PHYLOGENY AND HISTORICAL BIOGEOGRAPHY OF LIMPETS OF THE ORDER PATELLOGASTROPODA BASED ON MITOCHONDRIAL DNA SEQUENCES

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ABSTRACT

Using new and previously published sequences of two mitochondrial genes (fragments of 12S and 16S ribosomal RNA; total 700 sites), we constructed a molecular phylogeny for 86 extant species, covering a major part of the order Patellogastropoda. There were 35 lottiid, one acmaeid, five nacellid and two patellid species from the western and northern Pacific; and 34 patellid, six nacellid and three lottiid species from the Atlantic, southern Africa, Antarctica and Australia. *Emarginula foveolata fujitai* (Fissurellidae) was used as the outgroup. In the resulting phylogenetic trees, the species fall into two major clades with high bootstrap support, designated here as (A) a clade of southern Tethyan origin consisting of superfamily Patelloidea and (B) a clade of tropical Tethyan origin consisting of the Acmaeoidea. Clades A and B were further divided into three and six subclades, respectively, which correspond with geographical distributions of species in the following genus or genera: (A1) north-eastern Atlantic (*Patella*); (A2) southern Africa and Australasia (*Scutellastra, Cymbula* and *Helcion*); (A3) Antarctic, western Pacific, Australasia (*Nacella* and *Cellana*); (B1) western to northwestern Pacific (*Patelloida*); (B2) northern Pacific and northeastern Atlantic (*Lottia*); (B3) northern Pacific (*Lottia* and *Yayoiacmea*); (B4) northwestern Pacific (*Nipponacmea*); (B5) northern Pacific (*Acmaea* and *Niveotectura*) and (B6) northeastern Atlantic (*Tectura*). Approximate divergence times were estimated using geological events and the fossil record to determine a reference date. Divergence of the two major clades likely occurred as far back as the early Cretaceous. The phylogeny also suggests that nine principal geographic clades were formed during the late Mesozoic to early Cenozoic in association with the disruption of Pangea, which gave rise to new oceans and seaways.

INTRODUCTION

The limpets of the order Patellogastropoda are of particular evolutionary interest, because they are recognized as a basal branch of the extant Gastropoda, as revealed by cladistic analysis of morphological characters (Haszprunar, 1988; Ponder & Lindberg, 1997). They are abundant and familiar inhabitants of intertidal rocky shores throughout the world oceans from tropical to polar regions, and they play an important role in littoral marine ecosystems (Branch, 1985a, b).

Historically, taxonomical studies of limpets have used external shell morphology, but the high degree of variability of these characters has led to taxonomic confusion. It has been recognized that the radula is a useful character for species-level distinctions (Pilsbry, 1891; Thiele, 1929). Other characters that have been successfully used to differentiate species are coloration of the foot and pallial tentacles (Evans, 1947; Bowman, 1981), coloration of the egg (Habe, 1944), sperm ultrastructure (Healy, 1988; Hodgson & Bernard, 1988; Hodgson, Ridgway, Branch & Hawkins, 1996), and karyotype of the chromosomes (Nakamura, 1987; Cervella, Ramella, Robotti & Sella, 1988).

Despite numerous studies on the taxonomy of limpets, evolutionary relationships among them are poorly known. This is largely due to the scant fossil record, reflecting that the high-energy condition of their habitat on intertidal rocky shores is unfavorable for preservation of shells. Even if a fossil shell is preserved, it is often difficult to make generic and even familial assignments based on the shell structure. However, MacClintock (1967) showed that shell microstructures were often sufficiently well preserved in fossil

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limpet shells and that they could be used for generic and familial classification. Thus, the diagnostic characters of shell microstructure provide reliable palaeontological evidence for estimating divergence times of clades (Lindberg & Hickman, 1986; Lindberg, 1988; Kase, 1994; Kase & Shigeta, 1996).

The first attempt to incorporate all available data from anatomy and shell ultrastructure in a cladistic analysis of patellids was that of Ridgway (1994); however, this work was largely limited to northern Atlantic species. Later, Ridgway, Reid, Taylor, Branch & Hodgson (1998) constructed a cladogram of species of Patellidae based on all available sets of informative morphological characters. In their analysis, geographical distributions were superimposed on the cladogram to infer historical biogeography. The resulting phylogenetic tree revealed the existence of several monophyletic clades which were treated taxonomically as genera: *Helcion, Cymbula, Scutellastra* and *Patella* in the superfamily Patellidae. This analysis also suggested that ancestral forms of the Patellidae had been widely distributed in southern African waters, and probably in the Tethyan Sea as well. Morphological analysis, however, has so far been unable to resolve the phylogeny of the Patellidae satisfactorily; some unsolved problems, such as the divergence time of the clades, remain.

Molecular techniques can provide important information about phylogenetic relationships and divergence times of evolutionary lineages and clades. A molecular phylogenetic study of Patellidae using mitochondrial 12S and 16S ribosomal RNA genes was done recently by Koufopanou, Reid, Ridgway & Thomas (1999). This study clarified, in some detail, the phylogenetic relationships and divergence times of geographical clades among the Patellidae in the Atlantic and southern Africa. Their area of origin, however, remained unclear in the analysis.

Furthermore, there were some incongruities between the results of the cladistic analysis (Ridgway *et al.*, 1998) and the molecular analysis (Koufopanou *et al.*, 1999).

Extant limpets show the highest diversity in southern Africa, Australia–New Zealand, the Japanese Islands and the west coast of North America (Powell, 1973; Lindberg, 1988), where kelp forests are well developed in the strong upwelling of nutrient-rich deep sea waters. As mentioned above, a great deal of effort has been made on the phylogeny of the Patellidae derived from the Atlantic, southern Africa and Australia. However, little progress has been made on the phylogenies of the Lottiidae and the Acmaeidae in the Pacific Ocean. In a comprehensive review of patellogastropods, Lindberg (1988) applied cladistic analysis to all available character sets, and presented the relationships of the Lottiidae and Acmaeidae from the eastern Pacific and the western Atlantic seacoasts of North America; however, no detailed consideration of the Patellidae was made. The purpose of the present study is to clarify the molecular phylogenetic relationships of the patellogastropods of the world oceans with particular reference to the Lottiidae in the Pacific and the Atlantic, using combined mitochondrial 12S and 16S sequence data from the present study and from previous authors (Koufopanou *et al.*, 1999). A scenario of the phylogenetic evolution and historical biogeography of the patellogastropods is presented by integrating molecular, morphological, paleontological and geological data.

MATERIAL AND METHODS

Collection of samples and DNA extraction

Table 1 lists the species and collection localities of specimens analysed by the present authors. Generic assignments were made following Lindberg (1986) and Sasaki (1998a, 1999a) with minor amendments. The new samples include most of the species of Lottiidae, two species of Patellidae and five species of Nacellidae from the Japanese Islands; two lottiid and three nacellid species from southeast Asia; seven lottiid and one acmaeid species from western North America; and two lottiid species from the northeastern Atlantic Ocean. In total, 44 species were newly sequenced (Table 1), and combined with 42 species sequenced by Koufopanou *et al.* (1999) (Table 2) for the molecular phylogenetic analyses. *Emarginula foveolata fujitai* (Fissurellidae) was used as the outgroup. All voucher specimens are deposited in the Laboratory of Geobiology, Department of Earth and Planetary Sciences, Nagoya University, Japan.

Living specimens obtained by field collection were fixed in 80% ethanol. Total DNA was extracted from a small fragment of the mantle or foot muscle tissue, either using High Pure PCR Template Preparation Kit (Roche) or a standard phenol/chloroform extraction method. In the latter procedure, a small amount of tissue was treated with 200 μl of TEN buffer (10 mM Tris, ph 8.0, 10 mM EDTA,10 mM NaCl), 20 μl of 10% SDS, and 40 μl of proteinase K (20 mg/ml) at 37°C for 1–2 h with gentle rotation, followed by phenol/chloroform and chloroform extractions, and ethanol precipitation.

PCR amplification and DNA sequencing

A fragment of the mitochondrial small-subunit ribosomal RNA gene (12S rRNA) and large-subunit ribosomal RNA gene (16S rRNA) were amplified using the 'universal' primer pair (Kocher, Thomas, Meyer, Edwards, Pääbo, Villablanca & Wilson, 1989) modified by Koufopanou *et al.* (1999): 12Sma (5'-CTGGGATTA-GATACCCTGTTAT-3') and 12Smb (5'-CAGAGAGTGACGG-GCGATTTGT-3'), and 16LRN13398 (5'-CGCCTGTTTAACAAAA-ACAT-3') and 16SRHTB (5'-ACGCCGGTTTGAACTCAGATC-3'), respectively. Owing to the difficulty of amplifying some template

Table 1. List of species newly sequenced in this study.

Species	Localities
Acmaea mitra Rathke, 1833	Boiler Bay, Washington, USA
Cellana grata (Gould, 1859)	1. Kaino, Mie, Japan
	2. Cape d'Aguilar, Hong Kong
Cellana nigrolineata (Reeve,1839)	Kaino, Mie, Japan
Cellana orientalis (Pilsbry, 1891)	Okinawa, Japan
Cellana testudinaria (Linnaeus, 1758)	1. Shinzato fishing port, Okinawa, Japan
	2. Vietnam
Cellana toreuma (Reeve, 1854)	1. Oga, Akita, Japan
	2. Cape d'Aguilar, Hong Kong
Lottia atrata (Carpenter, 1857)	Campus Point, California, USA
Lottia cassis (Eschscholtz, 1833)	Akkeshi, Hokkaido, Japan
Lottia digitalis (Rathke, 1833)	Cattle Point, Washington, USA
Lottia dorsuosa (Gould, 1859)	Morozaki, Aichi, Japan
Lottia emydia (Dall, 1914)	Aininkappu, Hokkaido, Japan
Lottia fenestrata (Reeve, 1855)	Strawberry Hill, Washington, USA
Lottia gigantea Sowerby, 1834	Royal Palms, Los Angeles, USA
Lottia kogamogai Sasaki & Okutani, 1994	Hiraiso, Ibaraki, Japan
Lottia langfordi (Habe, 1994)	Goshikinohama, Kochi, Japan
Lottia limatula (Carpenter, 1864)	Coal Oil Point, California, USA
Lottia lindbergi Sasaki & Okutani, 1994	Akkeshi, Hokkaido, Japan
Lottia luchuana (Pilsbry, 1901)	1. Ogimi, Okinawa, Japan
	2. Cape d'Aguilar, Hong Kong
Lottia pelta (Rathke, 1833)	Cattle Point, Washington, USA
Lottia persona (Rathke, 1833)	Cattle Point, Washington, USA
Lottia scabra (Gould, 1846)	Royal Palms, Los Angeles, USA
Lottia scutum (Rathke, 1833)	Cattle Point, Washington, USA
Lottia sp. cf. *borealis* (Lindberg, 1982)	Akkeshi, Hokkaido, Japan
Lottia tenuisculpta Sasaki & Okutani, 1994	Kaino, Mie, Japan
Lottia testudinalis (Müller, 1776)	1. Coal Oil Point, California, USA
	2,3,4. Millport, UK
Nipponacmea concinna (Lischke, 1870)	Morozaki, Aichi, Japan
Nipponacmea fuscoviridis (Teramachi, 1949)	Akasyouzaki, Fukui, Japan
Nipponacmea gloriosa (Habe, 1944)	Kamo, Shizuoka, Japan
Nipponacmea habei (Sasaki & Okutani, 1994)	Yamada, Iwate, Japan
Nipponacmea nigrans (Kira, 1961)	Hiraiso, Ibaraki, Japan
Nipponacmea radula (Kira, 1961)	Gesuzima, Kagoshima, Japan
Nipponacmea schrenkii (Lischke, 1868)	Akasyouzaki, Fukui, Japan
Nipponacmea teramachii (Kira, 1961)	Owase, Mie, Japan
Niveotectura pallida (Gould, 1859)	Aininkappu, Hokkaido, Japan
Patelloida saccharina lanx (Reeve, 1855)	Oga, Akita, Japan
Patelloida saccharina saccharina (Linnaeus, 1758)	Katsuren, Okinawa, Japan
Patelloida saccharinoides (Habe & Kosuge, 1996)	Cape d'Aguilar, Hong Kong
Patelloida striata (Quoy & Gaimard, 1834)	Katsuren, Okinawa, Japan
Patelloida pygmaea (Dunker, 1860)	Tahara Bay, Aichi, Japan
Patelloida pygmaea conulus (Dunker, 1861)	Tahara Bay, Aichi, Japan
Scutellastra flexuosa (Quoy & Gaimard, 1834)	Tanabe, Wakayama, Japan
Scutellastra optima (Pilsbry, 1904)	Takarazima, Kagoshima, Japan
Tectura virginea (Müller, 1776)	1,2. Millport, UK
Yayoiacmea oyamai (Habe, 1955)	Omaezaki, Shizuoka, Japan

Classification and nomenclature after Lindberg (1986) and Sasaki (1998a, 1999a) with minor amendments by the present authors.

Table 2. List of species analysed by Kofopanou *et al.* (1999), with localities.

Species	Localities
Cellana capensis (Gmelin, 1791)	Cape Vidal, South Africa
Cellana pricei Powell, 1973	O Le Pupu, Upolu, Western Samoa
Cellana solida (Blainville, 1825)	Oford, Tasmania, Australia
Cellana taitensis (Röding, 1798)	Pointe de Tapahi, Tahiti, French Polynesia
Cellana tramoserica (Holten, 1802)	Mollimook, NSW, Australia
Cymbula adansonii (Dunker, 1853)	Longstrand, Swakopmund, South Africa
Cymbula canescens (Gmelin, 1791)	St Helena
Cymbula compressa (Linnaeus, 1758)	Kommetjie, Cape Town, South Africa
Cymbula granatina (Linnaeus, 1758)	Kommetjie, Cape Town, South Africa
Cymbula miniata (Born, 1778)	1. Kommetjie, Cape Town, South Africa
	2. East London, South Africa (form *sanguinans* Reeve, 1854)
Cymbula oculus (Born, 1778)	Kommetjie, Cape Town, South Africa
Cymbula safiana (Lamarck, 1819)	1. Mocamedes, Angola
	2. Estepona, Spain
Helcion concolor (Krauss, 1848)	East London, South Africa
Helcion dunkeri (Krauss, 1848)	Bloubergstrand, Cape Town, South Africa
Helcion pectunculus (Gmelin, 1791)	Kommetjie, Cape Town, South Africa
Helcion pruinosus (Krauss, 1848)	Dalebrook, Cape Town, South Africa
Nacella concinna (Strebel, 1908)	Signy Island, Antarctic
Patella aspera Röding, 1798	1. Madeira, Portugal
	2. Dancing Ledge, Dorset, UK
	3. Santa Maria, Azores, Portugal
Patella caerulea Linnaeus, 1758	Estepona, Spain
Patella candei d'Orbigny, 1839	Santa Maria, Azores, Portugal
Patella depressa Pennant, 1777	Zahara los Atunes, near Tarifa, Spain
Patella ferruginea Gmelin, 1791	Isla di Gorgona, off Livorno, Italy
Patella lugubris Gmelin, 1791	Pedra de Lume, Sal Island, Cabo Verde
Patella pellucida Linnaeus, 1758	Swanage, Dorset, UK
Patella rustica Linnaeus, 1758	1. Barcelona, Spain
	2. Madeira, Portugal
Patella vulgata Linnaeus, 1758	Dancing Ledge, Dorset, UK
Patelloida latistrigata (Angas, 1865)	Oford, Tasmania, Australia
Scutellastra aphanes (Robson, 1986)	Munster, South Africa
Scutellastra argenvillei (Krauss, 1848)	Kommetjie, Cape Town, South Africa
Scutellastra barbara (Linnaeus, 1758)	Kommetjie, Cape Town, South Africa
Scutellastra chapmani (Tenison-Woods, 1875)	Ulladulla, NSW, Australia
Scutellastra cochlear (Born, 1778)	Kommetjie, Cape Town, South Africa
Scutellastra exusta (Reeve, 1854)	Cape Vidal, South Africa
Scutellastra flexuosa (Quoy&Gaimard, 1834)	Taga Blowholes, Savaii, Western Samoa
Scutellastra granularis (Linnaeus, 1758)	Kommetjie, Cape Town, South Africa
Scutellastra laticostata (Blainville, 1825)	Kalbarri, Western Australia
Scutellastra longicosta (Lamarck, 1819)	Kommetjie, Cape Town, South Africa
Scutellastra mexicana (Broderip&Sowerby, 1829)	Mexico
Scutellastra miliaris (Philippi, 1848)	Moçâmedes, Angola
Scutellastra obtecta (Krauss, 1848)	Cape Vidal, South Africa
Scutellastra peronii (Blainville, 1825)	Gerringong, NSW, Australia

Classification and nomenclature after Ridgway *et al.* (1998).

DNA from the Pacific species using 12Sma, a new primer 12S97L (5'-AAC(C/T)CAAAG(A/G)ACTTGGCGGT-3') was developed based on the aligned sequences of the Patellidae. PCR amplification was performed in 25 μl of reaction volume containing 10 mM Tris–HCl ph 8.3, 50 mM KCL, 1.5 mM MgCl$_2$, 200 μM dNTPs, 0.2 μM each primer, 0.5 mg/ml BSA (Sigma), 2 units Taq polymerase (Takara), and 1 μl of template DNA solution. The cycling parameters for amplification consisted of an initial denaturation for 3 min at 94°C; followed by 30 cycles of denaturation for 45 s at 94°C, annealing for 90 s at 56°C, and extension for 120 s at 72°C; and ended with a 5 min extension at 72°C. Amplification products were purified using High Pure PCR Product Purification Kit (Roche). Purified amplification products were used as templates sequencing using Applied Biosystems BigDye vr.3 dye terminator cycle sequencing kit. Sequencing reaction products were purified using the ethanol precipitation and analyzed on an ABI PRIZM 377 DNA Sequencer, following the manufacturer's protocol.

Sequence analysis and phylogeny reconstruction

The sequences of the 12S and 16S from 40 patellid species and two lottiid species listed in Table 2 with localities, have already been published (Koufopanou *et al.*, 1999) and have accession numbers AF058174-AF058271 in GenBank. The sequence of only one fragment was available for *Cymbula granatina* and *Helcion pectunculus* (12S only), and *Scutellastra mexicana* (16S only).

The 12S and 16S sequences determined in this study (Table 1) and from Koufopanou *et al.* (1999) (Table 2) were aligned using ClustalX (Thompson, Gibson, Plewniak, Jeanmougin & Higgins, 1997), with minor manual adjustments. Analyses were carried out using PAUP 4.0 version b10 (Swofford, 1998). The phylogenetic tree was constructed by the neighbour-joining (NJ) method (Saitou & Nei, 1987) based on a matrix consisting of genetic distance calculations according to Kimura's two-parameter method (Kimura, 1980). Bootstrap analysis with 1000 replications was used to assess the stability of each node. In order to obtain an estimation of approximate divergence times, the tpcv program in the Lintre package (Takezaki, Rzhetsky & Nei, 1995) was used. To construct a linearized tree, sequences from species that have evolved significantly faster or slower than the average (at the confidence level of 1%) were excluded. Heights of nodes and the standard errors of the resultant linearized tree were converted into divergence times using a reference date of 38 Ma from the palaeontological record (Lindberg & Hickman, 1986).

RESULTS

We determined the nucleotide sequences of a fragment of 12S (ranging from 300 to 420 bp) and 16S (ranging from 483 to 728 bp) ribosomal RNA genes for the 44 species of patellogastropoda listed in Table 1. The sequences for each gene region have been deposited in DDBJ and Gen Bank under accession numbers AB106425-AB106520 and AB107900-AB107909.

The lengths of the alignments for the 12S and 16S fragments were 511 and 940 bp, respectively. After removal of sequence-ambiguous regions, 288 and 412 bp were retained for 12S and 16S, respectively, leaving a total of 700 bp in the combined data set. Excluding the outgroup, pairwise Kimura's two parameter distances based on the combined regions ranged from 0.8% (for *Scutellastra aphanes* and *Scutellastra obtecta*) to 42.5% (for *Tectura virginea* and *Cellana nigrolineata*).

Phylogenetic trees were constructed by the neighbour-joining (NJ) method based on the three data sets, namely 12S, 16S, and combined 12S and 16S sequence data sets, with *Emarginula foveolata fujitai* as an outgroup. In the 12S and the 16S trees (Fig. 1A, B), two major monophyletic clades, the Patelloidea

(Patellidae and Nacellidae) and the Acmaeoidea (Lottiidae and Acmaeidae), were supported with high bootstrap values. There was a high degree of agreement between the two trees, but some minute incongruences were found. In particular, *Acmaea mitra*, *Niveotectura pallida*, *Tectura testudinalis*, *Tectura fenestrata* and *Yayoiacmea oyamai* were placed on different branches of the tree. These rearrangements may be due to short sequence length in 12S and the more highly conserved 16S.

In the combined 12S and 16S tree (Fig. 2), seven monophyletic subclades were also supported with high bootstrap values, comprising species classified in the following genera: *Nacella* and *Cellana*; *Scutellastra*, *Cymbula* and *Helcion*; *Patella*; *Patelloida*; *Lottia*; *Lottia* and *Yayoiacmea*; *Nipponacmea*; *Acmaea* and *Niveotectura*; and *Tectura*. Analysis of the combined data set indicated a high degree of agreement with the 16S data set, except that *Acmaea mitra* and *Niveotectura pallida* did not constitute a clade in the 16S tree, but with higher bootstrap values supporting each clade (Fig. 2).

DISCUSSION

Molecular phylogenetic trees and classification of the Patellogastropoda

In the tree of the combined sequences (Fig. 2), monophyly of each of the two major clades corresponding taxonomically to the Patelloidea and the Acmaeoidea was strongly supported by high bootstrap probabilities, 97% and 100%, respectively. These clades are also clearly distinguished by other morphological characters (Powell, 1973; Lindberg, 1981, 1988; Lindberg & McLean, 1981; Sasaki, 1998b). For example, while the Acmaeoidea have a single left ctenidium, the ctenidia are absent and replaced by 'secondary' pallid gills in the Patelloidea. In the Acmaeoidea, radular teeth have solid, simple basal plates; the lateral tooth is wide and short; and rachidian teeth are lacking. In the Patelloidea, radular teeth have morphologically complex basal plates and consist of more numerous, long and narrow teeth.
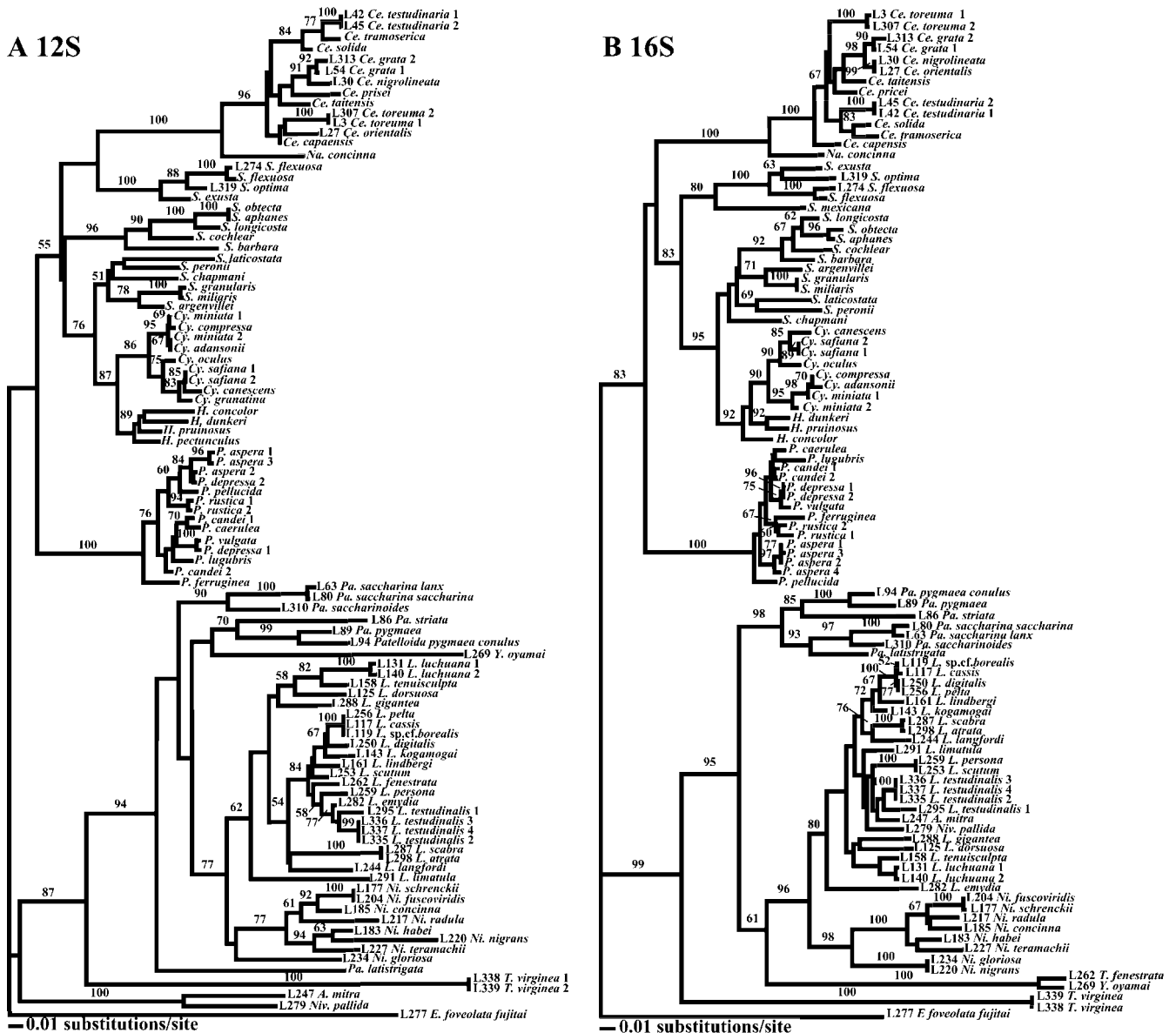

Figure 1. Neighbour-joining trees resulting from separate analyses of 12S rRNA (288 bp) (**A**) and 16S rRNA (412 bp) (**B**) data with bootstrap values (based on 1000 replications) shown above each branch. *Emarginula foveolata fujitai* was used as the outgroup. Generic abbreviations: *A.*, *Acmaea*; *Ce*, *Cellana*; *Cy.*, *Cymbula*; *E.*, *Emarginula*; *H.*, *Helcion*; *L.*, *Lottia*; *Na.*, *Nacella*; *Ni.*, *Nipponacmea*; *Niv.*, *Niveotectura*; *P.*, *Patella*; *Pa.*, *Patelloida*; *S.*, *Scutellastra*; *T.*, *Tectura*; *Y.*, *Yayoiacmea*.
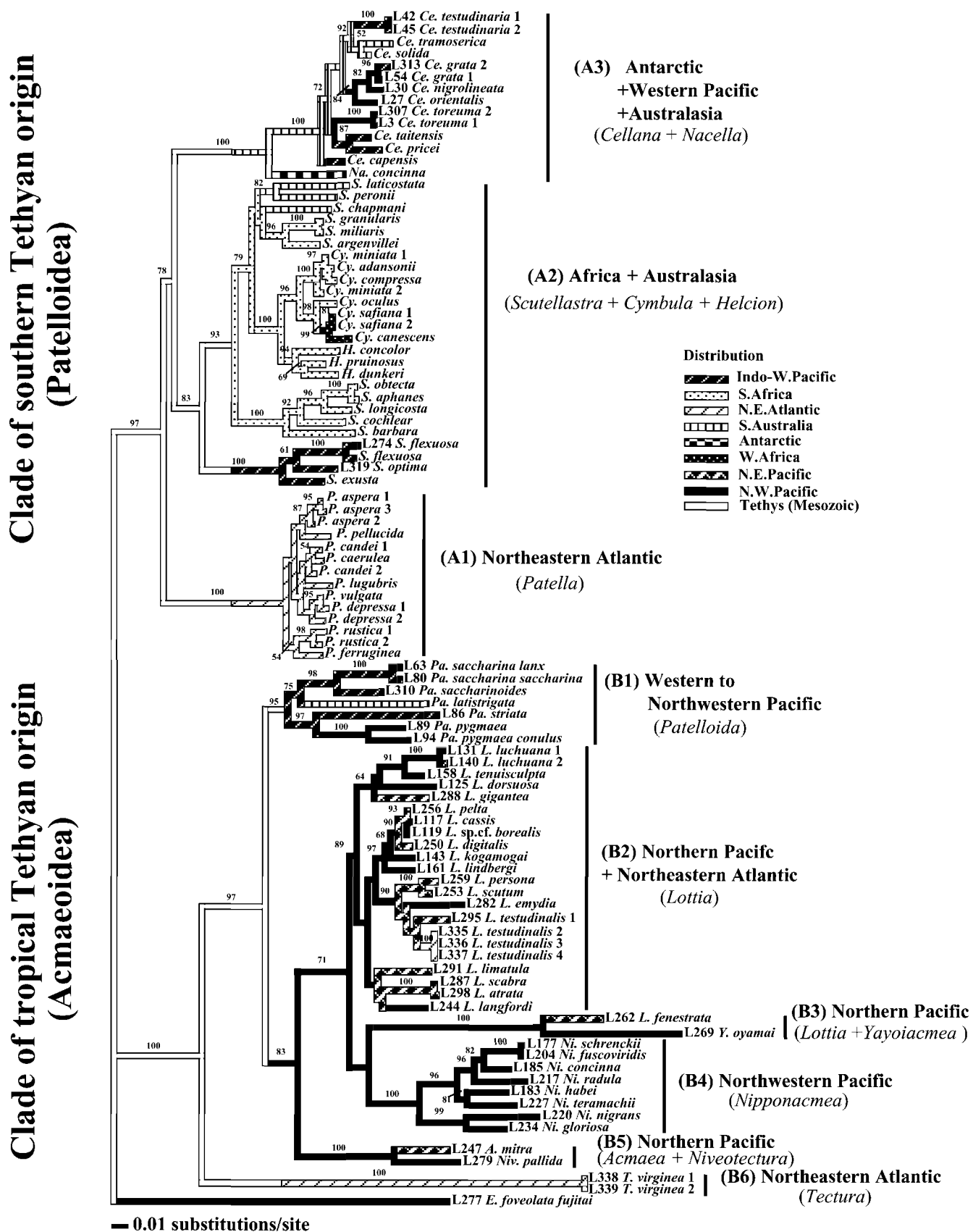
Clade of southern Tethyan origin (Patelloidea)

Clade of tropical Tethyan origin (Acmaeoidea)

(A3) Antarctic +Western Pacific +Australasia
(*Cellana + Nacella*)

(A2) Africa + Australasia
(*Scutellastra + Cymbula + Helcion*)

Distribution

▨▨▨ Indo-W.Pacific
⌑⌑⌑ S.Africa
▧▧▧ N.E.Atlantic
⫿⫿⫿ S.Australia
▨▨▨ Antarctic
▨▨▨ W.Africa
▨▨▨ N.E.Pacific
■■■ N.W.Pacific
☐☐☐ Tethys (Mesozoic)

(A1) Northeastern Atlantic
(*Patella*)

(B1) Western to Northwestern Pacific
(*Patelloida*)

(B2) Northern Pacifc + Northeastern Atlantic
(*Lottia*)

(B3) Northern Pacific
(*Lottia +Yayoiacmea*)

(B4) Northwestern Pacific
(*Nipponacmea*)

(B5) Northern Pacific
(*Acmaea + Niveotectura*)

(B6) Northeastern Atlantic
(*Tectura*)

■ **0.01 substitutions/site**

Figure 2. Neighbour-joining tree resulting from the combined analysis of 12S and 16S (700 bp) data with bootstrap values (based on 1000 replications) above each branch. Geographical distributions are superimposed on the tree. *Emarginula foveolata fujitai* was used as an outgroup. For generic abbreviations refer to Figure 1. The generic classifications were made after Lindberg (1986), Sasaki (1998a, 1999a) and Ridgway *et al.* (1998) with minor amendments (see text).

Among the Patelloidea, relationships between the Patellidae and the Nacellidae similar to those reported in Koufopanou *et al.* (1999) were identified. Based on morphological analysis, Ridgway *et al.* (1998) recognized four principal clades in the Patellidae corresponding to the genera *Scutellastra*, *Cymbula*, *Helcion* and *Patella*. Molecular phylogenetic analysis, however, revealed that *Scutellastra* is a paraphyletic group (Koufopanou *et al.*, 1999; this study).

According to Lindberg & Vermeij (1985), *Patelloida* consists of at least two subclades. One subclade (clade B1 in Fig. 2) includes species characterized by low to medium shell profiles, strong radial ribs or many fine riblets, reduced third lateral teeth, and habitats including various substrata. The other subclade, which has been called the *Patelloida profunda* group by Christiaens (1975) and Lindberg & Vermeij (1985), includes species characterized by medium to high shell profiles, many ribs, equal-sized lateral radular teeth, and habitats limited to calcareous substrata. The former group consists of two subclades which were clearly identifiable in the molecular phylogenic tree based on the combined sequences (Fig. 2).

In the combined 12S and 16S tree, species assigned to *Tectura* by Lindberg (1986) are placed in three different clades, each supported with high bootstrap values. The group including *Tectura scutum* is closely related to species of *Lottia*, and occurs in a nested clade of *Lottia*. The species of this group (*T. scutum*, *T. persona*, *T. emydia* and *T. testudinalis*) are herein assigned to the genus *Lottia*, with which they share similar radular teeth. *Tectura fenestrata*, a representative of the second group of *Tectura*, is closely allied with *Yayoiacmea oyamai* (clade B3). *Tectura virginea* (clade B6), the type species of *Tectura*, forms the basal branch in the Acmaeoidea.

The genus *Nipponacmea* was proposed for the Japanese lottiid limpets previously assigned to the genus *Notoacmea* based on shell structure, anatomical characters and endemism to the Japonic zoogeographical province (Sasaki & Okutani, 1993). Lindberg (1986) first pointed out that the genus *Notoacmea* is restricted in geographic distribution to New Zealand and Australia, and reclassified North American species from *Notoacmea* to *Tectura* based on differences in shell structure. The integrity of *Nipponacmea* was strongly supported by a bootstrap value of 100% (clade B4). Its two distinct subclades also had high bootstrap values, but are not morphologically distinguishable from each other.

The clade consisting of *Acmaea mitra* and *Niveotectura pallida* was supported with a high bootstrap value (100%) (clade B5). *Acmaea mitra*, the type species of the genus *Acmaea*, is classified as Acmaeidae, and *Niveotectura pallida* is allocated to the Lottiidae, under currently accepted classification schemes (Lindberg & Marincovich, 1988; Sasaki, 1999b). *Niveotectura pallida* is here transferred from the Lottiidae to the Acmaeidae. This is supported by the similarity of the radula in both species, which each shows three pairs of uniform lateral teeth. Therefore the species in this clade are easily distinguishable from others found in the Pacific Ocean.

Estimation of divergence times of clades

To estimate approximate divergence time of the clades, a linearized tree was constructed. Six taxa, *Tectura virginea*, *Tectura fenestrata*, *Yayoiacmea oyamai*, *Niveotectura pallida*, *Acmaea mitra* and *Nipponacmea nigrans*, were excluded from the tree since they have evolved significantly faster than the other taxon at the 99% level. Heights of nodes and the standard errors of the resulting linearized tree can be converted into divergence times if at least one reference date is available. In the present study, we set the divergence between *Cellana* and *Nacella* at 38 Ma (Fig. 3, Table 3). In a molecular phylogenetic study of the patellid limpets, Koufopanou *et al.* (1999) also used 38 Ma as a minimum

Table 3. Divergence times (Ma) estimated from the combined 12S and 16S sequence data set.

Divergent events of clades	Heights in the linearized tree	Estimated divergence time (Ma)
Tropical Tethyan *vs* southern Tethyan	0.181 ± 0.014	143.3 ± 11.1
Northeastern Atlantic (A1) *vs* Africa + Australasia (A2) + Antarctic + western Pacific + Australasia (A3)	0.128 ± 0.012	101.3 ± 9.5
Africa + Australasia (A2) *vs* Antarctic + western Pacific + Australasia (A3)	0.115 ± 0.010	91.0 ± 7.9
Divergence of *Nacella* and *Cellana* associated with the opening of the seaway between Antarctica and Australia and the first ocurrence of *Cellana* in the Pacific	0.048 ± 0.007	38
Western to northwestern Pacific (B1) *vs* other Pacific elements (B2), (B3), (B4)	0.093 ± 0.009	73.6 ± 7.1
Northwestern Pacific (B4) *vs* Northern Pacific + northeastern Atlantic (B2)	0.076 ± 0.008	60.1 ± 6.3

The heights and their standard errors were estimated using the tpcv program implemented in the Lintre package (Takezaki *et al.*, 1995). The heights were converted into divergence times based on the reference date of 38 Ma for the divergence of *Nacella* and *Cellana* (see text).

age for this evolutionary event, based on occurrences in the fossil record and the geological age of *Cellana ampla* from the Upper Eocene of Oregon (Lindberg & Hickman, 1986; Lindberg, 1988). However, they entertained some uncertainty about the reliability of using the fossil to support the age of the clade, since Lindberg & Hickman (1986) had used doubtfully synapomorphic characters of shell microstructure to assign the fossil to the genus *Cellana*. At the same time, they suggested the potential importance of using *Nacella* to date the divergence of *Cellana* and *Nacella*, since this genus is defined by a synapomorphic character of shell microstructure. In this respect, re-examination of taxonomic relationships based on the shell microstructure of fossil *Cellana* from the Upper Eocene of Antarctica (Stilwell & Zinsmeister, 1992) and New Zealand (Beu, Maxwell & Brazier, 1990) holds promise. *Nacella*, an austral, cold-water sister genus of the tropical *Cellana*, now has a limited distribution around Antarctica and the southernmost part of South America (Ubaldi, 1985a, b). The divergence of *Nacella* and *Cellana* was very likely associated with the founding of the proto-Antarctic Circumpolar Current following the breaking off of Australia from the Antarctic continent in the early Late Eocene. The full opening of the seaway at 38 Ma led to the sharp cooling of the surface water in the Southern Hemisphere and the development of the first glaciers in Antarctica (van Andel, 1985). These major geological and climatic events may have promoted the divergence of *Nacella* and *Cellana*, two clades that adapted to the austral cold waters and northern tropical to subtropical warm waters of the Indo-Pacific, respectively. Fossil *Cellana* from the late Eocene in Oregon may mark the appearance of *Cellana* on the west coast of North America. Hence, we used 38 Ma to mark the divergence of *Nacella* and *Cellana*, but with a slightly different interpretation than was used by Koufopanou *et al.* (1999).
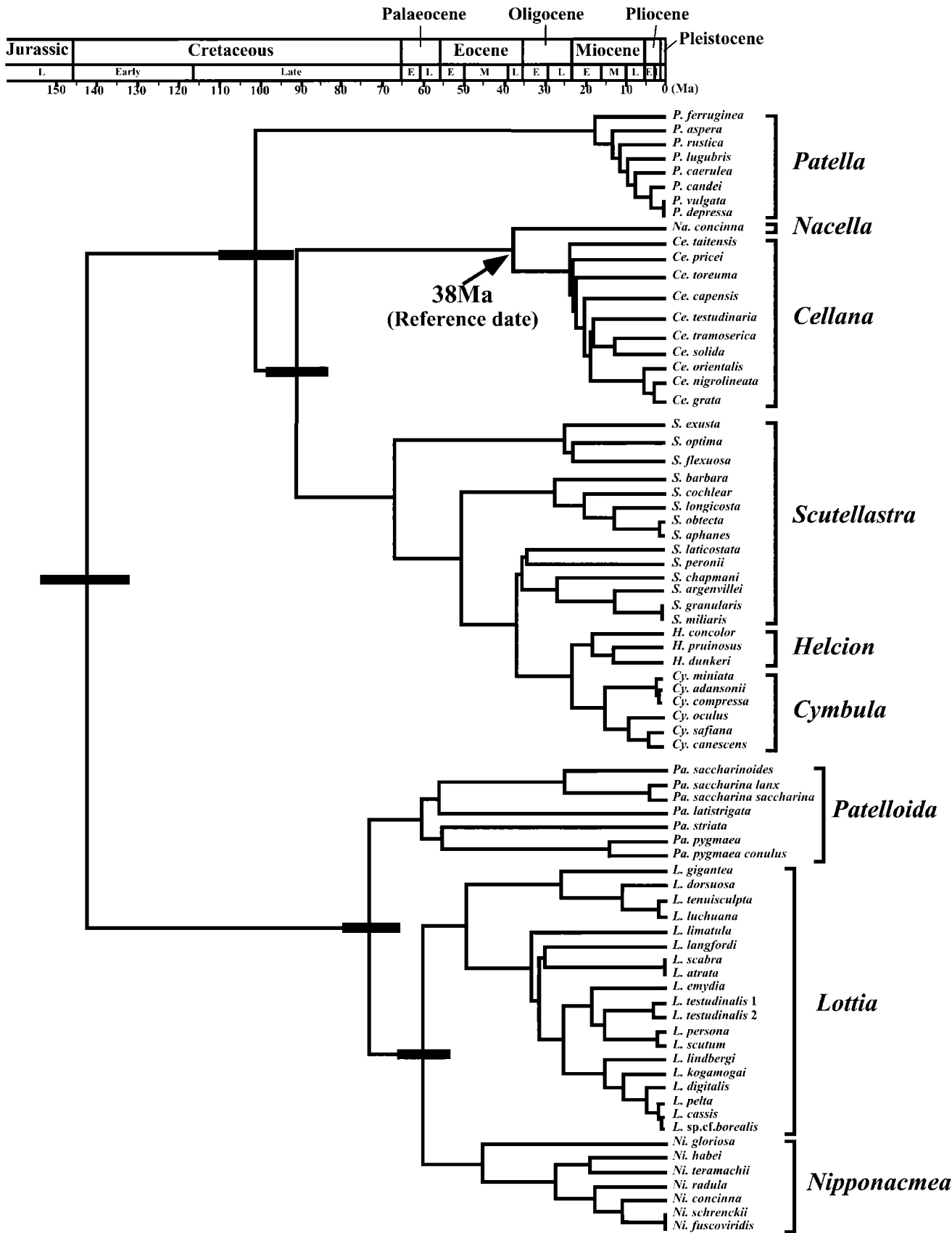
Figure 3. A linearized tree made by the tpcv program implemented in the Lintre package (Takezaki *et al.*, 1995) showing the estimated divergence times of the clades using a reference date of 38 Ma for the divergence of *Nacella* and *Cellana*. The outgroup and some other taxa were removed (see text). Solid bars indicate the standard error of the estimated age. For generic abbreviations refer to Figure 1.

Phylogenetic evolution and historical biogeography

Using a dated phylogeny, the geographical distribution of extant species, and the fossil record, it is possible to reconstruct the historical biogeography and probable processes of phylogenetic evolution of a taxonomic group (Collins, Frazer, Palmer, Vermeij & Brown, 1996; Reid, 1996; Reid, Rumbak & Thomas, 1996; Koufopanou *et al.*, 1999). In Figure 2, the geographical distributions of the taxa have been superimposed on the molecular phylogenetic tree. The most striking finding in our molecular phylogenetic analyses is that the Patellogastropoda consists of two groups (designated clade A and clade B for convenience). Clades A and B are comprised of three and six subclades, respectively, with the following geographical distributions: A1 in the northeastern Atlantic; A2 in Africa and Australasia; A3 in Antarctica, Australasia and the western Pacific; B1 in the western to northwestern Pacific; B2 in the northern Pacific and northeastern Atlantic; B3 in the northern Pacific; B4 in the northwestern Pacific; B5 in the northern Pacific; and B6 in the northeastern Atlantic.

Clade A is taxonomically composed of the Patelloidea (Patellidae as a paraphyletic group and Nacellidae as a mono-phyletic group), and their centre of origin was possibly in peri-Gondwana of the Tethys Sea, if we adopt parsimony criteria when ranges of the subclades are superimposed on the tree (Fig. 2). On the other hand, clade B consists of the Acmaeoidea (Lottiidae as a paraphyletic group and Acmaeidae as a mono-phyletic group). The clade of *Tectura virginea* (clade B6) forms the basal branch of clade B. This suggests that the centre of origin of clade B was possibly in the Tethys Sea. The divergence of these two major clades dates back to the early Cretaceous based on estimations made with the molecular clock (Fig. 3). In the Cretaceous, the Tethys Sea was opened widely to the east to connect with the Pacific Ocean (Fig. 4A). Based on the palaeogeography of the Cretaceous period, the centre of origin of clade A was likely located on the northern margin of Gondwanaland, facing the Tethys Sea. The centre of the clade B was likely on the southern margin of Eurasia, facing the tropical Tethys Sea. Consequently, we designate clade A the clade of southern Tethyan origin (hereafter, the southern Tethyan clade), and clade B the clade of tropical Tethyan origin (hereafter, the tropical Tethyan clade).

The Cretaceous is one of the most remarkable times in earth history. The South Atlantic Ocean as well as the Pacific Ocean
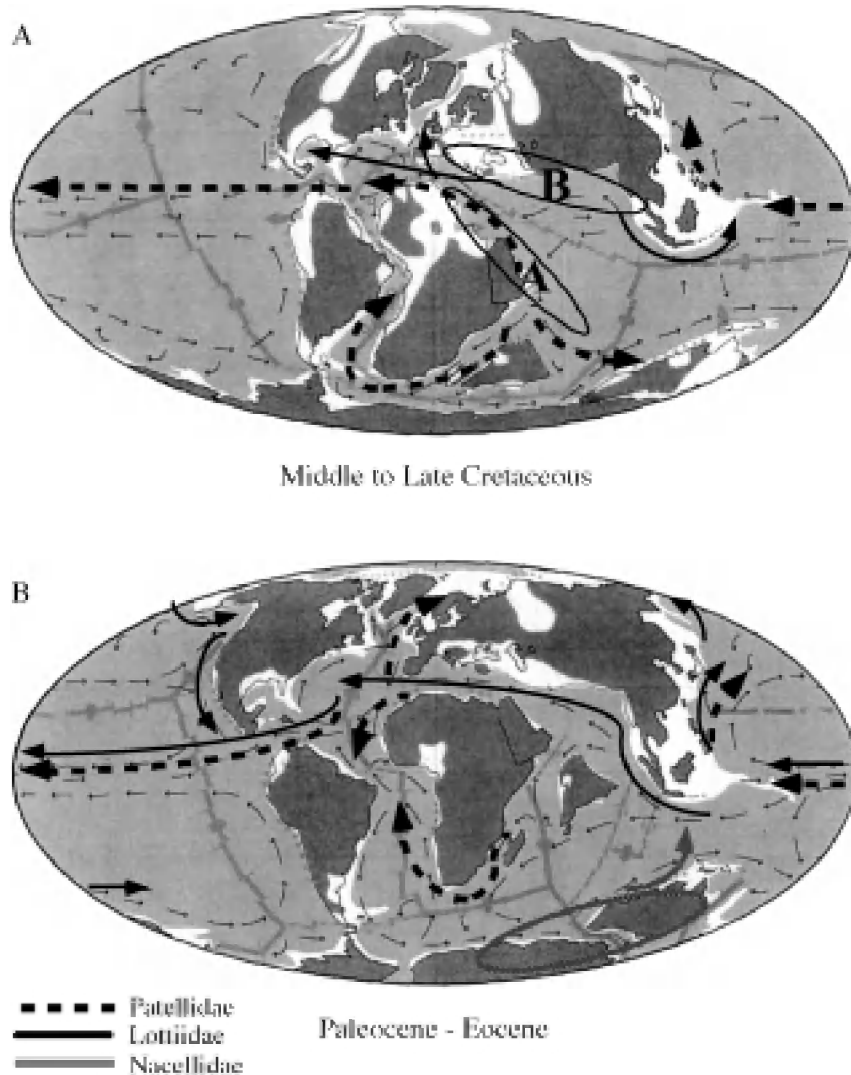

Figure 4. Palaeogeographic maps showing the inferred places of origin and migration routes of Patellogastropoda. **A.** The areas of origin and migration routes of the Patellidae and the Lottiidae in the Middle to Late Cretaceous. **B.** Migration routes of the Patellidae, Nacellidae and Lottiidae in the Palaeocene-Eocene. (Base map adapted from Haq, 1984.)

opened and spread, and the break up of Gondwanaland was accompanied by modification of the Tethys Sea. Consequently, the circumequatorial seaway was established when the Tethyan Sea became connected with the Pacific Ocean through the Caribbean Sea (Fig. 4A). During this period, the dispersion of ancestral species to new territories was accomplished through migration along coastlines or transport of larvae by ocean currents. Within the southern Tethyan clade, the *Patella* clade (clade A1) diverged from the ancestral species first (Figs 2, 3). This was explained by the invasion of ancestral species of *Patella* to the central Atlantic. However, the *Patella* species currently found in the northeastern Atlantic underwent a more recent radiation, perhaps in the Neogene, based on the minimal molecular and morphological divergence among these species (Ridgway *et al.*, 1998; Koufopanou *et al.*, 1999). *Cymbula* and *Helcion* migrated southwestwards along the African coastline; *Helcion* is still found in southern Africa and *Cymbula* has dispersed northwards through colonization of the West African coast. *Scutellastra* was widely distributed in the Indo-Pacific and around southern Australia and southern Africa. The implication that *Scutellastra* originally had a Tethyan distribution and extended its distribution westwards by riding the intense paleoequatorial current is supported by the presence of *Scutellastra mexicana*, the basal branch of the *Scutellastra flexuosa* clade species (Fig. 1B), in the Eastern Pacific (Koufopanou *et al.*, 1999). The divergence time of the *Scutellastra flexuosa* clade is estimated to have been as early as 70 Ma (Fig. 3). *Scutellastra* fossils have been identified from the Upper Cretaceous of northern Japan. *Patella soyaensis*, described by Kase & Shigeta (1996) from the upper Campanian (75 Ma) of the Late Cretaceous of Hokkaido, Japan, was assigned to *Scutellastra* based on shell microstructure by Ridgway *et al.* (1998). Other species from the Middle Miocene and Lower Pliocene of Japan (Kase, 1994) can also be assigned to the genus *Scutellastra* based on shell microstrucure. This suggests that *Scutellastra* reached Japan during the Cretaceous either via dispersion in the current turning north from the palaeoequatorial current or by rafting on ammonites (Akpan, Farrow & Morris, 1982; Kase, Shigeta & Futakami, 1994). More recent dispersal from the western to eastern Pacific is unlikely because the prevailing currents, together with the great distance between Polynesia and South America make, 'Ekman's barrier' almost impenetrable to most shallow-water marine invertebrates of the western Pacific (Vermeij, 1987). In summary, a recent derivation of *Scutellastra mexicana* from Indo-West Pacific *Scutellastra* by eastward dispersal (Lindberg & Hickman, 1986; Ridgway *et al.*, 1998) is very unlikely, as already discussed by Koufopanou *et al.* (1999).

The Nacellidae split from the Patellidae, during the Cretaceous. The Nacellidae clade, consisting of *Nacella* and *Cellana*, probably originated on the coast of peri-Gondwanaland comprising southern Africa, Australia and Antarctica. As already discussed, the divergence of the tropical genus *Cellana* and the austral, cold-water genus *Nacella* was likely in the late Eocene (Fig. 3). *Cellana* is now widely distributed in the Indo-Western Pacific region and shows greatest diversity in Australasia. All available evidence suggests that the Nacellidae accomplished radiation in the warm water regions during the Neogene after extending their distribution northwards from the Antarctic region.

Among the tropical Tethyan clade, the clade of *Tectura virginea* (clade B6) diverged first (Figs 1B, 2). The clade is now restricted to the northeastern Atlantic.

During the Cretaceous, *Patelloida* extended its distribution westwards from its origin in the tropical Tethys Sea (Fig. 4A). The earliest fossil of this genus have been found in the Upper Cretaceous (Albian) of England (Akpan *et al.*, 1982), and the fossil dating to the Late Cretaceous (Campanian) was found in California (Lindberg, 1983). Furthermore, *Patelloida* species

commonly occurred in the near shore sediments in North America and Europe during the Tertiary (MacClintock, 1967; Lindberg, 1983; Lindberg & Hickman, 1986). This suggests that the *Patelloida* clade (clade B1), with its origins in the tropical Tethys Sea, reached Europe and North America during the Late Cretaceous. At that time, migration of marine organisms in an east to west direction was probably much easier than north to south dispersions, due to the prevailing circumglobal palaeoequatorial currents existing at that time.

Among the clades originating in the western Pacific (clades B2–B5), *Acmaea mitra* and *Niveotectura pallida* (clade B5) diverged first. This clade is restricted to the North Pacific. Fossils of *Niveotectura* are found in the Miocene to Pleistocene sediments in the North Pacific ranging from Alaska (Lindberg & Marincovich, 1988) to northern Japan (Hase, 1965; Kaseno & Matsuura, 1965, Iwai & Shiobara, 1969; Shikama & Masujima, 1969).

Our phylogenetic analyses (Figs 2, 3) suggest that the *Lottia* clade (clade B2) diverged along with the *Nipponacmea* clade (clade B4) in the late Palaeocene. Judging from the molecular phylogenetic tree, migrations took place on both sides of the Pacific. The Aleutian-Commander arc of islands linking Alaska and Kamchatka provides a dispersal corridor for North Pacific species (Vermeij, Palmer & Lindberg, 1990). This is demonstrated by the close relationship between northwestern Pacific species (*Lottia cassis, Lottia* sp. cf. *borealis*) and northeastern Pacific species (*Lottia pelta, Lottia digitalis*) shown in Figure 2. In addition, northwestern Pacific species may have extended their ranges to the northeastern Pacific through the transport of larvae in currents that formed the northern part of a large gyre in the North Pacific (Fig. 4B).

The *Nipponacmea* clade (clade B4) most likely diverged during the Palaeocene and has remained in the Northwestern Pacific since then. Fossils of this genus from the Pliocene have been found in northwestern Honshu, Japan (Noda, 1973).

During a warm period of the Tertiary, *Cellana* and *Patelloida* extended their ranges of distribution (Lindberg & Hickman, 1986; Lindberg, 1988). However, these genera disappeared in Europe and North America following the start of a cool period (Lindberg, 1988).

Relatively recent geological events may have also impacted modern distributions of Patellogastropoda: the closing of the Panamic portal about 3.5 Ma (Keigwin, 1978), the closing of the Tethys Sea in the Miocene, and the opening of the Bering Strait during the Pliocene. In particular, 295 molluscan species took part in the interchange between the North Pacific and Arctic-Atlantic basins, after the opening of the Bering Strait (Vermeij, 1991). The Pacific lottiid, *Lottia*, immigrated into the Atlantic by transarctic migration (Carlton, Vermeij, Lindberg, Carlton & Dudley, 1991; Vermeij, 1991). As is clearly shown in the molecular phylogenetic trees (Figs 1B, 2), *Lottia testudinalis* living in the northern Atlantic is an immigrant from the Pacific. The fossil record of the genus *Lottia* is known from the Late Pliocene of California (Lindberg, 1988).

These hypotheses of molecular phylogeny and historical biogeography lead us to conclude that the modern geographic distribution patterns of patellogastropod limpets were established during the late Mesozoic and Cenozoic in association with plate tectonics and climatic changes following the breakup of Pangea. Changes in global geography and related oceanographic patterns are reflected in the biogeography of limpets.

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