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Title: The *Patelloida profunda* group: Phylogenetics, biogeography, morphology and molecular evolution among a widespread, closely-related group of limpets (Gastropoda: Lottidae).

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Dr. Gustav Paulay, Chairperson, Thesis Committee

Studies on the origin and diversification of Indo-West Pacific (IWP) biota are hampered by the frequently sympatric distribution of related species. Limpets of the *Patelloida profunda* group are exceptional in retaining largely allopatric ranges, which together with their predominant restriction to calcareous shores, make them a promising group to address questions of IWP diversification. In the Pacific, the group is basically confined to tectonically uplifted islands where emergent fossil reefs provide suitable substrata. Both tectonism and sea-level fluctuations alter the distribution and connectedness of these habitats, and have provided ongoing opportunities for speciation. Using 16S and COI mtDNA sequence data from most *P. profunda* group members, several other *Patelloida*, and taxa from another patellogastropod family, I test hypotheses about the origins and present distributions of these limpet species based on phylogenetic relationships. Results show a deep split in the Paleogene between Pacific and Indian ocean clades. Differentiation among some Pacific taxa is shallower and consistent with Plio-Pleistocene sea level fluctuations as a driving mechanism. Preliminary trends indicate deeper divergences among Indian Ocean taxa compared with Pacific taxa, which is consistent with the comparatively few karstic environments available in the former. Results raise questions about the phylogenetic boundaries of the *P. profunda* group, the genus *Patelloida*, and patellogastropod families in general.

The *Patelloida profunda* group: Phylogenetics, biogeography, morphology and molecular evolution among a widespread, closely-related group of limpets (Gastropoda: Lottidae)

by

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"knowledge and understanding reside on the shores of distant islands and not only on the shelves of libraries"

Stoddart 1992

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INTRODUCTION

Explaining the overwhelming organismal diversity on the planet has been a daunting problem that scientists have faced for centuries (Darwin 1859, Wallace 1859, Hutchinson 1959, Cox 1993, Miles and Dunham 1993, Barraclough et al. 1998). The mechanisms that generate biodiversity have been especially difficult to document in the ocean, due to its vast size and inaccessibility (Palumbi 1997, Paulay 1997). However, molecular phylogeography may provide a way to identify marine speciation events (Valentine and Jablonski 1983, Rosen 1988, Palumbi 1992, McMillan and Palumbi 1995, Paulay 1997, Bermingham and Moritz 1998). Although traditionally applied to intraspecific-level questions, molecular phylogeography seeks to determine how historical factors influence the geographic distribution of gene lineages (Avise et al. 1987, Avise 1998). Molecular tools allow direct access to heritable character information (genes), which can be used to construct phylogenies and estimate divergence times. These estimates can then be correlated with major historical vicariant events, inferred from the geological record, to identify potential speciation mechanisms (Avise 1994).

In phylogeographic studies of marine taxa, it has proven difficult to determine sister species' relationships, estimate divergence times and identify biogeographic events consistent in timing with speciation events. This is because 1) the relationships of closely related species can be difficult to determine, and often remain unresolved (e.g. Blum 1989, McMillan and Palumbi 1995), 2) the timing of speciation events has proven difficult to estimate (e.g. Starnes 1988, Pandolfi 1992, Springer and Williams 1990) and 3) the geography of speciation events is often difficult to detect because many are obscured by

secondary sympatry, often due to subsequent dispersal after speciation (e.g. Benzie 1993, Kabat 1996, Palumbi et al. 1997, Barraclough and Vogler 2000, Williams 2000). Few studies in the marine realm have been able to satisfy these three criteria to produce a cohesive statement regarding the phylogeography of a group to date (G. Paulay pers. comm.). Choosing a phylogeographically powerful group, which is one that will be useful in testing biogeographical hypotheses of speciation, is crucial when undertaking phylogeographic studies (C. Meyer unpubl. for the definition of phylogeographic power).

These three criteria are satisfied by the *Patelloida profunda* group, a closely related subset of species within the genus *Patelloida*. Relationships among *P. profunda* group members are expected to be resolvable because 1) *P. profunda* sister-species are discernible based on preliminary molecular analyses (L. Kirkendale, pers. obs.), 2) secondary range overlap is absent or likely uncommon, as the recognized taxa (after Lindberg and Vermeij 1985) are distributed allopatrically across all three major ocean basins (Figure 1 and Table 1) a pair of putative geminate species within the *P. profunda* group, which occur on the two sides of the Isthmus of Panama, may provide a framework for estimating divergence times within the group (Lessios 1979). Thus, the *P. profunda* group is expected to satisfy the three criteria for an ideal phylogeographic study.

Numerous morphological methods have been used to deduce phylogenetic relationships among closely related molluscan species. These include analyses of external and internal shell characters, including shell microstructure, and soft anatomical and radular features.

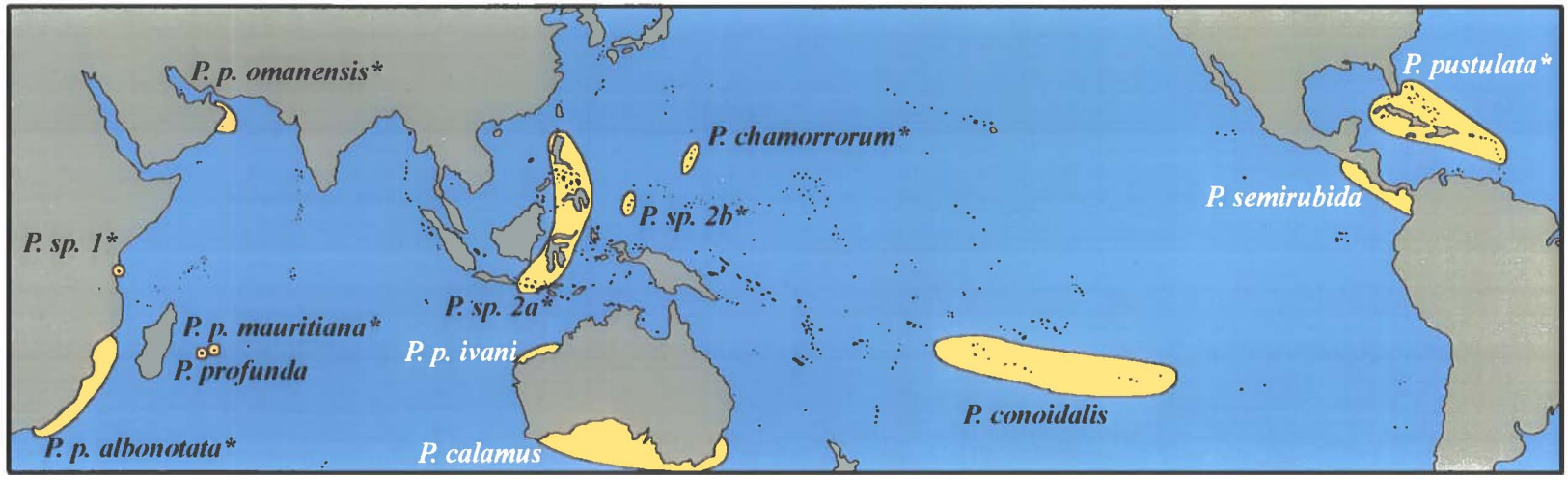


Figure 1. Known geographic distribution and sampling locales for the "*Patelloida profunda* group", after Lindberg and Vermeij (1985). Species' names in black indicate taxa that inhabit limestone substrates, and names in white refer to taxa whose habitat is unknown. Asterisks indicate taxa for which molecular data were obtained.

Table 1. Summary of sampling localities, molecular results and morphological analyses for patellogastropod taxa included in this study.

Molecular Status	Taxa	Locality	No. of individuals sequenced (COI/16S)	Radula type	Habitat specificity
Ingroup	*Patelloida profunda mauritiana*	Mauritius	2/2	1	karst
Ingroup	*Patelloida profunda albonotata*	South Africa	2/2	1	calcareous substrata
Ingroup	*Patellioda profunda omanensis*	Gulf of Oman	2/2	1	calcareous substrata
Ingroup	*Patelloida sp. 1*	Zanzibar	2/2	1	karst
Ingroup	*Patelloida chamorrorum*	Southern Marianas Islands	3/3	1	karst
Ingroup	*Patelloida sp. 2a*	Indonesia (Bali)	2/2	1	karst
Ingroup	*Patelloida sp. 2a*	Philippines	3/2	1	karst
Ingroup	*Patelloida sp. 2a*	Indonesia (Sulawesi)	2/2	1	karst
Ingroup	*Patelloida sp. 2b*	Palau	2/1	1	karst
Ingroup	*Patelloida pustulata*	Caribbean	1/1**	unknown	karst
Not tested	*Patelloida profunda*	Reunion	2/2	2	unknown
Not tested	*Patelloida profunda ivani*	Northwest Australia	0/0	1*	unknown
Not tested	*Patelloida calamus*	South Australia	0/0	1 or 3*	unknown
Not tested	*Patelloida conoidalis*	Southeast Polynesia	0/0	1	karst
Not tested	*Patelloida semirubida*	Panama	0/0	unknown	unknown
Outgroup	*Patelloida sp. 3*	Christmas Island	4/3	1	karst
Outgroup	*Patelloida sp. 4a*	Philippines and Palau	3/3	1	karst
Outgroup	*Patelloida sp. 4b*	Indonesia (Sulawesi)	2/2	1	karst
Outgroup	*?Notoacmaea sp. 1*	Tonga	0/1	2	karst
Outgroup	*?Asteracmaea sp. 1*	Reunion	2/2	2	unknown
Outgroup	*Yayoiacmaea oyamai*	Japan	0/0	2	CCA
Outgroup	*Patelloida saccharina*	Philippines and Japan	1/2	3	unknown
Outgroup	*Patelloida pygmaea*	Japan	0/1	3	unknown
Outgroup	*Patelloida striata*	Philippines	0/1	3	unknown
Outgroup	*Patelloida heteromorpha*	Australia	2/2**	3*	unknown
Outgroup	*Patelloida alticostata*	Australia	1/1**	3*	unknown
Outgroup	*Cellana tudor*	?Australia	1/1**	unknown	unknown
Outgroup	*Cellana sp. 2*	Australia	1/1**	unknown	unknown
Outgroup	*Cellana sp. 4*	Australia	1/1**	unknown	unknown
Outgroup	*Cellana rota*	Gulf of Oman	1/1	unknown	unknown

Radular type:1= 23032, lateral teeth are robust and almost equal in strength: 2= 03030, similar to 1 but without marginals: 3=23032, third lateral tooth is smaller than the other lateral teeth (Morphological results from Kase, unpublished)

* after Ponder and Creese 1980

** sequence data provided by Brian Simison, UC Berkeley

CCA refers to crustose coralline algae

Of these, radular characters are commonly utilized as useful morphological tool(s) to identify clades and estimate relationships among species (Lindberg 1988, 1998). Although radulae can be ecophenotypically variable in some groups (Lindberg 1988, Reid and Mak 1999), they have also proven to be taxonomically conserved and informative in others (Kool 1987, Bradner and Kay 1996).

The primitive radular character state for the subfamily Patelloidinae, to which *Patelloida* belongs, is possession of two pairs of marginal teeth (Lindberg 1998). Specialization is hypothesized to have resulted in a complete loss of marginal teeth within some members of both the Patelloidinae and the Lottinae, the only other subfamily of the Family Lottidae (Lindberg 1998). Although shell morphology has been referred to as virtually useless for distinguishing among patelloidinae species (Lindberg 1998), shell microstructure, developed by MacClintock (1967), is considered phylogenetically informative at certain levels (T. Kase, pers. comm.). The most significant trend in shell microstructure in the Lottidae is the loss of the calcitic foliated shell layer (Lindberg 1988, 1998). Because shells can persist as fossils, unlike soft tissues that usually degrade rapidly after death, they are especially useful in palaeontological studies. Paradoxically, patellogastropods have a generally poor fossil record (Lindberg 1988, 1998). This is thought to be a consequence of few preservational opportunities in high energy nearshore environments, which they usually inhabit (Koufopanou 1999).

Patelloida profunda was described from Reunion in the western Indian ocean by Deshayes in 1863 (Lindberg and Vermeij 1985). The *P. profunda* group was first recognized and delineated by Christaens (1975) and included eight species/subspecies: *P.*

profunda profunda, P. conoidalis, P. calamus, P. profunda albonotata, P. profunda mauritiana, P. profunda ceylanica, P. profunda omanensis, and P. profunda ivani, which exhibit similar shell and radular characters. Group members possess a shell that is grey-white, uniformly conical and solid, with brown radial rays and prominent, rounded radial ribs. The exterior brown radial rays are visible internally (at the shell margin), and the interior apex of the shell has an orange-brown callosity with a white muscle scar. *Patelloida conoidalis* is the notable exception to the previously outlined description, as it has a plain white shell with rounded radial ribs, but lacks the brown radial rays. Radular teeth, which are continuously replaced in conveyor-belt fashion, are mounted on a radular ribbon, and are used to scrape the substrate to gather food. In the *P. profunda* group, the radula are arranged in a 2-3-0-3-2 (two marginals, three laterals and no median tooth) formula or pattern (Christaens 1975). Because all taxa analyzed generally exhibited these features, the group was believed to have arisen from a common ancestor (Christaens 1975).

Lindberg and Vermeij (1985) described *P. chamorrorum*, from the southern Marianas Islands, which was allied to the *P. profunda* group along with four other newly assigned taxa, *P. pustulata, P. semirubida* and two undescribed *Patelloida* species (one from Java/New Guinea, and the other from Palau). *Patelloida chamorrorum* exhibited the radular morphology common to the group (2-3-0-3-2), but was distinct from other group members because it possessed lateral extensions on the third lateral tooth (Lindberg and Vermeij 1985). The authors further described the group as being primarily restricted to karstic shorelines and/or calcareous debris, and briefly proposed that each island, where a

member of the group was found, would yield a distinct species (Lindberg and Vermeij 1985). This prediction was likely made because of the expected poor dispersal capabilities due to a short-lived, non-feeding, planktonic larval stage, a condition plesiomorphic (shared, ancestral) for patellogastropod taxa (Lindberg 1988, 1998).

Morphological studies of closely related gastropod taxa are often constrained by the absence of synapomorphic (shared, derived) characters. Molecular tools, which utilize rapidly evolving gene regions, have allowed many evolutionary relationships among recently diverged taxa to be characterized because they add these types of crucial characters to analyses (Avise et al. 1987, Bermingham and Lessios 1993, Avise 1994, Baverstock and Moritz 1996). Several inter- and intraspecific phylogenetic questions about gastropods have been tackled successfully using 16S ribosomal RNA (rRNA) and cytochrome oxidase I (COI) genes in mitochondrial DNA (mtDNA) (Reeb 1995, Reid et al. 1996, Reid and Geller 1997, Koufopanou 1999). The 16S gene is the large (1274 base pair (bp)) subunit rRNA in mtDNA, which is thought to be relatively conserved (Palumbi 1996) and therefore may be useful in resolving deeper divergences among *P. profunda* group members. It is plausible that species of the *P. profunda* group may have diverged long ago, due to ancient speciation events, which would result in high levels of sequence divergence. Characterization of the relationships among group members, if divergences are relatively old, should be feasible by comparison of sequence data among taxa from this gene region.

The 16S gene region can be inappropriate when low levels of sequence divergence among closely-related are found. This region may not be changing fast enough, and thus

accrue enough mutations, to successfully resolve relationships among recently diverged taxa. In cowries, preliminary results indicate that mutation rates are twice as fast in the cytochrome oxidase I (COI) gene region, and thus it provides a better basis for differentiating closely related taxa, compared with the 16S gene region (C. Meyer, unpubl.). The COI gene (1573 bp long) is a subunit of the cytochrome oxidase complex of the electron transport chain (Alberts et al. 1996). Most importantly, the COI gene region may resolve relationships among closely related taxa that 16S cannot, as well, it will provide an additional estimate of the relationships among taxa.

Extant *Patelloida profunda* group members are predominantly associated with high intertidal, calcareous environments (Lindberg and Vermeij 1985). During high sea stands or transgressions, like today, populations would have become restricted to those islands (usually tectonically-uplifted) where exposed limestone substrata persisted, usually in the form of raised reefs. During periodic low sea stands or regressions, a multitude of suitable habitats would have been available as reefs became emergent around all islands, where they existed during high sea stands. This would have provided a series of stepping stones between groups of uplifted islands (where populations could persist at high sea stands) and facilitated dispersal and gene flow. Recurrent high sea stands would have resulted in the extinction of populations on tectonically stable or subsiding islands, while simultaneously isolating survivors on tectonically-uplifted islands, which retained karstic shores. The late Pliocene and Pleistocene was a time of frequent and large scale transgressions and regressions, which greatly impacted shallow-water habitats, communities and populations, as exemplified by the previously outlined sequence of events

(Myers 1989, Paulay 1991). This scenario would be supported if divergences among group members were within the past 2.6 My, when sea-level fluctuations were the most pronounced (Haq et al. 1987, Hallam 1994).

Ancient biogeographic events are more difficult to reconstruct than recent biogeographic events. This is because evidence necessary to understand these occurrences becomes increasingly obscured with time. Thus, less is conclusively known about tectonic processes, which are prevalent biogeographic mechanisms >10 Mya, compared with sea-level fluctuations, which were most pronounced in the Plio-Pleistocene, approximately 2.6 Mya. Needless to say, tectonic events may drive speciation and are a plausible cause of allopatric speciation events older than approximately 10 My (Rosen 1988, Hallam 1994, McMillan and Palumbi 1995). *Patelloida* is an ancient genus of patellogastropod limpets that first appeared in the Cretaceous of the Paris Basin (Figure 1) (Lindberg 1988). The fossil record of the *P. profunda* group indicates a "clear and definite Tethyan distribution in space and time"(Vermeij and Lindberg 1985). The ancient origin and persistence of the group raises the possibility that tectonic events have altered the distribution, and possibly contributed to speciation, among its' members.

Speciation driven by tectonic mechanisms would be supported if sister-species' pairs exhibit high levels of sequence divergence, which are concordant in timing with previously hypothesized vicariance events. If speciation within the group is a result of several biogeographic mechanisms, for example, ancient biogeographic events and relatively recent sea-level fluctuations, then sister-species' divergences should be highly

variable (Valentine and Jablonski 1983, Rosen 1988). These biogeographic mechanisms, operating over vastly different timescales led to the formulation of two hypotheses, which are:

H_1: Speciation among *Patelloida profunda* group members has occurred relatively recently and is due to sea-level fluctuations, which were most intense during the Plio-Pleistocene, from approximately 2.6 Mya.

H_2: Speciation among *Patelloida profunda* group members has occurred due to a number of biogeographic processes, and will be highly variable in timing.

To test these hypotheses, molecular data from both the 16S and COI gene regions were analyzed to produce a phylogenetic hypothesis of sister-species' relationships among the *Patelloida profunda* group members and outgroup taxa. Morphological studies of the radula and shell were also conducted and compared with molecular findings. Well-supported clades identified by the phylogenetic hypothesis were calibrated using an appropriate molecular clock to estimate the timing of observed divergences. Biogeographic events, concordant in timing with observed divergences among *Patelloida profunda* group members, are then discussed as possible mechanisms of speciation.

MATERIALS AND METHODS

Specimen Acquisition

Members of the *Patelloida profunda* group have a worldwide distribution, ranging from South Africa to the Caribbean (Figure 1). As many species as possible were collected by myself and with the help of numerous shell collectors and scientists (Table 1). Of the 12 group members outlined by Lindberg and Vermeij (1985), including two proposed new species, seven members were available for this study (Table 1). Specimens were received from Reunion, the type locality of *P. profunda*, which were initially identified as *P.profunda*, but after further scrutiny, were allied to a closely related, but relatively obscure and poorly known group within the Lottidae (*Asteracmaea*). Additional members were also included that morphogically resembled other *P. profunda* group members from the Philippines, Zanzibar, and Bali. Although numerous contacts were established, neither Australian taxon required, *P. profunda ivani* from Northwestern Australia, nor *P. calamus* from temperate Southern Australia, were received. Specimens of *P. pustulata* were not obtained, but molecular sequence data for both gene regions was provided by Brian Simison, UC Berkeley. Although both *P. semirubida* (Panama), and *P. conoidalis* (French Polynesia) were obtained, preservational problems precluded successful DNA extraction. Morphological studies were conducted for *P. conoidalis*, because many preserved specimens were available, but not *P. semirubida*, because only two specimens were obtained and these were kept for genetic analysis.

Total Genomic DNA Extraction

Total genomic DNA was obtained from live or preserved muscle tissue using modifications of standard protocols (Sambrook et al. 1989, Dessauer et al. 1996). Tissue was cut into small pieces, ground with a mortar and pestle, placed in an isolating medium (12.5 ul of a 20 nm solution of proteinase K and 1 ml CTAB detergent) and incubated overnight at 65°C. DNA was extracted using standard chloroform protocol and precipitated using ammonium acetate (half-volume of 7.5 M) and ethanol (3 volumes of ice-cold 95%) for at least 30 minutes. The pellet was washed with ethanol (500 ul of ice-cold 70%), dried overnight and suspended in 50 ul of distilled and deionized water (ddH_2O). DNAzol methodology was also used and involved a similar protocol as that for CTAB extractions, except that tissue was gently shook overnight (via an orbital shaker), was not incubated, and 20 ul of a 10 nm proteinase K solution was used. After extraction, the pellet was eluted in 200 uL of ddH_20 (for further details of DNAzol extraction procedure see Chomczynski et al. 1997).

Amplification of 16S and COI Gene Regions

Universal primers were used to amplify a region of the COI and 16S gene using the polymerase chain reaction (PCR) (Palumbi 1996). Primer sequences used were as follows:

LCO1490	5'-GGTCAACAAATCATAAAGATATTGG-3' (FOLMER)
HCO2198	5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (FOLMER)
16Sar	5'-CGCCTGTTTATCAAAAACAT-3' (PALUMBI)
16Sbr	5'-GCCGGTCTGAACTCAGATCACGT-3' (PALUMBI)

Reactants included 1 uL of genomic DNA template, which was diluted 1:150 with ddH_20 if the DNA was a CTAB extraction, or used undiluted if the DNA template was a DNAzol extract (genomic yields were generally higher with the CTAB extraction, compared to DNAzol). Other reactants included 31.8 uL ddH_20, 5 uL of PCR buffer (Taq or Tfl depending on the enzyme used)(Perkin Elmer), 5 uL of dNTPs (10 mM stock), 2 uL of each primer (10 uM stock), 3 uL of $MgCl_2$ solution (25 mM stock, Perkin Elmer), and 0.2 uL TAQ or TFl enzyme (Perkin Elmer). Reactants for both gene regions were run for 35-37 cycles with the following parameters: an initial denaturation for 60 seconds at 95°C, then further denaturation for 40 seconds at 94°C, annealing for 35-40 seconds at 38-45°C (COI) or for 35 seconds for 50-55°C (16S), followed by extension for 60 secs at 72°C. The PCR product was visualized by electrophoresis on a 1-2% TBE or TAE agarose gel, which was stained with ethidium bromide, and photodocumented. Multiple PCR products, indicated by double bands, were subjected to increased annealing temperatures during subsequent PCR rounds. Successful PCR products were cleaned for cycle sequencing using Wizard PCR Preps (Promega), following described protocol. Verification of cleaned PCR products occurred in the same manner as for initial PCR products.

Sequencing of 16S and COI Gene Regions

Sequences were generated using a LI-COR Automatic Sequencer following manufacturers' recommendations (LI-COR Inc. 1993). Thermal cycle sequencing, using the Sanger-dideoxy chain termination method, was conducted using an Epicentre standard kit and the PCR (Sambrook et al. 1989).

Alignment of 16S and COI Gene Regions

Forty-two operational taxonomic units (OTUs) were included in combined 16S and COI analyses; of these, 35 were common to both data sets (Table 1). *?Notoacmaea* sp. 1. (Tonga), *P. striata*, *P. pygmaea* and *P. saccharina* B were only sequenced successfully for 16S and only one individual each of *P.* sp. 3 (Christmas I.), *P.* sp. 2b (Palau) and *P.* sp. 2a (Philippines), were sequenced successfully for COI (Table 1). Sequences generated in both directions for at least one individual of each taxon, if available, were aligned using AlignIR with gap penalties set to ten and gap length penalty set to five (Licor 1993). Alignments among individuals were adjusted by eye. Cytochrome oxidase I sequence was translated to amino acids based on the invertebrate mitochondrial code to aid in alignment of conflicting regions (Maddison and Maddison 1997).

Two areas within the hypervariable loop region of 16S (126 bp and 58 bp, respectively) were difficult to align among distantly related outgroup taxa (Table 1). Many studies that utilize sequence data from 16S gene regions exclude hypervariable areas due to alignment ambiguity (Koufopanou 1999, Romano and Palumbi 1996, Smith and Paterson 1995). Topologies with and without the two areas specified above were compared. Although some fine-scale resolution was lost among closely related taxa after the removal of hypervariable regions, major relationships among groups were not altered. Thus, variable sites in 16S were excluded from subsequent analyses. This did not result in the exclusion of any data from among the ingroup, but did allow for less ambiguous alignments among outgroup taxa. Primer ends were also excluded from both 16S and

COI data sets. The final data set included 599 bp from the COI gene region and 369 bp from the 16S gene region (968 bp total).

Phylogenetic Analysis of Sequences

Outgroup members were used to root the phylogeny and included taxa from both inside and outside *Patelloida* (Table 1). In all analyses, gaps were treated as missing states and character states were treated as unordered. Transitions (ts) and transversions (tv), calculated using LogDet distance settings, were graphed to identify potential biases and visualize general trends, in both data sets (PAUP* Swofford 1998). Differential ts/tv ratios were tested using multiple weighting schemes to address these biases (user-specified 3:1, 5:1, 10:1) (PAUP * Swofford 1998). Clades of interest were not altered by differential weighting, thus sequence data were equally weighted in subsequent analyses. A partition-homogeneity test indicated that 16S and COI data sets were congruent and could be combined (Farris et al. 1995). Phylogenetic relationships among taxa were inferred from the aligned sequences using maximum parsimony methods (PAUP * Swofford 1998). Parsimony informative sites included 181 of 369 included characters for 16S, and 336 of 599 included characters for COI. Phylogenies were tested for robustness by bootstrapping (1000 replicates, fast stepwise addition, PAUP* Swofford 1998) and clade robustness was estimated using decay indices (TreeRot v.2 Sorenson 1999).

Estimation of a Molecular Clock

Recent literature has alluded to the considerable rate variation of molecular clocks (Harrison 1991, Avise 1994, Hillis et al. 1996, Reid et al. 1996). Ideally, appearances in the fossil record should be used, as independent molecular clock calibrations, to estimate the timing of speciation events. However, because of the poor fossil record for patellogastropods, this option was not available. When attempting to test various speciation hypotheses with widely divergent timeframes (e.g., distinguishing Eocene/Miocene versus Plio-Pleistocene divergences), which this study aims to accomplish, a moderate degree of precision is sufficient (Palumbi 1997).

A maximum-likelihood analysis was run to test for clock-like substitution among OTUs of the Pacific and Indian Ocean subclades (D and E), using *P. pustulata* (Caribbean) as an outgroup for rooting purposes (Huelsenbeck and Rannala 1999, PAUP* Swofford 1998). Clock-like behaviour among ingroup clades from the Indian and Pacific basins could not be rejected for 16S or COI gene regions ($p>0.05$ for 16S and $p>0.1$ for COI)(PAUP* Swofford 1998). Transitions and transversions in the 16S gene region and the COI gene region (both third position and first and second positions combined) were used to estimate sequence divergence 1) between taxa from the Indian and Pacific subclades, and 2) among taxa from the Pacific Ocean (absolute number of changes)(PAUP* Swofford 1998).

This preliminary result justified the application of an "appropriate" molecular clock, from as closely related taxa as possible, to minimize the problems of rate heterogeneity. Rate heterogeneity is often most pronounced when clocks are generated

from, and then applied to, very different taxa (such as a mouse and an elephant). One reason for this is due to very divergent metabolic rates among relatively unrelated taxa (Hillis et al. 1996).

Patelloida semirubida (Panama), the proposed sister-species to *P. pustulata* (Caribbean), was not obtained during the course of this study. Therefore, a molecular clock could not be generated from within the group, as was intended. Molecular clock calibrations generated from two gastropods, *Littorina* and *Cypraea* were thus used (Reid et al. 1996, C. Meyer, unpubl.). Average divergence estimates, for the substitutions and groups outlined previously, were calibrated using; 1) a 16S molecular clock for transitions and transversions in littorines (0.2043%/My and 0.084%/My, respectively) (Reid et al. 1996), and cowries (0.250%/My and 0.058%/My, respectively) (C. Meyer, unpubl.), and 2) COI third position transitions and transversions in cowries (1.3%/My and 0.32%/My, respectively) (C. Meyer, unpubl.) to estimate the timing of these divergences. Molecular clock estimates are very similar between littorines and cowries for both transitions and transversions in the 16S gene region, which indicates minimal rate heterogeneity between these two taxa.

Morphology

Comparisons of radular tooth morphology and shell microstructure using scanning electron microscopy (SEM) were conducted for as many sequenced taxa as possible by Dr. Tomoki Kase and his student Mr. Kano Yasunori at the National Science Museum in Tokyo, Japan. Shell microstructure was uninformative for ingroup differentiation, because

it did not vary among group members, which were distributed across multiple clades. However, radular findings are reported, because they exhibited phylogenetically informative trends (see Table 1).

RESULTS

Molecular Evolution

Several criteria concerning the molecular evolution of the two gene regions analyzed in this study, were addressed before sequence data were used to estimate phylogenetic relationships and divergence times, among taxa. All but one have already been discussed in the materials and methods section, however, they are briefly summarized together here. These criteria, which were satisfied during the course of phylogenetic analysis, include, but are not limited to: 1) an accurate alignment among gene regions, 2) estimation of congruency between data sets/gene regions, 3) inclusion or exclusion of hypervariable loop regions, 4) appropriate weighting of substitutions (ts/tv ratio), 5) inclusion of appropriate outgroup taxa for rooting purposes, and 6) tests of clock-like behaviour among each data set. The outgroup criterion (5), which was briefly discussed in the materials and methods, is more thoroughly reviewed in the discussion.

Nomenclatural Issues

Several taxa that were unidentifiable and may represent new species were included in this study. These include ?*Asteracmaea* sp. 1, ?*Notoacmaea* sp. 1, and *Patelloida* spp. 1-4 (Table 1). These taxa represent limpets from both the ingroup and outgroup from throughout the Pacific and Indian oceans. The designation of new species or subspecies, if unidentified taxa are confirmed as such, will be conducted by Dr. Kase and me, after examination of type specimens and further morphological and molecular verification.

Molecular Phylogenetics

In the study of phylogenetics, trees are used to depict the hypothesized relationships among taxa, and can be generated from molecular (such as nucleotide sequence data, as in this study) or morphological, or a combination of both, data sets. Many methods of tree reconstruction are available, including both distance (i.e. neighbour-joining) and character-based (i.e. maximum parsimony), approaches (Swofford et al. 1996). Distance methods measure the differences among molecules as a single variable, whereas character data measures differences as a series of discrete variables (the characters, in this case are the changes or substitutions in 16S and COI sequence data), each with many states (Swofford et al. 1996). The trees produced in this study were estimated using maximum parsimony, a well-established, character-based approach, which operates under the principle of parsimony (that is, the simplest reconstruction is also the most likely) (Swofford et al. 1996)(PAUP* Swofford 1998).

Under maximum parsimony, two trees were estimated (or recovered) for COI data, nine for 16S data and 12 for the combined (16S/COI) data. The branches or relationships, common to all such maximally parsimonious trees for each gene region (16S, COI and combined), were used to produce one tree, also for each gene region, called a strict consensus tree. For each branch of the strict consensus tree, bootstrap and decay values were calculated to estimate how well the data (numbers of changes or substitutions among taxa) supported the individual branches generated under maximum parsimony. The branches of the strict consensus tree do not indicate the amount of change that has occurred over time among taxa, however, in phylograms, which are another type

of tree, the number of changes (or substitutions) among taxa are conveyed by the branch lengths. One of the trees generated using maximum parsimony methods was chosen as representative of the hypothesized relationships among taxa, and reported as a phylogram. Both of these two types of trees are reported in this study, however the strict consensus trees, including the bootstrap and decay indices, form the basis for phylogenetic discussions among members of the *Patelloida profunda* group. In contrast, phylograms (generated for each gene region) form the basis of molecular clock estimates.

Four of five clades/subclades (A, B, D and E, Figures 2-4) were consistently highly supported by sequences from 16S, COI and combined gene regions in maximum parsimony analyses (>90%/≥5, bootstrap and decay, respectively). These are 1) an outgroup clade (A) and subclade (B), and 2) two ingroup subclades, one from the Indian Ocean (D) and the other from the Pacific Ocean (E). The outgroup clade (A) includes more distantly related members of the genus, including *Patelloida heteromorpha*, *P. saccharina* and *P. alticostata* (as well as *P. striata* and *P. pygmaea* for 16S sequence data only), and subclade B, which includes individuals of *P.* sp. 3 (Christmas I.), *P.* sp. 4a (Palau and Philippines) and *P.* sp. 4b (Sulawesi) (Table 1 and Figures 2-4). Subclade D includes the Indian Ocean *P. profunda* group members: *P. p. omanensis* (Gulf of Oman), *P. p. albonotata* (South Africa), *P. p. mauritiana* (Mauritius) and *P.* sp. 1 (Zanzibar) and subclade E includes the Pacific Ocean *P. profunda* group members: *P. chamorrorum*, *P.* sp. 2b (Palau) and *P.* sp. 2a (Philippines, Bali and Sulawesi) (Table 1 and Figures 2-5).

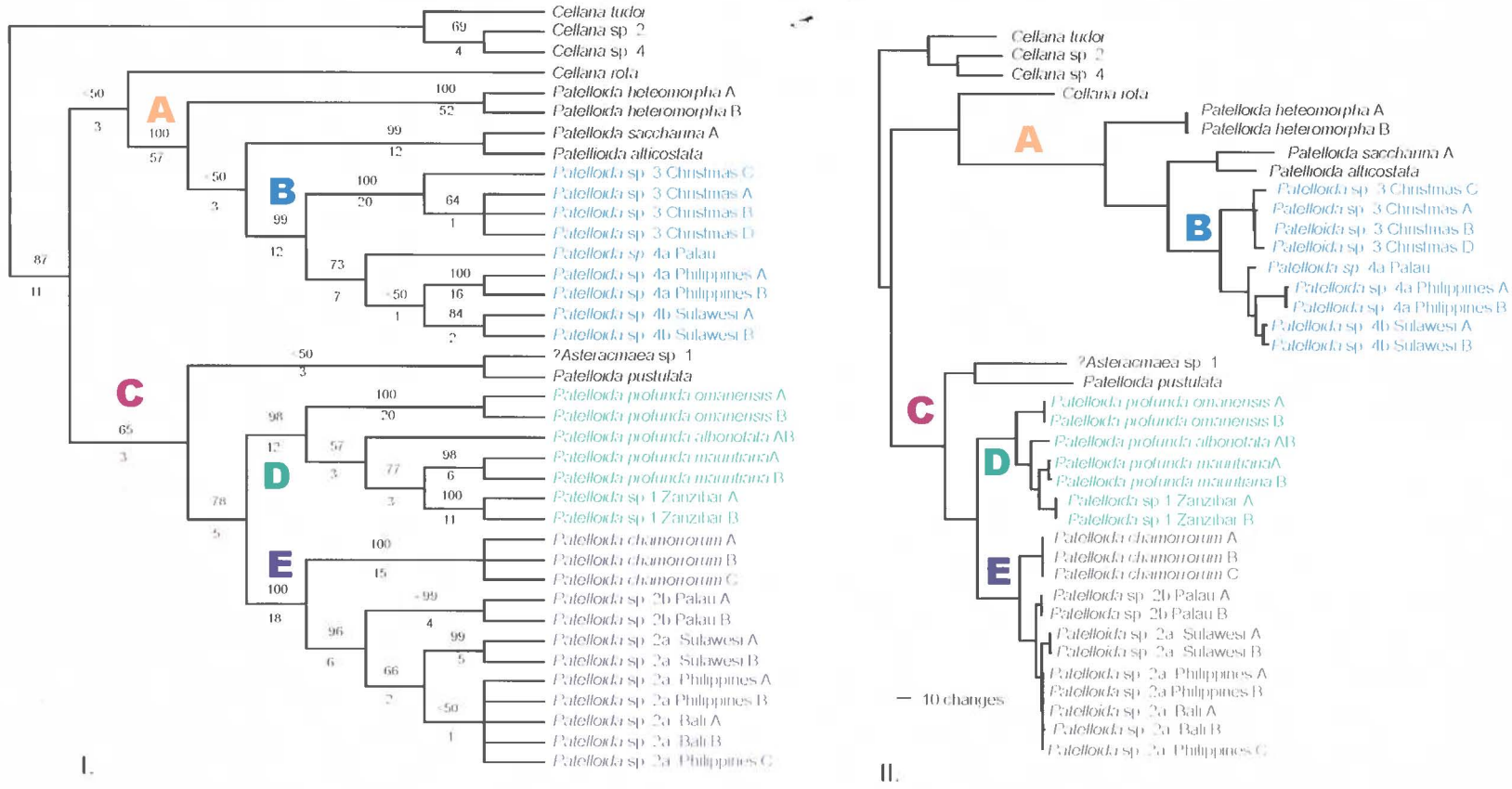


Figure 2. I. Strict consensus of two most parsimonious trees for COI. *Cellana* spp. are used as the outgroup to root the tree. Bootstrap values for 1000 replications are reported above branches and decay results below. A. refers to the outgroup clade, B. to the outgroup subclade, C. to the ingroup clade, and D. and E. to Indian and Pacific ingroup subclades, respectively. II. Phylogram of one of the two most parsimonious trees for COI. Letters A-E refer to clades outlined in 2.I., and changes refer to the absolute number of changes.

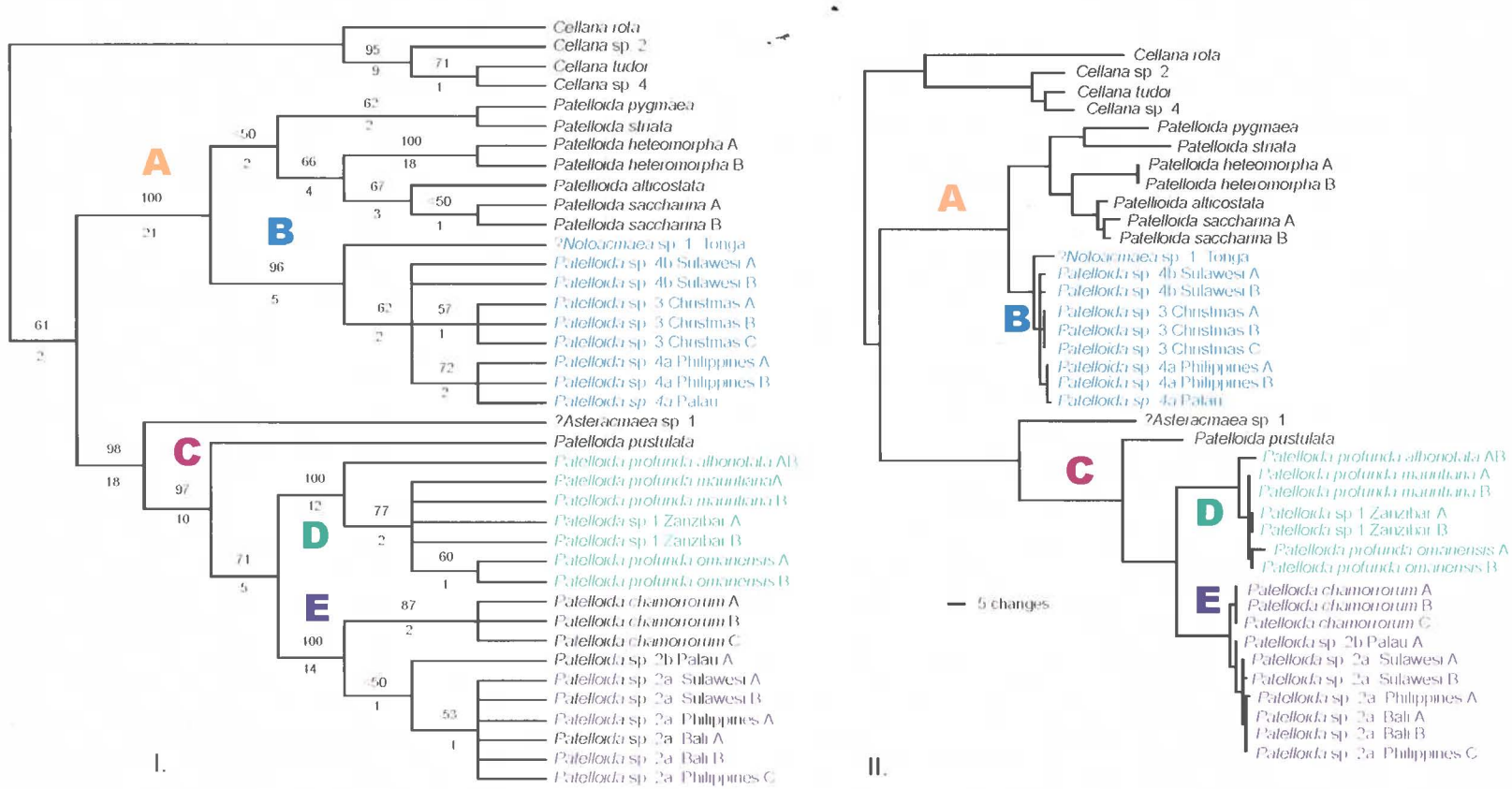


Figure 3. I. Strict consensus of nine most parsimonious trees for 16S, excluding variable sites. *Cellana* spp. are used as the outgroup to root the tree. Bootstrap values for 1000 replications are reported above branches and decay results below. A. refers to the outgroup clade, B. to the outgroup subclade, C. to the ingroup clade, and D. and E. to Indian and Pacific ingroup subclades, respectively. II. Phylogram of one of nine most parsimonious trees for 16S. Letters A-E refer to clades outlined in 3.I., changes refer to absolute number of changes.

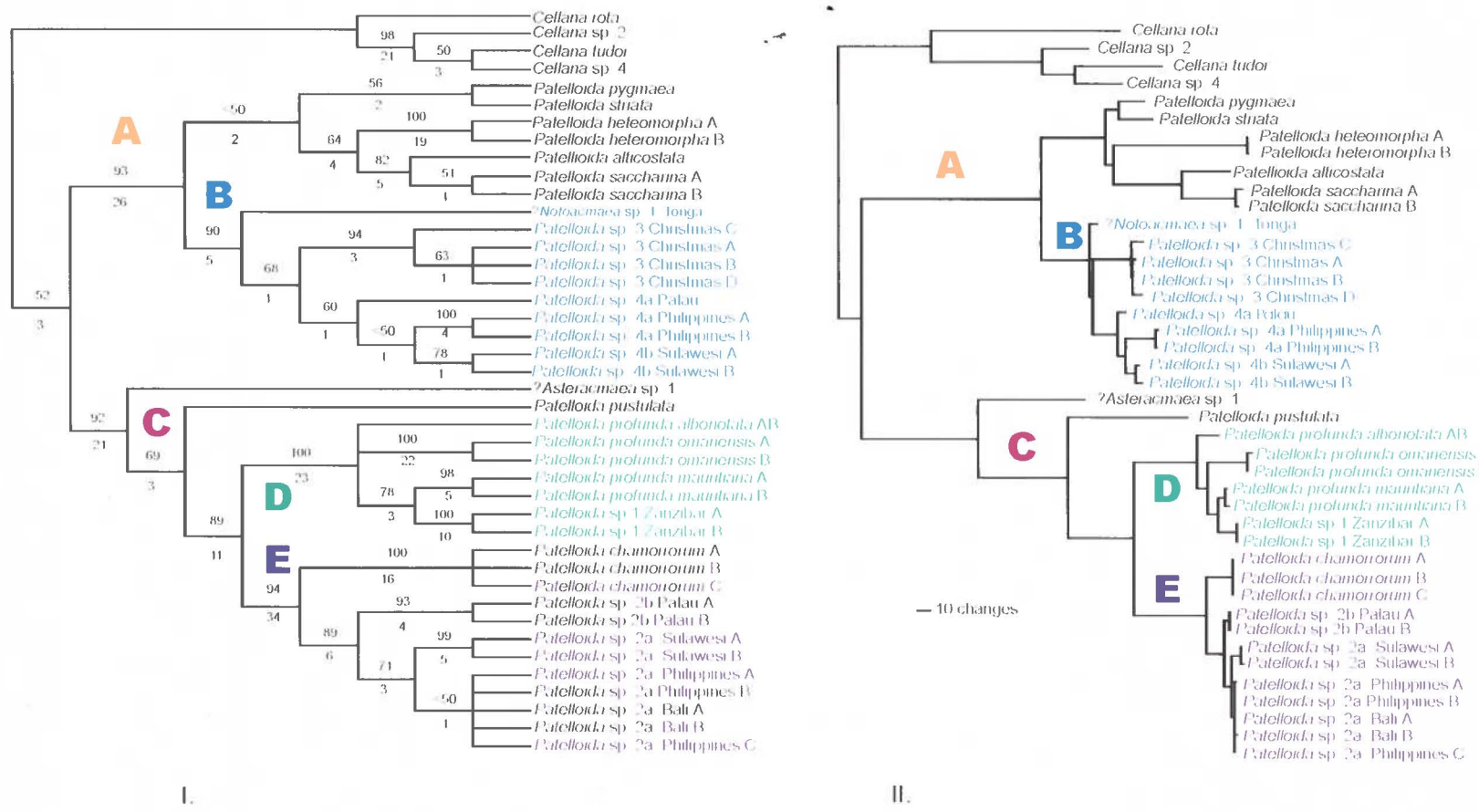


Figure 4. I. Strict consensus of 12 most parsimonious trees for 16S and COI gene regions. *Cellana* spp. are used as the outgroup to root the tree. Bootstrap values for 1000 replications are reported above branches and decay results below. A. refers to the outgroup clade, B. to the outgroup subclade, C to the ingroup clade, and D. and E. to Indian and Pacific ingroup subclades, respectively. II. Phylogram of one of 12 most parsimonious trees for 16S and COI. Letters A-E refer to clades outlined in 4.I., and changes refer to absolute number of changes.

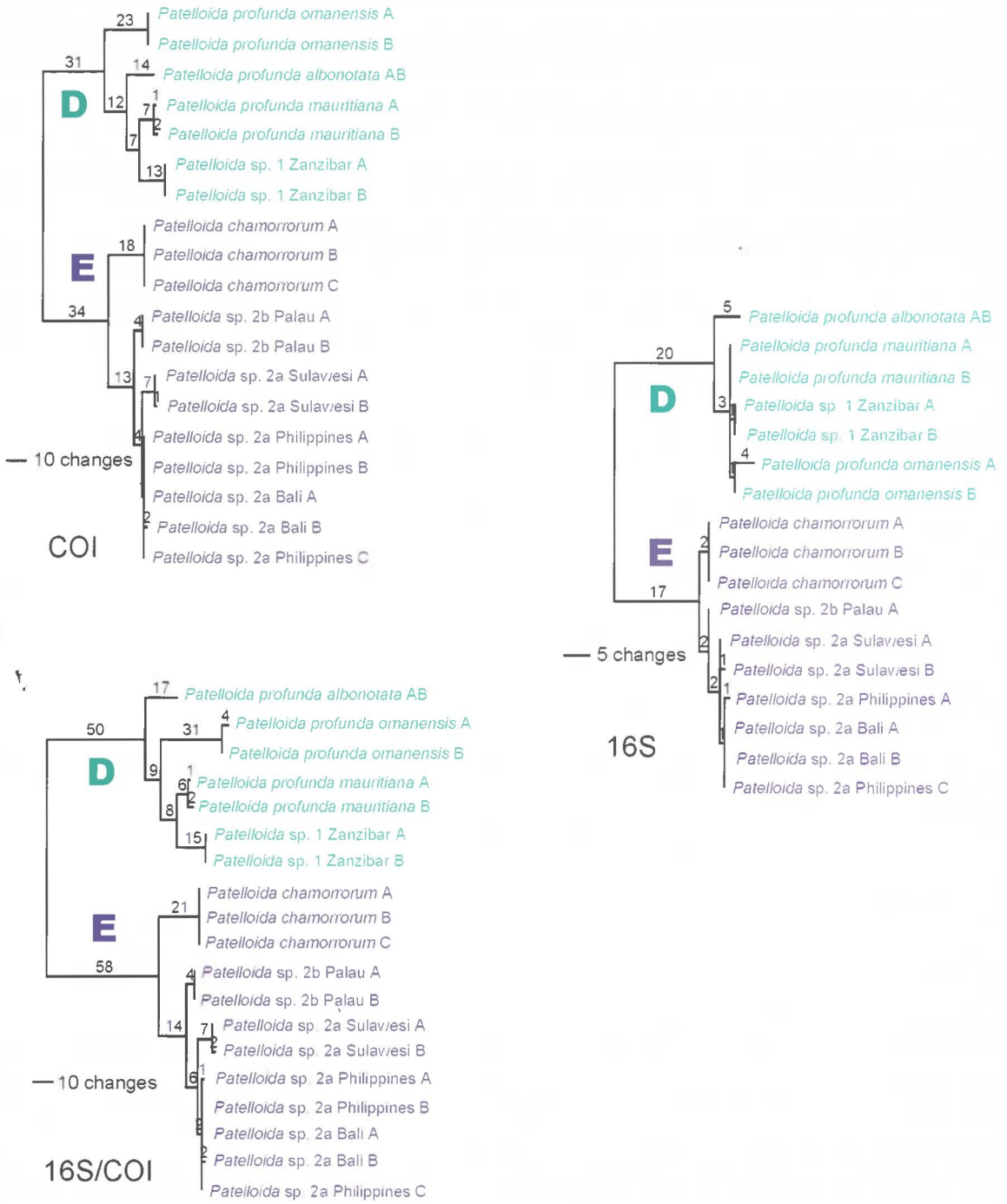


Figure 5. Phylogram of absolute number of changes among ingroup members from the Indian and Pacific subclades for COI (1/2), 16S (1/9) and combined (16S and COI) gene regions. The values in parentheses refer to the number of trees presented, compared to the total number of most parsimonious trees generated.

A fifth clade (C) corresponds to the *Patelloida profunda* group ("ingroup") as outlined by Christaens (1975), Lindberg and Vermeij (1985), and briefly discussed by Ponder and Creese (1980) (Table 1 and Figures 2-4). It includes subclades D and E, as well as the most distantly related member of the *P. profunda* group, *P. pustulata* (Caribbean). The monophyly of the ingroup is highly supported by analyses of the 16S gene region (97%/10 bootstrap and decay, respectively)(Figure 3). However, Clade C is not well-supported by the combined data set (69%/3, bootstrap and decay, respectively) (Figure 4) and based on COI data, ?*Asteracmaea* sp. 1, which is usually sister to the ingroup (in 16S and combined analyses with 98%/18 and 92%/21, bootstrap and decay, respectively) is included in Clade C (65%/3, bootstrap and decay, respectively)(Figure 2). However, the close (sister-taxon) relationship between *P. pustulata* and ?*Asteracmaea* sp. 1, which led to the inclusion of ?*Asteracmaea* sp. 1 in Clade C based on COI, is poorly supported (<50%/3 bootstrap and decay, respectively) (Figure 2).

As mentioned previously, within the ingroup subclade (C), two highly-supported subclades (D and E), which correspond to Indian and Pacific *Patelloida profunda* ingroup subclades, respectively, were resolved (Figures 2-5). Comparisons between these two ingroup subclades generally revealed higher average sequence divergence estimates (branch lengths) among Indian Ocean taxa than among Pacific Ocean taxa (based on absolute number of changes) (Table 2 and Figure 5). Although divergence estimates (or branch lengths) between the two subclades were relatively consistent for each gene region, a large range of individual divergence times (or branch lengths) for taxa within both

Table 2. Sequence divergence estimates (percent divergence for absolute number of changes) (above), and timing of divergences, (Mya) (below) among and between Indian and Pacific ingroup taxa

	Pacific taxa				Indian taxa		
	COI ti	COI tv	16S ti	16S tv	COI ti	COI tv	16S ti
Indian taxa	21.64-22.28%*	17.78-18.14%	7.84-7.99%	3.36-3.44%	11.12-15.20%	2.77-4.34%	0.59-1.52%
Pacific taxa	8.37-11.97%	0.70-0.93%	0.74-1.02%	n/a			
	COI ti Cowrie**	COI tv Cowrie**	16S ti Littorine/Cowrie**	16S tv Littorine/Cowrie**	COI ti Cowrie**	COI tv Cowrie**	16S ti Littorine/Cowrie**
Indian taxa	16.9*	56.1	38.7 / 31.6	40.5 / 58.6	10.2	11.4	5.4 / 4.4
Pacific taxa	7.8	2.6	4.3 / 3.5	n/a			

N. B. COI transitions and transversions are for the third position only and all divergence estimates include 95% confidence intervals

*this relatively low number is likely due to saturation of transitional changes in this quickly evolving gene region

**cowrie or littorine refers to the clock used to estimate the timing of divergences

subclades was observed (Figure 5). For example, within the Pacific ingroup, *Patelloida chamorrorum* is very divergent (16.78-17.33% and 1.15-1.33% sequence divergence, based on COI third position, and 16S, transitions, respectively) from all other Pacific taxa sampled (Table 3 and Figure 5). Variation among the remaining Pacific taxa, which include individuals of *P.* sp. 2a from Bali, Sulawesi, and the Philippines and *P.* sp. 2b from Palau, was much less (3.17-4.35% to 0.37-0.54% sequence divergence based on COI third-position and 16S transitions, respectively) (Table 3 and Figure 5).

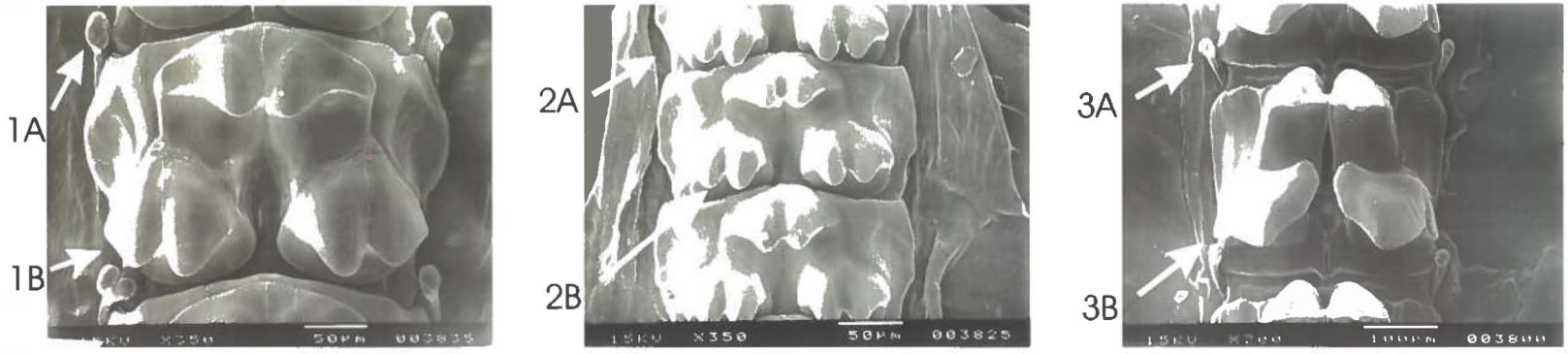
Analysis of Radular Characters

Radular character-state transitions, based on SEM analyses of radulae, were mapped onto a simplified consensus tree (which was common to all gene regions) to determine where they occurred and to see if they supported the tree. Three groups, which are referred to as Forms 1, 2 and 3, were identified on the basis of radular dentition (Figure 6). Form 1 is a 2-3-0-3-2 radula (2 marginals, 3 laterals and no median tooth), the ancestral condition in the subfamily Patelloidinae, with equal-sized laterals (Figure 6). Form 2 is a 0-3-0-3-0 radula (no marginals, 3 laterals and no median tooth), again, with equal-sized laterals (Figure 6). This is a derived condition exhibited by taxa from both the Lottinae and Patelloidinae, within the family Lottidae (Lindberg 1998). Form 3 is also considered derived, like Form 2, but exhibits only a slight modification of the primitive condition (2-3-0-3-2 radular dentition or Form 1). The third lateral tooth is smaller, rather than equal in size, to other laterals (T. Kase, unpubl.) (Figure 6).

Table 3. Sequence divergence estimates (percent divergence for absolute number of changes) (above), and timing of divergences, (Mya) (below) among Pacific ingroup taxa.

	Pacific taxa*	
	COI ti	**16S ti**
Patelloida chamorrorum	16.78-17.33%	1.15-1.33%
Pacific taxa*	3.17-4.35%	0.37-0.54%
	COI ti	**16S ti**
	Cowrie	**Littorine/Cowrie**
Patelloida chamorrorum	13	6.1 / 5.0
Pacific taxa*	2.9	1.9 / 1.6

N. B. COI transitions are for the third position only and all divergence estimates include 95% confidence intervals
* excluding *P. chamorrorum*
**cowrie or littorine refers to the clock used to estimate the timing of divergences



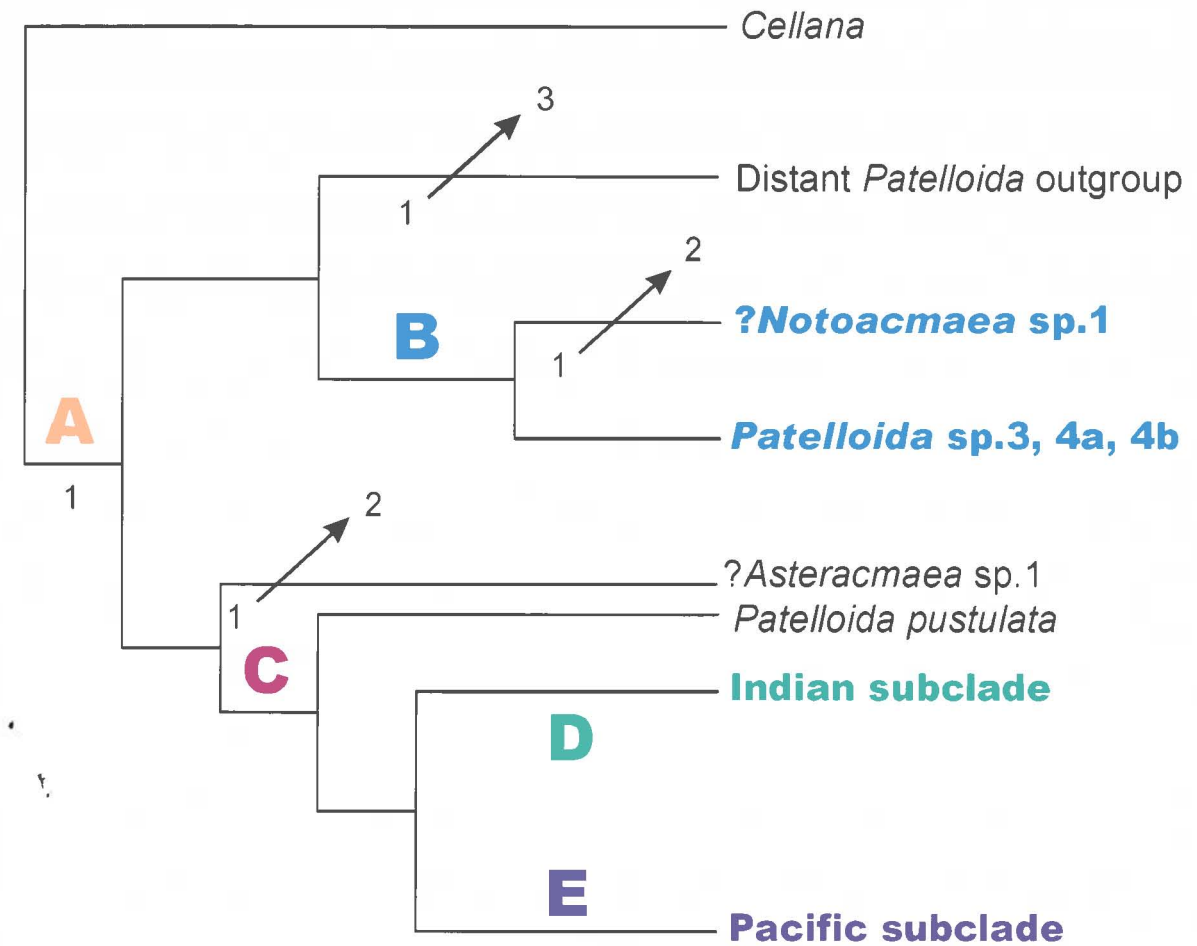
Patelloida chamorrorum ?*Asteracmaea* sp. 1 *Patelloida striata*

Figure 6. The three radular forms exhibited by patellogastropod taxa included in this study. Form 1 is typified by equal laterals and two pairs of marginals, form 2 has equal laterals but no marginals, and form 3 exhibits unequal laterals and two pairs of marginals. All photos were taken by Mr. Kano using SEM at the National Museum, Tokyo, Japan. Numbers refer to forms (1-3) and letters to marginals and laterals (A and B, respectively).

Radular characters are only phylogenetically-informative for describing relationships among outgroup taxa included in this study. Radular form 1 is exhibited by all *Patelloida profunda* group members (Clade C), as identified by molecular markers, as well as three additional taxa: *P.* sp. 3 (Christmas), *P.* sp. 4a (Palau and Philippines) and *P.* sp. 4b (Sulawesi)(Table 1 and Figure 6). In all molecular analyses these three taxa fall within Subclade C of the outgroup (Figure 7). Radular form 2 is exhibited by three species; *Yayoiacmaea oyamai* (not sequenced), ?*Notoacmaea* sp. 1(Tonga), and ?*Asteracmaea* sp.1 (Table 1 and Figure 6). ?*Notoacmaea* sp. 1 belongs to the outgroup subclade (B), and ?*Asteracmaea* sp. 1 is sister to the ingroup clade (C)(Figure 7). Radular form 3 is exhibited by *P. striata*, *P. pygmaea* and two individuals of *P. saccharina* (Figure 6). These three taxa comprise a monophyletic portion of the distantly related outgroup clade (A)(Table 1 and Figure 7).

Biogeography

The wide range of sequence divergence estimates, after calibration with both cowrie and littorine molecular clocks for transitions and transversions in COI and 16S gene regions, indicate that divergence times among taxa within the ingroup clade (C) are highly heterogeneous (Tables 2 and 3). Divergence times between ingroup subclades (D and E), occurred roughly 31-59 Mya, indicating an ancient split between taxa distributed within the Pacific and Indian Ocean basins (excluding divergence and clock estimates for COI transitions, which are likely saturated at this relatively deep divergence) (Table 2). This places the timing of this divergence firmly in the Palaeogene.



Legend

Form 1: 2 pairs marginal teeth, equal sized laterals
Form 2: no marginal teeth, equal sized laterals
Form 3: 2 pairs marginal teeth, unequal sized laterals

Figure 7. The Radular Story: Morphological results mapped onto major clades present in all molecular trees. The character change from one to three is informative, because it is synapomorphic (shared, derived) and provides support for the monophyletic status of the distant *Patelloida* outgroup. The character change from one to two is uninformative because it is homoplastic, and occurs in two different branches of the phylogeny (convergence). The ancestral state one is uninformative because it is plesiomorphic (shared, ancestral), many taxa in multiple clades possess this radular form. Bolded letters refer to clades (as in Figures 2-5).

In comparison, divergence estimates indicate relatively recent splits among Indian and Pacific Ocean taxa (subclades D and E), from 4.4-11.4 Mya (among Indian) and 2.6-7.8 Mya (among Pacific)(Table 2). Further characterization of divergence times among Pacific taxa, yield estimates of an older split for *P. chamorrorum* from *P.* sp. 2a (Sulawesi, Philippines, Bali) and *P.* sp. 2b (Palau) (5.0-13 Mya) compared with much more recent divergences among these latter Pacific taxa (1.6-2.9 Mya) (Table 3). These latter divergences likely occurred during the Plio-Pleistocene.

These results indicate a wide range of divergence time estimates and driving forces of speciation among *Patelloida profunda* group members. They lend support to hypothesis two, which predicted that speciation among group members would be variable in timing and therefore a result of more than one biogeographic process. Hypothesis one is rejected, because divergence among group members is not solely a result of Plio-Pleistocene events from ~2.6 Mya.

DISCUSSION

Molecular Phylogenetics

The present study of the *Patelloida profunda* group (ingroup or Clade C) includes the most geographically widely separated members, as well as representatives from all three major ocean basins (Table 1 and Figure 1). The monophyly of the ingroup is highly supported based on 16S data, but less well-supported by combined and COI data sets (Figures 2-5). Based on data from the COI gene region, *P. pustulata* was allied with ?*Asteracmaea* sp. 1 (Figure 2). Although this finding is weakly supported (<50%/3, bootstrap and decay, respectively), it is a trend recovered by phylogenetic analysis of the given data set. This association may be a result of substitution saturation in the COI gene region at this relatively deep node in the phylogeny. Because COI changes relatively quickly, for example, in relation to 16S, it becomes overloaded with change and either uninformative or misinformative at recovering the correct relationship among very divergent taxa. Misinformative saturation can result in long-branch attraction, which occurs when high amounts of change make it difficult to distinguish between homologous (phylogenetically-informative similarities due to descent from a common ancestor) and homoplastic (phylogenetically-uninformative similarities due to convergence, parallelisms, or reversals, instead of common ancestry) changes. This can lead to unrelated taxa, which exhibit high amounts of change relative to other taxa included in the phylogeny, erroneously paired together (Swofford et al. 1996). The likelihood of long-branch attraction between these two taxa is supported by their extremely divergent raw sequence

data, which necessitated placing large inserts (hyphens) into sequence data from all other *P. profunda* group members, to make alignment possible. Long-branch attraction between these taxa will be tested in the future, as taxa closely-related to *P. pustulata* and *?Asteracmaea* sp. 1, become available.

A related concern involves resolving the identity of *?Asteracmaea* sp. 1, because of its close relationship to the *Patelloida profunda* group. The radular findings suggest that this taxon from Reunion, initially identified as *P. profunda*, is most similar to an obscure group of lottids in the genus *Asteracmaea*, a potentially close relative of *Patelloida*. Species from this genus lack marginal teeth, are generally very small, and consequently have been little studied (Lindberg 1998).

Sequence data from additional, key taxa would help to better resolve relationships at relatively deep and critical points in this preliminary phylogeny. Sequence data for both gene regions from *Patelloida semirubida* (Panama), the hypothesized sister-species of *P. pustulata*, will aid in further characterization of the position of *P. pustulata*. Inclusion of the other missing *P. profunda* group members; *P. profunda*, *P.conoidalis* and the two Australian group members, *P. profunda ivani* and *P. calamus*, which may have diverged relatively early from other group members, is crucial. Additional outgroup representatives in other closely related patellogastropod families, such as *Yayoiacmaea oyamai* (in the genus *Asteracmaea*), and e.g. *Lottia luchuana,* from the subfamily Lottinae, will further resolve the relationship of *P. profunda* group members to closely related outgroup taxa. Sequence data from a more conserved area, for example a nuclear gene region, may also

help to better delineate these relatively deep divergences, and most importantly, break up long-branches that may spuriously unite unrelated taxa.

Two monophyletic ingroup subclades, which correspond to *Patelloida profunda* Indian and Pacific Ocean ingroup members (Christaens 1975, Lindberg and Vermeij 1985), were highly supported by all three data sets (Figures 2-5). Sequence divergence estimates (branch lengths) between these two clades are very similar, and indicate a single speciation event that resulted in the split between taxa distributed in the two ocean basins (Figures 2-5). Comparisons within each subclade indicate higher levels of average sequence divergence among taxa from the Indian, compared to the Pacific ingroup subclade, which may indicate longer divergence times among the former (Table 2 and Figures 2-5).

Divergence estimates were further characterized among taxa within the Pacific ingroup subclade. Among Pacific taxa, *Patelloida chamorrorum* was much more divergent, and likely diverged earlier from other Pacific taxa (*P.* sp. 2a from Bali, Sulawesi, and the Philippines and *P.* sp. 2b Palau), than the latter diverged from each other (Table 2). Sequences from additional Indo-Malay taxa are necessary to better characterize preliminary intra- and inter-specific divergences revealed in this study. For example, because of small sample sizes, it is unclear whether Pacific taxa (excluding *P. chamorrorum*) are genetically isolated, or whether gene flow is occuring. Sequences from the cytochrome-B gene region, which may mutate faster than COI based on comparisons of divergence rates in other gastropod groups, may be useful at delineating relationships among these taxa (Collins et al. 1996).

The choice of outgroups to root a phylogeny is one of the most important and sensitive steps in phylogenetic analyses (Swofford et al. 1996). In this study, four species of *Cellana* (Superfamily Nacelloidea, Family Nacellidae) were used to root the phylogeny. Sequence from the COI gene region indicates that *Cellana* may not be monophyletic (Figure 2). Although it was a poorly supported relationship (<50/3 bootstrap support and decay, respectively), and may be due to long-branch attraction as suspected for *P. pustulata* and ?*Asteracmaea* sp. 1, *C. rota* grouped with a clade of *Patelloida*. Visual inspection of raw sequence data indicates that the *P. profunda* ingroup exhibited greater sequence similarity with *Cellana* species, than with other representatives from within the genus *Patelloida*. A similar finding has also been documented between other *Cellana* and *Patelloida* taxa (B. Simison, pers. comm.).

Previous studies, based on morphological analyses, have suggested a close relationship between *Cellana* and *Patelloida*. Although *Cellana* was traditionally included within the family Patellidae, in the superfamily Patelloidea, it has been moved to a different family and suborder (Nacellidae and Nacellinae, respectively) (Lindberg 1988). Based on a morphological study, MacPherson (1955) suggested that *Cellana* was intermediate in radular characters between the superfamilies Acmaeoidea and Patelloidea (Lindberg 1998) and that *Cellana* might be the stock of Patellidae from which the Acmaeidae (to which *Patelloida* was previously allied, before Lindberg (1986b) reinstated the senior synonym Lottidae for almost all taxa assigned to the Acmaeidae) sprang.

The sequence similarity between *Patelloida* and *Cellana* was an exciting, albeit, unexpected finding of this study, which may have important implications for phylogenetic relationships at the generic and familial levels. Additional taxon sampling of other patellogastropod families to break up long sister-group lineages, as observed between *P. pustulata* and *?Asteracmaea* sp. 1, will allow better characterization of the relationship of *Cellana* and *Patelloida*.

Analysis of Radular Characters

Morphological data provide independent, but limited, additional insight into relationships among taxa included in this study. The primitive character state for the subfamily Patelloidinae is possession of two pairs of marginal teeth (Lindberg 1998). Three taxa in Clade B, besides members of the ingroup, exhibited this character state (radular form 1), which is phylogenetically uninformative because it is symplesiomorphic (Table 1 and Figures 6 and 7). Radular form 2 was exhibited by three taxa; *?Asteracmaea* sp. 1, *Yayoiacmaea oyamai*, and *?Notoacmaea* sp.1 (Table 1 and Figure 6). This derived condition, which is characterized by the loss of both pairs of marginal teeth, occurs in two different clades in all trees reported and thus appears to be homoplastic (specifically, convergent) and phylogenetically uninformative (Figure 7). Radular form 3 was another derived character state that showed a minor departure (the third lateral tooth was smaller in size relative to the other lateral teeth) from the primitive condition (Form 1)(Table 1 and Figure 6). The change to this radular character state adds support to a clade comprised of *atelloida. pygmaea*, *P. striata*, *P. heteromorpha*, *P. alticostata* and *P.*

saccharina, which was unsupported based on COI sequence data, and weakly supported in combined and 16S analyses (<50/2 bootstrap and decays, respectively for 16S and combined gene regions)(Figure 7). Although this change was synapomorphic and thus, phylogenetically informative, the focus of this study was not on intra-clade relationships among outgroup taxa.

Many informative morphological characters are available, even given the relatively primitive anatomy of taxa within *Patelloida,* which are not routinely analyzed (Sasaki 1999). For resolving ancient relationships among these groups, relatively conserved characters, such as gut looping patterns, counts of lateral tooth plates, and embryological studies would be appropriate (Lindberg 1988, Sasaki 1999). Studies to determine reproductive strategies/anatomy of members of the *P. profunda* group, may offer additional morphological characters useful in phylogenetic analyses, as well. This knowledge may also yield information about the duration of the planktonic larval stage (veliger) and larval dispersal capabilities of group members, which would be useful in understanding how present distribution patterns arose.

Biogeography

Numerous studies to date have provided evidence of recent divergence and/or speciation between Indian and Pacific taxa (MacMillan and Palumbi 1995, Williams and Benzie 1998, Benzie 1999, Williams 2000), this study is one of the few to have conclusively documented a much older divergence between taxa distributed in the two ocean basins. An ancient divergence between Indian and Pacific Ocean taxa is evident by

the rough estimates of divergence times for substitutions in both gene regions (31.4 to 59.3 Mya) (Table 2). This relatively old divergence, in the Eocene or Oligocene, is not inconsistent with the fossil record, which indicates that *Patelloida* appeared in the Cretaceous (Lindberg and Vermeij 1985). However, because earlier biogeographic events are more difficult to reconstruct than later events, it is difficult to determine the process(es) that resulted in this ancient split between Indian and Pacific Ocean taxa. A combination of processes, including severing of previously-contiguous populations and extinctions due to TECO events (tectonics, eustatic, climatic and oceanographic influences) as well as dispersal, may have resulted in the distribution of extant group members (Rosen 1988). Although it is difficult to conclusively identify events concordant in timing with the observed split, these results do substantially predate intensification of sea-level fluctuations in the late Plio-Pleistocene as a driving mechanism.

In this study, higher levels of average sequence divergence, which correspond to older divergence times, were observed among Indian Ocean ingroup taxa (subclade D), compared with their Pacific Ocean counterparts (subclade E)(Figures 2-5). This finding was based on sequence data from the COI gene region, which is most appropriate at resolving relationships among closely-related taxa (Table 2). This trend has also been observed in two studies of asteroids, where higher levels of allozyme differentiation among Indian Ocean, compared with Pacific Ocean, populations of both *Acanthaster planci* and *Linckia laevigata* were reported (Williams and Benzie 1998, Benzie 1999). It was suggested that this may be a result of the relative lack of suitable habitat, specifically, reefs and islands in the Indian Ocean, compared with the Pacific Ocean.

This explanation is also applicable to the *Patelloida profunda* group, which is restricted to calcareous substrates, generally found on reefs and emergent islands. The reduced habitat availability in the Indian, compared with the Pacific Ocean, where numerous islands may serve as stepping stones to connect populations (e.g. Indo-Malay region) may have led to greater isolation of populations because of increased limitations to gene flow, and thus to a potentially older origin of species' boundaries among Indian Ocean taxa (Figure 1). Greater taxon sampling throughout the Indian and Pacific Oceans must be conducted to more thoroughly address whether higher levels of divergence are consistently observed among taxa from these two basins.

Although a number of recent divergences of Indo-West Pacific fish, crustacean and echinoderm taxa have been attributed to Plio-Pleistocene sea-level fluctuations over a range of geographic scales (McMillan and Palumbi 1995, Lavery et al. 1996, Palumbi 1997, Benzie 1999 and Williams 2000), few recent divergences have been documented over any geographic scale among gastropod molluscs (G.P. pers. comm.). Divergence among *Cellana* spp. in Hawaii is thought to be relatively recent, however, this has not been conclusively demonstrated (Reeb 1995). Certain groups of cowries, as well as insular Pacific *Astralium* species, have exhibited divergences concordant in timing with sea-level fluctuations within the last 2.6 Mya (C.P.M. unpublished and G.P. unpublished).

In this study, young divergences (~1.6-2.9 Mya) were documented among Pacific ingroup taxa, excluding *Patelloida chamorrorum* (Table 3). These divergence estimates are consistent in timing with a vicariance hypothesis, which involves recent sea-level

fluctuations in the Plio-Pleistocene, from approximately 2.6 Mya. Numerous transgressions and regressions were known to occur at this time, which may have alternately stranded and reconnected populations, resulting in the shallow divergences reported among Indo-Malay ingroup taxa, including *P.* sp. 2a (Sulawesi, Bali and Philippines) and *P.* sp. 2b (Palau) (Myers 1989, Paulay 1991). Further, shallow-water karstic habitats of continental margins and islands, prevalent in the Indo-Malayan triangle, were likely to have been greatly affected by these events. Additional population-level analyses of these taxa will determine whether these taxa are genetically isolated, or whether gene flow is ongoing.

Highly variable levels of sequence divergence and timing of speciation were revealed among *Patelloida profunda* group members in this study, which lends support to hypothesis two. This finding is consistent with two other molecular phylogenetic studies of molluscs, which also showed variable levels of sequence divergence and timing of speciation events among taxa. A recent molecular study of giant clams has revealed divergences from the Pliocene to the Miocene (approximately 5-20 Mya) among species of *Tridacna* and *Hippopus* in the Indo-west Pacific (Schneider and O'Foighil 1999). An ongoing study of cowries has revealed divergences that are consistent in timing, not only with older, biogeographic events, but also with Plio-Pleistocene mechanisms (C. Meyer, pers. comm.). Molecular sequence data from studies of the 16S gene region of the archaeogastropod *Astralium* spp., as well as morphological results, reveal high levels of structure among populations on neighbouring archipelagoes in Oceania. These analyses

have revealed both Miocene and Plio-Pleistocene divergences among insular taxa in the Pacific (G. Paulay, pers. comm.).

Phylogeographic studies to date have generally reported recent divergences among taxa, across different geographic scales, which have been largely attributed to sea-level fluctuations in the Plio-Pleistocene (McMillan and Palumbi 1995, Lavery et al. 1996, Palumbi 1997, Benzie 1999 and Williams 2000). However, the emerging picture from studies of molluscs suggests a much more complex scenario. Myriad biogeographic forces, including dispersal and vicariance events, operating over widely-divergent timeframes, from the Palaeocene to the Plio-Pleistocene, likely drive divergence, and ultimately, speciation among Indo-Pacific taxa.

CONCLUSIONS

Sequence divergence estimates for the ingroup, which was strongly supported as monophyletic based on maximum parsimony analyses of sequence data from the 16S gene region, are highly variable. Divergence times range from a Palaeogene split between Indian and Pacific ingroup taxa, to Plio-Pleistocene divergences among certain Indo-Pacific ingroup taxa. Together, these preliminary findings lend support to hypothesis two, which proposed that divergences would be a result of many biogeographic driving agents, such as Plio-Pleistocene sea-level fluctuations, tectonically-driven vicariance and dispersal events. To better characterize these trends, additional taxa must be included at many levels including 1) the addition of more distantly related taxa to improve outgroup analyses and address problems of suspected long-branch attraction, and 2) population-level analyses to determine whether gene flow is ongoing among closely-related Indo-Malay taxa.

Another future area of study will be to intensively sample *Patelloida conoidalis* on different islands, throughout its' French Polynesian range. It will be interesting to determine if speciation occurred relatively recently, perhaps due to sea-level fluctuations, as found for some insular Pacific *Astralium* taxa, or has been a result of a more ancient divergence, as reported for other *Astralium* taxa, as well as between *Tectarius viviparus* and *T. niuensis* (Reid and Geller 1997).

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