotypic families, Cristatellidae and Pectinatellidae, displayed twice the amount of variation as all other species combined. Because of the lack of sufficient informative sites parsimony analysis could not be used in the analysis. Maximum likelihood and distance matrix analyses both suggest a new phylogenetic tree that runs contrary to the traditional view. At the base of the tree nearest the outgroup are all globular colonies with large, hooked statoblasts and large lophophores. At the top of the tree are the branching, tubular colonies, with relatively smaller statoblasts and lophophores. These results cloud the already subjective view of phylactolaemates being related in some way to the ancient marine Stenolaemates or Ctenostomes, both of which exhibit tubular colonies. In fact, the aligned DNA sequences of phylactolaemate bryozoans show greater similarity to phoronids than to gymnolaemates. They also suggest unexpected evolutionary trends among phylactolaemate statoblasts: spiny, self-inflating statoblasts of Pectinatellidae and Cristatellidae appearing early, followed by the general morphological simplification of Plumatellidae and Fredericellidae.

1 INTRODUCTION

Freshwater bryozoans (Class Phylactolaemata) constitute a well-defined group of sessile invertebrates. With about 80 described species inhabiting a wide range of freshwater habitats, they are among the most common metazoans living on submerged substrates. Like their marine counterparts, phylactolaemates are exclusively modular in structure, composed of many identical zooids all freely sharing a common coelom (Wood 2001). The group displays an impressive diversity of colony morphology, ranging from diffuse branching tubules to compact, globular masses. Freshwater bryozoans often cause serious fouling of irrigation pipes and water cooling systems (Wood & Marsh 1998, Smith et al. this volume). Several species are implicated as final hosts to a serious myxozoan parasite of salmonid fish (Canning et al. 1999).

Among all of the so-called lophophorate animals (bryozoans, phoronids, and brachiopods) only the phylactolaemates and a handful of ctenostome bryozoan species occur in fresh water. One of their adaptations to a freshwater habitat is the asexual

production of dormant structures called statoblasts. These enable populations to survive drought, cold temperatures, and other unfavorable conditions; they also serve as effective disseminules. Statoblast morphology is distinctive for each family and often provides diagnostic features for species identification.

Phylactolaemate bryozoans have long been regarded as the most primitive of the living bryozoans (Hyman 1959, Ryland 1970). This view is based on features believed to pre-date modern marine species: cylindrical zooid shape, muscular body wall, bilateral lophophore symmetry, and monomorphic zooids.

From this standpoint, evolutionary trends within the class seem fairly clear. Fredericellids, with their diffuse tubular structure, and structurally simple statoblasts would be closest to the ancestral type (Cori 1941, Brien 1960, Lacourt 1968, Ryland 1970, Willmer 1990). With this starting point, the evolutionary trends shown in Table 1 would include:

- Increased compactness of colonies leading to a consolidation and fusion of zooids.
- Lophophores with steadily increasing tentacle numbers.

Table 1. Summary of morphological features distinguishing the five families of Phylactolaemata. The traditional view of phylactolaemate evolution assumes progression from left to right

Condition	Fredericellidae	Plumatellidae	Pectinatellidae	Lophopodidae	Cristatellidae
Colony form	Branched tubes, diffuse.	Branched tubes, diffuse to appressed.	Sac-like, thick walled, zooids crowded.	Sac-like, thick walled, zooids crowded.	Sac-like, thick walled, zooids crowded.
Zooid spacing	Widely spaced	Widely spaced to compact.	Compact	Compact	Compact
Tentacle	Circular arrangement	U-shaped	U-shaped	U-shaped	U-shaped
Statoblasts	Simple, bean-like	2 types: Floating (self-inflating or sessile with peripheral spines	Floating only (self-inflating)	Floating only (not self-inflating) with spines radiating from center.	Floating only (self-inflating);
Approximate number of known species	4	70	1	5	1

• Increased complexity of statoblasts, starting with simple, bean-like structure of fredericellids, followed by development of two functional types in plumatellids, finally leading to the appearance of spiny, dual-function types in the globular colonies.

This scheme is tacitly acknowledged in most recent books and articles where phylactolaemate bryozoans are described. Typically, Fredericellidae is treated first, followed by Plumatellidae, the progression always ending with Cristatellidae (Lacourt 1968, Bushnell 1974, Geimer & Massard 1986, Wood 1989, Smith 1989, Ricciardi & Reiswig 1994).

Among the three non-tubular families (Cristatellidae, Lophopodellidae, Pectinatellidae) there is no clear indication of the most likely lineage. All three families produce relatively large, free statoblasts with projecting hooks or spines. In Cristatellidae the spines originate from a central area (fenestra) while in other families they originate along the periphery. Statoblasts of Lophopodidae become buoyant only after desiccation, while those of Pectinatellidae and Cristatellidae are inflated to achieve buoyancy prior to their release. Unfortunately, the two latter families are each represented by only a single species, so the systematic information they can provide is limited.

This study is based on 18S ribosomal DNA extracted from nine species of phylactolaemate bryozoans. There were two components of the work. First, we wanted to see to what extent

molecular data could contribute to an understanding of family relationships in phylactolaemate bryozoans. Second, we sought to explore the relationship of phylactolaemate bryozoans to other lophophore-bearing invertebrates based on their 18S ribosomal DNA sequences.

For the phylactolaemates specifically, we wanted to test the idea that fredericellid species lie closest to the common ancestor. In addition, we hoped for clarification on the systematic position of the *Hyalinella punctata*, a species that shares important features with both Plumatellidae and Lophopodidae (Wood 2001). Finally, we sought a better understanding of the relationships among Pectinatellidae, Lophopodidae, and Cristatellidae, the so-called >higher= phylactolaemates (Mukai & Oda, 1980).

2 METHODS

Nine bryozoan species were used in this study, representing the five major families: Fredericellidae (*Fredericella sultana* and *F. indica*), Plumatellidae (*Plumatella reticulata*, *P. fungosa*, and *Hyalinella punctata*), Lophopodidae (*Lophopodella carteri* and *Asajirella gelatinosa*), Pectinatellidae (*Pectinatella magnifica*), and Cristatellidae (*Cristatella mucedo*). All were collected in England except *Pectinatella*, which came from Ohio, USA. We placed specimens in filtered water to clear the gut of contaminants, then narcotized them with menthol and preserved them in 100% ETOH. Several polypides were removed from each colony for DNA extraction using Qiagen Qiaamp DNA mini kit.

Oligonucleotide primers for amplification (as synthesized, 5' to 3', F=forward, R=reverse) were as follows: 18S fragment 1 F, TCCCAGCTCCAATAGCG; R, GCAGCAACTTTAATATACGC; fragment 2 F, ATTCTTAGATCGTCGCAAG; R, AGAGTCTCGGTTATCG. The following PCR master mix (1x) was used: 5.2 l ddH₂O, 2.0 l 10x

buffer, 2.0 l DNTP (2 M), 2.4l MgCl (25 M), 1.0 l primer #1, 1.0 l primer #2, and 0.4 l TAQ. A 20 l PCR reaction was run comprising of 14 l of the master mix and 6 l of DNA. The reaction mixture was then placed into a thermocycler and run through 32 cycles of the following program: 93°C denaturing (45 seconds), 55°C annealing (45 seconds), 72°C extension (2 minutes), 4°C holding. Amplified samples were then electrophoresed using a 0.8% agarose gel at 120 volts (1x TBE), stained, then cut from the gel. The DNA was purified from the gel using Qiagen QIAquick Gel Extraction Kit, concentrated, and sent to a third party sequencing house for direct sequencing.

To build a tree based on the 8 phylactolaemate species we used *Phoronis australis* (GenBank AF119079) and *Phoronis hippocrepia* (U08325) as a combined outgroup. Sequences were initially aligned by ClustalW, and further alignment was done manually using BioEdit (Hall 1999). The analyzed data set had 1454 to 1459 nucleotides depending on the species. Approximately 1352 (92.9%) of these were constant, 80 (5.5%) were parsimony uninformative, and only 23 (1.6%) parsimony informative. By including the outgroup species the number of parsimony-informative sites rose to 95 (6.5%). The sequences did not differ significantly in base composition ($t = 1.200$, $df = 22$, $P \text{ value} = 0.2430$).

To compare the phylactolaemates with other lophophore-bearing phyla we selected DNA sequences from 4 phylactolaemate species, each representing a different family. These were aligned together with 18S data from other lophophorates acquired from GenBank. Listed by taxon along with GenBank accession numbers, these included 3 phoronids: (*Phoronis australis* (AF119079), *Phoronis hippocrepia* (U08325), and *Phoronis psammophila* (U36271); 2 brachiopods: *Terebratulina retusa* (U08324), and *Megerlia truncata* (U08321); 3 cheilostome gymnolaemates, *Caberea borvi* (AF119082), *Electra bellula* (AF499744), and *Schizoporella* (AF499743); and 1 ctenostome gymnolaemate, *Alcyonidium gelatinosum* (X91403). Two cnidarian species were selected to serve as a combined outgroup: *Aurelia auritus* (AY039208) and *Anemonia sulcata* (No. X53498).

Alignment was performed with ClustalW and BioEdit as described above. The alignment had 1402 to 1439 characters of which 59% were constant, 23% parsimony uninformative, and 18% parsimony-informative.

We conducted phylogenetic analyses using PAUP* 4.0b8 (Swofford 2000). For maximum parsimony 1000 heuristic searches were performed using random addition sequence. Characters were weighted equally and unordered, and gaps were treated as missing. For the phylactolaemate species

Modeltest version 3.5 (Posada & Crandall 1998) indicated that sequence data best fit the K80 model with discrete approximation of the gamma distribution (K80+G, shape parameter 0.2047; Kimura 1980). The best fit for combined lophophorate data was the TrN model with discrete approximation of the gamma distribution (TrN+G, 4 rate categories, shape parameter 0.3807; Tamura & Nei 1993).

3 RESULTS

3.1 *Phylactolaemates*

In a comparison of the 5 phylactolaemate families the number of exclusive nucleotide substitutions was highest among the Lophopodidae (16) and Pectinatellidae (13) and lowest among the Fredericellidae (0) and Plumatellidae (4). Of course the phoronid outgroup had many more with 69.

The exclusion of all sites with insertions or deletions left 189 variable sites. Removing the cnidarian species left only 23 sites that could be informative for parsimony analysis. Eighteen of these 23 sites involved base substitutions occurring exclusively in the two lophopodid species. Among the 23 sites there was not single a substitution involving Pectinatellidae, Cristatellidae, or Pectinatellidae, which together comprise 60% of the phylactolaemate families under consideration. Consequently these sequences were not accessible to parsimony analysis, and the tree shown in Fig. 1 reflects the failure to distinguish these families by parsimony. However, the phylogenetic tree in Fig. 1 does clearly separate lophopodid and plumatellid species, placing *Hyalinella* solidly among the plumatellids. Bootstrap numbers involving the three inaccessible families are understandably low.

Distance matrix analysis using UPGMA suggested a somewhat different phylogenetic tree (Fig. 2). Here Lophopodidae and Pectinatellidae are positioned at the base of the tree, with successive nodes leading to Cristatellidae, and finally a Plumatellidae-Fredericellidae complex. As expected, the two lophopodid and fredericellid species remained solidly clustered in all analyses. *Hyalinella punctata* was still joined with the Plumatellidae, far from the Lophopodidae.

3.2 *Lophophorates*

In a rough comparison of lophophorate sequences both brachiopods and phoronids had the greatest number of nucleotides matching the phylactolaemate bryozoans (93%). Phylactolaemates were 91% similar to Cheilostomes and 85% to the cnidarian outgroup.

Parsimony analysis clustered all species solidly within their respective groups (Fig. 3). However, the

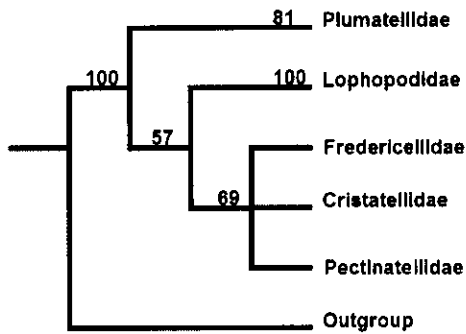


Figure 1. Parsimony analysis of 18S rDNA sequence data for 5 families (9 species) of phylactolaemate bryozoans using two phoronid species as the outgroup. The scarcity of parsimony-informative sites limits the significance of the tree for Pectinatellidae, Cristatellidae, and Fredericellidae. Bootstrap values are based on 1000 heuristic search replicates performed using random addition sequence.

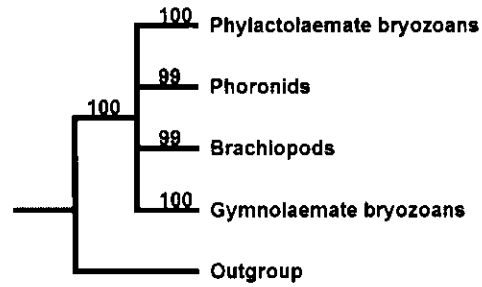


Figure 3. Parsimony analysis of 18S rDNA sequence data for 14 lophophore-bearing species representing 4 distinct groups, with 2 cnidarian species serving as the outgroup. Bootstrap values are based on 1000 heuristic search replicates performed using random addition sequence.

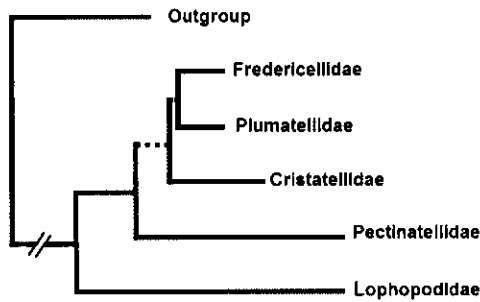


Figure 2. Phylogenetic tree suggested by distance analysis (UPGMA) of 18S rDNA sequence data for 5 families (9 species) of phylactolaemate bryozoans using two phoronid species as the outgroup. Dotted lines have an undefined length.

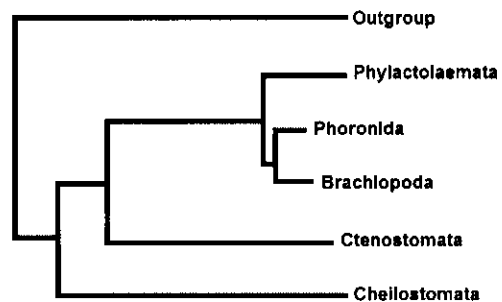


Figure 4. Phylogenetic tree suggested by distance analysis (UPGMA) of 18S rDNA sequence data for 14 lophophore-bearing species representing 4 distinct groups, with 2 cnidarian species serving as the outgroup.

majority-consensus tree could not suggest lineages among them at the 50% level. Distance matrix methods were consistent in suggesting a tree that places phylactolaemate bryozoans close to phoronids and brachiopods and quite distant from Gymnolaemata (Fig. 4).

4 DISCUSSION

4.1 *New interpretation of phylactolaemate phylogeny*

This is the first attempt to study family phylogeny in freshwater bryozoans using the tools of molecular genetics. Interpretation of the data is somewhat complicated by the fact that two of the five families, Cristatellidae and Pectinatellidae, are each represented by only a single species. This effectively precludes

parsimony analysis, because none of the variations in their sequences are shared with other species in the study.

Pectinatellidae and the combined species of Lophopodidae had by far the greatest number of exclusive nucleotide substitutions. This suggests a long, isolated history for each group, which is consistent with their placement at or near the base of the distance matrix trees. By contrast, the plumatellids and fredericellids, all showing little genetic variation, are interpreted as being more recently evolved.

The revised scheme of family relations within the Phylactolaemata runs contrary to systems previously proposed (Hyman 1959, Ryland 1970, Wood 1983). They suggest that:

- Early phylactolaemate bryozoans could have been compact and thick-walled colonies with

relatively large zooids. Diffuse colonies of branching tubules would have appeared later, together with a general reduction in zooid size.

• The earliest statoblasts were uninflated, of course, but they were lophopodid, not fredericellid statoblasts. Statoblast self-inflation appeared with Pectinatellidae, and was subsequently lost in Fredericellidae (along with the statoblast annulus) and *Hyalinella punctata*.

Implications of this scheme are discussed below with respect to colony and statoblast morphology.

4.2 Colony morphology

Among the marine (gymnolaemate) bryozoans, branching tubules and cylindrical zooids are typical of Stenolaemata and Ctenostomata. Both of these are ancient groups, represented in marine deposits from the Ordovician (Boardman et al. 1983; Cheetham & Cook 1983). They pre-date the calcified, box-like zooids of Cheilostome bryozoans which erupted in the late Cretaceous and are still dominant today. It may be this progression from diffuse, branching tubules to a more compact colony form that has driven the notion of a similar trend in phylactolaemate bryozoans.

However, there is otherwise no reason to consider compact colonies any more advanced than diffuse, tubular ones. If the ancestral phylactolaemate were a solitary animal, then the development of modular form might well have begun with tightly clustered units. New zooids, formed by asexual budding and failure to separate, could remain together in close proximity, perhaps generating a colony-wide pattern of feeding currents as in modern *Pectinatella* (Mukai 1998).

One danger to tightly clustered zooids in freshwater habitats is the risk of losing the entire colony to desiccation if water levels drop. Those phylactolaemate colonies with the most closely knit zooids (*Pectinatella*, *Lophopodella*, *Cristatella*) reduce that risk somewhat with colony motility. In laboratory studies, for example, *Lophopodella carteri* can move downward as much as 1 cm/day ahead of a falling water level (Riley, pers. com.). More diffuse colonies are permanently sessile, but can grow long branches in any direction, extending the life of at least a portion of the colony when the water level drops.

4.3 Statoblasts

The phylogenetic trees suggested by 18S rDNA data indicate that the lophopodid statoblast is the most primitive. Although it sinks upon release from the colony, the wide annulus of sclerotized chambers traps air upon drying, providing effective buoyancy when the statoblast is returned to water. It had been proposed by Wood & Marsh (1996) that the initial lack of buoyancy was an adaptation protecting

statoblasts from harsh tropical sunlight and warm surface water. While this may still be true, the new phylogenetic tree offers a different explanation for the absence of self-inflation.

Until recently the sharp differences between the statoblasts of fredericellid and plumatellid species appeared not to support close proximity of these families. In fredericellids the statoblast is a simple capsule, while the plumatellid statoblast adds elegant sclerotized structures that either provide buoyancy or else cement the capsule firmly to the substratum. However, new studies on living colonies of the fredericellid *Internectella bulgarica* confirm that this species also produces free statoblasts capable of self-inflation and buoyancy, much like those of plumatellids (Wood, unpublished). A second type of statoblast in this species is sessile with a distinct annulus similar to the plumatellid sessoblast. (Gruncharova, 1968, Wiebach, 1974). In all other respects, *Internectella* retains the classic features of a fredericellid, with sparse colonies of stringy tubules, widely spaced zooids, and circular lophophores. A common species in Southeast Asia, *Internectella bulgarica* represents a compelling link between Fredericellidae and Plumatellidae.

In all other fredericellid species, the sessile, bean-like statoblast has been thought by some to represent a primitive condition (Cori 1941, Brien 1960, Lacourt 1968, Ryland 1970, Willmer 1990). In our interpretation, this simplification is derived. The outer periblast of fredericellid statoblasts is gone or at least reduced. The minutely jagged, keel-like basal ring that helps secure some fredericellid statoblasts to the substratum could well be a remnant of the basal periblast of plumatellid sessoblasts.

The proposed new tree places Pectinatellidae and Lophopodidae in some proximity. These are the only two families in which the statoblast periphery is adorned by various sizes of hooks, and so the adjacent lines are not surprising. Cristatellidae also has peripheral hooks, but they originate from centrally located spines, so their presence is apparently independent in this line. Minute peripheral hooks are seen in statoblasts of the plumatellid, *Swarupella andamanensis* (Rao et al. 1985) but the significance of this feature is not apparent.

4.4 Phylactolaemates, phoronids, and brachiopods

The suggestion of significant evolutionary distance between phylactolaemate and gymnolaemate bryozoans is not novel. Hyman (1959) noted similarities between phylactolaemates and *Phoronis ovalis*, the only modular phoronid species. Jebram (1973) added a supporting argument based on the similar adoral orientation of new buds in these two groups, exactly opposite from gymnolaemates. Mundy et al. (1981) nicely summarized these points,

listing true body wall musculature and details of lophophore ontogeny as features not shared by gymnoalaemates. Bachus & Banta (2002) further noted the parallel between yolk-bearing peritoneal cells forming fat bodies in phoronids and the yolk peritoneal cells that accumulate in phylactolaemate statoblasts. Finally, Zimmer (1997) described the unusual lecithotrophic larva of *P. ovalis*, which appears to be more similar to the phylactolaemate larva than to the typical actinotroch of other phoronids. Taken together, these diverse observations build an interesting case for a common ancestry of phoronids and phylactolaemate bryozoans. The 18S rDNA analysis offered here links Phylactolaemata to a phoronid-brachiopod ancestor, but provides no further detail.

If phylactolaemates were derived from phoronids or a phoronid root why is there no evidence from previous, well-documented molecular studies? The answer could lie in the selection of species and methods. The 18S rDNA analysis by Halanych et al. (1995) chose a single phylactolaemate (*Plumatella repens*) to represent all bryozoans; Cohen & Gawthrop (1996) included only one species of gymnoalaemate (and found the phylactolaemates genetically closer to priapulids). Other studies have used a variety of assumptions and methods of analysis producing widely different phylogenetic trees.

The findings presented here are far from conclusive. The small number of species in most phylactolaemate families does not allow much resolution. In retrospect, the 18S region of rDNA proved to be rather uniform and may not have been the best place to explore bryozoan phylogeny, (although it was an area where good lophophore data were available). Certainly a complicating factor here may be the inhibition of meiotic crossing over in certain phylactolaemates, leading to an accumulation of favorable mutations and variable rates of rDNA evolution in different parts of the genome (Bachus & Banta 2002). Additional kinds of evidence need to be explored, including new regions of the genome and chromosome morphology. For example, Bachus & Banta (2002) noted that morphology of the NOR chromosome in *Cristatella* appears to be more similar to that of *Fredericella* than that of *Plumatella*. They also saw a heteromorphism in the *Pectinatella* chromosome in which suggested links to either Fredericellidae or Plumatellidae, or to both (B.T. Bachus, pers. com.).

5 CONCLUSIONS

Analysis of about 1,450 sequential nucleotides in 18S ribosomal DNA of phylactolaemate bryozoans suggests that compact, gelatinous colonies with large

zooids and spiny, free statoblasts are closest to the ancestral type. Diffuse colonies with branching tubules and sessile statoblasts appear to be more recent.

In a comparison of rDNA from species representing cheilostomes, ctenostomes, phoronids, brachiopods, and phylactolaemates, the latter group was more closely linked with the phoronids/brachiopod line than with any of the others. While far from conclusive, this result adds to a growing list of diverse reports distancing phylactolaemates from gymnoalaemate bryozoans.

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