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Phylogeny and relationships of pleurotomariid gastropods (Mollusca: Gastropoda): an assessment based on partial 18S rDNA and cytochrome *c* oxidase I sequences

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Abstract

The phylogenetic position of the ancient family Pleurotomariidae within the Molluscan class Gastropoda, as well as the relationships of its Recent genera and species, were assessed using an iterative, two-gene (18S rDNA and cytochrome *c* oxidase I) approach to phylogeny reconstruction. In order to orient the Pleurotomariidae within Gastropoda, partial 18S rDNA sequences were determined for 7 pleurotomariid and 22 other gastropods that span the major groups within the class as well as for one cephalopod and two polyplacophorans, which serve as outgroups. Cladistic analyses of a sequence of approximately 450 base pairs (bp) near the 5′ end of the 18S rDNA support the monophyly of the following higher gastropod taxa: Patellogastropoda, Vetigastropoda, Neritopsina, Apogastropoda, and its subclades Caenogastropoda and Heterobranchia. The 18S rDNA sequences and 579 bp of cytochrome *c* oxidase I (COI) analyzed separately and together, indicate that Pleurotomariidae are included within Vetigastropoda but comprise a clade that is the sister group to the other families referred to this order. Monophyly of the Pleurotomariidae is also supported by the unique presence of seven separate inserts (ranging in length from 1 to 68 bp) within the V2 variable region of the 18S RNA. Relationships of the genera and species within Pleurotomariidae are fully resolved using "total molecular evidence" consisting of partial sequences of 18S rDNA and COI and including data on length variation within the inserts.

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Introduction

The superfamily Pleurotomarioidea is recorded from Upper Cambrian deposits (Knight et al., 1960; Tracey et al., 1993) and comprises one of the oldest undisputed gastropod lineages. Its diversity at all taxonomic levels was greatest during the Paleozoic, when these animals were the most numerous, varied, and abundant of all gastropods. Pleurotomarioideans were conspicuous components of shallow-water marine communities throughout the Paleozoic and Mesozoic, but Cenozoic fossils are rare, occurring in deep-water facies (for review, see Hickman, 1976). Of the approximately 1500 species that have been described, only 24, all members of the family Pleurotomariidae, are living today. The Recent species are limited to tropical and temperate latitudes, occurring along the margins of continental tectonic plates that bound the western shores of the Atlantic, Pacific, and Indian Oceans as well as along southeastern Asia, northwestern Australia, and intervening islands (for review, see Anseeuw and Goto, 1996). All living species are members of upper continental slope communities (approx. 100–1000 m), most are restricted to hard substrates and steep to vertical slopes, and tend to be the numerically dominant, large gastropods in these habitats (Harasewych et al., 1988).

Pleurotomarioideans are most readily identified on the basis of having dextral, conispiral shells with narrow, generally deep emarginations or "slits" along their outer lips that give rise to a spiral band or selenizone along or near the periphery of the shell (Figure 1). The discovery of the first living pleurotomariid in 1856 prompted an era of intense anatomic scrutiny of these rare "living fossils," with every new collection of specimens generating a se-

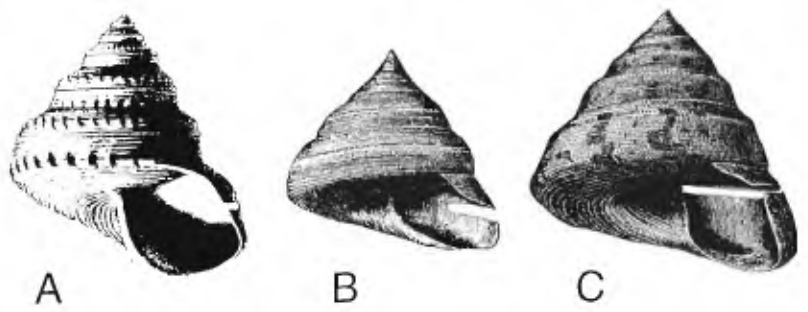


Figure 1. Representatives of fossil and living Pleurotomariidae. (A) *Pleurotomaria anglicus* (Sowerby, 1818), type species of *Pleurotomaria*, Jurassic, France. (B) *Perotrochus quoyanus* (Fischer and Bernardi, 1856). Recent, Lesser Antilles. (C) *Entemnotrochus adansonianus* (Crosse and Fischer, 1861), Recent, Lesser Antilles. Reproduced from Knight et al., 1960, pl. 131 (A) and from Dall, 1889, pl. 37 (B and C).

ries of publications (for review, see Bayer, 1965). Pleurotomariids have since been regarded almost universally as the most primitive living gastropods (e.g., Thiele, 1929; Taylor and Sohl, 1962; Boss, 1982; Vaught, 1989) and appeared as such in paradigms of gastropod evolution (e.g., Bouvier, 1887; Yonge, 1947; Morton and Yonge, 1964; Graham, 1985; Salvini-Plawen, 1985; Brusca and Brusca, 1990; for a detailed review, see Bieler, 1992). The combination in pleurotomarioideans of an asymmetrically coiled shell and symmetrically paired pallial organs (including gills, auricles, kidneys, and hypobranchial glands) has been regarded as transitional between extinct, planispirally coiled, bilaterally symmetrical ancestors, generally believed to be bellerophonts, and asymmetrical modern gastropods.

Interest in gastropod phylogeny has had a renaissance over the past two decades, prompted in large part by discoveries of primitive, previously unknown groups, primarily from deep-sea hydrothermal vents, and fueled by developments of molecular techniques and software for cladistic analysis, as well as by the more widespread application of the principles of phylogenetic systematics. The first of the major works to reassess gastropod evolution was that of Golikov and Starobogatov (1975). These authors recognized that the patellogastropod limpets, together with some Paleozoic groups, manifested a series of archaic characters that were neither shared with nor could be derived from other gastropods. They placed these taxa in the subclass Cyclobranchia to segregate them as an early, monophyletic offshoot from the remaining gastropods, and considered the pleurotomarioideans to be the most primitive of the Recent forms among the remaining gastropod taxa.

Salvini-Plawen (1980) divided the basal gastropods into the Vetigastropoda, in which he included the Pleurotomarioidea (Pleurotomariidae + Scissurellidae + Haliotidae + Fissurellidae), Cocculinoidea, Trochoidea, and the extinct Macluritoidea and Murchinsinoidea, as well as two other "early side-branches," the patellogastropod limpets and the Neritopsina. Following a rapid succession of articles providing data on new suites of anatomic and ultrastructural characters as well as on newly discovered taxa, Haszprunar (1988) published a hypothesis of gastropod phylogeny in which he proposed the successive emergence of 13 higher taxa (Figure 2A). More recently, Ponder and Lindberg (1996, 1997) published parsimony-based phylogenetic analyses of Gastropoda based on anatomic characters (Figure 2B) that differed from Haszprunar's hypothesis in the composition and placement of several key families and orders, but concurred in placing the patellogastropod limpets as the sister taxon to the remaining gastropods and the pleurotomariids as closely related to the trochids.

Few DNA sequence-based phylogenies containing more than five gastropods have been published to date (Emberton et al., 1990; Tillier et al., 1992, 1994, 1996; Rosenberg et al., 1994, 1997), all based on small portions (approx. 150–700 bp) from various domains within the large ribosomal molecule (28S rRNA). Most focused on evolutionary relationships of higher taxa within Gastropoda, but several (Tillier et al., 1992, 1994; Rosenberg et al., 1994, 1997) spanned the class as a whole. These studies, for the most part, supported the monophyly of Vetigastropoda and Apogastropoda (*sensu* Ponder and Lindberg, 1997 = Caenogastropoda + Heterobranchia), but differed in the placement of such basal groups as the Neritopsina (with Bivalvia in Rosenberg et al., 1994, 1997) and the patellogastropod limpets. Relationships of the patellogastropod limpets have proved especially difficult to resolve, as they emerge as a sister group to all other Gastropoda (Tillier et al., 1992), as a sister group to either Vetigastropoda, Neomphalina + Apogastropoda,

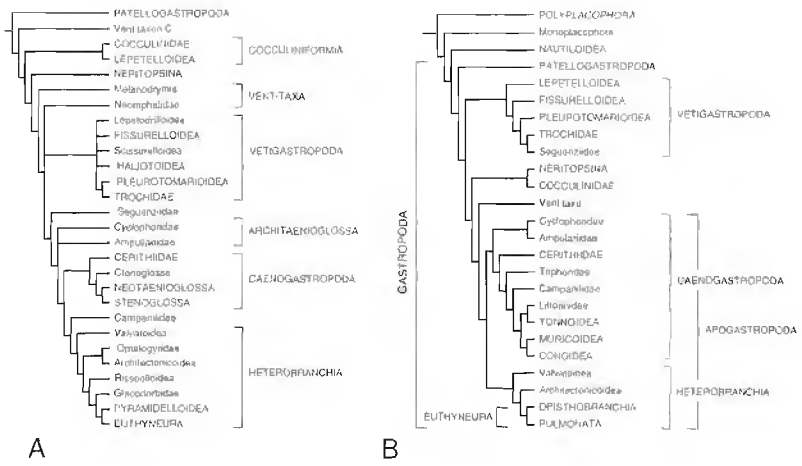


Figure 2. Recent morphology-based hypotheses of gastropod phylogeny. (A) Phylogenetic reconstruction of Haszprunar (1988, Figure 5), with nomenclature and taxon rank modified/updated to be comparable with that of (B) Ponder and Lindberg (1996a, Figure 11.3D). Taxa listed in uppercase are represented in the present study (see Table 1). The "Hot Vent C" limpets reported by Haszprunar (1988) were subsequently described as family Neolepetopsidae by McLean (1990) and included in Patellogastropoda by Ponder and Lindberg (1996, 1997).

or Heterobranchia (Tillier et al., 1994), or within Caenogastropoda (Rosenberg et al., 1997), depending on such variables as outgroup, included taxa, and the algorithm used for tree construction. In the only study to contain a pleurotomariid (Rosenberg et al., 1994), the single species emerged within Caenogastropoda at the base of the Neogastropoda. Sequences of the small subunit ribosomal RNA (18S rRNA) molecule have been determined for relatively few (<5) gastropods, generally in the context of broader studies such as the evolution of the Metazoa (e.g., Field et al., 1988; Ghiselin, 1988; Winnepenninckx et al., 1994).

The composition of the superfamily Pleurotomarioidea has also undergone considerable revision during the course of renewed scrutiny. Knight et al. (1960) included within the Pleurotmarioidea 20 extinct families as well as the living families Pleurotomariidae, Scissurellidae, and Haliotidae, a scheme followed in most contemporary classifications (e.g., Hyman, 1967; Hickman, 1984; Vaught, 1989). Salvini-Plawen (1980) added Fissurellidae to Pleurotomarioidea, which he included in Vetigastropoda. Boss (1982) added Neomphalidae, but removed Fissurelloidea as a separate superfamily. In a reassessment of the Vetigastropoda, Salvini-Plawen and Haszprunar (1987) identified other anatomic characters to support the monophyly of Vetigastropoda, which they considered to include the superfamilies Fissulrelloidea, Trochoidea, and Pleurotomarioidea (containing the families Pleurotomariidae, Scissurellidae, and Haliotidae), with Neomphalidae appended as "*incertae sedis*." These authors noted that no synapomorphies could be found to unite the pleurotomarioidean families, and that Pleurotomarioidea was most likely polyphyletic. Most recently, Ponder and Lindberg (1996, 1997) added the superfamily Lepetelloidea and the family Senguenziidae to the Vetigastropoda, removed the Neomphalidae, grouping it with other "Vent taxa," and considered Pleurotomarioidea to be the sister taxon of Trochidae + Seguenziidae (see Figure 2B).

The family Pleurotomariidae is of Triassic (Ladinian) origin (Tracey et al., 1993). The majority of the dozen genera included in this family are extinct, and all have been defined typologically on the basis of the presence or absence of a few easily recognizable shell characters used sequentially. Most modern authors concede that generic distinctions are not always clear and that many taxa are not readily assigned into existing genera (Cox, 1960; Hickman, 1976; Szabó, 1980). The genus *Pleurotomaria* Defrance, 1826, is generally regarded as having ranged from the lower Jurassic to the Lower Cretaceous (Knight et al., 1960), while the genera *Perotrochus* Fischer, 1885, *Entemnotrochus* Fischer, 1885, and *Mikadotrochus* Lindholm, 1927, have been proposed for living pleurotomariids. Some authors regard these as, at best, subgenera of *Pleurotomaria* (e.g., Dall, 1889; Hickman, 1976, 1984; Abbott and Dance, 1982), while most others (e.g., Knight et al., 1960; Bayer, 1965; Benfrika, 1984; Vaught, 1989; Endo, 1995; Anseeuw and Goto, 1996) accord some or all of them generic rank. A synopsis of available information on living Pleurotomariidae has recently been published (Anseeuw and Goto, 1996). To date, there have been no published studies of phylogenetic relationships within the family.

The principal objectives of this study are to reconstruct the major outlines of gastropod evolution on the basis of partial sequences of the 18S rDNA

gene and to assess the phylogenetic position and composition of the superfamily Pleurotomarioidea on the basis of these data. Corroboration and better resolution of the relationships of the genera and species within the family Pleurotomariidae are provided by analyses of sequence data from the mitochondrial cytochrome *c* oxidase subunit I gene.

Results

Partial sequences comprising approximately 450 base pairs (bp) near the 5′ end of the 18S rDNA gene were determined for 27 gastropods, one cephalopod, and one polyplacophoran, and aligned with homologous regions of the complete gene sequences of one polyplacophoran and two gastropods obtained from GenBank (see Table 1). The portion of the gene used in this study corresponds to positions 60 to 515 of the 18S rRNA sequence of *Onchidella celtica* as reported by Winnepenninckx et al. (1994, p. 101). Among the taxa included in the present study, the length of the gene between these two positions varied considerably (430 bp in *Xenophora* to 537 bp in *Perotrochus midas*) owing to the presence of insertions and deletions, especially in the regions of the helices 10, E-10-1, and 11 (see Winnepenninckx et al. 1994, Figure 1). The large insertion in this region that was found in all Pleurotomariidae is of questionable homology to the smaller E-10-1 insertions found in *Nautilus* and in the patellogastropod and cocculiniform limpets. Therefore, the alignment encompassing all taxa spans 653 positions (Figure 3), although the longest constituent sequence is 537 bp. Seven autapomorphic, single-nucleotide insertions were excluded from phylogenetic analyses, as were 10 regions, ranging in length from 1 to 171 bp, that could not be reliably aligned (Figure 4B). Of the remaining 392 alignable sites, 199 were constant and 73 were parsimony uninformative. Cladistic analysis of the 120 informative characters using the branch and bound algorithm of PAUP 4.0.0d42 produced 4656 most parsimonious trees with a length (L) of 433, consistency index (CI) of 0.621, retention index (RI) of 0.744, and rescaled consistency index (RC) of 0.481. The strict consensus of these trees is illustrated in Figure 5A. Results of bootstrap and jackknife analyses (1000 replicates) are superimposed on the consensus tree. Characters were reweighted by the maximum value of the rescaled consistency (RC) indices using a base weight of 1, and reanalyzed using the branch and bound algorithm to yield 60 most parsimonious trees of length 229.95 (CI = 0.779; RI = 0.845; RC = 0.659). The strict consensus of these trees, with bootstrap and jackknife support for nodes, is shown in Figure 5B.

Quantitative comparisons of the 18S rDNA sequence-based phylogeny with morphology-based

Table 1. Locality data, tissues extracted, voucher specimen information, and sequence accession numbers for taxa used in this study.

Taxon	Collection locality	Tissue	Voucher material	Sequence accession number 18S	Sequence accession number COI
Class Polyplacophora					
Acanthopleura japonica (Lischke, 1873)	GenBank ex Winnepenninckx et al., 1993			EMBL X70210	
Cryptochiton stelleri (Middendorff, 1847)	Bamfield, B.C., Canada	Buccal muscle	USNM 888657	GSDB L78876	
Class Cephalopoda					
Nautilus scrobiculatus Lightfoot, 1786	Papua, New Guinea	Buccal muscle	USNM 885678	GSDB L78877	
Class Gastropoda					
Patellogastropoda					
Acmaea mitra Rathke, 1833	Bamfield, B.C., Canada	Buccal muscle	USNM 888640	GSDB L78878	
Cellana nigrolineata (Reeve, 1854)	Minabe, Japan	Buccal muscle	USNM 888623	GSDB L78879	
Cocculiniformia					
Cocculinidae					
Cocculina messingi McLean & Harasewych, 1995	Bahamas	Buccal muscle	USNM 888655	GSDB L78880	
Lepetelloidea					
Notocrater houbricki McLean & Harasewych, 1995	Bahamas	Whole animal	USNM 888656	GSDB L78881	
Neritopsina					
Nerita versicolor Gmelin, 1791	Big Pine Key, FL, USA	Buccal muscle	USNM 888658	GSDB L78882	
Neritina reclivata (Say, 1822)	Big Pine Key, FL, USA	Buccal muscle	USNM 888659	GSDB L78883	

Table 1. Continued

Taxon	Collection locality	Tissue	Voucher material	Sequence accession number 18S	Sequence accession number COI
Vetigastropoda					
Fissurelloidea					
Diodora cayenensis (Lamarck, 1822)	Sebastian Inlet, FL, USA	Buccal muscle	USNM 888660	GSDB L78884	GSDB L78908
Haliotoidea					
Haliotis rufescens Swainson, 1822	Bamfield, B.C., Canada	Buccal muscle	USNM 888642	GSDB L78885	
Trochidae					
Astraea caelata (Gmelin, 1791)	Berry Is., Bahamas	Buccal muscle	USNM 888603	GSDB L78886	GSDB L78909
Cittarium pica (Linné, 1758)	Jamaica	Buccal muscle	USNM 888661	GSDB L78887	
Pleurotomarioidea					
Entemnotrochus adansonianus (Crosse & Fischer, 1861)	Guadeloupe	Buccal muscle	USNM 888647	GSDB L78888	GSDB L78910
Entemnotrochus rumphii (Schepman, 1879)	Amami-O-Shima, Japan	Buccal muscle	USNM 888698	GSDB L78889	GSDB L78911
Perotrochus quoyanus (Fischer & Bernardi, 1856)	Guadeloupe	Buccal muscle	USNM 888646	GSDB L78890	GSDB L78915
Perotrochus lucaya F.M. Bayer, 1964	Bahamas	Buccal muscle	USNM 888619	GSDB L78891	GSDB L78916
Perotrochus maureri Harasewych & Askew, 1991	Charleston, SC, USA	Buccal muscle	USNM 888662	GSDB L78892	GSDB L78914
Perotrochus midas F.M. Bayer, 1965	Goulding's Cay, Bahamas	Buccal muscle	USNM 888645	GSDB L78893	GSDB L78913
Perotrochus teremachii Kuroda, 1955	Japan	Buccal muscle	USNM 888617	GSDB L78894	GSDB L78912
Caenogastropoda					
Cerithiidae					
Cerithium atratum (Born, 1778)	Sebastian Inlet, FL, USA	Buccal muscle	USNM 888663	GSDB L78895	GSDB L78907
Xenophoroidea					
Xenophora exutum Reeve, 1843	Minabe, Japan	Buccal muscle	USNM 888631	GSDB L78896	
Tonnoidea					
Fusitriton oregonense (Redfield, 1848)	Dutch Harbor, AK, USA	Buccal muscle	USNM 888634	GSDB L78897	
Muricoidea					
Oliva sayana Ravenel, 1834	Ft. Pierce, FL, USA	Buccal muscle	USNM 888605	GSDB L78898	
Conoidea					
Hastula cinerea (Born, 1778)	Ft. Pierce, FL, USA	Buccal muscle	USNM 888611	GSDB L78899	
Heterobranchia					
Pyramidelloidea					
Fargoa bushiana Bartsch, 1909	Ft. Pierce, FL, USA	Whole animal	USNM 888638	GSDB L78900	
Euthyneura					
Opisthobranchia					
Haminoea antillarum (d'Orbigny, 1841)	Ft. Pierce, FL, USA	Buccal muscle	USNM 888664	GSDB L78901	
Aplysia dactylomela (Rang, 1828)	Minabe, Japan	Buccal muscle	USNM 888624	GSDB L78902	
Pulmonata					
Onchidella celtica (Cuvier, 1817)	Genbank ex Winnepenninckx et al., 1994			EMBL X70211	
Physa heterostropha Say, 1822	Ithaca, NY, USA	Buccal muscle	USNM 888613	GSDB L78905	
Limicolaria kambeul (Bruguière, 1792)	Genbank ex Winnepenninckx et al., 1992			EMBL X66374	
Limax maximus Linné, 1758	Silver Spring, MD, USA	Buccal muscle	USNM 888604	GSDB L78906	

EMBL indicates European Molecular Biology Laboratory Data Library; GSDB, Genome Sequence Data Bank; USNM, mollusk collection, National Museum of Natural History, Smithsonian Institution.

phylogenetic hypotheses are based on reanalyses of sequence data for subsets of shared taxa (shown in upper-case letters in Figure 2) and subsequent manipulation of tree structure using MacClade (Maddison and Maddison, 1992). Resulting increases in tree length and indices are reported in Table 2.

To better resolve the relationships among the Vetigastropoda, a subset of taxa that included the seven pleurotomariid and four nonpleurotomariid vetigastropods, as well as the two Neritopsina and *Cerithium* and *Hastula* (as representatives of Caenogastropoda), was reanalyzed. Although the alignment of nucleotides was not altered, elimination

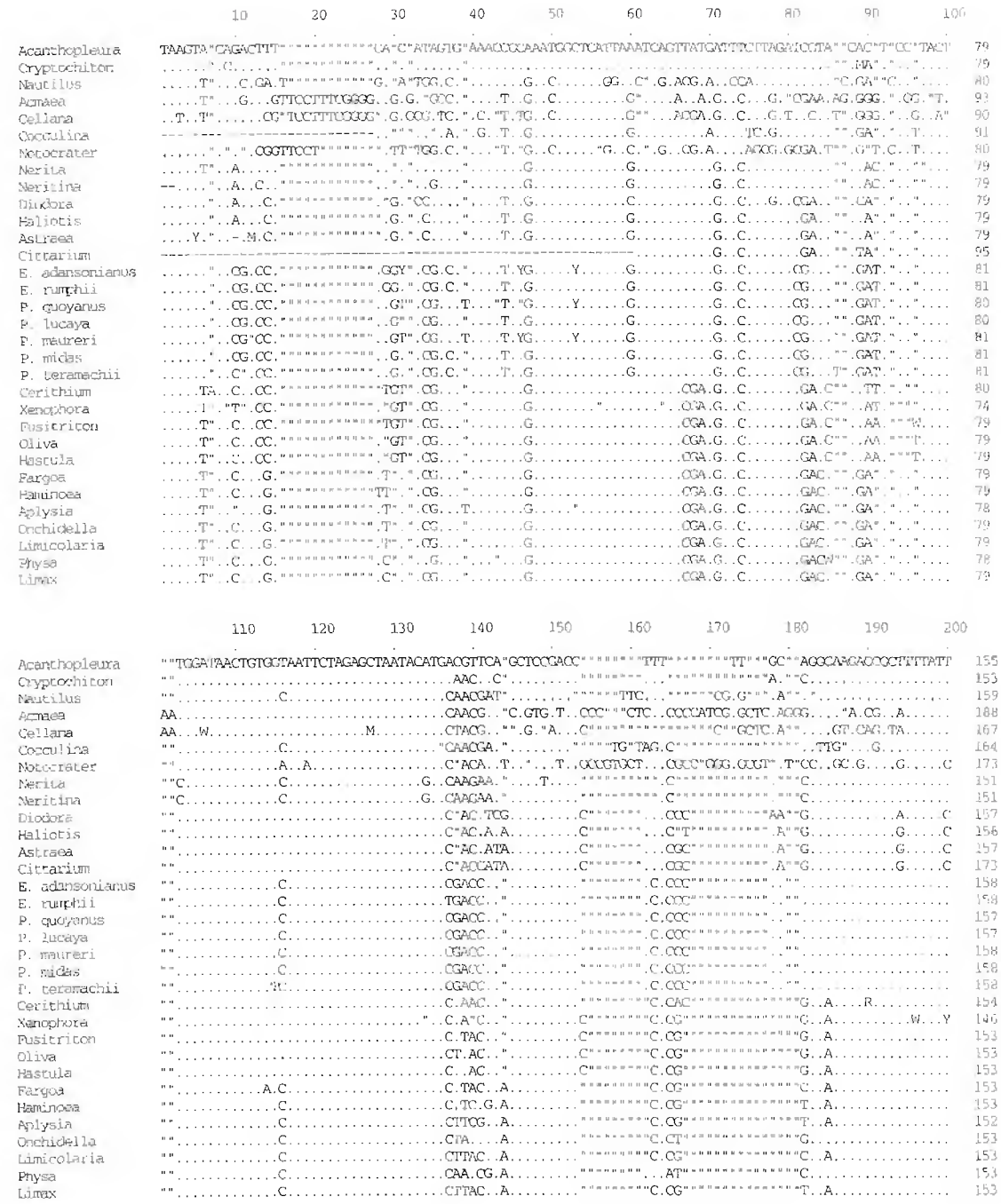


Figure 3. Aligned partial sequences of the molluscan 18S rDNA gene spanning helices 6–17, and corresponding to positions 60–515 of the 18S rRNA sequence of *Onchidella celtica* as reported by Winnepenninckx et al. (1994, p. 101). Among the included taxa the length of the gene between these two positions ranges from 430 to 537 bp owing to the presence of insertions and deletions, especially in the regions of the helices 10, E-10-1, and 11. All sequences were confirmed by at least two sequencing reactions in one direction and a third sequencing reaction in the opposite reaction. Ambiguous base assignments are noted using IUPAC symbols. Dots (·) represent nucleotides identical to those of *Acanthopleura*, dashes (–) represent missing data, and quotation marks (") represent gaps inserted during alignment.

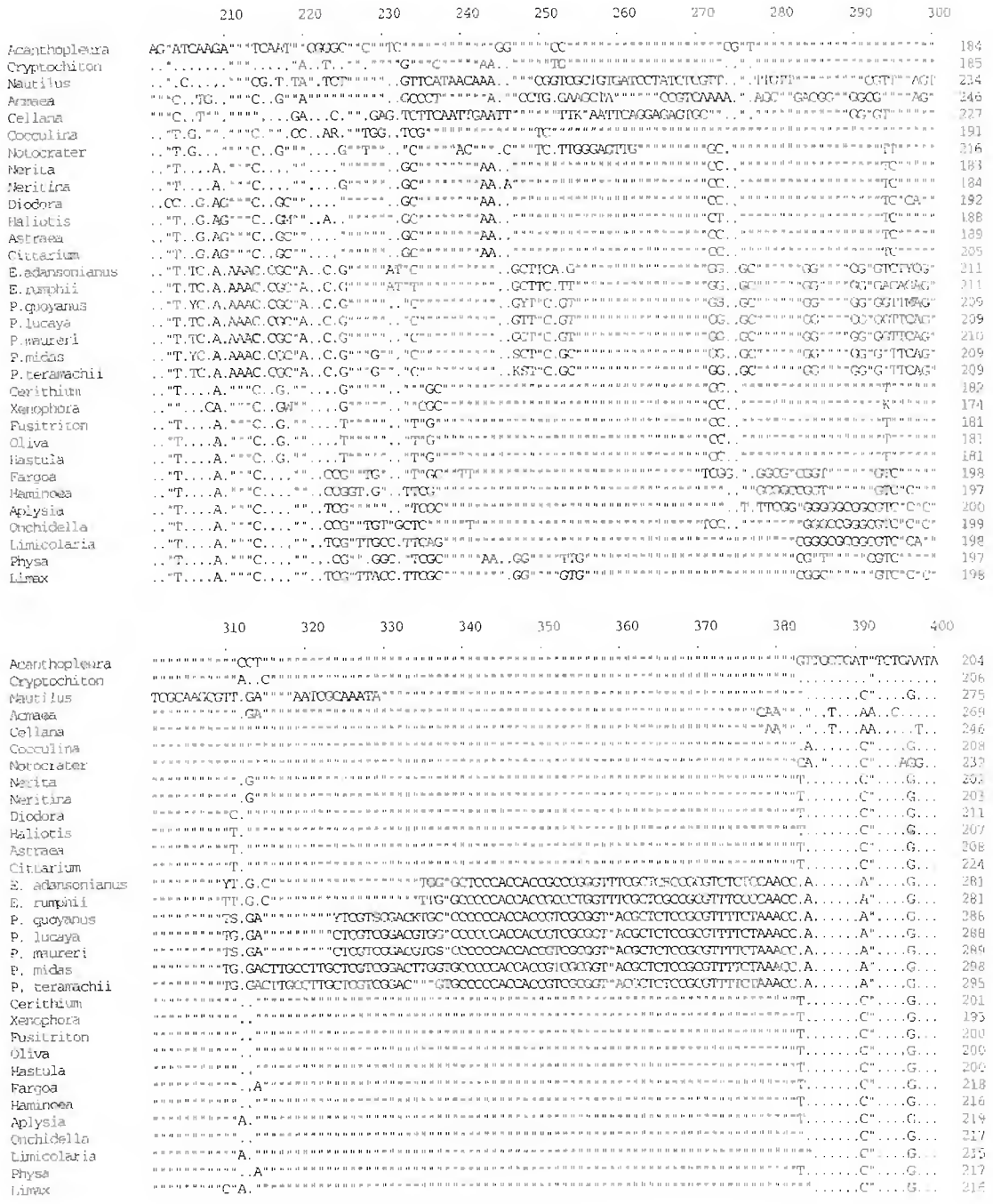


Figure 3. Continued

of taxa that contained putatively nonhomologous insertions enabled the alignment to be condensed to 556 positions. All of the regions excluded from the original analysis could be unambiguously aligned, although not all were present in all taxa. All Pleurotomariidae were found to contain seven insertions in the V2 region (De Rijk et al., 1992) that appear to be unique to the family. These insertions, which vary in length from 1 to 68 nucleotides, are concentrated in the helices 10 and E-10-1 (Table 3, Figure 4C). Two of the seven insertions vary in length among pleurotomariid taxa, each appearing to contain one or more insertions or deletions. Five autapomorphic, single-nucleotide insertions from

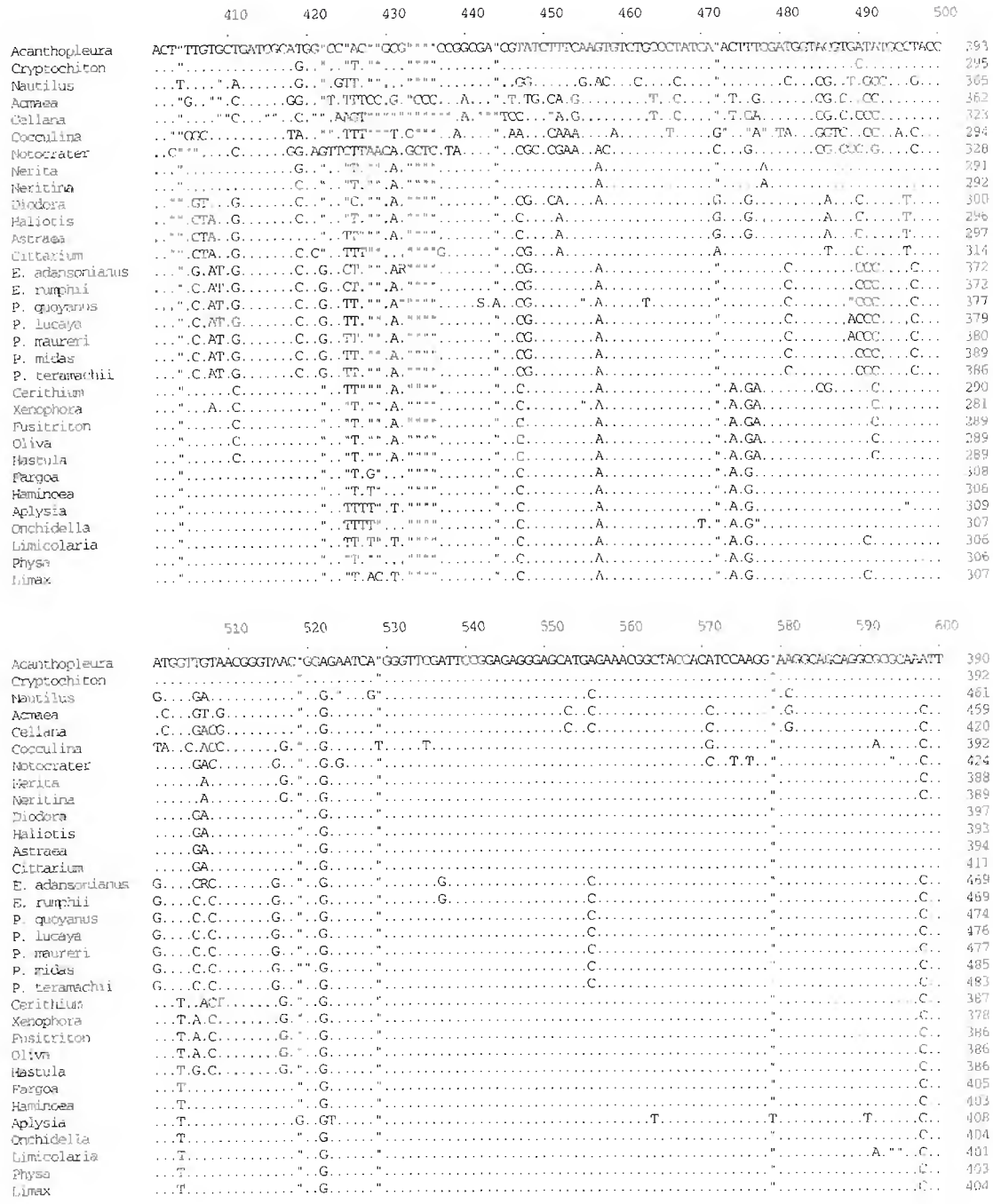
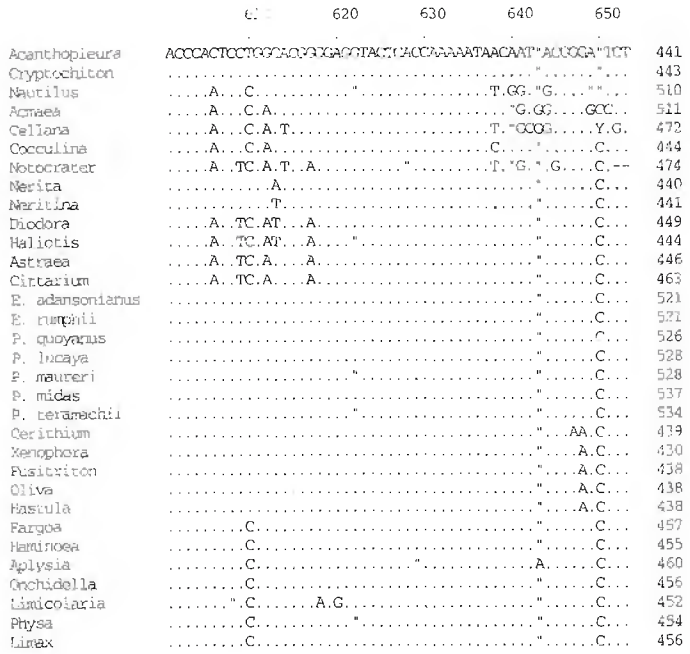


Figure 3. Continued

other portions of the sequence were excluded from all analyses. An initial maximum parsimony analysis (exhaustive search option, 94 informative characters) was performed on data from which the seven insertions (totaling 94 nucleotides) unique to Pleurotomariidae were excluded. Figure 6A illustrates the strict consensus of the resulting 36 most parsimonious trees (L = 168; CI = 0.851; RI = 0.914; RC = 0.778). Figure 6B shows the strict consensus of the six most parsimonious trees (L = 196; CI = 0.872; RI = 0.918; RC = 0.801) produced when the analysis was repeated with the seven insertions included in the data set (105 informative characters).

Figure 7 contrasts the phylogenetic information contained in the length polymorphisms of these in-

Figure 3. Continued



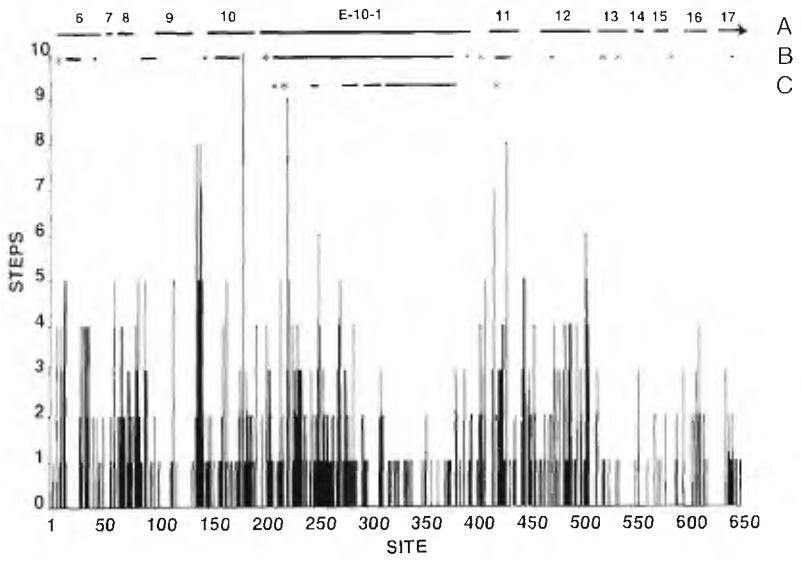


Figure 4. Distribution of variable sites along the 18S rDNA gene among the 32 taxa in this study (Table 1). (A) Positions of helices (from Winnepenninckx et al., 1994, Figure 1). (B) Positions of bases excluded from phylogenetic analyses due to uncertain homology or alignment (bars) or because they were autapomorphic insertions (*). (C) Location of the seven insertions unique to Pleurotomariidae. Asterisks (*) indicate single base insertions.

sertions with that contained in their sequence. Of the 94 aligned positions in the inserts, only 11 are parsimony-informative, producing 9 trees (L = 27; CI = RI = RC = 1.0). A consensus tree is shown in Figure 7A. Figure 7B illustrates the strict consensus of three trees (L = 22; CI 0.864; RI = 0.885; RC = 0.764) based on an analysis of the distribution of the 19 insert segments (Figure 8) among pleurotomariid taxa.

Alignment of the partial sequences for the mitochondrial cytochrome *c* oxidase I gene (Figure 9) produced an unambiguous alignment corresponding to positions 32 to 611 of the COI sequence of *Drosophila yakuba* (Folmer et al., 1994, Table 2). Of the 579 nucleotides, 333 were constant and 59 were parsimony-uninformative. Analysis of the 187 informative cites using the exhaustive search algorithm produced a single most parsimonious tree (L = 540; CI = 0.669; RI = 0.581; RC = 0.388), shown in Figure 10A with bootstrap and jackknife support for nodes superimposed.

The "total molecular evidence" data matrix consisted of 1154 characters (556 18S rDNA + 579 COI + 19 insert segments), of which 755 were constant

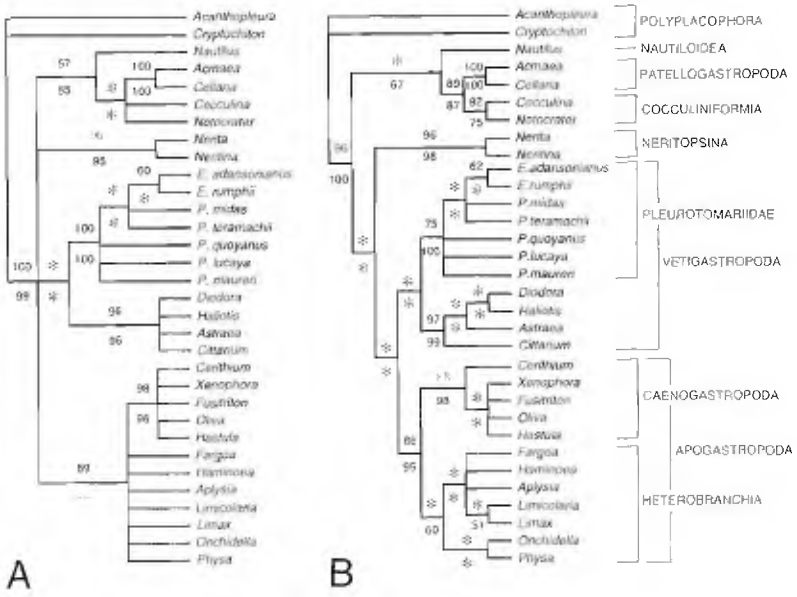


Figure 5. Phylogenetic relationships among major groups of Gastropoda, *Nautilus*, and two Polyplacophora based on approx. 450 bp of 18S rDNA sequence from which autapomorphic insertions and regions of uncertain alignment have been removed. (A) Consensus of the 4656 shortest trees (L = 443; CI = 0.621; RI = 0.744; RC = 0.481) resulting from a branch and bound search. (B) Consensus of 60 shortest trees (L = 229.95; CI = 0.779; RI = 0.845; RC = 0.659) produced by a branch and bound search of a data set in which characters were reweighted by the maximum value of the rescaled consistency index (base weight = 1). Bootstrap proportions given as percentages above the nodes and jackknife proportions given as percentages below the nodes. An asterisk (*) indicates that the node was not supported in a 50% majority rule bootstrap or jackknife consensus tree.

Table 2. Comparisons of 18S rDNA sequence-based phylogeny of the class Gastropoda with contemporary, morphology-based hypotheses.*

Phylogenetic hypothesis	Tree length	CI	RI	RC	Taxa (*N*)
Haszprunar (Figure 2A)	360	0.65	0.53	0.34	14
18S rDNA tree†	338	0.69	0.61	0.42	14
Ponder & Lindberg (Figure 2B)	371	0.63	0.50	0.32	15
18S rDNA tree‡	338	0.69	0.62	0.43	15

*To facilitate comparisons, trees were pruned to contain the same sets of taxa. When higher taxa were represented by multiple species in the 18S data set, a single representative was selected with the objective of minimizing the number of deletions and ambiguous base assignments. Patellogastropoda is represented by *Cellana*; Neritopsina by *Nerita*; Pleurotomariidae by *Perotrochus lucaya*; Trochidae by *Astraea*; Euthyneura and Opisthobranchia by *Haminoea*; Pulmonata by *Onchidella*. Polyplacophora were excluded from the data set, and *Nautilus* was selected as the outgroup. Comparisons are based on taxa identified by Upper Case in Figure 2.

†A single most parsimonious tree resulted from an analysis using the reduced subset of taxa.

‡Three equally parsimonious trees differing only in the topology of taxa within Caenogastropoda resulted from an analysis using the reduced subset of taxa. One of these trees, which agreed with the generally accepted topology of Caenogastropoda included in Ponder and Lindberg (Figure 2B), was used as the basis for comparison.

and 275 were parsimony-informative. An exhaustive search yielded a single most parsimonious tree (L = 712; CI = 0.729; RI = 0.658; RC = 0.479), shown in Figure 10B. Bootstrap and jackknife values are given for each node.

Discussion

Maximum parsimony analyses of the conservative, unambiguously aligned portions of the 18S rDNA sequence data resulted in a phylogenetic hypothesis for the class Gastropoda (Figure 5) that is, for the most part, similar to those based on morphologic data (Figure 2). Quantitative comparisons (Table 2) indicate that the phylogenetic hypotheses advocated by Haszprunar (1988) are, as a whole, more congruent with the molecular data than those published by Ponder and Lindberg (1996, 1997). On the basis of molecular data, living Gastropoda represented in our study are divided into four groups,

Table 3. Insertions within the 18S rDNA gene that are unique to Pleurotomariidae.*

Insertion	Length (bp)	Position in aligned sequence (Figure 3)	Segment
1	3	211–213	1
2	1	220	2
3a	3	247–249	3
3b	1	250	4
3c	4	251–254	5
4	7	276–277, 284–285, 290–291, 293	6
5	6	296–299, 310	7
6a	1	314	8
6b	1	315	9
6c	8	316–323	10
6d	10	324–333	11
6e	1	334	12
6f	2	335–336	13
6g	1	337	14
6h	1	338	15
6i	20	339–358	16
6j	1	359	17
6k	23	360–382	18
7	1	422	19

*Positions are referenced to the aligned sequences shown in Figure 3. Insertions 3 and 6 vary in length among pleurotomariid taxa, and have been subdivided into segments that could be scored as either present or absent in individual taxa (Figure 8) and serve as the basis for the cladogram in Figure 6B.

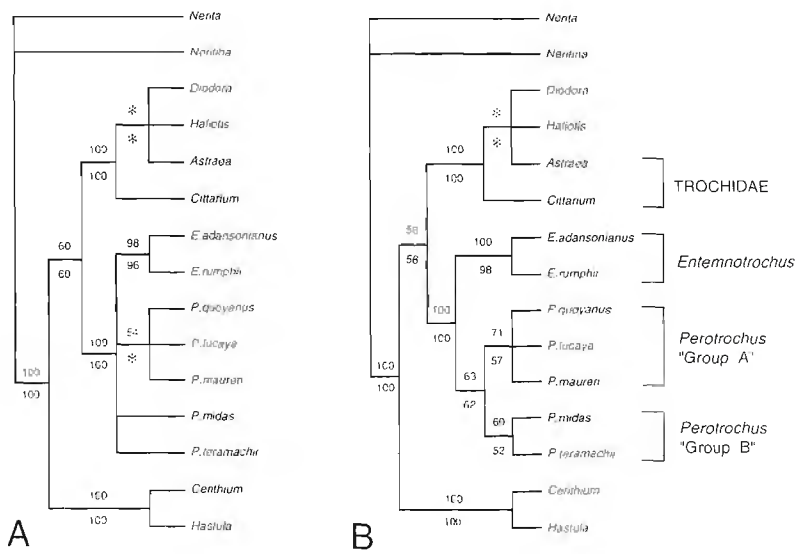


Figure 6. Phylogenetic relationships among Vetigastropoda, Neritopsina, and Caenogastropoda based on 18S rDNA sequences. Five autapomorphic, single-nucleotide insertions are excluded from analyses. (A) A consensus tree of 36 most parsimonious trees (L = 168; CI = 0.851; RI = 0.914; RC = 0.778) resulting from a branch and bound search of the data set from which the seven insertions (94 nucleotides) unique to Pleurotomariidae have been excluded. (B) Consensus of the six most parsimonious trees (L = 196; CI = 0.872; RI = 0.918; RC = 0.801) produced when the analysis was repeated with the seven insertions included in the data set. Bootstrap proportions given as percentages above the nodes and jackknife proportions given as percentages below the nodes. An asterisk (*) indicates that the node was not supported in a 50% majority rule bootstrap or jackknife consensus tree.

the limpets, the Neritopsina, the Vetigastropoda, and the Apogastropoda, each representing radiations within an extinction-resistant clade (Erwin and Signor, 1991) with early Paleozoic origins (Tracey et al., 1993). Relationships between these clades are not robustly resolved, suggesting that diver-

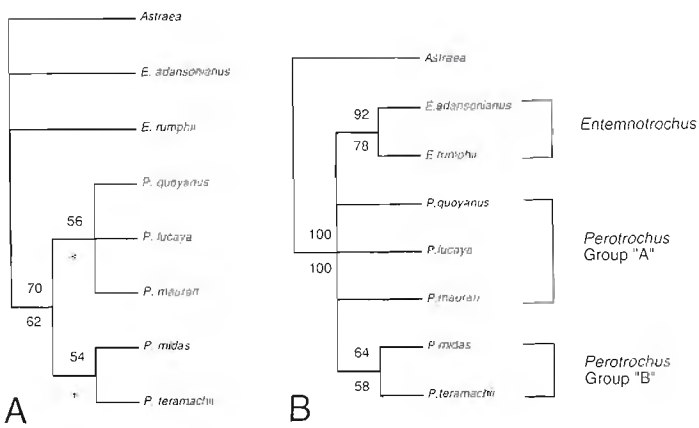


Figure 7. A comparison between the phylogenetic information contained in the length polymorphisms of the insertions unique to Pleurotomariidae with that contained in their sequence. (A) Consensus of 9 trees (L = 27; CI = RI = RC = 1.0) based only on sequences of the inserts. (B) Consensus 3 trees (L = 22; CI = 0.864; RI = 0.885; RC = 0.764) based on an analysis of the distribution of insert segments among pleurotomariid taxa. Bootstrap proportions given as percentages above the nodes and jackknife proportions given as percentages below the nodes. An asterisk (*) indicates that the node was not supported in a 50% majority rule bootstrap or jackknife consensus tree.

Insert Segment	1 2 3 4 5 6 7 8 9 10	11 12 13 14 15 16 17 18 19
Astraea	− − − − − − − − − −	− − − − − − − − −
E. adansonianus	+ + + + + + + + + −	− − + + − + + + +
E. rumphii	+ + + + + + + + + −	− − + + − + + + +
P. quoyanus	+ + + − + + + + − −	+ + + + − + − + +
P. lucaya	+ + + − + + + + − −	+ + + + − + − + +
P. maureri	+ + + − + + + + − −	+ + + + − + − + +
P. midas	+ + + − + + + + + +	+ + + + + + − + +
P. teramachii	+ + + − + + + + + +	+ − − + + + − + +

Figure 8. The distribution of 19 insert segments among Pleurotomariidae. These insert segments (characterized in Table 3) are absent in *Astraea* and all nonpleurotomariid taxa within our study. Inserts are scored as present (+) or absent (−) without regard to their sequence.

gences were ancient and rapid. Philippe et al. (1994) calculated that rapid (≤40 Million-year) radiations are beyond the limits of resolution of the entire 18S rDNA gene.

Perhaps the most anomalous result of our study is the grouping of the cephalopod *Nautilus* in a clade together with the patellogastropod and cocculiniform limpets. Although the node connecting *Nautilus* to the limpet taxa is not strongly corroborated by either bootstrap or jackknife proportions, moving *Nautilus* to its accepted position as a sister group to the Gastropoda increases the length of the tree by 9 steps. The monophyly of patellogastropod limpets is strongly supported (bootstrap and jackknife proportions of 100). The two species in our study contain uniquely shared insertions (e.g., positions 16–27, 32, 86, 643) and a deletion (position 62) that provide additional, independent support for their monophyly. Our data do not strongly support the monophyly of Cocculiniformia, but do indicate that Cocculinidae and Lepetelloidea are more closely related to each other and to the patellogastropod limpets than to Neritopsina or Vetigastropoda, respectively, as suggested by Ponder and Lindberg (1996, 1997).

Manipulation of the tree to produce a monophyletic Cocculiniformia emerging basal to Neritopsina (Haszprunar hypothesis) increases the tree length by 9 steps. Placing *Cocculina* as a sister taxon to Neritopsina lengthens the tree by 11 steps, while making the lepetelloidean limpet *Notocrater* the basal vetigastropod increases the tree length by 17 steps. The most parsimonious placement of *Notocrater* within Vetigastropoda is as sister taxon to the nonpleurotomariid vetigastropods, which increases tree length by 12 steps. Although data are lacking for *Cocculina*, *Notocrater* contains a portion of the large patellogastropod insert (positions 16–20), adding support for its relationship to the patellogastropod limpets.

It should be noted that all the taxa grouped in this clade are anomalous in containing insertions that are highly variable in sequence and length, difficult to align, of uncertain homology, and are absent in other gastropods as well as in the chitons. Although these insertions were excluded from the data set and did not contribute to the structure of the tree, we conjecture that these taxa may have undergone inversions, translocations, or changes other than point mutations in this region of the gene that are not homologous among all taxa. Branch

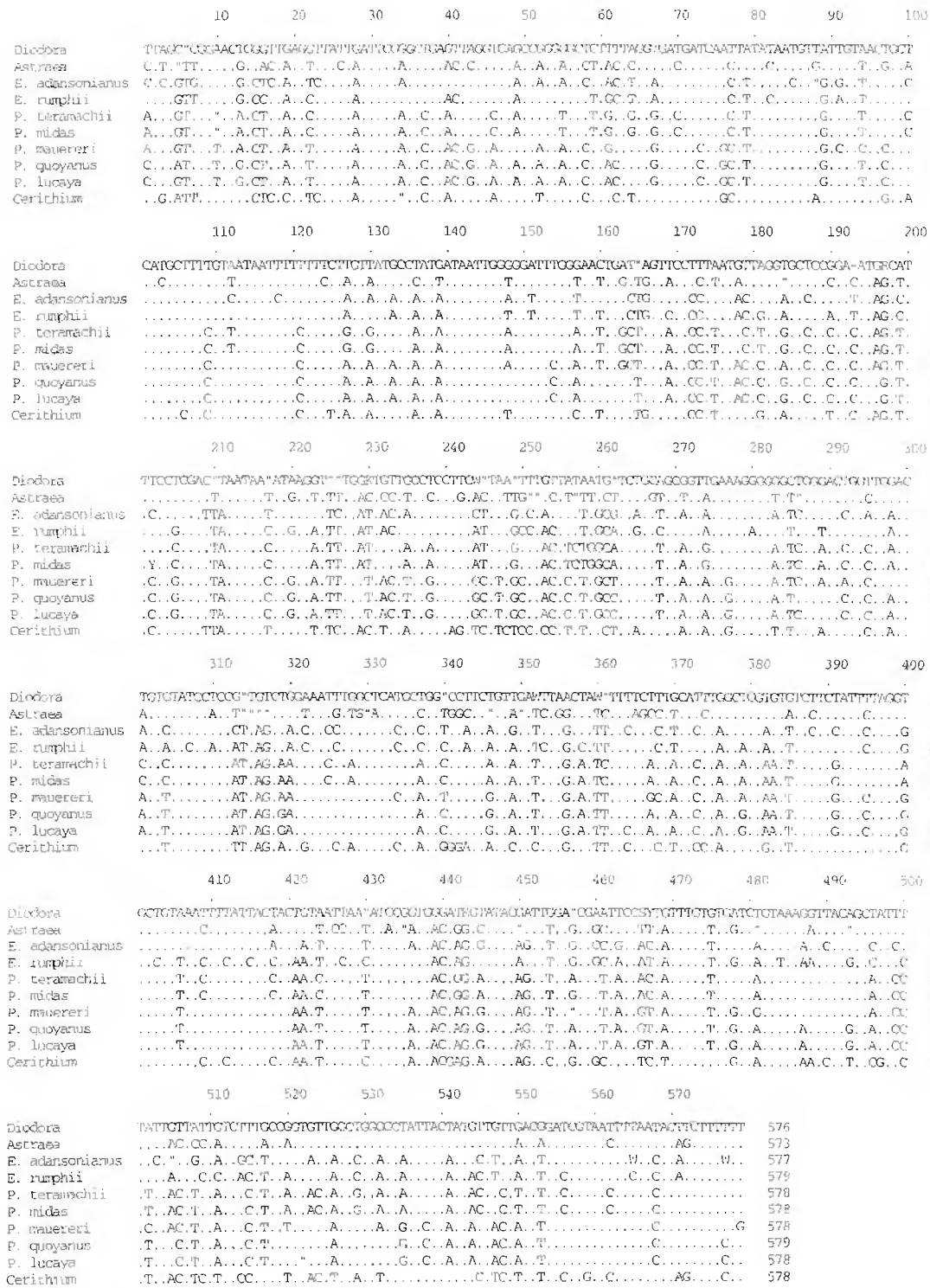


Figure 9. Aligned partial sequences of the molluscan mitochondrial cytochrome *c* oxidase subunit I gene corresponding to positions 32 to 611 of the COI sequence of *Drosophila yakuba* (Folmer et al., 1994, Table 2). All sequences were confirmed by at least two sequencing reactions in each direction. Ambiguous base assignments are noted using IUPAC symbols. Dots (·) represent nucleotides identical to those of *Diodora*, and quotation marks (") represent gaps inserted during alignment.

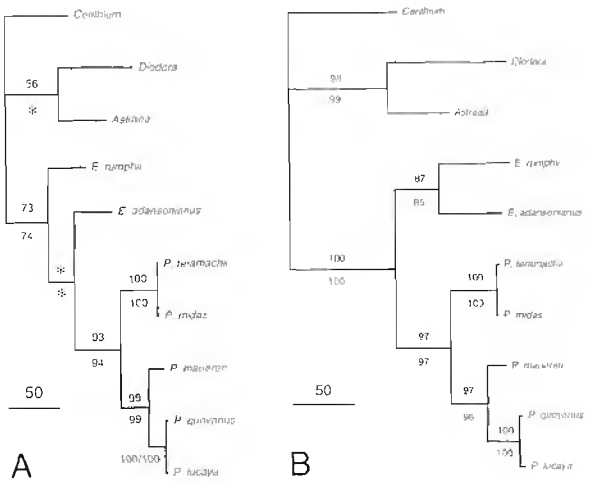


Figure 10. Phylogenetic relationships among Recent pleurotomariid species and genera. (A) The single most parsimonious tree resulting from an analysis (exhaustive search option) of 579 bp from the mitochondrial cytochrome *c* oxidase I gene (L = 540; CI = 0.669; RI = 0.581; RC = 0.388). Bootstrap proportions given as percentage above the node and jackknife proportions given as percentage below the node; (*) indicates that the node was not supported in a 50% majority rule bootstrap or jackknife consensus tree. (B) The single most parsimonious tree resulting from an analysis (exhaustive search option) of the "total molecular evidence" of 1154 characters (556 18S rDNA + 579 COI + 19 insert) (L = 712; CI = 0.729; RI = 0.658; RC = 0.479). Bootstrap proportions given as percentages above the nodes and jackknife proportions given as percentages below the nodes.

lengths for these taxa are two to three times as long as for other species in the tree, raising the possibility that they may be grouped on the basis of long branch attraction. Several authors (e.g., Termier and Termier, 1968; Shileyko, 1977; Ponder and Lindberg, 1997; Tillier et al., 1992) have speculated that the patellogastropod limpets may not be gastropods, and suggested that the monophyly of the Gastropoda requires further corroboration. However, cocculiniform limpets, which together with the patellogastropod limpets comprise a monophyletic group in our study, have been regarded as unambiguous gastropods in all previous classifications.

The monophyly of the Neritopsina is strongly supported, perhaps in part because both representatives in our study are members of the same subfamily (Neritinae). The position of the Neritopsina within Gastropoda is less certain and strongly influenced by the outgroup. When Polyplacophora served as the outgroup, Neritopsina emerged basal to Vetigastropoda, as hypothesized by Haszprunar (1988) and Hickman (1988), and supported by limited 28S RNA sequences (Rosenberg et al., 1994). However, when Polyplacophora were excluded from the data matrix and *Nautilus* was specified to be the outgroup, Neritopsina emerged between Vetigastropoda and Apogastropoda as advocated by Ponder and Lindberg (1996, 1997). Thus, 18S sequence data appear insufficient to resolve conclusively the relationships of Neritopsina.

The monophyly of the Pleurotomariidae is strongly supported by 18S sequence data as well as by the presence of seven insertions unique to the family. Contrary to the predictions of the morphology-based phylogenies, the Pleurotomariidae emerge as the sister group of the remaining vetigastropods, which also comprise a highly robust clade, rather than as a sister group to the family Trochidae. Remarkably, the monophyly of the Vetigastropoda, which is well supported by morphologic evidence (Salvini-Plawen and Haszprunar, 1987; Ponder and Lindberg, 1996, Figure 11.2), is but weakly supported by sequence data (bootstrap and jackknife proportions < 50% with both raw and reweighted characters), suggesting an ancient divergence between the two clades. Although Pleurotomarioidea date to the Cambrian, the remaining taxa appear later—Trochoidea first appear in the Ordovician, and earliest records of Haliotoidea and Fissurelloidea are from the Triassic (Tracey et al., 1993).

Reanalyses of the 18S rDNA sequence data from a subset of taxa for which the sequences could be reliably aligned in their entirety (Figure 6) resulted in an identical division of Vetigastropoda into pleurotomariid and nonpleurotomariid clades, with each clade strongly supported by bootstrap and jackknife proportions of 100. However, monophyly of the Vetigastropoda remains weakly supported. The relationships of the pleurotomariid taxa to each other as well as to other vetigastropods, predicted by the 18S sequences, were confirmed by sequence from the mitochondrial gene for cytochrome *c* oxidase I (Figure 10A). The phylogeny based on this gene sequence confirms the monophyly of the Pleurotomariidae and supports the monophyly of the nonpleurotomariid vetigastropods, specifically refuting a close relationship between Pleurotomariidae and Trochidae.

There have been differing opinions among morphologists about the relationships of vetigastropod taxa. McLean (1984) commented that the Trochoidea "share so many features with the Pleurotomariidae that their derivation from the group is readily perceived." However, Hickman (1984)

noted that the radula of Pleurotomariidae "had little in common with any living gastropod, including the [then included] two other pleurotomariodean families [Scissurellidae, Haliotidae] of more recent geological appearance." While McLean (1984) hypothesized that the Haliotidea are the "limpet-like derivatives" of the Pleurotomarioidea, Haszprunar (1985) noted that Haliotidae and Trochoidea are further linked by shared aberrant chemosensory structures.

In the light of the molecular evidence, the morphologic data and analyses of vetigastropod phylogeny should be reappraised with special attention to the precise composition of higher taxa and the resulting distribution of characters. The usage of Pleurotomarioidea has been inconsistent in molecular as well as morphologic studies. Tillier et al. (1994), for example, concluded that "Fisurelloidea is the sister group of Pleurotomarioidea and Trochoidea" based on 28S rDNA sequences, because they used *Haliotis* as the exemplar of Pleurotomarioidea. Thus, what appears to be a contradictory finding is, in fact, concordant with our results.

Within Pleurotomariidae, the monophyly of the genus *Entemnotrochus* was well supported by 18S sequences, even without data contained in the insertions. The insertions were required to corroborate the monophyly of *Perotrochus,* as well as its small-shelled (*Perotrochus* "Group A," Bayer, 1965) and large-shelled (*Perotrochus* "Group B," Bayer, 1965) clades. Information contained in the sequences of the seven inserts unique to Pleurotomariidae was sufficient to distinguish *Perotrochus* and to resolve it into small-shelled and large-shelled groups, while length polymorphisms of these inserts were able to differentiate *Entemnotrochus* from the large-shelled *Perotrochus* (Figure 7). By contrast, the nonpleurotomariid vetigastropod families could not be resolved by 18S sequence data. *Cittarium*, which emerged anomalously at the base of the clade, could be returned to its predicted position as sister taxon to *Astraea* by increasing tree length by only one step.

Cytochrome *c* oxidase I sequence data strongly support the monophyly of the genus *Perotrochus* and of its constituent subclades *Perotrochus* "Group A" and *Perotrochus* "Group B," and are capable of resolving closely related, parapatric species. Manipulation of tree topology to make the genus *Entemnotrochus* monophyletic increases tree length by one step. The single most parsimonious tree resulting from the exhaustive search analysis of the "total molecular evidence" is extremely robust and entirely concordant with the morphology-based hypotheses of Bayer (1965). The inclusion of both Japanese (*P. teramochii*) and Caribbean (*P. midas*) large-shelled species in a clade separate from the small-shelled species indicates that the divergence between *Perotrochus* "Group A" and "Group B" occurred before the closing of the Tethys during the Oligocene.

The clade comprising Apogastropoda, with each of the constituent groups (Caenogastropoda and Heterobranchia) broadly represented, is well supported by the 18S rDNA sequence. Monophyly of the Caenogastropoda is more robustly supported than that of the Heterobranchia. Although unsupported in the strict consensus tree, Heterobranchia emerges as a weakly supported clade in bootstrap and jackknife analyses (61% bootstrap and 61% jackknife proportions). The seven heterobranch taxa in this study contain 15 to 20 more nucleotides in the E-10-1 helix than do any of the caenogastropods, providing further evidence for their monophyly in the form of unique insertions. Resolution of phylogenetic relationships within Apogastropoda is the subject of ongoing research, and will be reported in a separate paper.

The gene coding for the small subunit ribosomal RNA (18S RNA) was the first to be used for sequence-based phylogenetic inference (Field et al., 1988), and has subsequently been applied primarily to resolve relationships of phyla and classes. Within Mollusca, this gene has been applied to studies of bivalve evolution (Rice et al., 1993; Kenchington et al. 1994), in which it has successfully resolved family and in some cases genus-level taxa. The present study, which represents the most dense and widespread sampling of taxa within a single molluscan class published to date, reveals the 18S gene to be capable of broad but uneven levels of taxonomic resolution, in some instances being able to resolve genera, in others failing to resolve superfamilies and orders. An empirical observation is that higher taxa that contain insertions are more finely resolved than those taxa that do not, even when the insertions are not included in the analyses. It further appears that larger insertions produce finer resolution than smaller insertions, but this hypothesis requires confirmation. A corollary of this conjecture is that the 18S gene should prove more useful for resolution of phylogenetic relationships among cephalopods, patellogastropod and cocculiniform limpets, and heterobranchs, all of which contain insertions, and less informative about relationships among Caenogastropoda and nonpleurotomarioidean vetigastro-

pods, which lack insertions. It is possible that Caenogastropoda and nonpleurotomarioidean vetigastropods may have insertions in other variable regions of the 18S gene that would increase the resolving ability of those regions.

The distribution of variable and conserved regions in the 18S RNA molecule was described by De Rijk et al. (1992) and later refined by Winnepenninckx et al. (1992). Kenchington et al. (1994) identified variable regions on a finer scale that were useful for resolving relationships within the class Bivalvia and noted a region of potential utility for studies of Gastropoda. They emphasized the necessity of determining the entire gene sequence for phylogenetic studies in order to capture data from all variable regions throughout the gene. Recognizing the inevitable conundrum of molecular systematists, Lecointre et al. (1993) assessed the comparative benefits of adding sequence length versus those of adding taxa and concluded that the impact of species sampling on tree topology and robustness is greater than the impact of sequence length. These authors recommended that sequencing efforts be modified to sequence fewer nucleotides per species while increasing the number of representatives of presumed monophyletic taxa and to include several outgroups, an approach that we have followed in the initial phase of this study. A dense sampling of taxa also allowed us to recognize patterns in the distribution and lengths of insertions and deletions among related taxa and to interpret these variations in a phylogenetic context.

The resolution achieved by combining data sets from multiple genes demonstrates the utility of an iterative, multistep, multigene approach to phylogeny reconstruction. In the initial step, the phylogenetic relationships of the taxon of interest (Pleurotomariidae) were assessed in a broad context. The 18S rDNA gene proved to be sufficiently variable to resolve many of the relationships among higher taxa within the molluscan class Gastropoda, especially in those taxa that contain one or more large insertions. Both sequences and length polymorphisms within the insertions further improved resolution. After monophyly of the Pleurotomariidae had been established and appropriate sister taxa identified, finer-scale relationships on the order of genera and species were resolved with supplemental sequence data from the more rapidly evolving mitochondrial gene. "Total molecular evidence," consisting of partial sequences of 18S rDNA and COI and including data on length variation within the inserts in the 18S rDNA gene, was sufficient to resolve fully the relationships of the genera and species within Pleurotomariidae. A similar approach may be fruitful for resolving relationships within groups such as Caenogastropoda that have proved refractory to resolution by 18S data. Alternatively, the addition of data for one or more slowly evolving genes may enhance the resolution of deeper branches within Gastropoda, Mollusca, and Metazoa.

Experimental Procedures

Sample collection and preparation

All of the species sequenced were collected expressly for this study, frozen while living, transported to the laboratory on dry ice, and maintained at −80°C until DNA was extracted. Specimens were collected by a variety of techniques including hand collecting, trapping, dredging, trawling, or use of the Harbor Branch Oceanographic Institution's JOHNSON-SEA-LINK and CLELIA manned submersibles. A list of taxa, collection localities, tissue extracted, and voucher specimen information are given in Table 1.

DNA extraction

Specimens were thawed and maintained on ice while the buccal muscles were dissected out. Small shreds of muscle (totaling 1–2 mm^3) were placed in a 1.5-ml Eppendorf tube containing 300 μl of CTAB buffer at 60°C and ground using a disposable pestle (Kontes catalog no. 749520) in an electric drill. When the animal was minute, the shell was crushed, shell fragments were removed with forceps, and the entire remaining tissue mass was used for extraction. Individual animals were used for all extractions. The extraction protocol follows Adamkewicz and Harasewych (1996), with the following modifications. The initial incubation at 60°C was extended to between one and two hours, and the tissue was ground again after 30 to 45 minutes. A second chloroform–isoamyl alcohol extraction was added. All centrifugation steps were extended to 10 minutes at 10,000 rpm. Absorbances at 260 and 280 nm were measured for all DNA preparations. When the ratio of absorbances at 260/280 nm was less than 1.5, the preparation was treated with proteinase K and reextracted.

Primer selection

Sequence data for taxa in Table 1 were obtained from a region of approximately 450 bp at the 5′ end of the small subunit of ribosomal DNA (18S rDNA) that contains the variable regions V1 and V2 (De

Rijk et al., 1992), reported by Kenchington et al. (1994) to be useful in inferring molluscan phylogeny. The primers (forward, 5′-GCCAAGTAGCATATGCTTGTCTC-3′, and reverse, 5′-AGACTTGCCTCCAATGGATCC-3′) were from Holland et al. (1991). Two additional, internal primers were designed by the authors for sequencing (forward, 5′-GCCGGCGACG(T/C)ATCTTTC-3′, and reverse, 5′-GAAAGAT(A/G)CGTCGCCGGC-3′). Sequence data for pleurotomariid taxa and selected outgroups were also obtained from a 579-bp fragment of mitochondrial cytochrome *c* oxidase subunit I (COI) using the "universal" primers for COI of Folmer et al. (1994) (forward, 5′-GGTCAACAAATCATAAAGATATTGG-3′, and reverse, 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′).

PCR amplification

PCR amplifications were performed using a Perkin-Elmer 480 thermal cycler. PCR reaction mixtures had a total volume of 50 μl, containing 200 to 500 ng of genomic DNA, 1.25 U of Amplitaq *Taq* polymerase (Perkin-Elmer), 200 μm of each dNTP, 0.25 μM of each primer, 1.5 mM $MgCl_2$, and 5 μl of a 10× Perkin-Elmer PCR reaction buffer. All PCR reactions were performed as "hot start" reactions with the following thermal cycler parameters: five minutes at 95°C, addition of *Taq* polymerase, 30 cycles of 45 seconds at 94°C for denaturing, two minutes at 50°C for annealing, and one minute at 72°C for extension. Following the 30 cycles, a five-minute final extension at 72°C was performed and the PCR reaction mixtures were then held at 4°C until use. For each PCR product 5 μl was evaluated on a 1% (w/v) agarose gel. If a PCR reaction was unsuccessful, 1 μl of the initial reaction mixture was used as template for a second round of amplification. The thermal cycler profile for reamplifications was the same as for the initial PCR with two exceptions: the number of cycles was decreased from 30 to 20 and the annealing temperature was raised from 50°C to 52°C. If a single band was observed, the reaction mixture was transferred to a Microcon-100 microconcentrator (Amicon, Inc.) for the removal of primers, unused dNTPs, and salts. An initial wash with 200 μl of 40% (v/v) isopropanol was performed prior to a wash with 450 μl of sterile, distilled water. Samples were concentrated to a final volume of 10 to 20 μl, and had a DNA concentration of 50 to 800 ng/μl.

DNA sequencing and data management

Purified PCR products (100–200 ng DNA per reaction) were sequenced on Applied Biosystems 373A automated DNA sequencers using fluorescence Dye Terminator Cycle Sequencing kits. Sequence data were edited and uploaded to a relational database called PHYLO. 18S rDNA target regions were sequenced and confirmed by at least two DNA sequencing reactions in one direction and a third reaction in the opposite direction. The COI target regions were sequenced and confirmed by at least two DNA sequencing reactions for each primer. Regions of ambiguity are noted using the IUPAC symbols. Sequence fragments for each gene were downloaded from PHYLO and assembled into a consensus sequence using modified TIGR Assembler software (Fleischmann et al., 1995). The consensus sequence was edited using a modified ABI Sequence Editor software. Sequences have been posted to the Genome Sequence Data Bank (URL http://www.ncgr.org), and accession numbers are included in Table 1.

Multiple sequence alignment

The confirmed consensus sequences from each organism were downloaded from PHYLO in multiple FASTA file format (Pearson and Lipman, 1988) and viewed with Genetic Data Environment (GDE) version 2.0 (Smith et al., 1994). The sequences were aligned using the multiple sequence alignment algorithm (MSA) of Sutton (Bult et al., 1997). For the protein coding gene, COI, the "protein assisted" feature of MSA was employed, which uses an optimal alignment of amino acid translations of the genes to guide the nucleotide alignment. Aligned sequences are available in nexus format via FTP at ftp.tigr.org/pub/data/gastropod_18s.aln (18S rDNA sequences) and ftp.tigr.org/pub/data/gastropod_col.aln (cytochrome *c* oxidase I), or from the corresponding author.

Phylogenetic analyses

Phylogenetic analyses using parsimony were conducted using PAUP 4.0.0d45 (Swofford, 1996; beta test version) on a Power Macintosh 6100/66. All characters were treated as unordered. For 18S rDNA sequences, autapomorphic insertions (present in a single taxon) and regions of uncertain homology, including insertions not present in all taxa (Figure 4B), were excluded from the data set prior to maximum parsimony analysis using the branch and bound search algorithm. In subsequent analyses, subsets of taxa with fewer regions of uncertain homology were analyzed using maximum parsimony and the exhaustive search option. Bootstrap and jackknife analyses (1000 replicates) were performed using the "fast" stepwise addition option.

To compare 18S rDNA sequence-based gastropod phylogeny with prevailing morphology-based phylogenetic hypotheses, subsets of the data matrix were created by pruning to contain taxa overlapping with the morphology-based trees under consideration (taxa appearing in upper case letters in Figure 2). When higher taxa were represented by multiple species in the molecular data set, a single representative containing the minimum number of deletions and ambiguous base determinations was selected. Following analysis (branch and bound option) of the reduced subset, the most parsimonious tree that most closely resembled the topology of the morphology-based tree to which it was being compared was imported into MacClade 3.01 (Maddison and Maddison, 1992), where branches were rearranged to correspond with their position in the morphology-based tree. Resulting changes in tree length and character indices were noted.

Phylogenetic signal contained in the seven insertions unique to Pleurotomariidae was assessed by analyzing only the information in these insertions. A phylogeny of the seven Recent Pleurotomariidae was calculated from these isolated insertion sequences. Individual insertions and deletions were then defined in terms of 19 segments (ranging from 1 to 22 nucleotides) that varied between the pleurotomariid taxa and scored as present or absent in each taxon regardless of sequence. A second analysis, based only on the presence or absence of these segments, was performed.

Cytochrome *c* oxidase I sequences of pleurotomariids and representative outgroups were analyzed using the maximum parsimony algorithm and the exhaustive search option. Bootstrap and jacknife analyses were performed as for 18S data. Preliminary analyses based on amino acid sequence, as well as first and second codons only, yielded poor resolution and were not pursued further.

Finally, the 18S sequences, cytochrome *c* oxidase subunit I sequences, and the presence/absence matrix for the 19 inserts were combined into a single "total molecular evidence" data matrix and analyzed using the exhaustive search option.

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