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## A molecular phylogeny of the Patellogastropoda (Mollusca: Gastropoda)

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**Abstract** Phylogenetic analyses of partial 18S rDNA sequences from species representing all living families of the order Patellogastropoda, most other major gastropod groups (Cocculiniformia, Neritopsina, Vetigastropoda, Caenogastropoda, Heterobranchia, but not Neomphalina), and two additional classes of the phylum Mollusca (Cephalopoda, Polyplacophora) confirm that Patellogastropoda comprises a robust clade with high statistical support. The sequences are characterized by the presence of several insertions and deletions that are unique to, and ubiquitous among, patellogastropods. However, this portion of the 18S gene is insufficiently informative to provide robust support for the monophyly of Gastropoda, or to address the division of the Gastropoda into the subclasses Eogastropoda (= Patellogastropoda + hypothetical coiled ancestors) and Orthogastropoda. These sequence data invariably group Patellogastropoda in a weakly supported clade with cocculiniform limpets, despite greater sequence divergences between Patellogastropoda and “Cocculini-

formia” than between the Patellogastropoda and Orthogastropoda. Partial 18S sequences support the inclusion of the family Neolepetopsidae within the superfamily Acmaeoidea, and refute its previously hypothesized position as sister group to the remaining living Patellogastropoda. This region of the 18S rDNA gene diverges at widely differing rates, spanning an order of magnitude among patellogastropod lineages, and therefore does not provide meaningful resolution of the relationships among higher taxa of patellogastropods. Data from one or more genes that evolve more uniformly and more rapidly than the 18S rDNA gene (possibly one or more of the mitochondrial genes) seem more likely to be informative about relationships within Patellogastropoda.

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**Supplementary material:** Aligned partial sequences of the gastropod 18S rDNA gene corresponding to positions 60–515 of the 18S rRNA of *Onchidella celtica* as reported by Winnepenninckx et al. (1994: *Nautilus* Suppl 2:p 101). Ambiguous base assignments are noted using IUPAC symbols. Dashes (–) represent gaps inserted during alignment, question marks represent missing data, periods represent identity to the sequence of the chiton *Acanthopleura japonica*. Available in electronic form on Springer-Verlag's server under <http://link.springer.de/link/service/journals/00227>.

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### Introduction

The patellogastropod limpets are conspicuous in intertidal, rocky shore faunas, achieving greatest abundance and diversity in temperate climates. Most limpets live in shallow water and feed on algae and seagrasses, but some inhabit sunken wood at bathyal and abyssal depths (Lindberg and Hedegaard 1996), and others have been discovered at hydrothermal vents and sulfide seeps (McLean 1990; Beck 1996). Because of their abundance and diversity, limpets have been used in studies in numerous biological disciplines, including ecology, embryology, sperm morphology, population genetics, and biogeography (e.g. Smith 1935; Abbott et al. 1968; Branch and Branch 1980; Hodgson 1996; Koufopanou et al. 1999). Due to their distinctive morphology and anatomical organization, patellogastropod limpets play a pivotal role in the ongoing reassessment of the evolutionary history of the molluscan class Gastropoda and the subphylum Conchifera (e.g. Haszprunar 1988; Bandel and Geldmacher 1996; Ponder and Lindberg 1996, 1997; Salvini-Plawen and Steiner 1996; Bandel 1997; Sasaki 1998).

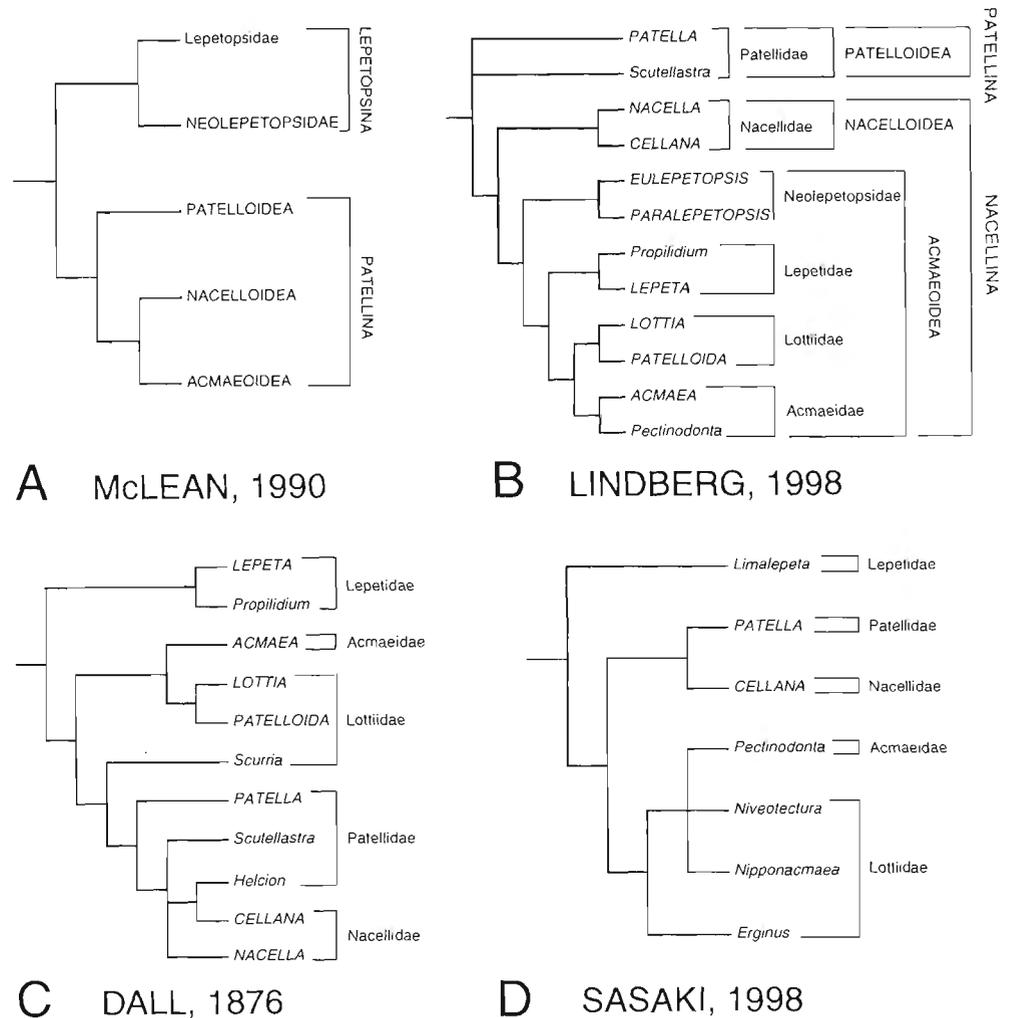
Limpets had been segregated from the coiled gastropods by the earliest molluscan taxonomists on the

basis of their low, conical shells (e.g. Lister 1678; Bunnani 1684; Linné 1758). During the gradual refinement of gastropod classification in the nineteenth century, systematists erected taxa such as Cyclobranchia (Cuvier 1817) and Docoglossa (Troschel 1861) to encompass the patellogastropod limpets, while excluding the various unrelated taxa with convergently derived, limpet-like shells (e.g. Fissurellidae, Calyptraeidae, Siphonariidae), usually on the criterion of the morphology of the radula. In the prevailing classifications of the time (e.g. Gray 1857; Fischer 1887; Pelsener 1906), patellogastropod limpets were generally placed at the base of taxa today included in the Gastropoda. In a significant departure from previous classifications, Thiele (1929) proposed a new arrangement of the Gastropoda based on the paradigm that paired mantle cavity organs constitute the primitive condition. He regarded the patellogastropod limpets to be closely related to the fissurellid limpets and included both groups in his order Archaeogastropoda. This arrangement has been accepted almost universally for over 50 years (e.g. Abbott 1974; Brusca and Brusca 1990; Ruppert and Barnes 1994). Modern analyses of gastropod phylogeny based on

morphological characters, begun by Golikov and Starobogatov (1975) and continued using cladistic principles (e.g. Haszprunar 1988; Ponder and Lindberg 1996, 1997; Sasaki 1998), invariably place the Patellogastropoda as the basal clade within Gastropoda. So profound are the morphological differences between limpets and the remaining gastropods (summarized by Lindberg 1998a; Sasaki 1998) that the subclass Eogastropoda was proposed to segregate the Patellogastropoda, together with their hypothesized, coiled ancestors, from the remaining gastropods, which were assigned to the subclass Orthogastropoda (Ponder and Lindberg 1996). Some authors (Termier and Termier 1968; Shileyko 1977) have speculated that patellogastropods might not be gastropods, but rather were independently derived from monoplacophorans.

Evolutionary relationships within Patellogastropoda have long been studied (e.g. Dall 1871, 1876; Fleure 1904; Lindberg 1988, 1998a; Lindberg and Hedegaard 1996; Sasaki 1998). While patellogastropods are readily sorted into groups (families/superfamilies) on the basis of highly concordant suites of shell ultrastructural, radular, and anatomical characters, recent hypotheses of

**Fig. 1A–D** Morphology-based hypotheses of patellogastropod relationships. Nomenclature and taxon rank have, in some cases, been modified to facilitate comparisons. Taxa listed in upper case are represented in this study. **A** McLean (1990: Fig. 1). **B** Lindberg (1998a: Fig. 15.31). **C** Dall (1876: Fig. 2); follows Lindberg (1998b) for modern equivalents of Dall's (1871, 1876) taxa. **D** Sasaki (1998: Fig. 104)



phylogenetic relationships among these groups differ significantly (Fig. 1B, D), despite their being based on broad suites of anatomical characters and cladistic methodology. Most notably, a distinctive, monophyletic lineage of limpets discovered at hydrothermal vents and hydrocarbon seeps was initially thought to be intermediate between patellogastropods and the remaining gastropods (Haszprunar 1988: Fig. 5, "Hot-Vent-C"), but subsequently was included within the patellogastropod clade as additional data became available (McLean 1990; Ponder and Lindberg 1996, 1997). McLean (1990) proposed the family Neolepetopsidae for these limpets based on their articulating radular teeth, and grouped them with extinct, Paleozoic limpets in the suborder Lepetopsina. McLean regarded the Lepetopsina as the sister taxon of the remaining patellogastropods (Fig. 1A) and the Neolepetopsidae as a possible phylogenetic relic of the Paleozoic surviving in deep-sea, sulfide-rich refugia such as vents and seeps (see McArthur and Tunnicliffe 1998). Other authors (Fretter 1990; Lindberg 1998a) advocated more recent origins via a closer relationship between the neolepetopsid limpets and the patellogastropod superfamily Acmaeioidea (Fig. 1B).

Relationships of the patellogastropod limpets have proven especially difficult to resolve using ribosomal sequence data, due largely to rapid evolutionary rates and long branches. In phylogenies based on partial 28S rRNA sequences, patellogastropod limpets emerge as a sister group to all other Gastropoda, or as a sister group to Vetigastropoda, Neomphalina + Apogastropoda, Heterobranchia, or within Caenogastropoda, depending on variables such as outgroup, included taxa, and algorithm for tree construction (Tillier et al. 1992, 1994; Rosenberg et al. 1997; McArthur and Koop 1999). Partial 18S rDNA sequences placed these limpets at the base of the Gastropoda, but in a clade that included a cephalopod and cocculiniform limpets (Harasewych et al. 1997). To date, sampling has been inadequate to examine relationships within Patellogastropoda.

The present study builds upon earlier work (e.g. Harasewych et al. 1997, 1998) in investigating the major features of gastropod evolution using sequences derived from the 5' end of the 18S rDNA gene, and in providing an independent set of data with which to test morphology-based hypotheses of gastropod phylogeny. We investigate relationships of and among patellogastropod limpets using a broader and more comprehensive taxonomic sampling than previously (Harasewych et al. 1997) in order to facilitate alignment and to improve determination of homology. Specifically, this study evaluates the utility of partial 18S rDNA sequence data to: (1) confirm the monophyly of the class Gastropoda and its subclasses Eogastropoda and Orthogastropoda, (2) ascertain whether "Cocculiniformia" group with Eogastropoda or Orthogastropoda, and (3) resolve phylogenetic relationships of family level taxa within Patellogastropoda, particularly the origins of the hypothesized relic family Neolepetopsidae.

## Materials and methods

Table 1 lists the taxa used in the present study, their collection locality, preservation history, tissue extracted, and voucher specimen information. Most taxa were collected specifically for this study, frozen while living, and maintained at  $-80^{\circ}\text{C}$  until DNA was extracted. Freshly collected specimens of *Nacella magellanica* were fixed in 95% ethanol prior to shipping. Data for *Lepeta caeca* and *Paralepetopsis floridensis* were obtained from museum specimens that had been fixed in formalin and stored in 70% ethanol. DNA was extracted from buccal muscles or entire individuals following the protocol of Harasewych et al. (1997). If this did not provide workable template for the polymerase chain reaction (PCR), as was the case for most formalin-preserved specimens, DNA was then extracted using the protocol of Chase et al. (1998). Newly designed oligonucleotide primers used for PCR amplification of approximately 450 bp of the 5' end of the 18S rDNA molecule were  $\Delta\text{GM-18F}$  (forward): 5' GCCAGTAGTCATATGCTTGTCTC and  $\Delta\text{GM-18R}$  (reverse): 5' AGACTTGCCCTCCAAT(A/G)GATCC. The entire fragment of 18S rDNA could not be amplified for all formalin-preserved specimens. In these cases, smaller fragments were amplified or secondarily re-amplified using several internal primers in combination with the primers listed above. These internal primers were: 18SR-1b (reverse): 5' GCTCTAGAATTACCACAG, 18SF-1c (forward): 5' GCATG(C/A)GAAACGGCTACCAC, 18SR-1c (reverse): 5' GTGGTAGCCGTTTC(T/G)CATGC. All oligonucleotide primers were also used in DNA sequencing reactions.

PCR amplifications were run in Perkin Elmer System 2400 or 9600 Thermal Cyclers using 50- $\mu\text{l}$  reactions containing 1  $\mu\text{l}$  of extracted DNA, 1.5 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$  each dNTP, 500 nM each primer, Promega reaction buffer, and 1 or 2 units *Taq* or *AmpliTaq* Gold DNA polymerase. Amplifications were performed using 5 min of denaturation at  $94^{\circ}\text{C}$ , 30 cycles of 45 s at  $94^{\circ}\text{C}$ , 2 min at  $55^{\circ}\text{C}$ , and 1 min at  $72^{\circ}\text{C}$  followed by a 5-min extension step at  $72^{\circ}\text{C}$ . Re-amplifications and amplification of small fragments were performed using 5 min of denaturation at  $94^{\circ}\text{C}$ , 30 cycles of 1 min at  $95^{\circ}\text{C}$ , 1 min at  $45^{\circ}\text{C}$ , and 1.5 min at  $72^{\circ}\text{C}$  followed by a 5-min extension step at  $72^{\circ}\text{C}$ . DNA sequences were obtained from amplification products using a Model 373 automated DNA sequencer (Applied Biosystems, Inc.).

The DNA sequences were aligned using the CLUSTALW program (Thompson et al. 1994), with verification and correction by eye in the context of the alignments of Harasewych et al. (1997, 1998). Phylogenetic analyses were performed using the computer program PAUP Release 4.0 beta 2 (Swofford 1998). The Polyplacophora (*Acanthopleura japonica* and *Cryptochiton stelleri*) and Cephalopoda (*Nautilus scrobiculatus*) were used as outgroups (after Runnegar 1996; Salvini-Plawen and Steiner 1996). Sequence ambiguities were treated as uncertainties; gaps were treated as missing data. For parsimony, all characters were treated as unordered and weighted equally. Bootstrap and jackknife analyses (1000 replicates) were performed using the "fast" step-wise addition option. Support indices (Bremer 1988) were calculated using TreeRot (Sorenson 1996). Pairwise comparisons of sequences were calculated using MEGA (Kumar et al. 1993).

Models for use in maximum likelihood searches were chosen by the use of the likelihood ratio test (LRT), after Sullivan and Swofford (1997) and Huelsenbeck and Crandall (1997). Five random-addition replicates were performed for maximum likelihood heuristic searches. The significance of differences in likelihood among different topologies was examined according to the test of Kishino and Hasegawa (1989), using the UNIX tester version of PAUP 4.0d66 (Swofford 1998).

## Results

Partial sequences representing approximately 450 base pairs (bp) from near the 5' end of the 18S rDNA gene

**Table 1** Locality data, tissue extracted, voucher specimen information, and sequence accession number for taxa used in the present study (*EMBL* European Molecular Biology Laboratory Data Library; *GB* GenBank; *GSDB* Genome Sequence Data Bank; *JC* Junciflora Collection, University of Victoria; *USNM* Mollusk Collection, National Museum of Natural History, Smithsonian Institution). Morphology-based classification of the Patellogastropoda (after Lindberg 1998a). † indicates sequence data from Harasewych et al. (1997)

| TAXON  | Collection locality                     | Tissue          | Voucher material | Sequence Accession Number 18S rDNA |
|--|---|-----------------|------------------|------------------------------------|
| CLASS POLYPLACOPHORA de Blainville, 1816   |   |                 |                  |                                    |
| <i>Acanthoplicaria japonica</i> (Lischke, 1873)  | Genbank ex Winnipeginickx et al. (1993) | Buccal muscle   | USNM 888657      | EMBL X70210                        |
| <i>Cryptochiton stelleri</i> (Middendorff, 1847)   | Bamfield, B.C., Canada                  |                 |                  | †GSDB L78876                       |
| CLASS CEPHALOPODA Cuvier, 1797   |   |                 |                  |                                    |
| <i>Nautilus scribiculatus</i> Lightfoot, 1786  | Papua, New Guinea                       | Buccal muscle   | USNM 885678      | †GSDB L78877                       |
| CLASS GASTROPODA Cuvier, 1797  |   |                 |                  |                                    |
| SUBCLASS EOGASTROPODA Ponder & Lindberg, 1996  |   |                 |                  |                                    |
| Order Patellogastropoda, Lindberg, 1986, = Onychoglossa G.O. Sars, 1878, = Docoglossa Troschel, 1861 |   |                 |                  |                                    |
| Suborder Patelina von Ihering, 1876  |   |                 |                  |                                    |
| Superfamily Patelloidea Rafinesque, 1815   |   |                 |                  |                                    |
| Family Patellicidae Rafinesque, 1815   |   |                 |                  |                                    |
| <i>Patella vulgata</i> Linné, 1758   | Bayona, Spain                           | Buccal muscle   | USNM 888670      | GB AF046046                        |
| Suborder Nacellina Lindberg, 1988  |   |                 |                  |                                    |
| Superfamily Nacelloidea Thiele, 1891   |   |                 |                  |                                    |
| Family Nacellidae Thiele, 1891   |   |                 |                  |                                    |
| <i>Nacella magellanica</i> (Gmelin, 1791)  | Punta Pyramide, Argentina               | Buccal muscle   | USNM 888000      | GB AF046047                        |
| <i>Cellana nigrolimitata</i> (Reeve, 1854)   | Minabe, Japan                           | Buccal muscle   | USNM 888623      | †GSDB L78879                       |
| Superfamily Acmaeoidae Forbes, 1850, = Lottioidea Gray, 1840   |   |                 |                  |                                    |
| Family Acmaeidae Forbes, 1850  |   |                 |                  |                                    |
| <i>Acmaea nitra</i> Rathke, 1833   | Bamfield, B.C., Canada                  | Buccal muscle   | USNM 888640      | †GSDB L78878                       |
| Family Lepetitidae Dall, 1869  |   |                 |                  |                                    |
| <i>Lepeta caeca</i> Müller, 1776   | Point Barrow, Alaska, 128 m             | Whole animal    | USNM 606042      | GB AF046048                        |
| Family Lottuidae Gray, 1840  |   |                 |                  |                                    |
| Subfamily Lottuiniae Gray, 1840  |   |                 |                  |                                    |
| <i>Lottia pelta</i> (Rathke, 1833)   | Bamfield, B.C., Canada                  | Buccal muscle   | USNM 888641      | GB AF046049                        |
| <i>Tectura scitum</i> (Rathke, 1833)   | Victoria, B.C., Canada                  | Foot and mantle | TC AGM-22        | GB AF046050                        |
| Subfamily Patelloidiniae Oliver, 1926  |   |                 |                  |                                    |
| <i>Patelloida saccharina laux</i> (Reeve, 1855)  | Amami-O-Shima, Japan                    | Buccal muscle   | USNM 888704      | GB AF 046051                       |
| Family Ncolepetopsidae McLean, 1990  |   |                 |                  |                                    |
| <i>Eulepetopsis vitrea</i> McLean, 1990  | Galapagos Rift Hydrothermal Vents       | Whole animal    | USNM 888727      | GB AF046052                        |
| <i>Paralepetopsis floridensis</i> McLean, 1990   | Florida Escarpment, SW Florida          | Whole animal    | USNM 860498      | 5'GB AF046053<br>3'GB AF046056     |
| SUBCLASS ORTHOGASTROPODA Ponder & Lindberg, 1996   |   |                 |                  |                                    |
| Order Cocculiniformia Haszprunar, 1987   |   |                 |                  |                                    |
| Superfamily Cocculinoidea Dall, 1882   |   |                 |                  |                                    |
| <i>Cocculina mesingi</i> McLean & Harasewych, 1995   | Grand Bahama Is., Bahamas               | Buccal muscle   | USNM 888655      | GB AF046054                        |
| Superfamily Lepetelloidea Dall, 1881   |   |                 |                  |                                    |
| <i>Notocater houbricki</i> McLean & Harasewych, 1995   | Grand Bahama Is., Bahamas               | Whole animal    | USNM 888656      | †GSDB L78881                       |
| Order Neritopsina Cox & Knight, 1960   |   |                 |                  |                                    |
| Superfamily Neritoidae Lamarck, 1809   |   |                 |                  |                                    |
| <i>Nerita versicolor</i> Gmelin, 1791  |   |                 |                  |                                    |
| <i>Neritina reclinata</i> (Say, 1822)  | Big Pine Key, FL, USA                   | Buccal muscle   | USNM 888658      | †GSDB L78882                       |
| <i>Septaria porcellana</i> (Linne, 1767)   | Big Pine Key, FL, USA                   | Buccal muscle   | USNM 888659      | †GSDB L78883                       |
| Order Vetigastropoda Salvini-Plawen, 1980  |   |                 |                  |                                    |
| Superfamily Fissurelloidea Fleming, 1822   |   |                 |                  |                                    |
| <i>Diadora carolinensis</i> (Lamarck, 1822)  | Sebastian Inlet, FL, USA                | Buccal muscle   | USNM 888660      | †GSDB L7888                        |
| Superfamily Haliotoidea Rafinesque, 1815   |   |                 |                  |                                    |

|  |                          |               |             |              |
|--|--------------------------|---------------|-------------|--------------|
| <i>Haliotis rufescens</i> Swainson, 1822                   | Bamfield, B.C., Canada   | Buccal muscle | USNM 888642 | †GSDB L78885 |
| Superfamily Trochoidea Rafinesque, 1815                    |                          |               |             |              |
| <i>Astraea caelata</i> (Gmelin, 1791)                      | Berry Is., Bahamas       | Buccal muscle | USNM 888603 | †GSDB L78886 |
| Superorder Apogastropoda Salvini-Plawen & Haszprunar, 1987 |                          |               |             |              |
| Order Caenogastropoda Cox, 1959                            |                          |               |             |              |
| Superfamily Ampullarioidea Gray, 1824                      |                          |               |             |              |
| <i>Pontacea bridgei</i> (Reeve, 1856)                      | Lake Worth, FL, USA      | Buccal muscle | USNM 888715 | GB AF046057  |
| Superfamily Cerithioidea Ferussac, 1819                    |                          |               |             |              |
| <i>Cerithium airatum</i> (Born, 1778)                      | Sebastian Inlet, FL, USA | Buccal muscle | USNM 888663 | †GSDB L78895 |
| Infraorder Neogastropoda Wenz, 1943                        |                          |               |             |              |
| Superfamily Buccinoidea Rafinesque, 1815                   |                          |               |             |              |
| <i>Buxycotpus spiratus pyruloides</i> (Say, 1822)          | Marvin Key, FL, USA      | Buccal muscle | USNM 888717 | GB AF046058  |
| Superfamily Conoidea Rafinesque, 1815                      |                          |               |             |              |
| <i>Haustoria cinerea</i> (Born, 1778)                      | Ft. Pierce, FL, USA      | Buccal muscle | USNM 888611 | †GSDB L78899 |
| Superorder Heterobranchia Gray, 1840                       |                          |               |             |              |
| Superfamily Pyramidelloidea Gray, 1840                     |                          |               |             |              |
| <i>Fargoa bushiana</i> Bartsch, 1909                       | Sebastian Inlet, FL, USA | Whole animal  | USNM 888638 | †GSDB L78900 |
| Superorder Opisthobranchia Milne Edwards, 1848             |                          |               |             |              |
| Superfamily Aplysioidea Rafinesque, 1815                   |                          |               |             |              |
| <i>Aplysia dactyloneta</i> Rang, 1828                      | Minabe, Japan            | Buccal muscle | USNM 888624 | †GSDB L78902 |
| Superorder Pulmonata Milne Edwards, 1848                   |                          |               |             |              |
| Superfamily Limacoidea Gray, 1824                          |                          |               |             |              |
| <i>Limax maximus</i> Linné, 1758                           | Silver Spring, MD, USA   | Buccal muscle | USNM 888604 | †GSDB L7890  |

were determined for eight patellogastropod limpets, one neritopsine, and two caenogastropods. Sequence from a second individual of the cocculiniform limpet *Cocculina messingi* was determined in order to extend previously published sequence for this taxon. We were unable to determine the sequence of a short internal region (32 bp) of the *Paralepetopsis floridensis* PCR products due to an internal sequencing compression and our limited sample of DNA template for PCR.

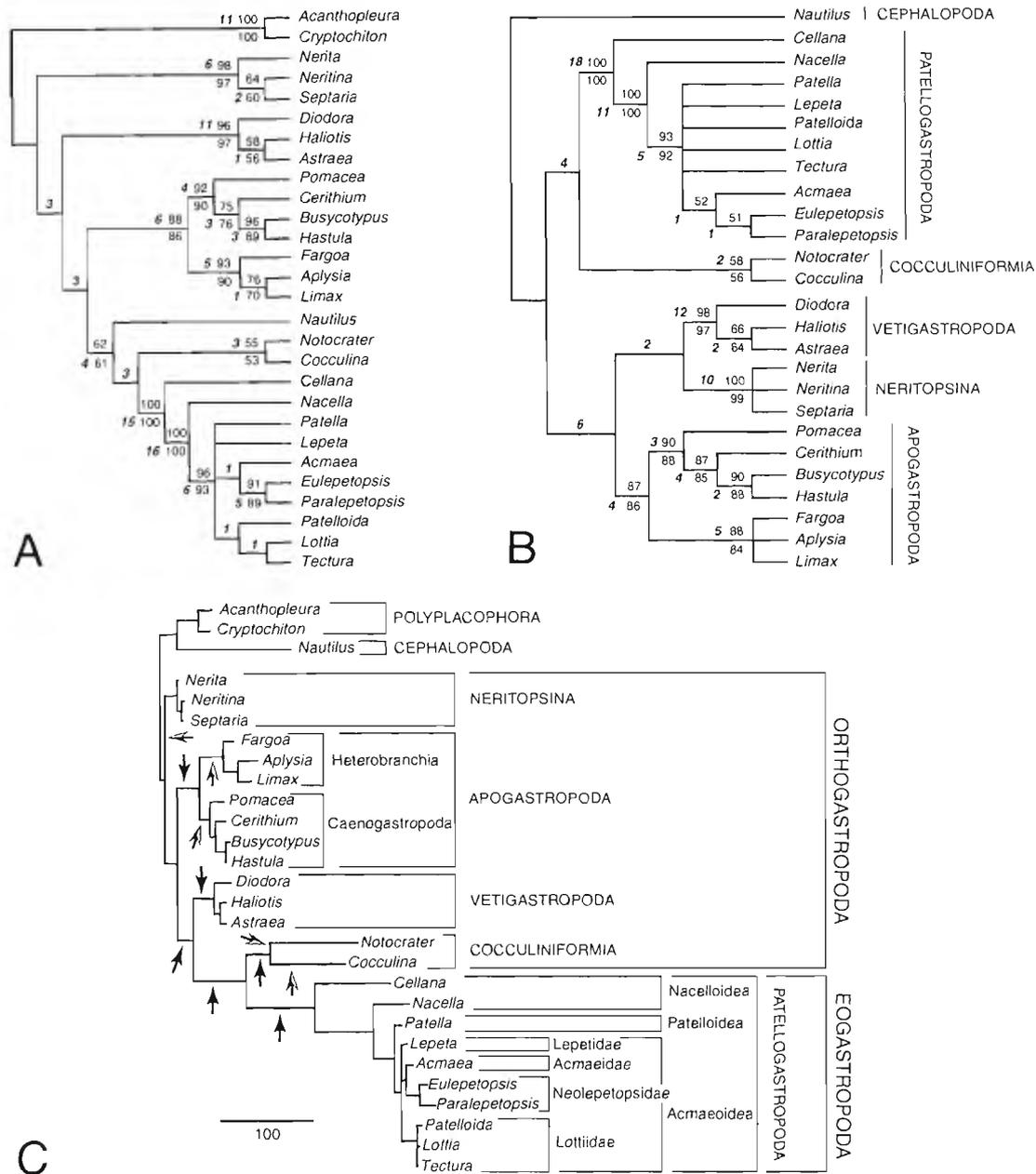
The new sequences were aligned against published sequences from two patellogastropod limpets, one cocculiniform limpet, and 11 additional gastropods, selected to include representatives of the other major gastropod groups (Neritopsina, Vetigastropoda, Caenogastropoda, Heterobranchia), as well as one cephalopod and two polyplacophorans, which served as nested outgroups (see Table 1 for sources). A NEXUS file of the alignment is provided as an appendix available as Electronic Supplementary Material at <http://link.springer.de/link/service/journals/00227>. The multiple sequence alignment spans 605 positions, but the length of this portion of the gene ranges between 430 bp (*Cocculina messingi*) and 523 bp (*Paralepetopsis floridensis*) among the taxa studied. Most of the length variation occurs in three regions of insertions. One is a 12-base insert in the terminal loop of Helix 6 (see Winnepenninckx et al. 1994), which occurs in all patellogastropods. Of the other taxa in the present study, only the cocculiniform limpets have a 2 to 6 base insert in this region. The second insert region (i2) is in the terminal loop of Helix 10. *P. floridensis* and *Notocrater houbrieki* have large, partially overlapping inserts in this region, while the remaining patellogastropods have shorter inserts. The third large insert (i3) is in the distal stem and loops of Helix E-10-1. *Nautilus scrobiculatus*, the patellogastropod limpets, and to a lesser extent the heterobranchs all have inserts in this region. These inserts could be aligned locally but not globally. Single-nucleotide insertions (aligned positions 7, 216, 236, 355, 470, 480, 530, 564) were excluded from all analyses, as were slightly larger regions unique to *N. scrobiculatus* (aligned positions 308–312, 315–316, 323–328, 332–333) and *N. houbrieki* (aligned positions 373–374). Of the remaining 580 positions, 268 were constant, and 197 were parsimony-informative.

Maximum parsimony analyses (branch and bound search) of these data using both ACCTRAN and DELTRAN character-state optimizations produced six most parsimonious trees ( $L = 646$ ;  $CI = 0.724$ ;  $RI = 0.848$ ). A strict consensus of these trees, together with bootstrap, jackknife, and Bremer support for nodes, is shown in Fig. 2A. Monophyly was supported (bootstrap proportions > 70%) for the major gastropod groups, Patellogastropoda, Neritopsina, Vetigastropoda, Apogastropoda, Caenogastropoda, and Heterobranchia, but only weakly indicated for Cocculiniformia. Patellogastropoda and Cocculiniformia emerged as sister taxa, as previously reported by Harszewych et al. (1997). The rooting of the Gastropoda was uncertain due to the internal placement of *Nautilus*

*scrobiculatus*. Repeating the analyses after excluding the two large inserts (i2 and i3) reduced the number of parsimony-informative sites to 143, and resulted in 105 most parsimonious trees (L = 459; CI = 0.680; RI = 0.855; RC = 0.581). The strict consensus of these trees was concordant with the previous consensus tree, except that the heterobranchs formed an unresolved trichotomy, the Lottiidae were no longer resolved, and bootstrap, jackknife, and Bremer support were diminished slightly for most nodes.

The distant emergence of the nested outgroups Polyplacophora and Cephalopoda under parsimony prompted us to repeat the analyses with the polyplacophorans deleted, because this distant outgroup be-

comes as a random outgroup, making the position of the root unreliable (Wheeler 1990). As Cephalopoda is recognized to be the sister taxon of Gastropoda in most modern phylogenetic classifications of Mollusca (e.g. Salvini-Plawen and Steiner 1996), *Nautilus scrobiculatus* was defined as the new outgroup and the analyses repeated. Of 580 positions, 190 were parsimony informative, yielding 12 most parsimonious trees (L = 608; CI = 0.748; RI = 0.859; RC = 0.643). The resulting strict consensus tree was identical in topology to Fig. 2A except for the position of the root. Contrary to the strict consensus tree, neither bootstrap nor jackknife estimates support the sister group relationship between Neritopsina and Vetigastropoda, but rather weakly



**Table 2** Evaluation of substitution models for one of the most parsimonious trees found (Fig. 3). Likelihood scores ( $-\ln L$ ) are shown. All scores not significantly worse than the most complex model (GTR + I +  $\Gamma$ ; likelihood ratio test,  $p > 0.05$ ) are in *bold*. The K2P + I +  $\Gamma$  model was rejected in subsequent iterations of tree estimation and model evaluation. Substitution models, each with, by definition, equal among-site rates of evolution; JC = Jukes and Cantor (1969), F81 = Felsenstein (1981).

|              | JC         | F81        | K2P               | HKY85             | GTR               |
|--------------|------------|------------|-------------------|-------------------|-------------------|
| Equal        | 3914.62210 | 3912.85818 | 3885.55896        | 3883.33064        | 3878.82914        |
| I            | 3792.93308 | 3790.85687 | 3760.47387        | 3758.31121        | 3757.13911        |
| $\Gamma$     | 3778.93886 | 3776.87239 | 3744.26706        | 3742.09018        | <b>3737.61648</b> |
| I + $\Gamma$ | 3777.29906 | 3775.14092 | <b>3742.76446</b> | <b>3740.47483</b> | <b>3736.40993</b> |

(bootstrap = 56, jackknife = 61) favor Neritopsina as the sister taxon to Apogastropoda. After further excluding the large inserts (i2 and i3), 138 of the remaining 447 characters were informative, yielding 210 trees ( $L = 429$ ;  $CI = 0.709$ ;  $RI = 0.867$ ;  $RC = 0.615$ ). The strict consensus of these 210 trees (Fig. 2B) was concordant with the previous consensus tree, except that the heterobranch clade formed a trichotomy and the Lotiidae were no longer resolved, and bootstrap, jackknife, and Bremer support were diminished slightly for most nodes.

One of the six most parsimonious trees contributing to Fig. 2A was used as a reference topology for evaluating different models of substitution. The most complex model (GTR + I +  $\Gamma$ ) produced the best likelihood score, but likelihood ratio tests illustrated that the simpler K2P + I +  $\Gamma$  model was not significantly worse for generating the reference topology from the data (Table 2). A heuristic search under maximum likelihood criteria using the K2P + I +  $\Gamma$  model was performed, and the resulting tree additionally subjected to model evaluation. This and subsequent iterations of tree estimation and model evaluation additionally rejected the fit of the K2P + I +  $\Gamma$  model of substitution. Heuristic searching under maximum likelihood criteria was thus

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**Fig. 2A–C** Phylogenetic analyses of patellogastropod limpets and other Gastropoda based on 605 aligned positions (430 to 523 bp) of 18S rDNA sequence. **A** Strict consensus of six most parsimonious trees produced when 25 bp of unique insertions were excluded from analysis ( $L = 646$ ;  $CI = 0.724$ ;  $RI = 0.848$ ;  $RC = 0.614$ ). **B** Strict consensus of 210 most parsimonious trees ( $L = 429$ ;  $CI = 0.709$ ;  $RI = 0.867$ ;  $RC = 0.615$ ) based on the same data as in A, except that the large inserts i2 and i3 were excluded, and the polyplacophorans (*Acanthopleura japonica* and *Cryptochiton stelleri*) were removed and *Nautilus scrobiculatus* specified as the outgroup. **C** Maximum likelihood analysis of phylogenetic relationships using the HKY85+I+ $\Gamma$  model (see “Results”) and the same data as in Fig. 3A. Strict consensus of the three most likely trees found ( $\ln L = -3731.42420$ ). Branch lengths are proportional to amount of evolutionary change. Unlike parsimony analysis, monophyly of the Gastropoda was supported. Arrows indicate positions to which outgroups were regrafted, under maximum likelihood criteria, to assess alternate rooting points of Gastropoda using the Kishino and Hasegawa test. Solid arrows indicate positions at which Polyplacophora and *N. scrobiculatus* were evaluated. Half-open arrows indicate positions at which only *N. scrobiculatus* was evaluated. See “Results” for discussion

K2P = Kimura (1980), HKY85 = Hasegawa et al. (1985), GTR = general time-reversible (see Swofford et al. 1996). Incorporation of among-site rate variation: I = a proportion of sites invariant (see Swofford et al. 1996).  $\Gamma$  = among-site rate variation varying according to a gamma distribution (see Swofford et al. 1996). All models were as implemented in the software PAUP 4.0d66 (Swofford 1998)

performed using the HKY85 + I +  $\Gamma$  model: transition bias, unequal base frequencies, a proportion of sites invariant, and the evolutionary rate of the remaining portion of sites varying according to a gamma distribution. Parameter estimates for this model were optimized by three iterations of tree estimation and model evaluation. Maximum likelihood searching under this model found three equally likely trees ( $\ln L = -3731.42420$ , Fig. 2C), all of which supported monophyly of the Gastropoda and placement of the root between the Neritopsina and the remaining Gastropoda. As with parsimony analyses, the major gastropod groups were monophyletic and the sister group to the Patellogastropoda was the Cocculiniformia. The only major differences were the movement of the Vetigastropoda closer to the Patellogastropoda–Cocculiniformia, and slightly improved resolution within the Patellogastropoda. The hypothesis of a molecular clock was rejected using the likelihood ratio test (HKY85 + I +  $\Gamma_{\text{clock}}$   $\ln L = -3859.60876$ ,  $p < 0.05$ ; Felsenstein 1981; Huelsenbeck and Crandall 1997). Additional analyses under likelihood criteria using only *Nautilus scrobiculatus* as an outgroup found an identical topology to that presented in Fig. 2C.

Pruning and regrafting of the outgroups, under maximum likelihood criteria, to alternate rooting points throughout the Gastropoda (Fig. 2C, arrows) illustrated that numerous rooting points were not significantly worse than the best rooting point (Kishino and Hasegawa test,  $p > 0.05$ ) and thus the root of the Gastropoda was not resolved. Searches using the same HKY85+I+ $\Gamma$  model found that the best tree without a sister relationship between the Cocculiniformia and Patellogastropoda was not significantly worse than the best overall topology (Kishino and Hasegawa test,  $p > 0.05$ ). However, the monophyly of the Patellogastropoda could not be rejected when compared to the best trees without monophyly (Kishino and Hasegawa test,  $p < 0.05$ ), and extensive pruning and regrafting could not reject the sister relationship of *Cellana nigrolineata* to all other sampled patellogastropods (Kishino and Hasegawa test,  $p < 0.05$ ). Resolution of internal patellogastropod relationships was weak – multiple internal topologies, except for the above placement of *C. nigrolineata*, were not significantly

worse than the best topology (Kishino and Hasegawa test,  $p > 0.05$ ). Additional analyses were performed under both parsimony (Fig. 3) and maximum likelihood (same results as in Fig. 2C), in which the taxa were limited to the Patellogastropoda and several proximal outgroups, treating gaps as missing characters, but these supported the same general conclusions as the above analyses. In all analyses Nacellidae was

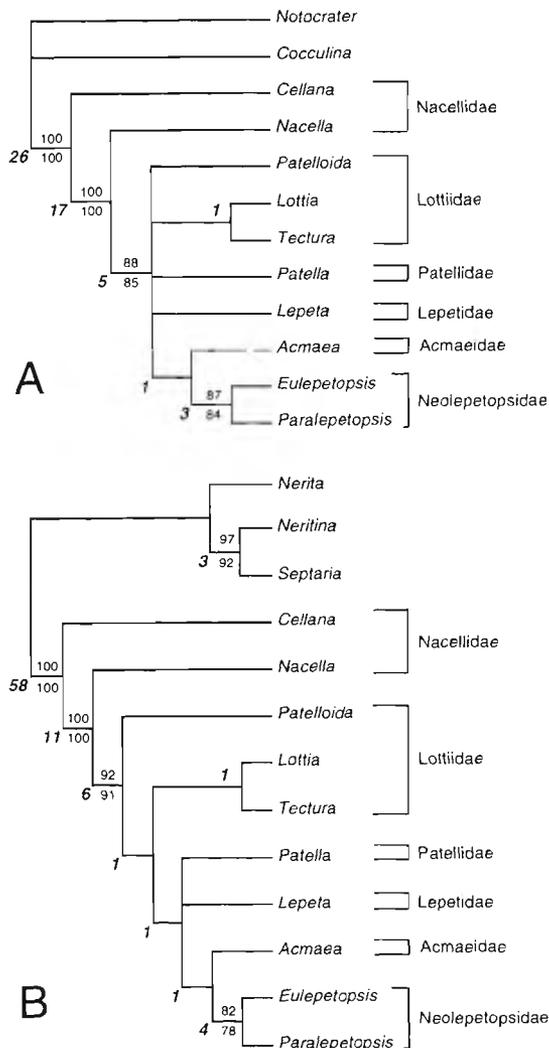
basal and paraphyletic and the Neolepetopsidae sister to *Acmaea mitra*.

## Discussion

Phylogenetic analyses of partial 18S rDNA sequences from species representing all living families of the order Patellogastropoda, most other major gastropod groups and two additional classes of the phylum Mollusca (Table 1) confirm that Patellogastropoda comprises a robust clade with high statistical support, and is characterized by the presence of several insertions and deletions that are unique to, and ubiquitous among patellogastropods. The magnitude of differences in the 18S gene (Table 3) between Patellogastropoda and the remaining gastropod groups exceeds differences among these groups, except for the cocculiniform limpets.

Parsimony analyses including chiton outgroups and all maximum likelihood analyses, incorporating among-site rate variation and more tolerance of long branch problems, both supported a sister relation between the Neritopsina and the remaining Gastropoda. This is contrary to the sister placement of Patellogastropoda to all remaining Gastropoda found in cladistic examinations of anatomical and ultrastructural information (e.g. Haszprunar 1988; Ponder and Lindberg 1996, 1997; Sasaki 1998). However, bootstrap support of parsimony hypotheses and regrafting tests of maximum likelihood hypotheses found little resolution of the inter-relationships of the major gastropod groups. On the basis of our 18S data we can make few predictions regarding the rooting of the Gastropoda and cannot robustly address the division of the class Gastropoda into the subclasses Eogastropoda and Orthogastropoda (Ponder and Lindberg 1996). Other data will be required to resolve this question.

While several extinct limpet taxa that first appeared during the Ordovician (Archinacellidae, Metoptomatidae, Macroscenella) or Mississippian (Lepetopsidae) have been attributed to the Patellogastropoda by some authors (e.g. Knight et al. 1960; McLean 1990; Tracey et al. 1993; Yochelson 1994), the earliest unequivocal patellogastropods, confirmed on the basis of distinctive shell microstructure, date from Triassic deposits (Bandel and Geldmacher 1996; Hedegaard et al. 1997). The considerable hiatus between the first fossil record of Patellogastropoda and the Cambrian origin predicted by their basal position in gastropod phylogeny has prompted a hypothesis (Ponder and Lindberg 1996, 1997; Lindberg 1998a) that patellogastropod limpets are derived from sinistrally coiled ancestors, possibly including members of the Paragastropoda (Linsley and Kier 1984). In both morphological and molecular data, numerous characters distinguish patellogastropods from orthogastropods (e.g. Ponder and Lindberg 1996, 1997; Harasewych et al. 1997; Sasaki 1998). As in a prior 18S-based phylogeny (Harasewych et al. 1997), the patellogastropods again emerge in a clade containing cocculiniform limpets in our analyses, despite greater sequence



**Fig. 3A, B** Maximum parsimony analyses of Patellogastropoda and their most proximal outgroups. **A** Strict consensus of 12 most parsimonious trees ( $L = 312$ ;  $CI = 0.949$ ;  $RI = 0.852$ ;  $RC = 0.808$ ) produced when *Notocrater houbrieki* and *Cocculina messingi*, either individually or together, were specified to be the outgroups to Patellogastropoda. **B** Strict consensus of three most parsimonious trees ( $L = 222$ ;  $CI = 0.950$ ;  $RI = 0.955$ ;  $RC = 0.908$ ) produced when Neritoidea (*Nerita versicolor* + *Neritina reclinata* + *Septaria porcellana*) was specified to be the outgroup. Selecting the vetigastropods *Diodora cayenensis* + *Haliotis rufescens* + *Astraea caelata* as the outgroup yielded three trees ( $L = 231$ ;  $CI = 0.939$ ;  $RI = 0.939$ ;  $RC = 0.882$ ) identical in topology and strict consensus, to those produced with Neritoidea as outgroup. Bootstrap proportions are given in percentage above, and jackknife proportions given in percentage below nodes supported at levels above 50%. Bremer support indices are shown in *bold italics*

differences between Patellogastropoda and the cocculiniform limpets than between Patellogastropoda and the other Orthogastropoda (Table 3).

Although the exact phylogenetic relationships of the cocculiniform limpets have been subject to varying interpretations, based on morphological data, they invariably emerge among taxa now included in Orthogastropoda. Haszprunar (1988) proposed the Cocculiniformia as a monophyletic sister group to the remaining orthogastropods. Ponder and Lindberg (1996, 1997) regarded Cocculiniformia as polyphyletic, referring cocculinoidean limpets to Neritopsina and lepetelloidean limpets as basal members of Vetigastropoda. More recently, Sasaki (1998) provided evidence that Cocculinoidea are more closely related to Vetigastropoda than to Neritopsina. Haszprunar (1988) noted that the patellogastropod family Neolepetopsidae and Cocculiniformia share a number of characters, including a symmetrical, limpet shell, a divided shell muscle, a shallow mantle cavity, and considerable variability in respiratory organs. While noting Lindberg's (1988) explanation that most of these characters are pedomorphic, Haszprunar (1988) considered it improbable that such pedomorphism was due to parallelism. However, he explicitly rejected the inclusion of the patellogastropod and cocculiniform limpets in a single clade because of differences in radular morphology. Patellogastropod limpets (including Neolepetopsidae) have a stereoglossate radula, a plesiomorphic feature they share with Polyplacophora and Tryblidiida (class Monopiacophora), while the cocculiniform limpets have a rhipidoglossate radula, which they share with Neritopsina and Vetigastropoda. Harasewych et al. (1997) reported the two patellogastropods in their 18S rDNA study to be most closely related to the cocculiniform limpets, but noted the presence of large insertions of questionable homology in these groups, and cautioned that the apparent close relationships of these taxa might be the consequence of long branch attraction. Our use of maximum likelihood criteria in combination with a substitution model incorporating among-site rate variation is the most robust approach to long-branch problems. Maximum likelihood also supports a sister relationship between the Cocculiniformia and Patellogastropoda, although this result was not robust when compared to alternative hypotheses. Base composition did not significantly differ among our sampled taxa ( $X^2$ ,  $p > 0.05$ ) and log-determinant approaches to phylogenetic reconstruction (Lake 1994; Lockhart et al. 1994) additionally supported a Patellogastropoda-Cocculiniformia sister grouping, albeit with low bootstrap support (not shown). Future studies should test the hypothesis of shared ancestry for the Patellogastropoda and Cocculiniformia.

Of the living families of patellogastropods, Patellidae and Acmaeidae have Triassic representatives, Lottiidae dates to the uppermost Lower Cretaceous, Lepetidae to the Middle Eocene, and Neolepetopsidae lacks a fossil record (Tracey et al. 1993). Although Nacellidae has

**Table 3** Numbers of base differences between Patellogastropoda and other groups of mollusks (based on 605 aligned positions, 25 bp of insertions present only in a single taxon excluded from analysis). Gaps and uncertain base calls removed from pairwise comparisons. Total number of nucleotide differences above the diagonal, transversions only below the diagonal (*PAT* Patellogastropoda; *GAS* Orthogastropoda-Cocculiniformia; *COC* Cocculiniformia; *CLA* non-gastropod mollusks (see Table 1 for included taxa))

|     | PAT   | GAS    | COC    | CLA    |
|-----|-------|--------|--------|--------|
| PAT | 0-78  | 85-109 | 97-127 | 88-122 |
| GAS | --    | 3-62   | 72-100 | 33-89  |
| COC |       | -      | 86     | 79-104 |
| CLA |       |        | -      | 74-78  |
| PAT | 0-42  | -      |        |        |
| GAS | 40-57 | 0-30   | -      |        |
| COC | 51-62 | 36-50  | 43     | -      |
| CLA | 45-72 | 14-53  | 38-56  | 36-40  |

been reported from the Lower Cretaceous of Australia (Powell 1973), these records are based on shell morphology, and are therefore tentative (Lindberg and Hickman 1986). The earliest record of Nacellidae confirmed by shell ultrastructure is from the Late Eocene (Tracey et al. 1993). Relatively few characters provide information on relationships of these higher taxa within Patellogastropoda. Phylogenetic relationships within Patellogastropoda based on partial 18S sequences are not, for the most part, robustly resolved, nor are they concordant with patterns previously hypothesized based on morphological characters (Fig. 1). The Patellidae and Acmaeidae, each with a fossil record extending to the Triassic, represent the oldest well-differentiated lineages within Patellogastropoda. The Patellidae are readily distinguished by numerous features, among them nine pairs of chromosomes, a pallial gill, a radula with a central tooth, and a shell that lacks a homogeneous calcitic layer. Acmaeidae have ten pairs of chromosomes, a ctenidium, a radula lacking a central tooth, and a shell with an outer homogeneous calcitic layer. The Lottiidae, which share chromosomal, anatomical, and radular features with Acmaeidae, are differentiated primarily on the absence of calcitic foliated structures in their shells (Lindberg 1998a). Lottiidae emerge as sister taxa of Acmaeidae in most classifications (Fig. 1B to D), dating this node to the Upper Cretaceous. Partial 18S sequences invariably, although weakly, join the two genera from the subfamily Lottiinae (*Tectura* + *Lottia*), and include a member of the subfamily Patelloidinae (*Patelloida*) in broader (Fig. 2) but not more taxonomically limited analyses (Fig. 3). However, these sequences are not capable of resolving relationships of Lottiidae to other acmaeoid limpets.

The monophyly of the family Neolepetopsidae (*Eulepetopsis* + *Paralepetopsis*) is strongly supported by partial 18S sequences, which consistently, although not robustly, place this family as sister taxon to Acmaeidae. These data contradict McLean's (1990) hypothesis of patellogastropod radular evolution, in which Neolepetopsidae, together with the extinct Paleozoic family

Lepetopsidae, comprise the sister group to the Patellina (Fig. 1A). Fretter (1990) regarded the anatomy and shell structure of Neolepetopsidae to indicate a closer affinity with Acmaeidea, which our data support. Morphological features (Fretter 1990; Lindberg 1998a) and molecular data (present study) support the inclusion of the family Neolepetopsidae within Acmaeidea. These results refute McLean's (1990) hypothesis of Paleozoic origins, antiquity, and refugial survival of the Neolepetopsidae at deep-sea, sulfide-rich hydrothermal vents and hydrocarbon seeps (reviewed by McArthur and Tunnicliffe 1998). While our sequence data group Neolepetopsidae with Acmaeidae, the presence of a radula with a pronounced central tooth in Neolepetopsidae (McLean 1990) suggests that it may be more remotely related to Acmaeidae than is Lottiidae, and thus possibly have a Mesozoic origin. As relationships between Neolepetopsidae and the Paleozoic family Lepetopsidae are conjectural (McLean 1990) and no longer supported, Lepetopsidae and therefore Lepetopsina are excluded from the clade containing Recent patellogastropods.

Perhaps because of their specialized deep-water habitat and small size, Lepetidae are characterized by features unique to the group, or by the reduction or loss of characters (e.g. ctenidium, osphradia, and associated ganglia) present in other limpets (Lindberg 1998a; Sasaki 1998). As a result, the relationships of this family are poorly understood and subject to widely varying interpretations. Based primarily on features of the radula, Dall (1876) and Sasaki (1998) consider Lepetidae to be the basal clade of Patellogastropods (Fig. 1C, D). Lindberg and Hedegaard (1996) and Angerer and Haszprunar (1996) concluded that Lepetidae are more closely related to Lottiidae than to Acmaeidae, while Lindberg (1998a) placed the Lepetidae as sister taxon to Acmaeidae + Lottiidae (Fig. 1B). Partial 18S sequences group Lepetidae with the remaining Acmaeidea (Fig. 2C), but are unable to resolve their relationship to Acmaeidae and Lottiidae (Figs. 2A to C, 3A).

The Nacellidae, with nine pairs of chromosomes, a pallial gill, a radula with a central tooth, and a shell with an outer homogeneous calcitic layer, have been grouped in a clade with Patellidae by earlier authors (e.g. Dall 1876; Powell 1973) (Fig. 1C), and, more recently, by Sasaki (1998) (Fig. 1D) on the basis of numerous shared characters, including pallial sensory organs, radular musculature, and odontophoral cartilages. Lindberg (1988, 1998a) (Fig. 1B) regarded the Nacellidae as the sister taxon of the Acmaeidea, arguing that similarities with Patellidae were shared primitive characters. A sister group relationship between Nacellidae and the Acmaeidea requires a Mesozoic origin for Nacellidae, while a Tertiary origin is consistent only with its derivation from Patellidae. In contrast to phylogenies based on morphological data, 18S sequence data invariably place the Nacellidae as a paraphyletic grade at the base of Patellogastropoda (Figs. 2, 3). Sequence differences among most patellogastropods are small (on the order of 0 to 2% sequence divergence). Neolepetopsidae differ from

other limpets at 1.5 to 4% of positions, while Nacellidae differ from other Patellogastropoda at about 5 to 15% of positions. The longest branch within Patellogastropoda is between *Cellana nigrolineata* and all other taxa in this clade. Given the very long branch between Patellogastropoda and the remaining gastropod taxa, the rooting of the Patellogastropoda along its longest internal branch may be artifactual. Patellogastropoda are separated from other Gastropoda by a very long branch in both 18S (present study) and 28S (McArthur and Koop 1999) ribosomal DNA sequences. An episodic change in rDNA co-evolution may have occurred during the early radiation of Patellogastropoda (see Friedrich and Tautz 1997), making both its phylogenetic placement and rooting difficult to resolve.

Partial sequences comprising a 450-bp portion near the 5' end of the 18S rDNA gene are adequate to confirm the monophyly of the Patellogastropoda, but insufficiently informative to provide robust support for the monophyly of Gastropoda, or to address the division of the Gastropoda into the subclasses Eogastropoda and Orthogastropoda (Ponder and Lindberg 1996). These sequence data invariably group Patellogastropoda in a weakly supported clade with cocculiniform limpets, despite greater sequence divergences between Patellogastropoda and "Cocculiniformia" than between the Patellogastropoda and Orthogastropoda. Partial 18S sequences support the inclusion of the family Neolepetopsidae within the superfamily Acmaeidea, and refute their previously hypothesized position as sister group to the remaining living Patellogastropoda. This region of the 18S rDNA gene diverges at widely differing rates, spanning an order of magnitude among different patellogastropod lineages, and therefore does not provide meaningful resolution of the relationships among patellogastropod taxa.

Koufopanou et al. (1999) recently published a study of the relationships and biogeography of the family Patellidae based on partial 12S and 16S rDNA sequences obtained from 34 of the 38 living species, and using nacellids and lottiids as outgroups. These partial gene sequences were unable to provide unequivocal support for the monophyly of the Patellidae, nor could they convincingly resolve the relationships among the patellogastropod subfamilies. Data from one or more genes that evolve more uniformly and more rapidly than the ribosomal genes studied thus far (possibly one or more of the mitochondrial protein-coding genes) seem more likely to be informative about relationships within Patellogastropoda.

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