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A New Species of Patelloida (Gastropoda: Lottiidae) from Western Bo Ron Ga Island, Northern Rakhine Coastal Region, Myanmar

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1. Introduction

Marine lottiid limpets occupy intertidal habitats in temperate to tropical latitudes worldwide (Edmonds and Vaughn, 2007). Being ecosystems, they can provide many goods and services by creating habitats then used by other species and can modify the physical and chemical environment, with major consequences for rocky shore populations, communities, and food webs (Cech et al., 2006).

Limpets of the genus Patelloida are a widespread and ecologically important group of gastropods (Dahlberg et al., 2003). The coastal areas along the Rakhine Coastal Region are species-rich regions of Patelloida limpets.

Three limpet families dominate in the coastal waters of Rakhine State; in order from the north in Bo Ron Ga Island, Bay of Hunters, to the south in the Andrew Bay these are Keyhole or Slit limpets (Family Fissurellidae), Lottiid

ABSTRACT

Lottiid limpets (Lottiidae, genus Patelloida) occur on the rocky shore of the upper intertidal zone in marine habitats, providing many ecosystem goods and services. In this study, the species identification of an "adulterant" limpet with a small body size, which is often misidentified as a "young individual" of other sympatric species, including P. saccharina and P. novacula, was determined for the first time, based on molecular markers (partial mitochondrial cox1 and rrnL genes), phylogenetic analysis, and morphometric approaches. This novel species, P. arakanensis, commonly known as the "boulder limpet" in Myanmar, appears to potentially influence the efficiency of *P. novacula*. Phylogenetic analyses confirm its status as the basal taxon of the Indo-Pacific Patelloida. A comparative study of the shell characteristics of P. arakanensis, and other Patelloida species revealed several distinctive morphological traits, including generally smaller body size, a deeply cupped shell that is convex in adults but flat in young individuals. Other distinctive features of the new species include growth concentric line traits that are unique compared with the sympatric *P. novacula* and *P. saccharina* species, such as a deeper growth line in the fast-growth phase after settlement, followed by a significantly shallower growth line and mass disperse during subsequent life stages. This study provides the basic information necessary for further ecological and population genetic studies on this new species.

> limpets (Family Lottiidae) and Nacellid true limpets (Family Nacellidae) (Naung-Naung-Oo, 2022).

> This study was initiated following a series of field investigations of the Estuarine and Marine Molluscs of Myanmar (Naung-Naung-Oo, 2022). The initial intention was to collect specimens of unidentified young Patelloida individuals for population genetic analysis. As the shell morphology of this species is phenoplastic and greatly affected by habitat and so employed partial mitochondrial cox1 gene sequencing as a molecular marker for species identification. According to descriptions from experienced local researchers, most non-Myanmar limpets are considered to be P. saccharina (the Broad-ribbed limpet), a species sympatric with P. novacula and commonly considered an "adulterant limpet" in Myanmar. The initial molecular data supported this claim; however, further studies unexpectedly identified some cox1 sequences that match neither those of P.

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saccharina, nor those of any other known species of the genus *Patelloida*. Therefore, it appears these individuals represent a hitherto undescribed species from this region.

The objective of this study was to determine the specific identity of this Arakanese limpet species using molecular markers (partial mitochondrial *cox1* and *rrnL* genes), phylogenetic analysis, and morphometric approaches. Morphological descriptions of shell characters and a brief record of the limpet life history obtained in this study will provide the basic information necessary for further ecological and population genetic studies of this new species.

2. Materials and Methods

2.1. Sample collection

Samples of the unidentified species of limpet were collected from western Bo Ron Ga Island (= Mye-Ngu-Kyun) in the northern Rakhine Coastal Region (Table 1). In field collection, individuals were sampled randomly selected on different rock boulders or ridges and transported to the laboratory alive.

2.2. DNA extraction, PCR amplification and sequencing

The genomic and mitochondrial DNA of each individual was extracted from adductor muscle or visceral mass using the TIANamp Marine Animals DNA kit (Tiangen, Beijing). A partial *cox1* segment was amplified by polymerase chain reaction (PCR) with primer pairs of COIL1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and COIH2198 (5'-TAAACTTCAGGGTGAACAAAAAAATCA-3'). The primer pair of 16sar (5'-CGGCTGTTTATCAAAAACAT-3') and 16sbr (5'-CCGGTCTGAACTCAGAATCACGT-3') was used to amplify the partial *rrnL* segment. PCR reactions were performed in a 25 µl volume with 0.5 µl template DNA

(approximately 30 ng), $0.5 \ \mu$ l 10 mM dNTP mix, $2.5 \ \mu$ l 10 × buffer (Mg²⁺ plus), 1 μ l of each primer (10 μ M), and 0.25 μ l (1 U) Ex*Taq* polymerase (Takara, Dalian, China). The PCR reactions were performed on an ABI Veriti thermal cycler (Applied Biosystems, California, USA) with the following parameters: pre-denaturation at 94 °C for 1 min, followed by 35 cycles of 94 °C for 20 sec, 45-55 °C annealing temperature (52 °C for *cox1* and 50 °C for *rrnL*) for 20 sec, extension at 72 °C for 1 min, and a final extension step at 72 °C for 5 min. PCR products were separated by electrophoresis on a 1% agarose gel, purified with QIAquick PCR Purification kit (QIAGEN, California, USA), and bi-directionally sequenced on an ABI 3730x1 DNA Sequencer (Applied Biosystems, California, USA).

2.3. Molecular identification and phylogenetic analysis

A basic local alignment search (nucleotide blast) was performed to find regions of local similarity between sequences in Genbank for each cytochrome c oxidase subunit I (cox1) and large mitochondrial ribosomal RNA subunit (rrnL) sequence examined in this study. To confirm the taxonomic status of the morphologically novel species obtained in this study, cox1 and rrnL sequences of the new species and those of other species of Patelloida, Lottia and Nipponacmea available from Genbank were subjected to the phylogenetic analysis (Table 2).

It is common to find several sequences representing different isolates of a species in Genbank. Thus, in order to simplify analysis and to avoid using incorrect sequences caused by misidentifications, RefSeq sequences, namely complete mitochondrial genomes proofed by Genbank staff, were used for several species for which they were available. MEGA 5 (Funk and Omland, 2003) was used for sequence alignments.

Location	Date	No. of individuals	Species (number, %)
Mye-Ngu-Kyun, Rakhine Coastal Region	Feb, 2020	85	P. novacula (56, 66%) P. arakanensis (25, 29%) P. saccharina (4, 5%)
Andrew Bay, Rakhine Coastal Region	Apr, 2014	55	P. novacula (31, 56%) P. mufria (24, 44%)
Sitaw, Taninthayi Coastal Region	Mar, 2021	97	P. novacula (31, 32%) P. arakanensis (55, 57%) P. saccharina (11, 11%)
Hmyaw-Yit, Taninthayi Coastal Region	Apr, 2018	33	P. novacula (33, 100%)

Maximum likelihood (ML), neighbour-joining (NJ), and maximum parsimony (MP) were employed for phylogenetic reconstructions. MP analyses were performed using Phylogenetic Analysis Using Parsimony, PAUP 4.0b10 (Swofford 2002), with a total of 1000 random addition searches using TBR (a tree-rearrangement: tree bisection reconnection). Bootstrap (BP) values were calculated from 1000 bootstrap replicates with 10 random additions per replicate in PAUP. NJ and ML analyses were performed using PhyML 3.0 online execution. We initially used the nacellid true limpets *Cellana orientalis* (Nacellidae) as an outgroup to investigate the relationship between the three limpet genera *Patelloida, Lottia* and *Nipponacmea*. All analyses yielded

topology indicating that *Patelloida* is a clade separate from *Lottia* and *Nipponacmea* (data not shown). Thus, in order to make maximum usage of alignment characters, we performed phylogenetic inferences for *Patelloida* species using *Lottia* spp. and *Nipponacmea* spp. as multiple outgroups.

2.4. Description of shell characters

Conchological distinctions used for the identification of other *Patelloida* limpets were employed for the description of shell features and characters of the new species (Mangel and Chamberlin, 2004), including shape and surface sculpture, external and internal shell colour, attachment area of valves, and position, colour and relative size of the adductor muscle scar.

3. Results

3.1. Molecular identification and phylogeny

The *cox1* gene was sequenced from a total of 270 limpet specimens and used to identify the limpet species by comparison with sequences available in Genbank (Table 2). Using a Blastn search, 151 sequences were identified as originating from *P. novacula*, 15 from *P. saccharina* and 24 from *P. mufria*; the remaining 80 sequences were nearly identical to each other but did not precisely match any sequences in the database (Table 1). These sequences are thought to identify a hitherto undescribed species, now

termed *Patelloida arakanensis*. The partial *rmL* gene was also sequenced from the mtDNA of these 80 individuals and used for phylogenetic analysis.

Among the 80 sequences of each gene, we found three intact haplotypes of rmL (hap 1–3) and seven (hap 1–7) haplotypes of cox1 (without indels), a variation pattern expected for gastropod mt genes. Sequences representing different haplotypes obtained in this study have been deposited in the Genbank under accession numbers NC2698938–NC2698965 (Table 3).

Table 2. List of the samp	les and sequences from	Genbank used in this study
	*	2

Species	Locality	cox1	rrnL
Lottia dorsuosa	Andaman Sea	KM221108.1	AB106502.1
Lottia pelta	Andaman Sea	AB238476.1	AB106491.1
Lottia scabra	Bay of Bengal	KJ006004.1	AB106504.1
Nipponacmea concinna	Bay of Bengal	AB238486.1	AB106511.1
Nipponacmea fuscoviridis	Bay of Bengal	AB263734.1	AB263733.1
Nipponacmea gloriosa	Andaman Sea	AB238488.1	AB106515.1
Nipponacmea nigrans	Andaman Sea	AB238490.1	AB106516.1
Nipponacmea schrenckii	Andaman Sea	AB238492.1	AB238364.1
Patelloida alticostata	Andaman Sea	AB238513.1	AB238382.1
Patelloida arakanensis	Bo Ron Ga I., Rakhine State, Myanmar	NC2698938	NC2698965
Patelloida conulus	Bay of Bengal	AB238514.1	AB196500.1
Patelloida corticata	Andaman Sea	AB287120.1	AB238384.1
Patelloida insignis	Andaman Sea		AB238386.1
Patelloida latistrigata	Bay of Bengal		AB238387.1
Patelloida lentiginosa	Bay of Bengal	AB238517.1	AB238388.1
Patelloida mimula	Andaman Sea		AB238389.1
Patelloida mufria	Bay of Bengal	AB287126.1	
Patelloida novacula	Andaman Sea	NC_503053.1	
Patelloida pygmaea	Bay of Bengal		AB161514.1
Patelloida saccharina	Andaman Sea	AY628326.1	
Patelloida saccharina lanx	Andaman Sea	AB238521.1	AB106487.1
Patelloida saccharinoides	Andaman Sea	AB238523.1	AB238392.1
Patelloida striata	Andaman Sea	AB161589.2	AB238394.1

Table 3. Systematic identification of new species

Taxa	ID description	Registration Log-No
Phylum	Mollusca Cuvier, 1795	(NCBI. 6447)
Class	Gastropoda Cuvier, 1795	(NCBI. 6448)
Order	Archaeogastropoda Thiele, 1925	(NCBI. 382158)
Family	Lottiidae Gray, 1840	(NCBI. 69676)
Genus	Patelloida Quoy & Gaimard, 1834	(NCBI. 72693)
Species	P. arakanensis	(NC2698938–NC2698965) new species

The data set based on *cox1* and *rrnL* genes contains a large number of informative characters for phylogenetic analysis. The *cox1* sequences include 574 aligned nucleotide positions, with 222 parsimony-informative characters, while 122 characters are informative in the *rrnL* dataset (of 406 bp total).

Although tree topologies and nodal supports are not completely identical between phylogenies based on the *rrnL* and *cox1* datasets, several important similarities can be seen, including 1) monophyly of the three limpet genera (*Patelloida*, *Lottia* and *Nipponacmea*); 2) separation of the Indian limpets (*P. conulus, P. exilis, P. rugosa* and *P. alticostata*) from the Indo-Pacific limpet group; and 3) resolution of the new species, *P. arakanensis*, as the most basal taxon in the Indo-Pacific limpet group (Fig. 1).

3.2. Systematic species description

3.2.1. Type measurements and deposition

The holotype specimen and one paratype specimen comprised of dry shells and tissues preserved in 95% ethanol have been deposited in the Museum of the Department of Marine Science, Sittway University, Rakhine State, Myanmar. The holotype and all paratype specimens were genetically identified. Shell measurements of the type materials are shown in Table 4.

3.2.2. Description of the holotype

The shell of *P. arakanensis* (Table 3) is solid, and opaque, with a variable and rather elevated shape. Outline roughly elongate-ovate, strongly scalloped, somewhat narrowing anteriorly. Apex subcentral, frequently eroded. External sculpture of 7 to 11 large, raised radial ribs that strongly

project at the margin giving the shell the appearance of a webfoot, and weaker riblets in the interstices. Main radial ribs are sometimes more numerous (up to 13 ribs in the form *saccharinoides*, and to 20 in the Australian subspecies *stella*).



Fig. 1. Phylogenetic reconstruction of *Patelloida* limpet using partial mitochondrial *rmL* and *cox1* gene sequences. The neighbor-joining (NJ) tree is shown. Bootstrap (BP) values were calculated for maximum likelihood (ML), NJ and maximum parsimony (MP) analyses. Nodes with high statistical support (BP \geq 75) from all three phylogenetic methods (i.e. NJ, ML and MP) are marked with a solid circle while moderate supports (50 \leq BP < 75) are marked with a rhombus. Three haplotypes of *rmL* (hap 1–3) and seven (hap 1–7) haplotypes of *cox1* of *P. arakanensis* were presented

Interior smoothish, with low radial undulations corresponding to the main outer sculpture. Colour: outside of shell greyish white, with dark grey or brown banding in the interstices of ribs, sometimes forming V-shaped marks towards the margin. Interior porcelaneous white, rimmed or spotted with black on the margin; apical region olive green, yellow or whitish and profusely speckled with brown spots or blotches (Fig. 2).

Table 4. Shell measurements	(mm)) of the type	materials
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Accession number	Width	Length	Depth	Notes
Holotype MSS.0001	2.21	17.28	6.5	Porcelaneous white adductor muscle scar
Paratype 1 MSS.0002	3.69	16.66	4.6	Yellowish white adductor muscle scar
Paratype 2 MSS.0003	6.39	17.20	6.2	Dark purple adductor muscle scar
Paratype 3 MSS.0004	5.04	20.91	5.1	White adductor muscle scar



Fig. 2. Types of Patelloida arakanensis collected from western Bo Ron Ga I., northern Rakhine Coastal Region

3.2.3. Variability in shell characters

Patelloida arakanensis generally has a small body size compared to other members of the genus. The shell morphology of this species is phenoplastic and greatly affected by niche, as seen in congeneric limpets. The inner shell is usually deeply cupped as seen in the holotype and paratype 2 specimens (Fig. 2). The outer shell is usually convex in adults and flat in young individuals. The attachment area is variably small, usually not more than half the shell height. The adductor muscle scars varied in colour from dark orange to yellowish white with light purple growth lines.

3.2.4. Anatomy

The anatomy of *P. arakanensis* is indistinguishable from that of other identified *Patelloida* species as described by Keegan, Gill and Ptashne (2007).

3.2.5. Ecology

P. arakanensis occurs both on rock boulders and in rock wall of tide pools, where individuals can be found on intertidal and subtidal rocks along the shoreline of Bo Ron Ga Island, Rakhine Coastal Region, that is, under marine conditions.

Animals with mature gonads are present during the period from April to September with spawning occurring primarily in (June-August). In Bo Ron Ga Island, three species, *P. novacula*, *P. arakanensis* and *P. saccharina*, appear to have strong niche overlap; in particular, the mass spawning time of *P. novacula* and *P. arakanensis* is almost identical. The lifehistory strategies of *P. novacula* and *P. arakanensis* may be distinguished in two ways in stages after larval attachment. *P. arakanensis* uses a fast-growth strategy during the first growth phase, and hence is likely to prevail in areas where it settles (e.g., on smooth rock surface).

3.2.6. Etymology

This is the first new species of the Lottiidae recorded from Bo Ron Ga Island (Lat. 19°55' N, Long. 92°58' E), northern Rakhine Coastal Region, Myanmar, and is thus named after the locality in which the limpet was originally found. In Rakhine, this limpet is known as the "Grin-Chat-Sue" because the shells of juvenile individuals on rock boulders are usually shaped like belly-button.

4. Discussion

The majority of known oyster species were described on the basis of shell morphology. However, phenotypic plasticity means that shell characters alone may not be adequate to identify species of *Patelloida*, particularly for young

individuals. Instead, both morphological and genetic characteristics should be considered when identifying species. The most recently described new species, *P. novacula*, was the first commercially important taxon with plastic morphology to be identified using genetic data (Shaw et al., 2005). Subsequently, both nuclear and mitochondrial genes have been used to distinguish species and infer phylogenetic relationships (Eisen, 1998; Forster and Symons, 2009). We note that the phenotypic plasticity of *P. arakanensis* shell morphology and its comparatively small body size prevented researchers from identifying this species previously. Instead, specimens of *P. arakanensis* were often misidentified as young individuals of other sympatric species, including *P. saccharina* and *P. novacula*.

Table 5. List of Patelloida species in different databases (Burgess and Jendrisak, 2002)

Species	WoRMS	WMSDB	Burgess and Jendrisak	eoL	NCBI	Distribution
P. alticostata (Angas, 1865)	\checkmark	-	-	-	\checkmark	Tropical Indo-Pacific
P. conulus (Dunker, 1861)	\checkmark	\checkmark	-	-	\checkmark	Tropical Indo-Pacific
P. corticata (Hutton, 1880)	\checkmark	-	-	-	\checkmark	Tropical Indo-Pacific
P. insignis (Menke, 1843)	\checkmark	-	-	-	\checkmark	Tropical Indo-Pacific
P. latistrigata (Angas, 1865)	\checkmark	\checkmark	-	\checkmark	\checkmark	Tropical Indo-Pacific
P. lentiginosa (Reeve, 1855)	\checkmark	-	-	-	\checkmark	Tropical Indo-Pacific
P. mimula (Iredale, 1924)	\checkmark		-	-	\checkmark	Tropical Indo-Pacific
P. mufria (Hedley, 1915)	\checkmark	\checkmark	-	\checkmark	\checkmark	South East Asia
P. novacula (Linnaeus, 1758)	-	-		-	\checkmark	Tropical Indo-Pacific
P. pygmaea (Dunker, 1860)	\checkmark	-	-	-	\checkmark	South East Asia
P. saccharina (Linnaeus, 1758)	\checkmark	\checkmark	\checkmark	-	\checkmark	South East Asia
P. saccharina lanx (Reeve, 1855)	\checkmark	-	\checkmark	\checkmark	\checkmark	South East Asia
P. saccharinoides (Habe and Kosuge, 1996)	\checkmark	-	-	\checkmark	\checkmark	South East Asia
P. striata (Quoy and Gaimard, 1834)		-	-	-		South East Asia

We identified P. arakanensis as a new species based on evidence from the following sources. First, its shell shape is similar to that of other Patelloida species. In particular, both inner shells have no commissural projections; this is a primary synapomorphy of the genus Patelloida, not shared by species from closely related genera such as Patelloida and Lottia. Secondly, mtDNA sequence-based phylogenetic analyses unambiguously confirm its taxonomic status as a member of the Indo-Pacific Crassostrea. Our inference is reasonable because 1) the molecular markers (i.e., partial cox1 and *rrnL* genes) used in this study have been widely and successfully used in phylogenetic reconstructions of marine gastropods including oyster drills, cowries and periwinkles (Pauling et al., 2006); and 2) molecular phylogeny inferred in this study unambiguously agrees with the morphological taxonomy of true limpets (i.e., Lottiidae family), particularly the well-supported monophyly of Indo- Pacific Patelloida, American Patelloida, Nipponacmea and Lottia. Additionally, studies of the morphological characteristics of the shells of P. arakanensis and other Indo-Pacific Patelloida species have shown that there are several distinctive traits that distinguish the new species from other known species.

In Myanmar, at least six *Patelloida* limpets have already been described based on morphological characters, including *P. mufria* (Hedley, 1915), *P. pygmaea* (Dunker, 1860), *P. saccharina* (Linnaeus, 1758), *P. saccharina lanx* (Reeve, 1855),

P. saccharinoides Habe and Kosuge (1965) and *P. striata* Quoy and Gaimard (1834) (Oo, 2022).

In order to determine whether P. arakanensisis a synonym of other non-Indo-Pacific Patelloida species, we reviewed all species of this genus listed in WoRMS (World Register of Marine Species), WMSDB (Worldwide Mollusc Species Data Base), eoL (Encyclopedia of Life), in Burgess and Jendrisak (2002), and NCBI (National Center for Biotechnology Information). All species listed in these databases have been confirmed by professional taxonomists and hence have reference value for Patelloida species worldwide. A total of 35 species are recognized in these sources (Table 5) and 14 of these species have DNA sequences available in Genbank. For the remaining 21 species, morphological descriptions, photographs, and other information (e.g., habitat and distribution) are available. These identifying characteristics of each species were carefully compared with those of the new taxon. The results showed that *P. arakanensis* differs in many aspects from each of these known Patelloida species.

The description of *P. arakanensis* provides valuable information for wild collection in Rakhine Coastal Region, Myanmar. The mass mortality of juveniles (commonly thought to be radial ripped limpets) on rock boulders was previously presumed to be caused by nutrient limitation due

to high-density shell settlement. We have now learned that the shell on rock boulders that suffer high mortality are primarily those of *P. arakanensis* rather than radial ripped limpets. No further study has been made, ecologically or physiologically, regarding the mechanism that triggers mass mortality of this species after the first fast-growing season. According to our preliminary investigation, the niche occupancy rate of *P. arakanensis* can reach as high as 100%.

5. Conclusion

This study provides the basis for future population genetics and ecological studies of *P. arakanensis*, a new member of the "true limpets" that plays a significant role in nearshore ecosystems. Furthermore, our findings reveal that the species diversity of Indo-Pacific limpets was hitherto underestimated and that mitochondrial DNA-based molecular diagnosis should be a powerful tool for future taxonomic work on this challenging group.

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