PRELIMINARY MOLECULAR PHYLOGENY OF THE RADULA-LESS DORIDS (GASTROPODA: OPISTHOBRANCHIA), BASED ON 16S MTDNA SEQUENCE DATA

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ABSTRACT

Maximum-parsimony analysis of the nucleotide sequence of the 16S mtDNA gene, obtained from several dorid nudibranch taxa, suggests that the radula-less dorids are likely to be a monophyletic group. However, there is little support for the most basal branches of the radula-less dorid clade. Most of the traditional groups within the radula-less dorids are monophyletic and for the most part well supported. These include the genera *Dendrodoris*, *Doriopsilla*, *Reticulidia*, *Phyllidiella* and *Phyllidia* (when *Fryeria* is included in *Phyllidia*). On the contrary, the family Dendrodorididae and the genus *Phyllidiopsis* are paraphyletic. Branch support for those arrangements that differ from the topology of previously published morphological trees is poor. Data obtained from the 16S mtDNA gene seem to be adequate to resolve the phylogenetic relationships of derived clades of radula-less dorids, but longer sequences are necessary to obtain more information at the most basal nodes.

INTRODUCTION

The radula-less dorids (Porostomata) constitute one of the traditional major groups of dorid nudibranchs. As a result of the loss of the radula, these animals have developed a number of anatomical transformations in the foregut, which is adapted for suctorial feeding on sponges. Historically, two groups of Porostomata have been distinguished based on differences in external morphology. Dendrodorididae O'Donoghue, 1924 includes animals with external appearance similar to other dorid nudibranchs, whereas Phyllidiidae Rafinesque, 1814 comprises species that lack a dorsal gill and have ventral respiratory leaves. The external morphology of members of the Phyllidiidae is unique among dorid nudibranchs.

Due to the remarkable external differences between the Phyllidiidae and the Dendrodorididae, several authors (Bergh, 1892; Eliot, 1903; O'Donoghue, 1929; Todd, 1983) have questioned the naturalness of the Porostomata, whereas other authors have supported the validity of this group (Pruvot-Fol, 1956; Schmekel, 1985). Baranetz & Minichev (1994), based on their personal interpretation of the evolution of the gill in nudibranchs, included the Dendrodorididae with the rest of the dorids with a dorsal gill and elevated the Phyllidiidae to a separate order. In addition, they divided the phyllidiids in two different suborders, Phyllidiina including animals with a dorsal anus and the new suborder Fryeriina for the animals with a ventral anus.

Chemical studies have shown that the natural products present in the Dendrodorididae and the Phyllidiidae differ in structure and origin (Cimino & Ghiselin, 1999). Phyllidiids accumulate terpene isocyanides obtained and transformed from the secondary metabolites of the sponges they eat. Dendrodorids also accumulate metabolites from sponges, but at the same time they are able to biosynthesize sesquiterpenoids *de novo*, a process that has not been documented in phyllidiids. Additionally, study of the ultrastructure of the spermatozoa of dendrodorids and phyllidiids apparently reveals no compelling reasons to associate them together (Healy & Willan, 1991). However, because there are no other groups of nudibranchs with a similar chemistry or sperm morphology to either dendrodorids or phyllidiids, neither chemical nor sperm data are conclusive in resolving this controversy.

Recently, authors have been using a phylogenetic approach to address the problem of the monophyly of the Porostomata. Brunckhorst (1993), based on a morphological data, concluded that the radula-less dorids are polyphyletic and proposed that the Dendrodorididae should be grouped with other dorids with a dorsal gill, retaining the Phyllidiidae at superfamilial rank. Brunckhorst's (1993) phylogeny is not conclusive because of the absence of enough comparative taxa. For his analysis, Brunckhorst (1993) included the major lineages of Phyllidiidae, but only a derived member of the Dendrodoridae (*Dendrodoris*) and a single radula-bearing dorid genus for comparison. No basal members of the Dendrodoridae or the radula-bearing dorids were included in the analysis.

Valdés & Gosliner (1999) published another phylogenetic hypothesis, this time including numerous members of dendrodorids, phyllidiids and a few other radula-bearing dorids. According to Valdés & Gosliner's (1999) phylogenetic hypothesis, the radula-less dorids constitute a monophyletic group supported by several synapomorphies. Within the radula-less dorids, the genus *Mandelia* Valdés & Gosliner, 1999, is the sister group of the other porostomes, which are arranged in two well-supported monophyletic clades, containing phyllidiids and dendrodorids, respectively. In addition, the radula-less dorids are an internal branch of the Cryptobranchia, which is the group containing dorid nudibranchs with a retractable gill.

Recently published molecular phylogenies including dorid nudibranchs, and using the 16S and 18S mtDNA genes (Thollesson, 1999a,b; Wollscheid and Wägele, 1999), have not been able to offer a definitive solution to the problem. However, Thollesson (2000) indicated that the sister-group relationship between one member of each group in his 16S mtDNA phylogeny suggests that the radula-less dorids could be a monophyletic group.

The aims of this paper are to test the monophyly of the Porostomata and the phylogenetic relationships within this group, using 16S mtDNA data from several species of radula-less dorids as well as several other comparative taxa. To complement previous studies, longer sequences of the 16S mtDNA gene belonging to a larger number of radula-less taxa have been obtained and analysed.

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MATERIAL AND METHODS

Specimens and DNA extraction

Most of the samples utilized for the present study were collected during the expedition Lifou 2000, to the Baie du Santal, Lifou, New Caledonia, organized by the Muséum National d'Histoire Naturelle of Paris and the IRF, Nouméa, New Caledonia. Additional samples were collected in different localities. Table 1 lists the taxa studied including localities and collection dates. All samples were preserved in ethanol 95%.

Tissues were digested for 3–4 h at 65°C with constant motion in 2 ml of lysis buffer (Tris HCl 100 mM at pH 8.0, EDTA 50 mM at pH 8.0, NaCl 10 mM, SDS 0.5%) containing 60 μg of proteinase K per ml. Digestion was followed by extraction, twice with phenol/$CHCl_3$ at pH 7.3 and once with $CHCl_3$. DNA was precipitated from the aqueous layer with 2.5 volumes of pure ethanol, washed in 80% ethanol, dried and resuspended in TE buffer (Tris 10 mM, EDTA 1 mM, pH 8.0).

PCR amplification and sequencing

Template DNA for PCR was prepared by diluting stock DNA with TE buffer to give spectrophotometric readings at 260 nm between 0.2 and 0.7 Å. Mitochondrial DNA was amplified from template DNA in 100-μl reactions using a hot-start method in a thermal cycler with a 7-min denaturing step at 94°C, primer annealing for 30 s at 46°C, and elongation for 1 min at 72°C with a final 7-min elongation step at 72°C. The primers used were the universal primers for the mitochondrial large ribosomal subunit (16S), 16sar-L (5′-cgcctgtttatcaaaaacat-3′) and 16sbr-H (5′-ccggtctgaactcagatcaccgt-3′), developed by Palumbi, Martin, Romano, McMillan, Stice & Grabowski (1991). PCR products were purified using Promega Wizard® PCR Preps DNA Purification System (Promega, Madison, WI) according to manufacturers' instructions.

Cycle sequencing was performed on the PCR products by using the Big Dye® (Perkin-Elmer, Norwalk, CT) reaction premix for 50 cycles of 96°C for 10 s, 45°C for 5 s and 60°C for 4 min. The nucleotide sequence was determined by using an ABI PRISM 3100 Genetic Analyser (Applied Biosystems, Norwalk, CT).

All sequences are deposited in Genbank (accession numbers AF430346-AF430371).

Sequence alignment

Complementary DNA strands were assembled and proof-read using Sequencher 3.1 for Macintosh (Gene Codes Corporation, Ann Arbor, MI). Sequences were aligned using the Clustal V algorithm (Higgins & Sharp, 1988) in the multiple alignment routine of Dnastar 3.06a for Macintosh. Subsequently, the alignment was improved with the utilization of secondary structural models to identify stem and loop regions. Secondary structure diagrams were modelled using the comparative sequence analysis method (Gutell, 1996), which is based on the premise that RNAs of closely-related taxa have very similar secondary and tertiary structures, regardless of the differences in nucleotide sequences. The secondary structure diagram of *Aplysia cervina* Dall & Simpson, 1901 by Medina & Walsh (2000) and *Thecacera pennigera* (Montagu, 1815) by Thollesson (1999a) were used as the structure model for the present study. The only non-canonical pairing allowed throughout the model was the wobble G-U pair, which is virtually as stable as an A-U pair (Chastain & Tinoco, 1991). Loop regions are identified following the numbering of Horovitz & Meyer (1995). The ambiguously

Table 1. List of species examined in this study, including sampling localities and collection dates.

	Locality	Date
Ingroup		
Dendrodoris tuberculosa (Quoy & Gaimard, 1832)	Lifou, New Caledonia	November 2000
Dendrodoris nigra (Stimpson, 1855)	Lifou, New Caledonia	November 2000
Dendrodoris denisoni (Angas, 1864)	Lifou, New Caledonia	November 2000
Dendrodoris albobrunnea Allan, 1933	Lifou, New Caledonia	November 2000
Dendrodoris elongata Baba, 1936	Lifou, New Caledonia	November 2000
Doriopsilla areolata Bergh, 1880	Asturias, Spain	July 1997
Doriopsilla gemela Gosliner, Schaefer & Millen, 1999	Baja California, Mexico	January 2000
Doriopsilla albopunctata (Cooper, 1863)	Baja California, Mexico	January 2000
Doriopsilla janaina Ev. Marcus & Er. Marcus, 1967	Costa Rica	January 2001
Phyllidia varicosa Lamarck, 1801	Lifou, New Caledonia	November 2000
Phyllidia elegans Bergh, 1969	Lifou, New Caledonia	November 2000
Phyllidia rueppelii (Bergh, 1869)	Hurghada, Egypt	September 1995
Phyllidia coelestis Bergh, 1905	Lifou, New Caledonia	November 2000
Phyllidiella pustulosa (Cuvier, 1804)	Lifou, New Caledonia	November 2000
Phyllidiella lizae Brunckhorst, 1993	Lifou, New Caledonia	November 2000
Phyllidiopsis cardinalis Bergh, 1875	Lifou, New Caledonia	November 2000
Phyllidiopsis sphingis Brunckhorst, 1993	Lifou, New Caledonia	November 2000
Phyllidiopsis striata Bergh, 1889	Lifou, New Caledonia	November 2000
Reticulidia halgerda Brunckhorst & Burn, 1990	Lifou, New Caledonia	November 2000
Reticulidia fungia Brunckhorst & Gosliner, 1993	Lifou, New Caledonia	November 2000
Outgroup		
Hexabranchus sanguineus Rüppell & Leuckart, 1831	Lifou, New Caledonia	November 2000
Archidoris pseudoargus (Rapp, 1827)	Asturias, Spain	July 1997
Peltodoris atromaculata Bergh, 1880	Ibiza, Spain	August 1996
Anisodoris nobilis (MacFarland, 1905)	Monterey, California	July 1999
Diaulula sandiegensis (Cooper, 1863)	Monterey, California	July 1999

aligned regions with this method were removed from the analysis. A total of 67 nucleotides were excluded from the analysis, in the following regions: 154–163; 173–185; 235–251; 432–454; 484–487. The aligned sequences have been deposited in TreeBASE (available at : http://www.treebase.org/) with accession numbers 5773–171224.

Phylogenetic analysis

The aligned sequences resulted in 497 nucleotides. Regions of ambiguous alignment were excluded from the final data set, which resulted in 430 nucleotides. Phylogenetic analysis under the parsimony criterion was performed with PAUP* 4.0 b8 (Swofford, 2001).

A heuristic parsimony analysis was performed with all sites weighted equally (TBR branch swapping option). One-hundred random starting trees were obtained via stepwise addition. Characters were polarized using the genus *Hexabranchus* as outgroup, based on the phylogeny by Wägele & Willan (2000), in which *Hexabranchus* is the sister taxon to the cryptobranch dorids. The reliability of clades in the shortest trees was assessed by using nonparametric bootstrapping (Felsenstein, 1985) with 1000 replicates, each executed as a heuristic search as described above. In addition, a Bremer analysis (Bremer, 1994) was conducted to estimate branch support. In cases where the number of possible trees exceeded computer memory, the strict consensus was calculated using the first 10,000 trees obtained.

RESULTS AND DISCUSSION

Secondary structure

Stems and loops in the secondary structure of mitochondrial rDNA have been coded and utilized as regular morphological characters in phylogenetic analysis (Lydeard *et al.*, 2000). Following the same approach, in the present study several differences have been observed in the secondary structure models of the taxa examined (Fig. 1). For the most part, differences

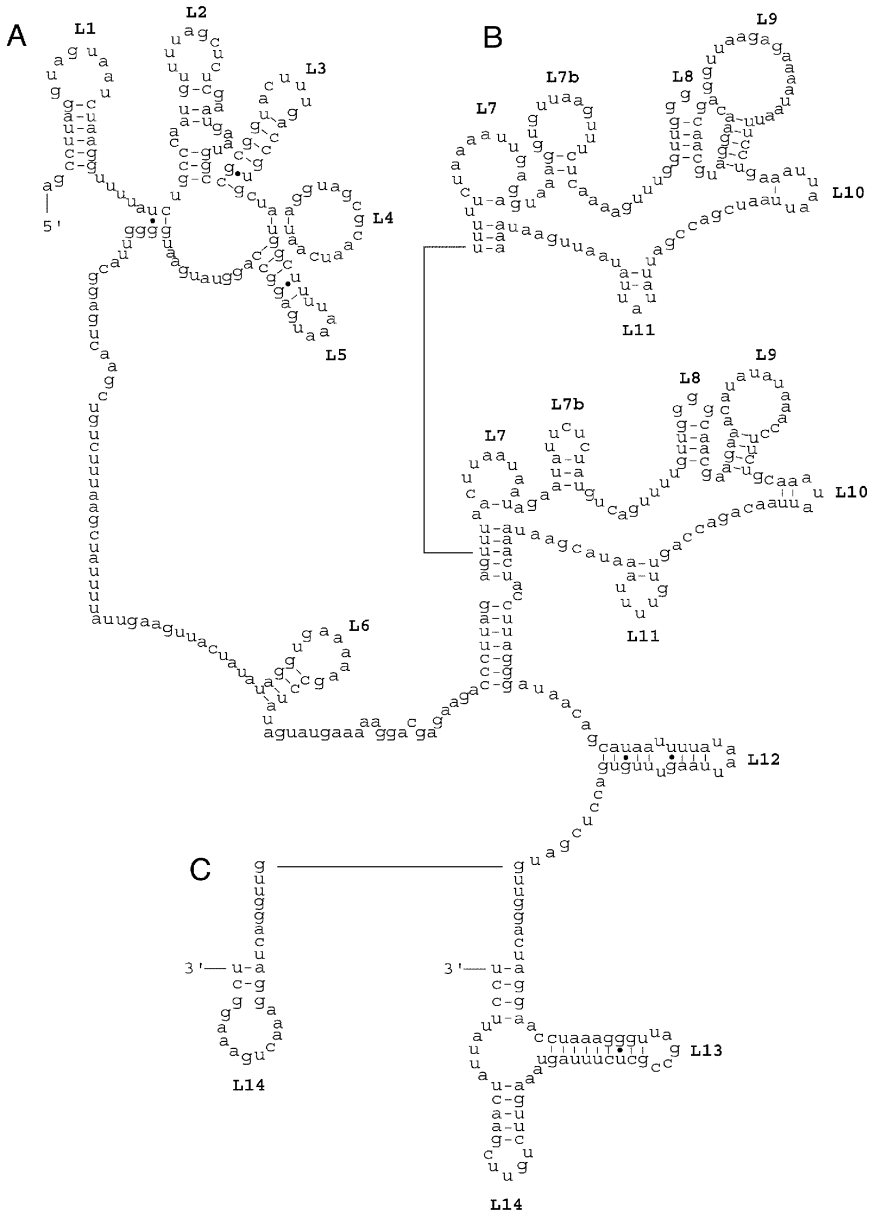

Figure 1. Secondary structure of the 16S rRNA. Loop numberings follows Horovitz & Meyer (1995). **A.** Entire sequence of *Dendrodoris denisoni*. **B.** Partial sequence of *Hexabranchus sanguineus* showing differences in the loops L7-L11 region. **C.** Partial sequence of *Diaulula sandiegesis* showing the missing L13 loop.

between taxa include minor variations in the length of some stems and the diameter of some loops. These characters do not appear to be sufficiently distinct to be phylogenetically informative. However, in the case of *Diaulula sandiegensis* the entire stem 13 is missing and loop 14 is considerably reduced. This structural arrangement differs greatly from that of other taxa examined and may constitute a phylogenetically informative character. Examination of the secondary structure in other species of *Diaulula* and close relatives will lead to a definitive conclusion.

The secondary structure of dorid nudibranchs differs from that of anaspidean opisthobranchs in having a largely reduced loop 10 and a possible additional loop, here called 7b. In addition, the region 9 that has been lost in *Aplysia* (Medina & Walsh, 2000), is present in dorid nudibranchs. All these structural changes, except for the loop 7b have been already detected by Thollesson (1999a) in nudibranchs. According to these preliminary data, it appears that the secondary structure of opisthobranchs molluscs could offer a number of useful characters for higher level phylogenies.

Phylogeny

The two parsimony trees obtained were 702 steps long (CI = 0.463, RI = 0.558). In the strict consensus tree (Fig. 2) the radula-less dorids are monophyletic, as are most of the traditional groups within this clade recognized by Brunckhorst (1993) and Valdés & Gosliner (1999). However, there is no bootstrap support for this node and the decay index is 1. The family Dendrodorididae and the genus *Phyllidiopsis* appear to be paraphyletic. In both cases there is little or no support for these nodes. Even though the topology of this gene tree differs from the phylogeny published by Valdés & Gosliner (1999) there are numerous similarities and the overall topology is similar.

In order to obtain more reliable phylogenies, examination of longer sequences and probably other genes is necessary. However, despite the small size of the 16S fragment examined, it appears to be useful for recovering the evolution of the derived radula-less dorids. In this case most of the traditional groups recognized by Valdés & Gosliner (1999) are monophyletic and strongly supported.

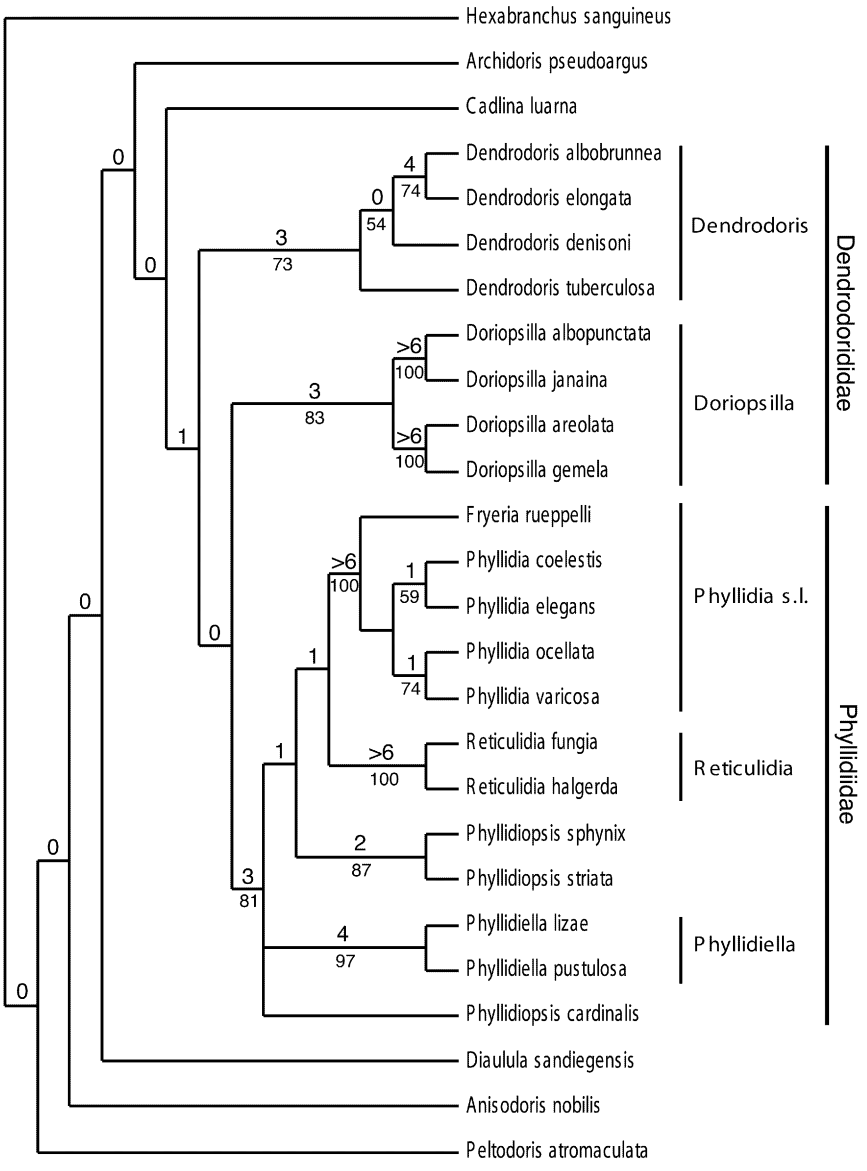

Figure 2. Maximum-parsimony tree of the radula-less dorids based on the 16S mtDNA gene. Decay indices are printed above each branch and bootstrap indices are printed below (50% majority rule). Traditional groupings are shown on the right side of the tree.

Radula loss and evolution of the anus position

The consensus tree obtained here is in agreement with the hypothesis proposed by Valdés & Gosliner (1999) that the radula was lost only once in the evolution of dorid nudibranchs. This hypothesis, which is clearly most parsimonious, has been opposed by Brunckhorst (1993) and other authors, who considered that the morphological differences between phyllidiid and dendrodorids nudibranchs justified the assumption of a different origin for these two groups. The utilization of modern phylogenetic systematics has shown that remarkable morphological differences are meaningless unless they are shared by other taxa. In the case of phyllidiid nudibranchs, the presence of ventral respiratory leaves is unique to this group and therefore uninformative for phylogenetic reconstruction under maximum parsimony.

The analysis of the present data set sustains the hypothesis proposed by Valdés & Gosliner (1999) that *Fryeria* species are members of the genus *Phyllidia* that have the anus displaced from a dorsal to a ventral position. Brunckhorst (1993) maintained the genus *Fryeria* as valid and distinct from *Phyllidia*, under the assumption that the position of the anus and other anatomical features of the type species were distinctive. However, phylogenetic analyses have shown that species of *Fryeria* are in fact members of *Phyllidia* with a ventral anus.

Congruence of molecular and morphological data sets

The major incongruence between morphological and molecular hypotheses of the radula-less dorids is the paraphyly of the Dendrodorididae in the molecular tree. However, this is poorly supported in both the Bremer and bootstrap analyses. Longer sequences are probably necessary to obtain more information at these nodes. Additionally, the genus *Phyllidiopsis* is not monophyletic when molecular characters are used, because *Phyllidopsis cardinalis* is at the base of the Phyllidiidae clade, and not nested with other members of *Phyllidiopsis*. According to Valdés & Gosliner (1999), *Phyllidiopsis* is monophyletic and supported by three synapomorphies: oral tentacles fused together; elongate buccal bulb; and elongate cerebro-buccal connective. On the other hand, there are some morphological differences between *P. cardinalis* and other members of the genus, such as the complex multicoloured dorsum with large and globose tubercles (Brunckhorst, 1993), which would support the molecular results. Further investigation of the phylogenetic relationships of *Phyllidiopsis* using different data sets is necessary to resolve the position of *P. cardinalis* and the possible paraphyly of this group.

The other traditional groups within the radula-less dorids are monophyletic and strongly supported. This molecular phylogenetic hypothesis is for the most part highly congruent with previous morphological analyses and the classification scheme proposed by Valdés & Gosliner (1999) for the radula-less dorids, and to some extent with the classification of the Phyllidiidae by Brunckhorst (1993).

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