

SYSTEMATIC REVISION OF *PATELLOIDA PYGMAEA* (DUNKER, 1860) (GASTROPODA: LOTTIIDAE), WITH A DESCRIPTION OF A NEW SPECIES

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ABSTRACT

Patelloida pygmaea (Dunker) and its closely allied species, *P. heroldi* (Dunker) and *P. conulus* (Dunker) have caused nomenclatural confusion because of their variable shell morphology and distinctive habitats. According to current nomenclature, these species of *Patelloida* have been synonymized and treated as one species with two ecological forms. *Patelloida pygmaea* lives on the shell of *Crassostrea gigas* (Ostreidae), *P. pygmaea* form *heroldi* occurs on intertidal rocks on sheltered shores and *P. pygmaea* form *conulus* is found on the shell of *Batillaria multiformis* (Batillariidae). Their taxonomic relationships and possible species status are, however, unclear. Using two mitochondrial genes (fragments of COI and 16S ribosomal RNA; total 1192 sites), we analysed 88 specimens of *P. pygmaea*, *P. pygmaea* form *heroldi* and *P. pygmaea* form *conulus* from 37 localities in East Asia. In the resulting molecular phylogenetic trees, the specimens of *Patelloida* fall into four clades with high bootstrap probabilities; these clades correspond taxonomically to *P. pygmaea*, *P. conulus*, *P. heroldi* and a fourth previously unrecognized taxon, *Patelloida ryukyuensis* n. sp., described here. A minimum-spanning network for 29 unique mitochondrial COI haplotypes obtained from 45 specimens in the same bay in central Japan form three distinct clusters, consisting of *P. pygmaea*, *P. conulus* and *P. heroldi*, respectively. This suggests that reproductive isolation has been established between each group. A detailed examination of radular and shell morphologies of the four taxa clarifies the morphological distinction between these species. The four species form a rather young clade in the genus *Patelloida* that diverged during the Pliocene. They provide an example of habitat segregation in a restricted marine environment.

INTRODUCTION

Patelloida pygmaea (Dunker, 1860) and its closely allied species, *P. heroldi* (Dunker, 1861) and *P. conulus* (Dunker, 1861) have caused nomenclatural confusion because of their variable shell morphology and habitats. *Patelloida pygmaea* lives on the shell of *Crassostrea gigas* (Ostreidae), *P. pygmaea* form *heroldi* occurs on rocks on sheltered rocky shores and *P. pygmaea* form *conulus* is found on the shell of *Batillaria* (Batillariidae) on intertidal mud flats, but occasionally also found attached to small boulders.

These were originally described as distinct species, *Patella (Acmaea) pygmaea* Dunker, 1860, *Patella heroldi* Dunker, 1861 and *Patella conulus* Dunker, 1861. *Patella heroldi* was thought to be synonymous with *Patella pygmaea* by Lischke (1869, 1871) and Pilsbry (1891, 1895). Pilsbry (1901) proposed *Acmaea heroldi* var. *signata* as an ecotype. However, some workers treated this species as a form of *Patella pygmaea*, while others treated it as a synonym. Habe (1944) placed *Patella pygmaea* in *Asteracmea* and *Patella heroldi* in *Collisella*, distinguishing them by coloration of the foot and egg. He considered *Patella conulus* to be synonymous with *Collisella heroldi*, based on shell morphology and coloration of the interior of the shell. Furthermore, he described *Patelloida (Asteracmea) lampanicola* as a new species based on the high conical shape of the shell and unique ecology living on the shell of *Batillaria*. But, later, he changed his mind and reduced *Patelloida lampanicola* to a synonym of *Patelloida conulus*, and assigned *Patelloida conulus* as a 'form' of *Patelloida pygmaea* (Habe, 1961). Habe (1964) considered *Patelloida pygmaea signata* (Pilsbry, 1901) to be a deep-water form of *Patelloida pygmaea*. Later, Kuroda, Habe & Oyama (1971) proposed

Chiazacmea pygmaea signatoides as a different ecotype of *Patelloida pygmaea*. Morton (1980) thought *Patelloida heroldi* to be synonymous with *Patelloida pygmaea*, but defined *Patelloida lampanicola* as a distinct species based on differences in radular teeth, shell morphology and behaviour. Based on examination of the lectotypes, Sasaki & Okutani (1994b) reduced *Collisella heroldi* to a synonym of *Patelloida pygmaea*, and noted that *Collisella heroldi* of Dunker is not what is called *Collisella heroldi* in Japan. Furthermore, they reclassified what is called *Collisella heroldi* in Japan into three distinct species, *Lottia kogamogai*, *Lottia tenuisculpta* and *Lottia lindbergi*, based on shell sculpture, radular teeth and basal plate morphology. Sasaki & Okutani (1994b) and Sasaki (1999) mentioned that the three species of *Patelloida* described by Dunker are all synonymous with *Patelloida pygmaea*, since they could not find any diagnostic characters to separate the species. In the most recent treatment of these limpets (Sasaki, 2000), the three species of *Patelloida* described by Dunker have all been synonymized, treated as *Patelloida pygmaea*, *Patelloida pygmaea* form *conulus* and *Patelloida pygmaea* form *heroldi*, while *Patelloida signatoides* was considered distinct, but the taxonomic relationships among them remain unclear.

Ecological forms within a species are known in some other groups of limpets (Jobe, 1968; Lindberg, 1979, 1981). The species inhabiting marine plants and algae tend to have highly specialized shells. The most striking example is the elongated and depressed form of *Lottia* (as *Notoacmea*) *depicta* on the leaves of the seagrass *Zostera* and its normal oval form attached to rocks on the shores of California, reported by Lindberg (1979). Similarly, elongate and laterally compressed shells are known in *Lottia alveus* living on *Zostera* (Carlton *et al.*, 1991), and in *Lottia* (as *Tectura*) *rosacea* and *Lottia* (as *Collisella*) *triangularis* attaching to branching coralline algae (Lindberg, 1981).

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Until recently, taxonomic decisions about individuals living in different habitats have been made by examining radular morphology. However, Simison & Lindberg (1999) resolved the taxonomic problem of the species complex of *Notoacmea fascicularis* by comparison of mitochondrial DNA sequences. Recently, Simison & Lindberg (2003) also clarified the species relationships between *Lottia strigatella* and *Lottia paradigitalis* using a molecular approach.

Thus, molecular techniques provide new tools for resolving taxonomic problems of limpets. In this paper, we used partial sequences of mitochondrial cytochrome-*c* oxidase I (COI) and large-subunit ribosomal RNA gene (16S rRNA) to resolve the taxonomic difficulties caused by the convergent and highly variable shell morphologies found in the species complex of *Patelloida pygmaea*.

MATERIAL AND METHODS

Field observation and collection of samples

Field observation and collection were done paying special attention to habitats of each species. We collected more than 500 specimens of *Patelloida pygmaea* (Fig. 1A, B), *Patelloida pygmaea* form *conulus* (Fig. 1C) and *Patelloida pygmaea* form *heroldi* (Fig. 1D) from embayments between Fukushima Prefecture, northeastern Japan and Okinawa Island, southwestern Japan, South Korea and Hong Kong. We could not find *P. pygmaea* form *conulus* in Hong Kong. Some specimens were donated by Dr Takenori Sasaki (University Museum, Tokyo University, Tokyo, Japan), Dr Katherine Lam (City University of Hong Kong, Hong Kong) and Dr Shinichi Sato (University Museum, Tohoku University, Sendai, Japan) (for all localities

see Fig. 2). As a result of the present phylogenetic analyses it became necessary to revise the specific classification of three forms of *Patelloida pygmaea* and one species of *Patelloida*. The revised names with locality information are presented in Table 1. All voucher specimens are deposited in the Laboratory of Geobiology, Department of Earth and Planetary Sciences, Nagoya University, Japan.

DNA extraction, PCR amplification and DNA sequencing

DNA was extracted from a single specimen from each locality, and two specimens from Tsuyazaki, Japan (Loc. S) and Myodo, South Korea (Loc. g) for constructing phylogenetic trees (Table 1). To construct the Minimum Spanning Network, DNA was also extracted from 15 specimens from each species living in Mikawa Bay, central Japan (Loc. F, G).

Living specimens were preserved in 80% ethanol. Total DNA was extracted from a 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 μ l of TEN buffer (10 mM Tris, pH 8.0, 10 mM EDTA, 10 mM NaCl), 20 μ l of 10% SDS, and 40 μ l 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.

Amplification of a part of the cytochrome-*c* oxidase I (COI) was achieved with the LCO1490 (5'-GGTCAACAAATCAT AAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGG TGACCAAAAAATCA-3') primer pair described by Folmer *et al.* (1994). Owing to the difficulty of amplifying some template

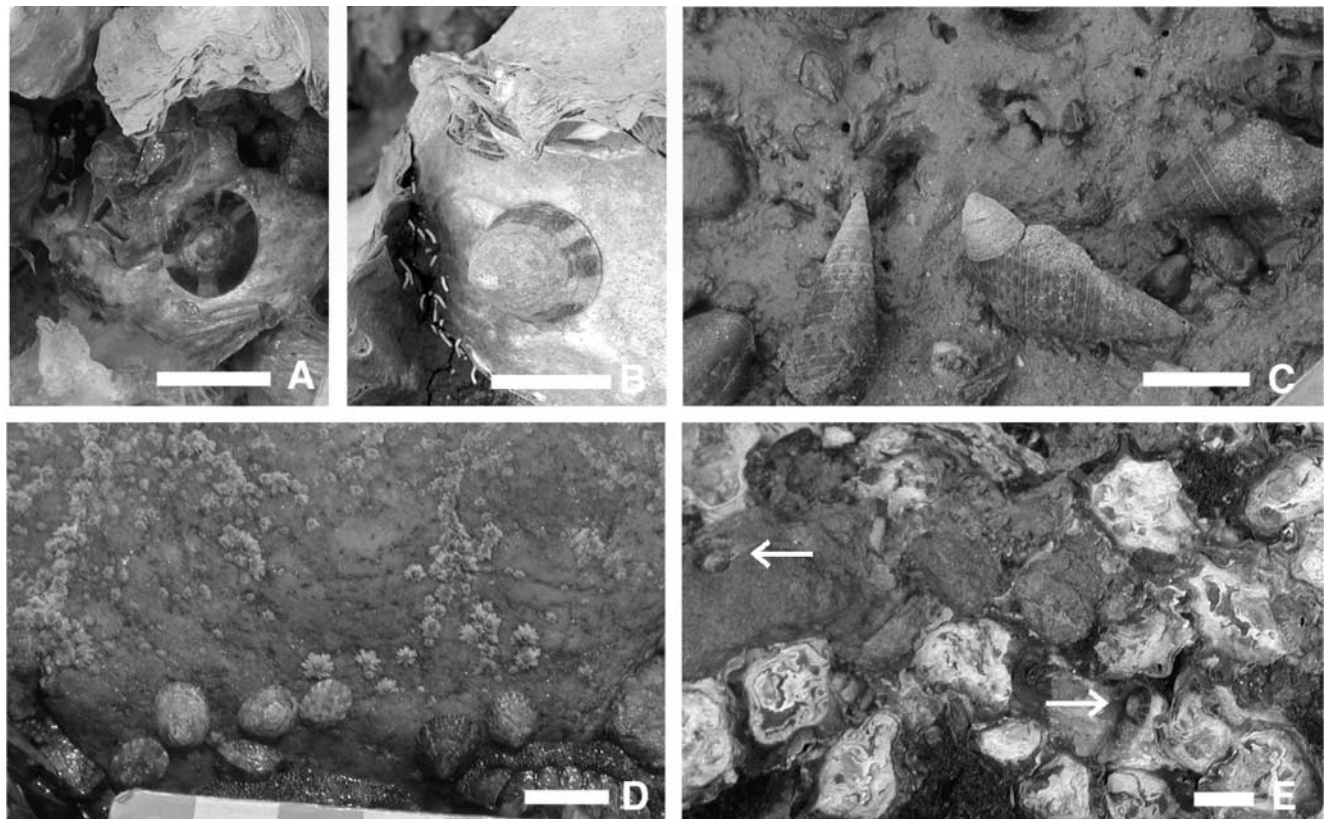


Figure 1. The ecology of *Patelloida*. **A, B.** Living *Patelloida pygmaea* on *Crassostrea gigas* at Shiokawa, Tahara, Aichi, Japan (Loc. G, see Fig. 2). **C.** Living *Patelloida conulus* on *Batillaria multiformis* at Shiokawa, Tahara, Aichi, Japan (Loc. G). **D.** Living *Patelloida heroldi* on the rocks at Nakanohama, Hazu, Aichi, Japan (Loc. F). **E.** Living *Patelloida ryukyuensis* n. sp. on *Saccostrea mordax* at Hanezi, Nago, Okinawa, Japan (Loc. c). Scale bar = 10 mm.

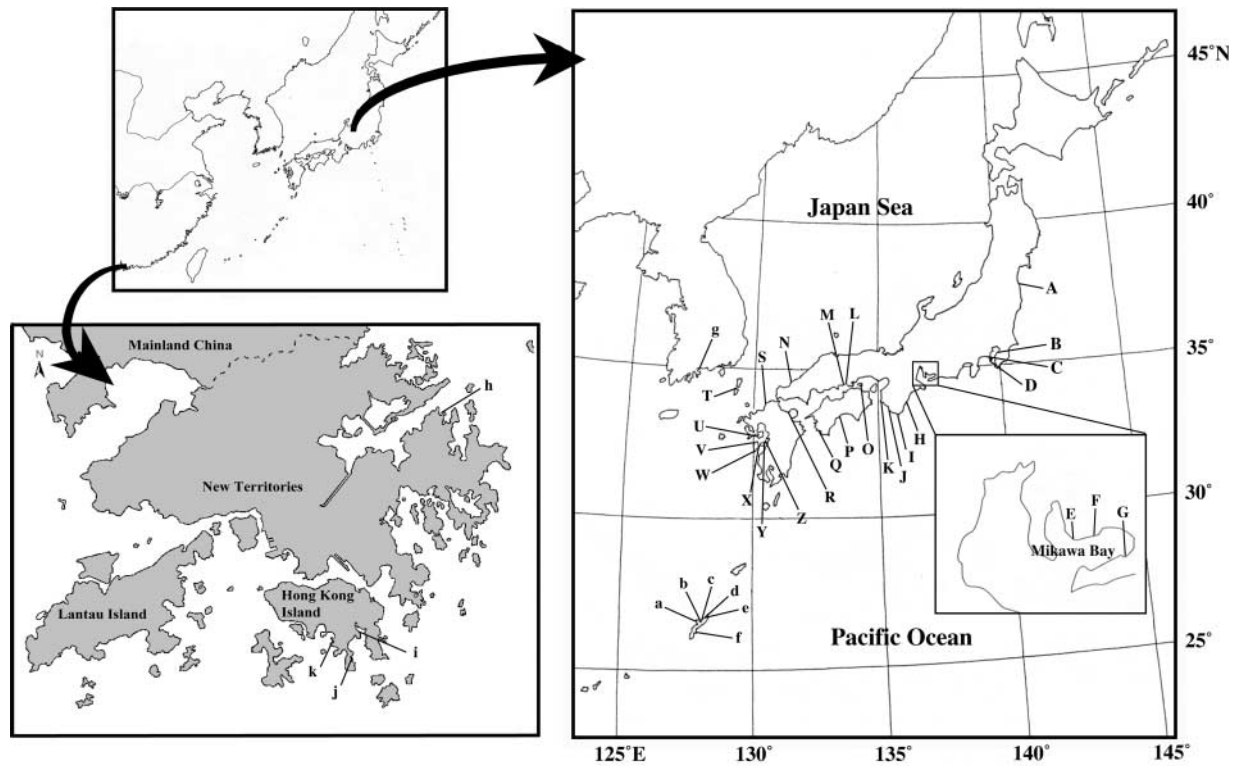


Figure 2. Localities of specimens used in the present study (Table 1).

DNA using LCO1490, COI30L (5'-CTATGTAYTTGCTTTT RGGGTTTTGGGG-3') was designed from aligned sequences of three species of *Patelloida*. A fragment of the mitochondrial large-subunit ribosomal RNA gene (16S rRNA) was amplified using the 16LRN13398 (5'-CGCCTGTTTAACAAAAACAT-3') and 16SRHTB (5'-ACGCCGGTTTGAAGCTCAGATC-3') primer pair designed by Kocher *et al.* (1989). PCR amplification was performed in 25 μ l of reaction volume containing 10 mM Tris-HCl pH 8.3, 50 mM KCL, 1.5 mM MgCl₂, 200 μ M dNTPs, 0.2 μ M each primer, 0.5 mg/ml BSA (Sigma), 2 units of Taq polymerase (Takara), and 1 μ l 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 54°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). Direct double-stranded cycle sequencing of 25–30 ng of COI and 16S PCR product was performed in both directions using the Applied Biosystems BigDye v. 3 dye terminator cycle sequencing kit. Cycle sequencing was performed using an Applied Biosystems GeneAmp PCR System 9700. The cycling parameters were 25 cycles of 10 s at 96°C, 5 s at 50°C, and 4 min at 60°C. Sequencing reaction products were purified using ethanol precipitation and analysed on an ABI PRISM 377 DNA sequencer.

Sequence analysis and phylogeny reconstruction

We determined the nucleotide sequences of a fragment of 16S rRNA ranging from 553 to 557 bp (*P. pygmaea*: 553 bp; *P. conulus*: 555 bp; *P. ryukyuensis* n. sp.: 556 bp; *P. heroldi*: 557 bp) and of COI exactly 631 bp. Sequences of 16S were aligned using ClustalX (Thompson *et al.*, 1997). Further minor manual adjustments to improve alignments were made by eye. Two areas within hypervariable loop regions of 16S were difficult

to align among the species. Regions where the alignment was ambiguous were excluded from the subsequent analyses as the sequences decrease the resolution of the phylogenetic analysis. After removal of sequence-ambiguous regions, 561 bp were retained for 16S. Sequence of COI was aligned using MacClade 4.03 (Maddison & Maddison, 2002) referring to translated amino acid sequence. Third codon positions of COI sequences were retained in all analyses.

The two genes were tested for congruence of phylogenetic signal using the incongruence length difference (ILD) test of Farris *et al.* (1995), as implemented by the partition homogeneity test in PAUP 4.0 version b10 (Swofford, 2002) (100 replicates). The partition homogeneity test detected no significant incongruity among gene partitions ($P = 0.30$). Sequences of 16S and COI were therefore combined and consequently a total of 1192 bp were used for constructing phylogenetic trees. Pairwise molecular distances of the partial 16S and COI genes were calculated by Kimura's two-parameter method (Kimura, 1980). Phylogenetic trees were constructed by the neighbour-joining (NJ) (Saitou & Nei, 1987), maximum parsimony (MP, unweighted) and maximum likelihood (ML) methods for 14 specimens of *Patelloida pygmaea* from 14 localities, 12 specimens of *Patelloida conulus* from 10 localities, eight specimens of *Patelloida heroldi* from eight localities and 10 specimens of *Patelloida ryukyuensis* n. sp. from 10 localities. Bootstrap analysis was used to assess the stability of each node. The outgroup comprised two species, *Patelloida striata* (Quoy & Gaimard, 1834) and *Patelloida signata* (Pilsbry, 1901). According to Nakano & Ozawa (2004), *Patelloida striata* is closely related to *Patelloida pygmaea* in the genus *Patelloida*. *Patelloida signata* used to be referred to as a deep-water form of *Patelloida pygmaea* (Habe, 1964). In the current nomenclature of limpets, this species is considered to be a distinct species, *Patelloida signatoides* (Sasaki, 2000). However, we believe *Patelloida signatoides* to be synonymous with *Patelloida heroldi signata* (Pilsbry, 1901), as shown by the

Table 1. Specimens and localities examined in this study for constructing phylogenetic tree.

Sample number	Species	Locality	In Figure 1	
L89	<i>Patelloida pygmaea</i> (Dunker, 1860)	Shiokawa, Tahara, Aichi, Japan	G	
L97		Esaki, Yamaguchi, Japan	N	
L107		Matsukawaura, Fukushima, Japan	A	
L108		Misho, Ehime, Japan	Q	
L111		Tsushima, Nagasaki, Japan	T	
L370		Kisarazu, Chiba, Japan	B	
L453		Samusaura, Shirahama, Wakayama, Japan	J	
L469		Tsuyazaki, Fukuoka, Japan	S	
L470		Kasaoka, Okayama, Japan	L	
L471		Matsunaga Bay, Fukuyama, Hiroshima, Japan	M	
L473		Hondo, Kumamoto, Japan	Y	
L474		Kawaura, Kumamoto, Japan	W	
L475		Nagaura, Matsushima, Kumamoto, Japan	Z	
L547		Jeonranam-do, Yeosu-shi, Myodo, South Korea	g	
L94		<i>Patelloida conulus</i> (Dunker, 1861)	Shiokawa, Tahara, Aichi, Japan	G
L463			Isshiki, Aichi, Japan	E
L464			Nagasu, Usa, Oita, Japan	R
L477	Matsunaga Bay, Fukuyama, Hiroshima, Japan		M	
L479	Nagaura, Matsushima, Kumamoto, Japan		Z	
L480	Hondo, Kumamoto, Japan		Y	
L482	Minamikushiyama, Nagasaki, Japan		U	
L483	Tomioka, Reihoku, Kumamoto, Japan		V	
L486	Tsuyazaki, Fukuoka, Japan		S	
L487	Tsuyazaki, Fukuoka, Japan		S	
L544	Jeonranam-do, Yeosu-shi, Myodo, South Korea		g	
L545	Jeonranam-do, Yeosu-shi, Myodo, South Korea		g	
L352	<i>Patelloida heroldi</i> (Dunker, 1861)		Yukinoura, Owase, Mie, Japan	H
L361			Izumo, Kushimoto, Wakayama, Japan	I
L364			Hayama, Kanagawa, Japan	C
L367			Usa, Tosa, Kochi, Japan	P
L430			Fukuda, Shodoshima, Kagawa, Japan	O
L431		Nakanohama, Hazu, Aichi, Japan	F	
L448		Yutsubo, Miura, Kanagawa, Japan	D	
L454		Mihama, Wakayama, Japan	K	
L371		<i>Patelloida ryukyuensis</i> n. sp.	Shiyoa fishing port, Okinawa, Japan	d
L372			Ogimi, Okinawa, Japan	e
L373	Yonashiro, Okinawa, Japan		f	
L450	Shinzato fishing port, Okinawa, Japan		a	
L465	Isagawa, Nago, Okinawa, Japan		b	
L466	Hanezi, Nago, Okinawa, Japan		c	
L518	Hoi Ha, Hong Kong		h	
L535	Repulse Bay, Hong Kong		i	
L536	Stanley, Hong Kong		j	
L540	Tai Tam Bay, Hong Kong		k	
L86	<i>Patelloida striata</i> (Quoy & Gaimard, 1834)	Ogimi, Okinawa, Japan	e	
L351	<i>Patelloida signata</i> (Pilsbry, 1901)	Hayama, Kanagawa, Japan	C	

photograph of the syntype specimen of *Patelloida heroldi signata* (Pilsbry, 1901) in Higo, Callomon & Goto (2001). The present phylogenetic analyses revealed that this species is clearly distinguished from *Patelloida pygmaea*. Thus, this specific name *Patelloida signata* is appropriate.

For examination of reproductive isolation, haplotype analysis based on the sequence of a fragment of COI from 15 individuals of each form of *P. pygmaea* collected from Mikawa Bay, central Japan (Fig. 2) was undertaken. The haplotypes

were defined using the ‘Redundant Taxa’ option in MacClade 4.03 (Maddison & Maddison, 2002), and the Minimum Spanning Tree (MST) (Kruskal, 1956; Prim, 1957) was constructed using ARLEQUIN version 2.0 (Schneider, Roessli & Excoffier, 2000). The MST is computed from the matrix of pairwise distances calculated between all pairs of haplotypes using a modification of the algorithm described in Rohlf (1973). The MST is convertible to a Minimum Spanning Network.

All sequences determined in this study have been deposited in DDBJ and GenBank under accession numbers AB161514–AB161632, AB196498–AB196511. The 16S sequence of *Patelloida striata* has already been published (Nakano & Ozawa, 2004), and has accession number AB106485.

Radular morphology

The radular was dissected and placed in 50% KOH at approximately 60°C for 5 min, and rinsed in distilled water. The radula was examined by scanning electron microscopy.

RESULTS

Molecular phylogeny

Pairwise molecular distances among 44 specimens (Table 2) show that intraspecific distance was low while interspecific distance was greater. In the all phylogenetic trees based on NJ, MP and ML (Fig. 3), the four clades were well supported (bootstrap greater than 90%) and correspond to *P. pygmaea*, *P. conulus*, *P. heroldi* and a new taxon, *P. ryukyuensis* n. sp.

The 29 unique mitochondrial COI haplotypes from 45 individuals obtained from Mikawa Bay, central Japan (Fig. 4) form three distinct clusters, consisting of *P. pygmaea*, *P. conulus* and *P. heroldi*. Interspecific distances were greater than intraspecific distances (Table 2). At this locality, *P. pygmaea* and *P. conulus* are found sympatrically.

Morphology

Examination of radula and shell of the four taxa discriminated by molecular phylogenetic analyses also revealed morphological distinction between the species. Although *P. heroldi* (Fig. 5E, F) and *P. pygmaea* (Fig. 5G, H) are similar in sculpture of the exterior surface of the shell, *P. heroldi* tend to be lower in profile than *P. pygmaea*. *Patelloida pygmaea* tends to have more convex slopes in its shell profile. Both taxa are similar in colour pattern of the shell. *Patelloida heroldi* exhibits broad brownish bands and a reticulate pattern in brown and greyish white, while *P. pygmaea* tends to lack a reticulate pattern. *Patelloida heroldi* is also distinguishable from *P. pygmaea* in the colour of the intermediate area between muscle scar and the shell margin: whitish blue in *P. heroldi*, white in *P. pygmaea*.

Patelloida pygmaea and *P. ryukyuensis* n. sp. (Fig. 5I–L) are closely similar, but with differences in the colour of the

shell and in body size. *Patelloida pygmaea* tends to be white with a few radial bands, while *P. ryukyuensis* n. sp. is tinged with brown. *Patelloida pygmaea* is larger and more convex than *P. ryukyuensis* n. sp.

Patelloida conulus (Fig. 5A–D) is highly variable in shell morphology and colour pattern. Although similar in shell colour and pattern to *P. heroldi*, *P. conulus* is characterized by having a high conical shell. The pattern of dark greenish brown with fine radial mottles has been found only in *P. conulus*. The intermediate area between the muscle scar and the shell margin varies from whitish blue like *P. heroldi* to white like *P. pygmaea*.

The radulae of these species are docoglossate, consisting of three pairs of lateral teeth and two pairs of marginal teeth. The radula of *P. heroldi* is readily distinguishable from that of others; the first lateral teeth are long and narrow, and the second lateral teeth are strongly elongated (Fig. 6A, B). The radula of *P. pygmaea* (Fig. 6E–H) is very similar to that of *P. ryukyuensis* n. sp. (Fig. 6I–L), and both taxa exhibit wide variation. Their radulae range from ‘slightly elongated’ to ‘rounded’ in form. In the slightly elongated form the first lateral teeth are long and narrow with a blunt cusp and the second lateral teeth are slightly elongated (Fig. 6E, F, I, J), while in the rounded form the first lateral teeth appear short and broad, and the second lateral teeth are rounded (Fig. 6G, H, K, L). The radula of *P. conulus* is also distinguishable from that of others; the first lateral teeth are short and broad with a blunt cusp and the second lateral teeth are trapezoid with rounded outer edges (Fig. 6C, D).

SYSTEMATIC DESCRIPTIONS

Family Lottiidae Gray, 1840
Genus *Patelloida* Quoy & Gaimard, 1834

Patelloida Quoy & Gaimard, 1834: 349 [type species by subsequent designation (Gray, 1847: 158), *Patelloida rugosa* Quoy & Gaimard, 1834].

According to Lindberg & Vermeij (1985), this genus consists of at least two subclades. One subclade includes species characterized by low to medium shell profiles, strong radial ribs or many fine riblets, reduced third lateral teeth, and with habitats including various substrata. The other clade, which has been called the *Patelloida profunda* group by Christiaens (1975) and Lindberg & Vermeij (1985), includes species characterized by

Table 2. Summary of range of distances for intraspecific and interspecific pairwise comparisons. Abbreviations; RY, Ryukyu Islands population; HK, Hong Kong population.

	16S	COI (all positions)	COI (excluding third codon position)
Intraspecific distances			
<i>P. pygmaea</i>	0.00%–0.74%	0.00%–1.12%	0.00%–0.48%
<i>P. conulus</i>	0.00%–0.73%	0.32%–1.93%	0.00%–0.72%
<i>P. heroldi</i>	0.18%–1.09%	0.00%–2.43%	0.00%–0.48%
<i>P. ryukyuensis</i> (RY + HK)	0.00%–10.37%	0.00%–10.05%	0.00%–1.93%
<i>P. ryukyuensis</i> (RY)	0.00%–1.83%	0.00%–0.64%	0.00%
<i>P. ryukyuensis</i> (HK)	0.54%–1.82%	0.00%–0.64%	0.00%–0.48%
Interspecific distances			
<i>P. pygmaea</i> vs <i>P. conulus</i>	11.04%–12.30%	19.07%–20.63%	2.45%–2.73%
<i>P. pygmaea</i> vs <i>P. heroldi</i>	11.02%–12.98%	19.20%–21.91%	2.94%–3.95%
<i>P. pygmaea</i> vs <i>P. ryukyuensis</i> (RY + HK)	13.18%–16.70%	19.64%–22.55%	3.95%–4.73%
<i>P. conulus</i> vs <i>P. heroldi</i>	11.56%–13.07%	20.18%–24.28%	2.43%–3.43%
<i>P. conulus</i> vs <i>P. ryukyuensis</i> (RY + HK)	13.12%–15.65%	16.87%–18.78%	2.68%–4.19%
<i>P. heroldi</i> vs <i>P. ryukyuensis</i> (RY + HK)	12.44%–13.99%	17.01%–19.27%	2.19%–4.72%

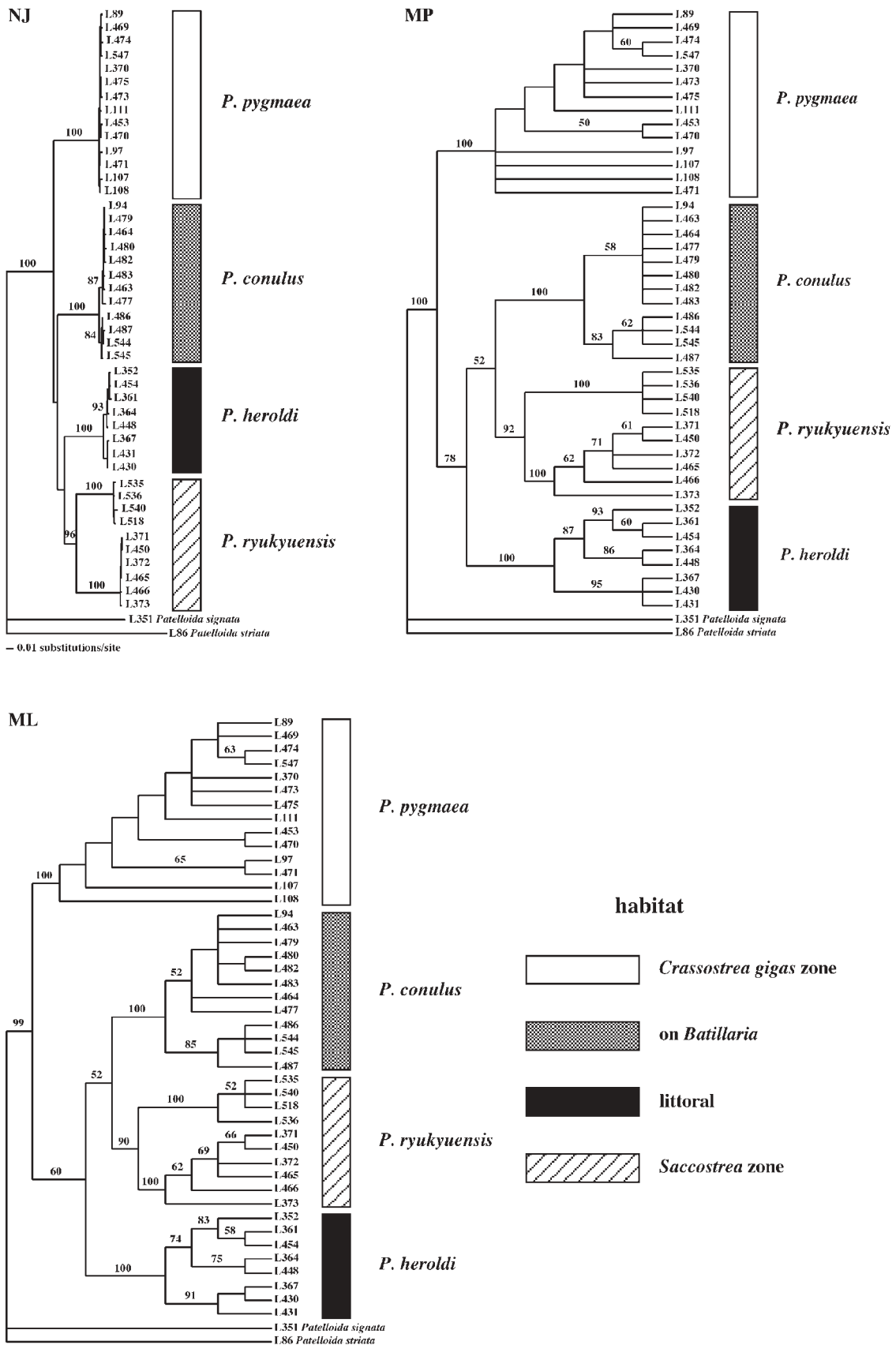


Figure 3. Three classes of phylogenetic tree (neighbour-joining, maximum parsimony, maximum-likelihood) resulting from the combined analysis of 16S and COI data (1192 bp) with bootstrap values (based on 1,000 replicates for NJ, 100 replicates for MP and ML) above each branch. *Patelloida striata* and *P. signata* were used as outgroups.

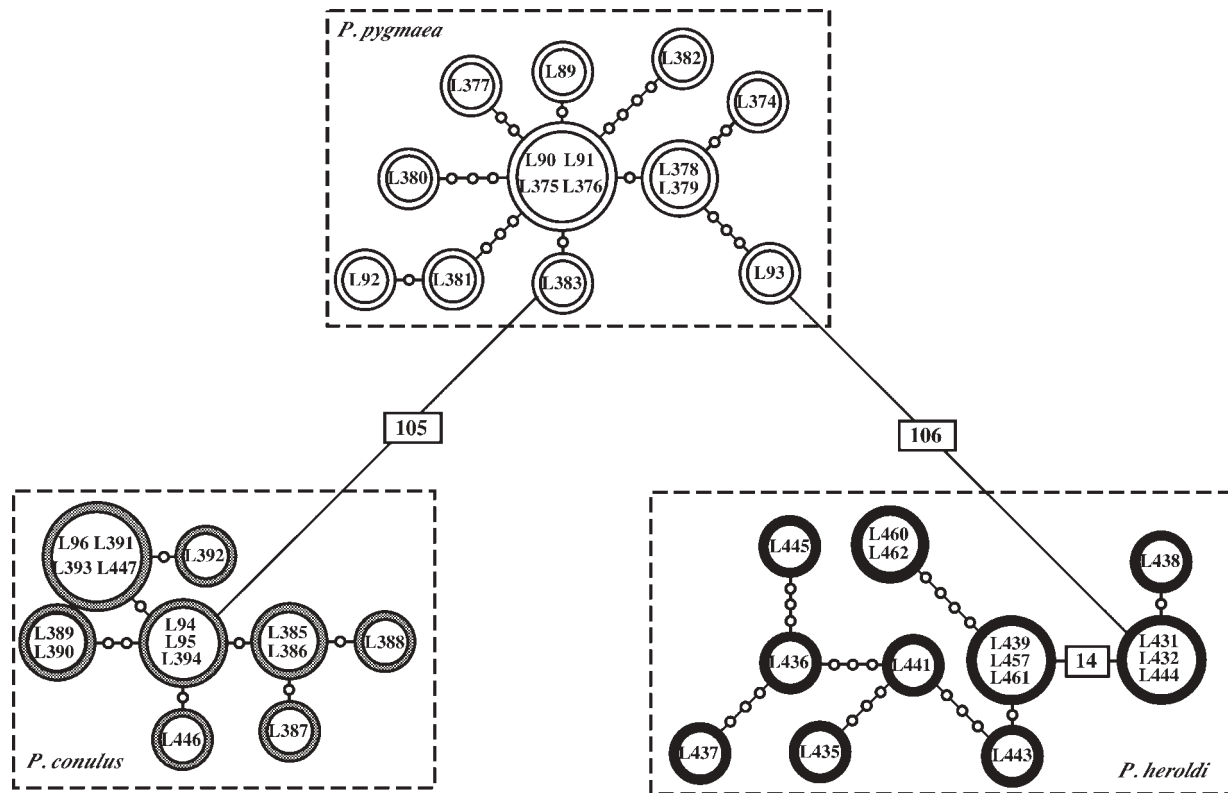


Figure 4. Minimum-spanning network for 29 unique mitochondrial COI haplotypes obtained from sample of 15 individuals of each species from Mikawa Bay. *Patelloida pygmaea* and *P. conulus* were collected in Shiokawa, Tahara, Aichi, Japan (Loc. G) and *P. heroldi* was in Hazu, Aichi, Japan (Loc. F). Open circles indicate inferred single mutation; numbers within square boxes indicate inferred number of mutation between species clusters.

medium to high shell profiles, many ribs, equal sized lateral radular teeth and habitats limited to calcareous substrata. The species discussed here belong to the former group.

***Patelloida conulus* (Dunker, 1861)**

(Figs 1C, 5A–D, 6C, D)

Patella conulus Dunker, 1861: 24, pl. 3, fig. 19 [Japan; neotype here designated NSMT-Mo 73598, 7.5 × 6.1 × 6.6 mm, from Shiokawa, Tahara, Aichi, Japan (Loc. G) (34°40' N, 137°18' E)]

Acmaea conulus—Pilsbry, 1895: 111.

Patelloida conulus—Higo, Callomon & Goto, 1999: 33.

Patelloida pygmaea form *conulus*—Sasaki, 2000: 28, fig. 10.

Patelloida (*Asteracmea*) *lampanicola*—Habe, 1944: 177 (Tanabe Bay, Wakayama, Japan; holotype NSMT Mo49770).

Patelloida (*Asteracmea*) *pygmaea lampanicola*—Habe, 1961: 5, pl. 3, fig. 6a.

Patelloida (*Chiazacmea*) *pygmaea lampanicola*—Habe, 1964: 7, pl. 3, fig. 6a. Habe & Kosuge, 1967: 7, pl. 4, figs 5–7.

Chiazacmea pygmaea lampanicola—Kuroda *et al.*, 1971: 25, pl. 8, figs 3, 4.

Patelloida pygmaea lampanicola—Okutani & Habe, 1975: 40.

Shell (Fig. 5A–D): Shell moderately thin, apertural margin very thin and fragile. Aperture circular, apex positioned centrally. Both anterior and posterior slopes from apex to margin are convex. Shell height variable from high to medium. Exterior sculpture delicate; faint radial riblets or fabric-like appearance. Colour pattern variable from dark greenish brown with fine radial mottling to greyish white with broad brownish radial bands or occasionally with reticulated pattern. Central area of shell inside muscle scar dark brown

or white. Intermediate area between muscle scar and shell margin white to bluish white. Interior margin narrow, reflecting exterior markings.

Radula (Fig. 6C, D): First lateral teeth short, broad, with blunt cusp. Second lateral teeth trapezoid, with rounded outer edge. Third lateral teeth reduced and rounded; separated from second lateral teeth except at bases. Two pairs of marginal teeth on radular membrane prominent and rounded.

Habitat (Fig. 1C): This species occurs mainly on the shell of *Batillaria multiformis* on sandy and muddy sedimentary shores at mid-intertidal level, but can also be found on the shell of *B. cumingii*, on small boulders and empty shells.

Distribution: Miyagi Prefecture to Kyushu, Japan and South Korea.

Remarks: This species is highly variable in shell morphology and colour. Consequently, the relationship between this species and *P. heroldi* has been confused. However, the species are not strictly sympatric; *P. conulus* occurs on sedimentary shores, whereas *P. heroldi* occurs on rocky shores.

The holotype of this species described by Dunker (1861) has been lost (Janssen, 1993; Janssen, personal communication), and we therefore designate a neotype.

***Patelloida heroldi* (Dunker, 1861)**

(Figs 1D, 5E, F, 6A, B)

Patella heroldi Dunker, 1861: 24, pl. 3, fig. 13.

Patelloida heroldi—Higo *et al.*, 1999: 33.

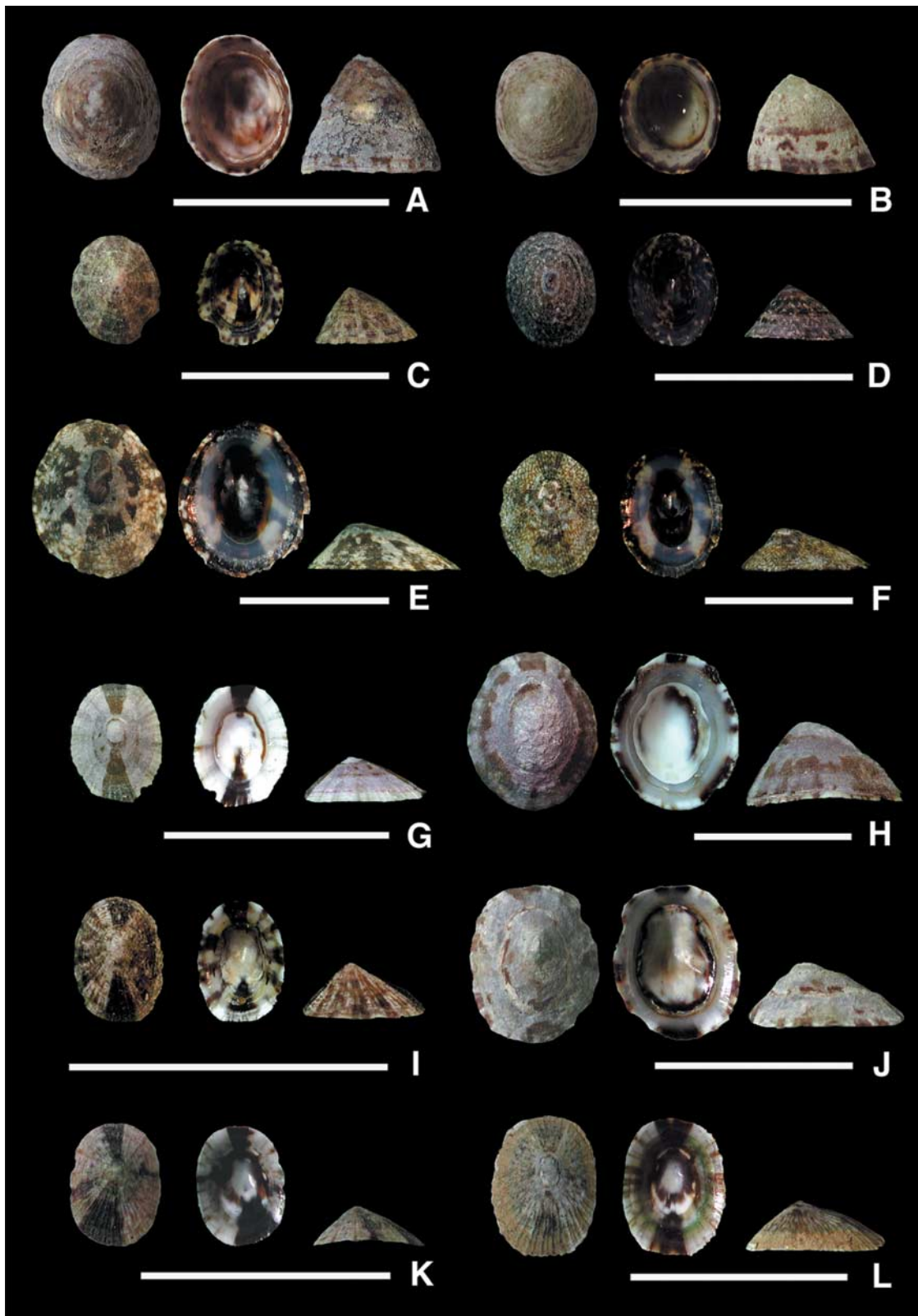


Figure 5. Shell morphology of *Patelloida* species. **A–D.** *Patelloida conulus*. **A.** Neotype, Shiokawa, Tahara, Aichi, Japan (Loc. G); 7.5 × 6.1 × 6.6 mm. **B.** Tsuyazaki, Fukuoka, Japan (Loc. S); 6.5 × 5.4 × 4.9 mm. **C.** Nagasu, Usa, Oita, Japan (Loc. R); 5.7 × 4.8 × 2.9 mm. **D.** Hondo, Kumamoto, Japan (Loc. Y); 6.1 × 4.9 × 3.2 mm. **E, F.** *Patelloida heroldi*. **E.** Yukinoura, Owase, Mie, Japan (Loc. H); 11.3 × 9.7 × 3.4 mm. **F.** Nakanohama, Hazu, Aichi, Japan (Loc. F); 9.2 × 7.0 × 2.8 mm. **G, H.** *Patelloida pygmaea*. **G.** Tsushima, Nagasaki, Japan (Loc. T); 6.2 × 4.8 × 2.0 mm. **H.** Shiokawa, Tahara, Aichi, Japan (Loc. G); 11.1 × 9.8 × 5.6 mm. **I–L.** *Patelloida ryukyuensis*. **I.** Holotype, Hanezi, Nago, Okinawa, Japan (Loc. c); 4.3 × 3.3 × 1.7 mm. **J.** Ogimi, Okinawa, Japan (Loc. e); 8.6 × 7.5 × 3.0 mm. **K.** Shinzato fishing port, Okinawa, Japan (Loc. a); 5.0 × 4.1 × 1.8 mm. **L.** Hanezi, Nago, Okinawa, Japan (Loc. c); 7.1 × 5.6 × 2.2 mm. Scale bar = 10 mm.

Patelloida pygmaea form *heroldi*—Sasaki, 2000: 28, fig. 8.
Patelloida (Asteracmea) pygmaea—Habe, 1944: 176 (not Dunker, 1860). Habe, 1961; 5, pl. 3, fig. 6 (not Dunker, 1860).
Patelloida pygmaea—Hirase & Taki, 1951: 56, fig. 4 (not Dunker, 1860).
Patelloida (Chiazacmea) pygmaea—Habe, 1964: 7, pl. 3, fig. 6. Habe & Kosuge, 1967, p. 7, pl. 4, fig. 8.
Chiazacmea pygmaea—Kuroda *et al.*, 1971: 24, pl. 8, figs 7, 8.
Patelloida pygmaea pygmaea—Okutani & Habe, 1975: 40.

Shell (Fig. 5E, F): Shell moderately thin, apertural margin very thin and fragile. Apex positioned in the anterior third of the shell. Apex often eroded and rounded. Anterior slope from apex to margin concave, posterior slope convex. Shell height medium to low. Exterior sculpture of numerous sharp radial ribs, typically broad brownish bands and reticulate pattern

in brown and greyish white. Central area of shell inside muscle scar brown. Intermediate area between muscle scar and shell margin bluish white. Exterior colour patterns showing through to interior surface. Interior margin broad, dark and reflecting exterior markings.

Radula (Fig. 6A, B): First lateral teeth long, narrow, with blunt cusp. Second lateral teeth strongly elongated, with blunt cusp; inner tooth margin concave, outer tooth margin convex. Third lateral teeth reduced, rounded triangular, separated from second lateral teeth except at bases. Two pairs of marginal teeth on radular membrane prominent and rounded.

Habitat (Fig. 1D): This species inhabits sheltered intertidal rocky shores. Vertical distribution ranges from mid to high intertidal zone.

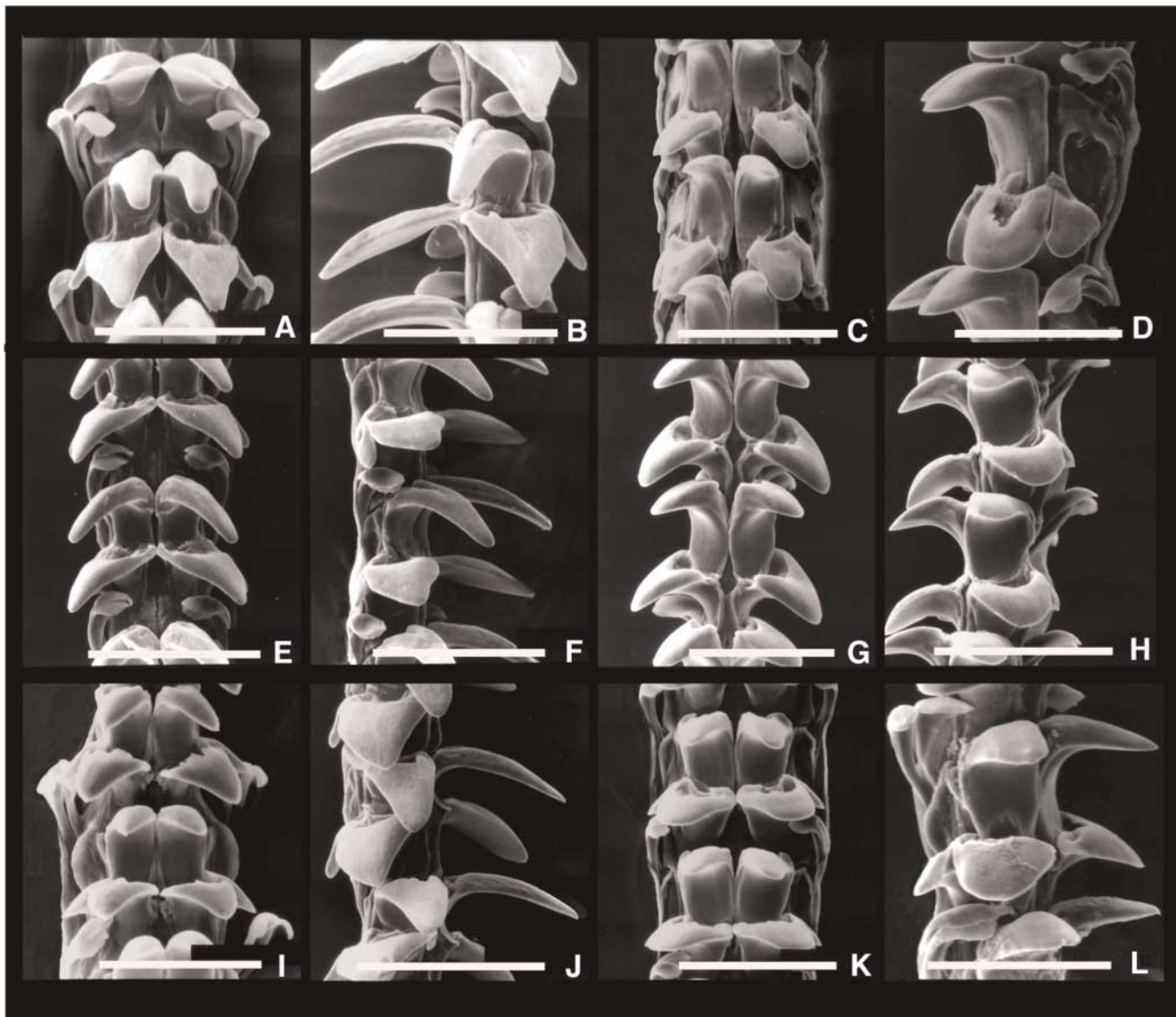


Figure 6. Radular morphology of *Patelloida* species. **A, B.** *Patelloida heroldi*. **A.** Dorsal view, Mihama, Wakayama, Japan (Loc. L). **B.** Lateral view, Nakanohama Hazu Aichi, Japan (Loc. F). **C, D.** *Patelloida conulus*. Dorsal (**C**) and lateral (**D**) view, Isshiki, Aichi, Japan (Loc. E). **E–H.** *Patelloida pygmaea*. Dorsal (**E**) and lateral (**F**) view, Kawaura, Kumamoto, Japan (Loc. W). Dorsal (**G**) and lateral (**H**) view, Hondo, Kumamoto, Japan (Loc. Y). **I–L.** *Patelloida ryukyuensis*. Dorsal (**I**) and lateral (**J**) view, Hanezi, Nago, Okinawa, Japan (Loc. c). Dorsal (**K**) and lateral (**L**) view, Hanezi, Nago, Okinawa, Japan (Loc. c). Scale bar = 100 μ m.

Distribution: Mutsu Bay and Ojika Peninsula to Kyushu, Japan and South Korea.

Remarks: Although *P. heroldi* was originally described as a distinct species (Dunker, 1861), it has commonly been identified as *P. pygmaea* (Dunker, 1860) as shown in the synonymy (Lischke, 1869, 1871; Pilsbry, 1891, 1895; Habe, 1971; Morton, 1980; Sasaki & Okutani, 1994b; Sasaki, 1999). Both species are clearly distinguished by the colour of the intermediate area of the shell interior and by radular morphology.

Note that the name *Collisella heroldi* has been misapplied (e.g. Kira, 1962; Habe & Kosuge, 1967) to species of *Lottia* (see Sasaki & Okutani, 1994b).

Patelloida pygmaea (Dunker, 1860)

(Figs 1A, B, 5G, H, 6E–H)

Patella (*Acmaea*) *pygmaea* Dunker, 1860: 234.

Patella pygmaea—Dunker, 1861: 24, pl. 3, fig. 20.

Patelloida (*Asteracmea*) *pygmaea*—Habe, 1944: 176.

Patelloida (*Chiazacmea*) *pygmaea*—Habe & Kosuge, 1967: 7, pl. 4, fig. 9.

Patelloida pygmaea pygmaea—Higo *et al.*, 1999: 33.

Patelloida pygmaea—Sasaki, 2000: 28, fig. 9.

Acmaea heroldi—Pilsbry, 1895: 111, pl. 6, figs 13–18 (not Dunker, 1861).

Patelloida pygmaea signata—Okutani & Habe, 1975: 40 (not Pilsbry, 1901).

Shell (Fig. 5G, H): Shell moderately thin, apex positioned in anterior third of shell. Both anterior and posterior slopes from apex to margin are convex. Shell height medium. Exterior sculpture of numerous sharp radial riblets. Colour pattern primarily white with a few radial brownish bands and lacking reticulate pattern. Central area of shell inside muscle scar typically white, occasionally brown in some specimens. Intermediate area between muscle scar and shell margin glossy white. Interior margin narrow, reflecting exterior markings.

Radula (Fig. 6E–H): Lateral teeth, especially second lateral teeth, variable from ‘slightly elongated’ to ‘rounded’ form. Slightly elongated form (Fig. 6E, F): first lateral teeth long, narrow with blunt cusp; second lateral teeth slightly elongated, inner tooth margin slightly concave, outer tooth margin convex; third lateral teeth reduced and rounded, separated from second lateral teeth except at bases; two pairs of marginal teeth on radular membrane are prominent and rounded. Rounded form (Fig. 6G, H): first lateral teeth short, broad with blunt cusp; second lateral teeth rounded, inner and outer tooth margins convex; third lateral teeth reduced and rounded, separated from second lateral teeth except at bases; two pairs of marginal teeth on radular membrane prominent and rounded.

Habitat (Fig. 1A, B): This species usually occurs on the shell of *Crassostrea gigas*, but it can also be found attaching to rocks in the *Crassostrea* zone.

Distribution: Miyagi Prefecture to Kyushu, Japan and South Korea.

Remarks: This species and *P. conulus* are sympatric on soft shores, but are clearly segregated in their habitat, *P. pygmaea* is attached to the shell of *Crassostrea gigas* and *P. conulus* to the shell of *Battilaria*. Morphologically, the species are distinguished by the shape and colour of the shell.

Patelloida ryukyuensis n. sp.

(Figs 1E, 5I–L, 6I–L)

Types: Holotype: NSMT-Mo 73592, 4.3 × 3.3 × 1.7 mm, Department of Zoology, National Science Museum, Tokyo; 5 paratypes NSMT-Mo73593–73597.

Type locality: Hanezi, Nago, Okinawa, Japan (Loc. c) (26°38′ N, 128°2′ E).

Shell (Fig. 5I–L): Shell moderately thin, apex positioned in anterior third of the shell. Anterior slope from apex to margin variable straight to slightly concave, and posterior slope convex. Shell height medium. Exterior sculpture of numerous sharp radial riblets. Aperture trapezoid with rounded outer edge. Colour pattern typically brown with a few radial dark brownish bands. Central area of shell inside of muscle scar white. Exterior colour patterns showing through to interior surface. Interior margin narrow and reflecting exterior shell. Aperture rounded to square.

Radula (Fig. 6I–L): lateral teeth, especially second lateral teeth, variable from ‘slightly elongated’ to ‘rounded’ form. Slightly elongated form (Fig. 6I, J): first lateral teeth long, narrow with blunt cusp; second lateral teeth slightly elongated, inner tooth margin slightly concave, outer tooth margin convex; third lateral teeth reduced and rounded, separated from second lateral teeth except at bases; two pairs of marginal teeth on radular membrane prominent and rounded. Rounded form (Fig. 6K, L): first lateral teeth short, broad with blunt cusp; second lateral teeth rounded, inner and outer tooth margins convex; third lateral teeth reduced and rounded, separated from second lateral teeth except at bases; two pairs of marginal teeth on radular membrane prominent and rounded.

Habitat (Fig. 1E): This species occurs in the *Saccostrea* zone on sandy bottoms. It is usually found on the shell of *Saccostrea mordax* in Ryukyu Islands and on *Saccostrea cucullata* in Hong Kong, but occasionally also on *Crassostrea angulata* or on rocks. Vertical distribution ranges from mid to high intertidal zone.

Distribution: Ryukyu Islands to Hong Kong.

Remarks: Although this species is very similar to *P. pygmaea*, these two species are distinguished by the colour of their shell and range of distribution. *Patelloida pygmaea* is white with a few radial brownish bands, while *P. ryukyuensis* n. sp. is tinged with brown. This species is smaller than *P. pygmaea*. *Patelloida pygmaea* is not found in the Ryukyu Islands or Hong Kong. Although the Hong Kong population forms a subclade in the clade of *P. ryukyuensis* n. sp. (Fig. 3), we assigned it *P. ryukyuensis* n. sp. since the individuals from Hong Kong were not distinguishable morphologically from those from the Ryukyu Islands.

DISCUSSION

Phylogeny and morphological evolution of Patelloida

Although the taxonomic relationships of the three species of *Patelloida* described by Dunker have been unclear, the results of this study provide unequivocal evidence of the distinctness of *Patelloida pygmaea*, *Patelloida heroldi*, *Patelloida conulus* and a fourth previously unrecognized taxon, *Patelloida ryukyuensis* n. sp. In the phylogenetic trees (Fig. 3), the four clades were strongly supported by high bootstrap probabilities (more than 90%). These

clades are clearly distinguished by their habitat, irrespective of their geographical distribution. Although *P. pygmaea* was thought to be distributed from Mutsu Bay and Ojika Peninsula in northeastern Japan to Okinawa in the south, and to Korea and China (Sasaki, 1999; Sasaki, 2000), the molecular phylogenetic trees revealed that the populations in Okinawa and Hong Kong can be distinguished from *P. pygmaea* as a distinct species. The minimum-spanning network of haplotypes from Mikawa bay (Fig. 4) shows three distinct clusters of *P. pygmaea*, *P. conulus* and *P. heroldi*, suggesting reproductive isolation between them.

These species are highly variable in shell morphology and colour pattern (Fig. 5). Ecological forms within a species are known in some groups of limpets (Jobe, 1968; Lindberg, 1979, 1981). The limpets living on sea grass and branching coralline algae have elongate and laterally compressed shells, for example *Lottia* (as *Notoacmea*) *depressa* (Lindberg, 1979), *Lottia alveus* (Carlton *et al.*, 1991) and *Lottia* (as *Tectura*) *rosacea* and *Lottia* (as *Collisella*) *triangularis* (Lindberg, 1981). Within the *P. pygmaea* group, *P. conulus* has a high-conical shell, and this may result from its attachment to the shell of *Batillaria*. *Notoacmea alta* attached to the shells of bivalves (especially *Branchidontes rostratus*) or gastropods also have a high-conical shell (Ponder & Creese, 1980).

Shell height variation is observed in many limpet species, and seems to be an ecophenotypic effect. For example, in the Japanese naecellid limpet *Cellana toreuma*, the ratio of height to length in samples from higher tidal levels is greater than in those from lower levels (Ino, 1935). This has been explained as a response to desiccation stress at higher levels (Segal, 1956; Vermeij, 1973; Branch, 1975) or to water turbulence (Simpson, 1985). The variation in the shell height of *P. heroldi* is likely to be affected by these ecological factors. However, the high-conical shell of *P. conulus* seems not to be explained by these factors. Because *P. conulus* usually has a high-conical shell in adult individuals, and its host *Batillaria* occurs intertidally on soft sediment, the tall shell of *P. conulus* may be a consequence of the limited space provided by its host species.

Colour pattern variation is also induced by ecological factors. In *Lottia* (as *Collisella*) *pelta* from California, individuals inhabiting the shells of *Mytilus* have blue-black shells, and those on brown algae are brownish, corresponding to the background colour (Lindberg, 1981). Eikenberry & Wickizer (1964) also reported *Lottia* (as *Acmaea*) *asmi* from *Mytilus* beds with black shells. *Patelloida pygmaea* on the shells of *Crassostrea* tend to have white shells without brown reticulate pattern, and *P. conulus* attaching to the shells of *Batillaria* are dark brown to black, corresponding to the shell colour of the host. Giesel (1970) has shown that *Lottia* (as *Acmaea*) *digitalis* has two major shell patterns, the type inhabiting colonies of the white goose-neck barnacle *Pollicipes polymerus* has a light shell with stripes, and the other found on rock surfaces has a dark shell with stripes. This cryptic effect is the result of selective visual predation by sea birds, and maintains the colour pattern polymorphism (Giesel, 1970; Hocky, Bosman & Ryan, 1987; Byers, 1989). Similar ecotypes are also known in some littorinid species dwelling among barnacles (Reid, 1993). On the other hand, *P. signata* and *Nipponacmea gloriosa* inhabiting coralline algae are reddish or reddish brown in colour (Sasaki & Okutani, 1993a). Both ecophenotypic (e.g. diet) and genotypic effects (natural selection by visual predator) have been invoked in these examples.

The morphological difference in radular teeth may have functional implications, being related to the diet (Steneck & Watling, 1982; Reid & Mak, 1999). Correlation between radular tooth morphology and diet has been reported in various groups of docoglossate limpets. Wood-eating species have saw-like teeth as in the genus *Pectinodonta* (Marshall, 1985) and *Potamacea fluviatilis* (Lindberg, 1990); kelp feeders have teeth with a broad, straight cutting edge as in *Discarria insessa* (Lindberg, 1988); species with blunt teeth feed on

coralline algae (Steneck, 1982; Lindberg & Vermeij, 1985; Sasaki & Okutani, 1993a, b). The radular morphology of *P. heroldi* with long, narrow lateral teeth (Fig. 6A, B) and of *P. conulus* with short, broad lateral teeth (Fig. 6C, D) may be adaptive in their habitats on hard and soft substrata, respectively. In *P. conulus*, the broad lateral teeth scrape the soft organic film from the shell of *Batillaria*. The radulae of *P. pygmaea* and *P. ryukyensis* n. sp. are variable and described as 'slightly elongated' or 'rounded' in form. Similar intraspecific variation is also known in *Nipponacmea habei* (Sasaki & Okutani, 1994a). The 'acute' and 'obtuse' forms of *Nipponacmea habei* are considered to be governed mainly by local micro-environmental factors and different diets. *Patelloida pygmaea* and *P. ryukyensis* n. sp. are commonly found attached to the shells of *Crassostrea gigas* and *Saccostrea*, respectively, but are also found on the rocks around oyster beds. These differences of microhabitat may produce the intraspecific variation in radular morphology. The individuals on *Crassostrea* or *Saccostrea* tend to exhibit the 'rounded' form, while those on rock tend to have the 'slightly elongated' form.

Habitat segregation of *Patelloida*

The genus *Patelloida* occurs on various substrata (Lindberg & Vermeij, 1985). Although the *Patelloida saccharina* group inhabits exposed rocky shores, the *P. pygmaea* group can be found on sheltered shores. The *Patelloida profundata* group appears to be restricted to high intertidal to supratidal calcareous substrata. These groups are phylogenetically distinct (Kirkendale & Meyer, 2004).

On the intertidal rocky shores where many limpets co-occur, their habitats are clearly subdivided. This has been demonstrated for species of *Lottia* by Haven (1973), Stimson (1973), Dehnel (1978), Sasaki & Okutani (1994b) and *Nipponacmea* by Sasaki & Okutani (1993a). However, there are few records of limpets living on other molluscs. Within the *P. pygmaea* group, *P. heroldi* and *P. pygmaea* co-occur on sandy shores with *Crassostrea*, and *P. pygmaea* and *P. conulus* can be found sympatrically on muddy shores with *Crassostrea* and *Batillaria*. But *P. pygmaea* and *P. conulus* are segregated on different hosts at the same tidal level, and *Patelloida heroldi* and *P. conulus* do not live together on the same shores. The species attaching to other molluscs possibly recognize their hosts by chemical means, as the veliger of *Crassostrea* recognizes adults (Knight-Jones, 1951). This facility is also found in barnacles, *Semibalanus balanoides* (Crisp & Meadows, 1962, 1963). Indeed, Morton (1980) and Kawai (personal communication) have demonstrated that the adult of *P. conulus* has a positive attraction to *Batillaria* in laboratory studies. It also appears that *P. conulus* may move from one host shell to another in order to obtain sufficient food, since this species is sometimes found on empty shells or small boulders.

The association of limpets living on other molluscs may provide symbiotic benefits for both species. *Patelloida conulus* on *Batillaria* keeps the host free from encrusting barnacles and rock oysters. In turn, *Batillaria* provide *P. conulus* with a hard substrate on sedimentary shores. In addition, breeding potential may be enhanced by bringing males and females into proximity, as suggested for *Capulus ungaricus* inhabiting *Turritella* (Thorson, 1965). Although *Crassostrea* and *Saccostrea* also provide *P. pygmaea* and *P. ryukyensis* n. sp. with a substrate, these species may rather use their bivalve host as a hiding place, as in the *Mytilus*-form of *Lottia* (as *Collisella*) *pelta* and *Lottia* (as *Collisella*) *asmi* (Lindberg, 1981).

Estimation of approximate divergence time and fossil record of *Patelloida*

The earliest fossil of this genus has been found in the Upper Cretaceous (Albian) of England (Akpan, Farrow & Morris, 1982),

and a fossil dating to the Late Cretaceous (Campanian) has been found in California (Lindberg, 1983). Furthermore, *Patelloida* species are common in near-shore sediments in North America and Europe during the Tertiary (MacClintock, 1967; Lindberg, 1983; Lindberg & Hickman, 1986). *Patelloida* reaches its highest diversity in the Australasian region at present. This genus is thought to have extended its distribution westwards from the tropical Tethys by riding the palaeoequatorial current and to have expanded northwards during a warm period (Nakano & Ozawa, 2004).

Within the *Patelloida pygmaea* group, *Acmaea schrenckii* has been reported by Yokoyama (1926) from the Lower Pleistocene in Sado Island in the Japan Sea and is assignable to *P. heroldi*. In addition, there are a few fossil records of this group in the middle to upper Pleistocene of Japan. Fossils of *P. conulus* have been found from the late middle Pleistocene at Oga Peninsula, northwestern Japan (Takayasu *et al.*, 1986), and of *P. pygmaea* from the late Pleistocene of the Hokuriku region, Japan Sea coast of central Japan (Matsuura, 1977). These young fossil records and the close morphological resemblance among the members of the *P. pygmaea* group may suggest that it is a rather young clade in the genus *Patelloida*.

Patelloida conulus consists of two subgroups corresponding to their geographical distributions (Fig. 3). The populations of the *P. conulus* of the Japan Sea side are genetically different from those of the Pacific side. Such intraspecific genetic variation has also been found in *Batillaria multiformis* (Kojima *et al.*, 2003) and *Turbo (Batillus) cornutus* (Kojima, Segawa & Hayashi, 1997), and was considered to have been caused by different warm currents, namely the Tsushima current on the Japan Sea side and the Kuroshio current on the Pacific side. This evidence suggests that the phylogenetic evolution of *P. conulus* may be closely linked with that of its host species *B. multiformis*. Hence, molecular phylogenetic analyses of the Japanese *Batillaria* based on the sequence of 12S and 16S ribosomal RNA (Ozawa, 2000) and cytochrome-*c* oxidase I (Kojima *et al.*, 2001) can be used to infer the age of the symbiotic relationships between *P. conulus* and its host species, *B. multiformis*. The oldest fossil of the lineage of *B. cumingi* has been reported from the lower Pliocene (*ca* 5 Ma) in the Sendai area, northeastern Japan (Ozawa, 2000). According to molecular clock estimates using this age as a reference date, divergence of *B. multiformis* and *B. flectosiphonata* occurred around 3 Ma (Ozawa, 2000). Assuming that the divergence of *P. conulus* was synchronized with that of its host species, *B. multiformis*, the members of the *P. pygmaea* species complex are likely to have diverged almost contemporaneously in the Pliocene.

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