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EVOLUTION OF SYMBIOSIS WITH *LINGULA* (BRACHIOPODA) IN THE BIVALVE SUPERFAMILY GALEOMMATOIDEA (HETERODONTA), WITH DESCRIPTION OF A NEW SPECIES OF *KOREAMYA*

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ABSTRACT

Many members of the bivalve superfamily Galeommatoidea have symbiotic associations with other marine benthic invertebrates. Among them, *Koreamya arcuata* (A. Adams, 1856) is distinctive because it is the only known bivalve symbiotic with brachiopods. Here we describe *Koreamya setouchiensis* n. sp. as the second example in this genus, based on specimens collected in and around the Seto Inland Sea, Japan. Similar to *K. arcuata*, this bivalve species attaches to the anterior end of the shell valve of living *Lingula anatina* Lamarck, 1801 by means of byssal threads. However, shell morphologies of the two bivalve species are clearly different; *K. setouchiensis* has an ovate shell, while *K. arcuata* has an elongated-triangular shell. These morphological differences are probably due to the difference in posture on the hosts. To understand how symbiotic association with *Lingula* evolved in Galeommatoidea, we performed molecular phylogenetic analyses using three nuclear (18S, 28S and H3) and one mitochondrial (COI) genes. The two *Koreamya* species with remarkably differently shaped shells were monophyletic, suggesting that their symbiotic associations with *Lingula* have the same evolutionary origin. Furthermore, the *Koreamya* clade formed a monophyletic group with anemone-associated galeommatoideans (*Nipponomontacuta actinariophila* and *Montacutona* sp.). This result and their morphological similarities suggest the possibility of host switching between sea anemones and *Lingula*.

INTRODUCTION

Galeommatoidea are a superfamily of tiny marine bivalves that have diversified greatly in shallow waters (Coan, Valentich Scott & Bernard, 2000; Bouchet *et al*., 2002; Paulay, 2003; Coan & Valentich Scott, 2012). Interestingly, many members of this bivalve group have symbiotic (mostly commensal) associations with marine benthic invertebrates (Boss, 1965; Morton & Scott, 1989). Symbiotic galeommatoideans usually live on the host's body or inside the host's burrow (Morton, 1988; Morton & Scott, 1989) and are considered to use these sites as a shelter from predators and to benefit from the water currents created by the hosts, which are rich in oxygen and organic particles (Morton, 1988; Morton & Scott, 1989; Goto, Hamamura & Kato, 2007). Except for a few exceptions (Ockelmann & Muus, 1978; Li & Ó Foighil, 2012), most symbiotic galeommatoideans use one to several closely related species as hosts (Sato *et al*., 2011), indicating that their host specificity is relatively high. In contrast, the host spectrum of galeommatoideans as a whole is surprisingly broad, including the phyla Arthropoda, Annelida, Echinodermata, Mollusca and Brachiopoda (Boss, 1965; Morton & Scott, 1989). Recent molecular phylogenetic study has shown that host switching among various host taxa has played a key role in diversification of this bivalve group (Goto *et al*., 2012). However, our knowledge of the evolutionary history of their host associations remains limited.

Among symbiotic galeommatoideans, *Koreamya arcuata* (Adams, 1856) is distinctive, because it is the only bivalve known to have a symbiotic association with brachiopods (Fernando & Fernando, 1983; Savazzi, 1991, 2001; Lützen, Hong & Yamashita, 2009; Sato *et al*., 2011). This bivalve lives attached by byssal threads to the shell valve of *Lingula*

(Lingulidae, Brachiopoda) (Savazzi, 2001; Lützen, Hong & Yamashita, 2009). *Lingula* are filter feeders with two shell valves and an elongated pedicle, and construct vertical burrows in shallow-water soft sediments (Emig, 2000). Various ectosymbionts of *Lingula* have been reported, but most are facultative symbionts (Hammond, 1984). In contrast, *K. arcuata* is an obligate symbiont (Savazzi, 2001; Lützen, Hong & Yamashita, 2009). It is an intriguing question as to how such a tight association with *Lingula* evolved in *Koreamya*.

A recent study suggested that *K. arcuata* is morphologically similar to *Montacutona* species, which are associated with sea anemones (Lützen, Hong & Yamashita, 2009). They share several morphological characteristics, such as a lithodesma in the hinge, a highly reduced outer demibranch and a particular type of sperm receptacle (Lützen, Hong & Yamashita, 2009). If *Koreamya* and *Montacutona* are truly closely related, host switching may have occurred between *Lingula* and sea anemones. On the other hand, *K. arcuata* was previously considered to be identical to the galeommatoidean *Curvemysella paula* (Adams, 1856) (Morton & Scott, 1989), which has a symbiotic association with hermit crabs (Morton & Scott 1989; Goto *et al*., 2007). This is probably because both bivalve species have crescent-shaped or elongated-triangular shells, which are apparently similar, although the two species clearly differ in their dentition and anatomical characteristics (Lützen, Hong & Yamashita, 2009).

In this study, we aim to understand how symbiotic association with *Lingula* evolved in Galeommatoidea, and report molecular phylogenetic analyses using two nuclear ribosomal genes (18S and 28S rRNA), one nuclear protein gene (histone H3) and one mitochondrial protein gene (cytochrome *c* oxidase subunit I, COI) of diverse galeommatoideans, including two *Koreamya* species, and eight outgroup species. In addition, we describe a new species of *Koreamya* based on the specimens collected in and around the Seto Inland Sea, Japan. Finally, we discuss the morphological and ecological adaptations of *Koreamya* to symbiotic association with *Lingula*.

MATERIAL AND METHODS

Sample and sequence data collection

We collected four specimens of tiny galeommatoidean bivalves attached to the shells of *Lingula anatina* Lamarck, 1801 in the muddy sand flats in and around the Seto Inland Sea, Japan, between 2009 and 2012 (Figs 1, 2A). Three of the bivalve specimens were stored in 100% ethanol and the other was stored in 70% ethanol after being fixed in 10% formaldehyde. Two of the three specimens stored in 100% ethanol were used for the molecular phylogenetic analyses (Tables 1, 2). In addition, total genomic DNA samples of four specimens of *Koreamya arcuata* extracted by Sato *et al*. (2011) were used for the molecular phylogenetic analyses (Tables 1, 2); two specimens were collected from *L. anatina* in Seondo-ri, Biin-myeon, Seocheon-gun, Chungcheognam-do, South Korea (Figs 1, 2B; Table 1), while the other two specimens were collected from *L. adamsi* Dall, 1873 in Woldoo, Yongjeong-ri, Hyeongyeong-myeon, Muan-gun, Jeollanam-do, South Korea (Fig. 1; Table 1) (Sato *et al*., 2011). Although the shell morphology of *K. arcuata* differs slightly between the two groups found on different *Lingula* species, the molecular analyses based on COI and ITS1 genes suggested that there is no genetic evidence to separate them (Sato *et al*., 2011). We used sequence data of galeommatoideans and outgroups from GenBank to reconstruct the molecular phylogeny (Table 2).

Molecular methods

Total genomic DNA was isolated from the bivalves following a previously described method (Goto *et al*., 2012). A small piece of soft tissue was homogenized in 800 μl of lysis buffer and incubated at 55°C overnight, after which 80 μl of saturated potassium chloride was added to the lysate. This solution was incubated for 5 min on ice and then centrifuged for 10 min. The

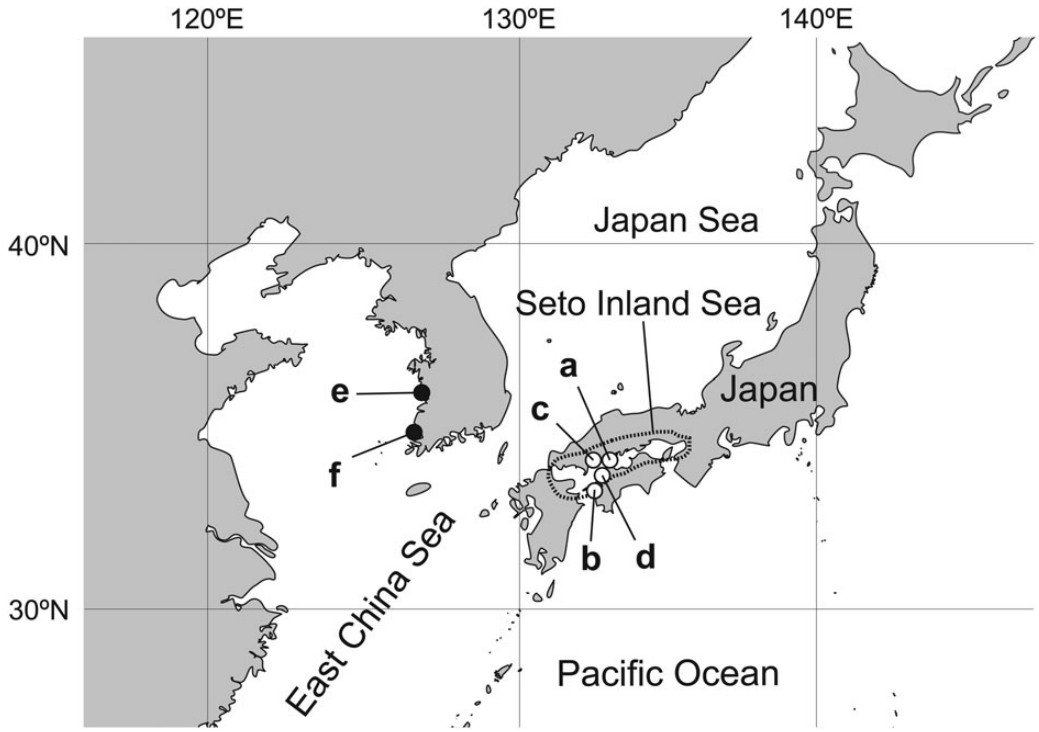

Figure 1. Sampling locations of *Koreamya setouchiensis* n. sp. (a–d) and *K. arcuata* (e, f). Japan: a, Hakatajima Island (type locality of *K. setouchiensis*); b, Maajiro; c, Nigata; d, Gogoshima Island. South Korea: e, Seondo-ri; f, Yongjeong-ri.

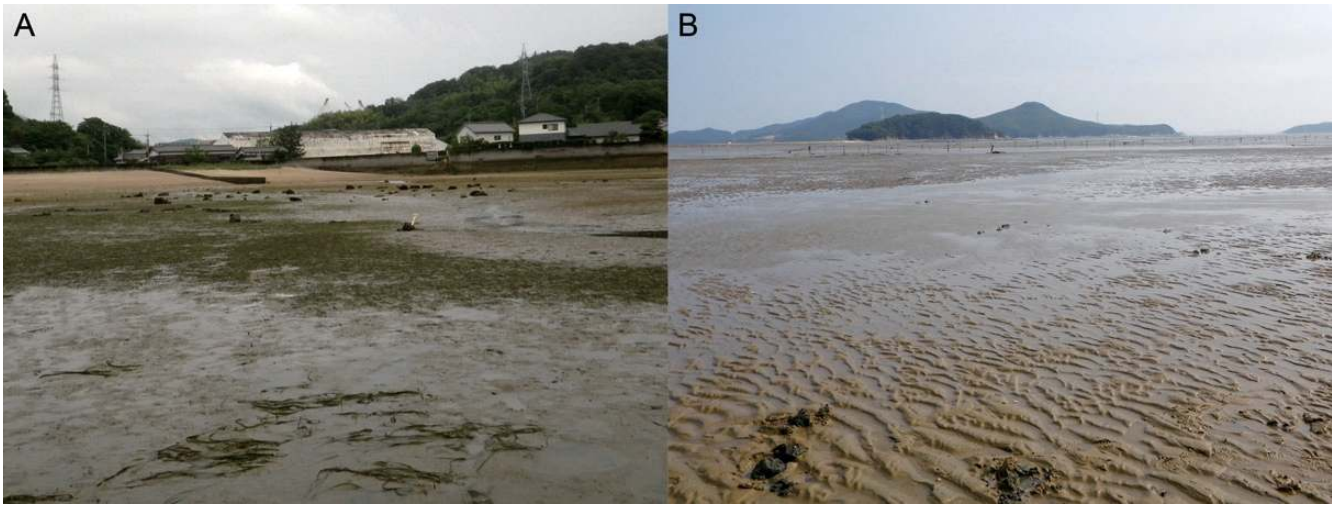

Figure 2. Habitat of *Koreamya setouchiensis* n. sp. and *K. arcuata*. **A.** Muddy sand flat at Hakatajima Island, Seto Inland Sea, Japan, type locality of *K. setouchiensis*. **B.** Mud flat at Seondo-ri, Biin-myeon, Seocheon-gun, Chungcheongnam-do, South Korea, sampling location of *K. arcuata*. (Photographs: **A**, R. Goto; **B**, S. Sato.)

Table 1. Sampling information of specimens from which sequences were newly obtained in this study.

Species	Code	Locality	Host
Koreamya setouchiensis	NSMT-Mo 78585	Maajiro, Yawatahama, Ehime, Japan	*Lingula anatina*
Koreamya setouchiensis	NSMT-Mo 78587	Gogoshima, Matsuyama, Ehime, Japan	*Lingula anatina*
Koreamya arcuata	TUMC-1190962-1	Seondo-ri, Seocheon-gun, South Korea	*Lingula anatina*
Koreamya arcuata	TUMC-1190962-2	Seondo-ri, Seocheon-gun, South Korea	*Lingula anatina*
Koreamya arcuata	TUMC-1190969-1	Yongjeong-ri, Muan-gun, South Korea	*Lingula adamsi*
Koreamya arcuata	TUMC-1190969-2	Yongjeong-ri, Muan-gun, South Korea	*Lingula adamsi*

supernatant (700 µl) was transferred to a new tube, cleaned once with a phenol/chloroform solution, and precipitated with an equal volume of 2-propanol. The DNA pellet was rinsed with 70% ethanol, vacuum-dried and dissolved in 100 µl TE buffer.

We sequenced fragments of the 18S, 28S, H3 and COI genes. Polymerase chain reactions (PCRs) were used to amplify ~1700 bp of 18S, ~1000 bp of 28S, ~350 bp of H3 and ~700 bp of COI. Amplifications were performed in 20-µl mixtures consisting of 0.4 µl of forward and reverse primers (20 µM each; primer sequences are provided in Table 3), 2.0 µl of ExTaq buffer, 1.6 µl of dNTPs (2.5 µM each), 0.1 µl of ExTaq polymerase (TaKaRa, Otsu, Japan) and 15.1 µl of distilled water. Thermal cycling was performed with an initial denaturation for 3 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at a gene-specific annealing temperature (Table 3) and 2 min at 72°C, with a final 3 min extension at 72°C. The sequencing reaction was performed using PCR primers and internal primers (Table 3) and a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and electrophoresed on an ABI 3130 sequencer (Applied Biosystems). The obtained sequences were deposited in the DDBJ/EMBL/GenBank databases with accession numbers AB907557–AB907575 (Table 2).

Phylogenetic analysis

Sequences of the 18S and 28S genes were aligned using the Muscle program (Edgar, 2004) with default settings in the software Seaview (Galtier, Gouy & Gaultier, 1996; Gouy, Guindon & Gascuel, 2010), while those of the H3 and COI genes were aligned without gaps. We employed Gblocks v. 0.91b (Castresana, 2000; Talavera & Castresana, 2007) to eliminate the ambiguously aligned regions in 18S and 28S alignments. Size of each gene prior and subsequent to treatment with Gblocks v. 0.91b is given in Table 4. Excluding ambiguous regions, the 18S, 28S, H3 and COI alignments contained 431, 245, 110 and 243 variable sites, respectively. Phylogenetic trees were constructed using the Bayesian and maximum likelihood (ML) methods. Bayesian analyses were performed using MrBayes v. 3.1.2 (Ronquist & Huelsenbeck, 2003) with substitution models chosen by Kakusan 4 (Tanabe, 2011). In the combined dataset, substitution parameters were estimated separately for each gene partition (Table 5). Two independent runs of Metropolis-coupled Markov chain Monte Carlo were carried out simultaneously, sampling trees every 100 generations and calculating the average standard deviation of split frequencies (ASDSFs) every 1,000 generations. Using the 'stoprule' option, analyses were continued until ASDSF dropped below 0.01, at which point the two chains were considered to have achieved convergence. As ASDSF was calculated based on the last 75% of the samples, we discarded the initial 25% of the sampled trees as burn-in. We confirmed that analyses reached stationarity well before the burn-in period by plotting the ln-likelihood of the sampled trees against generation time. For the ML analysis, model selection and tree search were conducted using the TreeFinder program (Jobb, Haeseler & Strimmer, 2004; Jobb, 2007). The robustness of the ML tree was evaluated by bootstrap analysis with 1,000 replications using the same program.

Table 2. GenBank accession numbers of the specimens used in this study.

Superfamily	Family	Species	18S rRNA	28S rRNA	H3	COI
Galeommatoidea	Galeommatidae	*Divariscintilla toyohiwakensis*	AB714745	AB714788	AB714831	AB714869
		Ephippodonta gigas	AB714746	AB714789	AB714832	AB714870
		Galeomma sp.	AB714747	AB714790	AB714833	AB714871
		Pseudogaleomma sp.	AB714748	AB714791	AB714834	AB714872
		Scintilla rosea	AB714749	AB714792	–	AB714873
		Scintilla aff. *hydatina*	AB714750	AB714793	AB714835	AB714874
		Scintilla sp.1	AB714751	AB714794	AB714836	AB714875
		Scintilla sp.2	AB714752	AB714795	AB714837	AB714876
	Lasaeidae	*Anisodevonia ohshimai*	AB714754	AB714797	AB714838	AB714878
		Arthritica japonica	AB714755	AB714798	AB714839	AB714879
		Brachiomya stigmatica	AB714753	AB714796	–	AB714877
		Byssobornia yamakawai	AB714756	AB714799	AB714840	AB714880
		Curvemysella paula	AB714757	AB714800	AB714841	AB714881
		Devonia semperi	AB714758	AB714801	AB714842	AB714882
		Entovalva lessonothuriae	AB714759	AB714802	AB714843	AB714883
		Kellia porculus	AB714760	AB714803	AB714844	AB714884
		Koreamya arcuata TUMC-1190962-1	AB907557*	AB907563*	AB907569*	AB474955
		Koreamya arcuata TUMC-1190962-2	AB907558*	AB907564*	AB907570*	AB474956
		Koreamya arcuata TUMC-1190969-1	AB907559*	AB907565*	AB907571*	AB474950
		Koreamya arcuata TUMC-1190969-2	AB907560*	AB907566*	AB907572*	AB474951
		Koeamya setouchiensis NSMT-Mo 78585	AB907561*	AB907567*	–	AB907573*
		Koeamya setouchiensis NSMT-Mo 78587	AB907562*	AB907568*	AB907573*	AB907574*
		Kurtiella aff. *bidentata*	AB714765	AB714808	–	AB714889
		Lasaea undulata	AB714761	AB714804	AB714845	AB714885
		Litigiella pacifica	AB714762	AB714805	AB714846	AB714886
		Melliteryx puncticulata	AB714763	AB714806	AB714847	AB714887
		Montacutona sp.	AB714764	AB714807	AB714848	AB714888
		Mysella vitrea	AM774519	AM779693	–	–
		Neaeromya rugifera	AB714766	AB714809	AB714849	AB714890
		Nipponomontacuta actinariophila	AB714767	AB714810	AB714850	AB714891
		Nipponomysella oblongata	AB714768	AB714811	AB714851	AB714892
		Nipponomysella subtruncata	AB714769	AB714812	AB714852	AB714893
		Paraborniola matsumotoi	AB714770	AB714813	AB714853	AB714894
		Peregrinamor gastrochaenans	AB714771	AB714814	–	AB714895
		Peregrinamor ohshimai	AB714772	AB714815	AB714854	AB714896
		Pseudopythina ochetostomae	AB714773	AB714816	AB714855	–
		Pseudopythina subsinuata	AB714774	AB714817	AB714856	AB714897
		Pseudopythina macrophthalmensis	AB714775	AB714818	AB714857	AB714898
		Pseudopythina aff. *ariake*	AB714776	AB714819	AB714858	AB714899
		Pseudopythina aff. *nodosa*	AB714777	AB714820	AB714859	AB714900
		Pythina deshayesiana	AB714778	AB714821	–	–
		Salpocola philippinensis	AB714779	AB714822	AB714860	AB714901
	Basterotiidae	*Basterotia carinata*	AB714780	AB714823	AB714861	AB714902
		Basterotia gouldi	AB714781	AB714824	AB714862	AB714903
		Basterotia sp.	AB714782	AB714825	AB714863	AB714904
Outgroups	Splecurtidae	*Azorinus minutus*	AB714783	AB714826	AB714864	AB714905
	Gastrochaenidae	*Gastrochaena cuneiformis*	AB714784	AB714827	AB714865	–
	Veneridae	*Irus mitis*	AB714785	AB714828	AB714866	AB714906
	Mactridae	*Meropesta nicobarica*	AB714786	AB714829	AB714867	–
	Solenidae	*Solen strictus*	AB714787	AB714830	AB714868	AB714907
	Solemyidae	*Solemya velum*	AF120524	AY145421	AY070146	U56852
	Nuculanidae	*Nuculana pella*	AY070111	AJ307553	AY070148	AY070138
	Trigoniidae	*Neotrigonia margaritacea*	AF411690	DQ279963	AY070155	U56850

*New sequences obtained for this study.

Table 3. Information on primers and PCR conditions used in this study.

Primer	Direction	Sequence 5′–3′	PCR condition	References
18SrRNA				
PCR amplification and sequencing			94°C 4 min (94°C 30 s, 55°C 30 s, 72°C 2 min) × 35, 72°C 5 min	
G01	Forward	CACCT GGTTG ATCCT GCCAG		Saunders & Kraft (1994)
G07	Reverse	AGCTT GATCC TTCTG CAGGT TCACC TAC		Saunders & Kraft (1994)
Sequencing				
G03	Forward	GTCTG GTGCC AGCAG CCGCG G		Saunders & Kraft (1994)
1155F	Forward	CTGAA ACTTA AAGGA ATTGA CGG		Wollscheid & Wägele (1999)
18d	Forward	CACAC CGCCC GTCGC TACTA CCGAT TG		Hillis & Dixon (1991)
18Sop	Reverse	GCTCC CTCTC CGGAA TCGAA CCC		Hoso *et al.* (2010)
G08	Reverse	GAACG GCCAT GCACC ACCAC C		Saunders & Kraft (1994)
28SrRNA				
PCR amplification and sequencing			94°C 4 min (94°C 30 s, 52°C 30 s, 72°C 2 min) × 40, 72°C 5 min	
D1	Forward	ACCCS CTGAA YTTAA GCAT		Colgan *et al.* (2003)
D3	Reverse	GACGA TCGAT TTGCA CGTCA		Vonnemann *et al.* (2005)
Sequencing				
D2F	Forward	CCCGT CTTGA AACAC GGACC AAGG		Vonnemann *et al.* (2005)
C2R	Reverse	ACTCT CTCTT CAAAG TTCTT TTC		Dayrat *et al.* (2001)
H3				
PCR amplification and sequencing			94°C 4 min (94°C 30 s, 52°C 30 s, 72°C 2 min) × 40, 72°C 5 min	
H3F	Forward	ATGGCTCGTACCAAGCAGACVGC		Colgan *et al.* (1998)
H3R	Reverse	ATATCCTTRGGCATRATRGTGAC		Colgan *et al.* (1998)
COI				
PCR amplification and sequencing			94°C 4 min (94°C 30 s, 50°C 30 s, 72°C 2 min) × 40, 72°C 5 min	
LCO1490	Forward	GGT CAA CAA TCA TAA AGA TAT TGG		Folmer *et al.* (1994)
HCO2198	Reverse	TAA ACT TCA GGG TGA CCA AAA AAT C		Folmer *et al.* (1994)

Table 4. Size of each gene fragment before and after treatment with Gblocks v. 0.91b.

Gene	Original length of alignment (bp)	Final length of alignment (bp)
18S rRNA	2030	1720
28S rRNA	1425	912
H3	328	328
COI	659	659

Table 5. Information on models of sequence evolution for Bayesian and maximum likelihood (ML) analyses.

Gene	Substitution model (Bayesian)	Substitution model (ML)
18S rRNA	K80 + Gamma	GTR + GI
28S rRNA	GTR + Gamma	GTR + GI
H3	–	GTR + GI
COI	–	GTR + GI
H3_1st	GTR + Gamma	–
H3_2nd	GTR + Gamma	–
H3_3rd	J69 + Homogenous	–
COI_1st	HYK85 + Gamma	–
COI_2nd	GTR + Gamma	–
COI_3rd	GTR + Gamma	–

Mapping of lifestyle and host taxon

To understand how symbiotic association with *Lingula* evolved in Galeommatoidea, we mapped information of the lifestyle (free-living or symbiotic), habitat and host taxon onto the phylogenetic tree. This information was based on the sampling data in the present and previous studies (Table 1; Sato *et al.*, 2011; Goto *et al.*, 2012). We used the sequence data of *Mysella vitrea* Laseron, 1956, which was analysed by Taylor *et al.* (2007). The voucher specimen of *M. vitrea* was collected by sieve from the sediment in an intertidal seagrass bed at Myorah, North Stradbroke Island, Moreton Bay, Queensland, eastern Australia, but no host information was available (J.D. Taylor, personal communication). In a previous study this species was found in a free-living state and also in symbiotic association with the ghost shrimp *Trypaea australiensis* Dana, 1852 in eastern Australia (Kerr & Corfield, 1998). Thus, we treated this species as a facultative commensal of *T. australiensis* (Fig. 8B).

SYSTEMATIC DESCRIPTIONS

Superfamily Galeommatoidea Gray, 1840

Family Montacutidae Clark, 1855

Genus *Koreamya* Lützen, Hong & Yamashita, 2009

Koreamya setouchiensis Goto, Ishikawa & Hamamura, new species

(Figs 2–6)

Type material: Holotype (Figs 3, 4, 6): National Museum of Nature and Science, Japan NSMT-Mo 78584. Shell length = 3.5 mm, shell height = 2.7 mm. The specimen was collected from *Lingula anatina* on an intertidal muddy sand flat of Hakatajima Island, Imabari, Ehime, Japan (Figs 1, 2A) on 4 July 2012. Shell length and width of the host *L. anatina* 18.5 mm

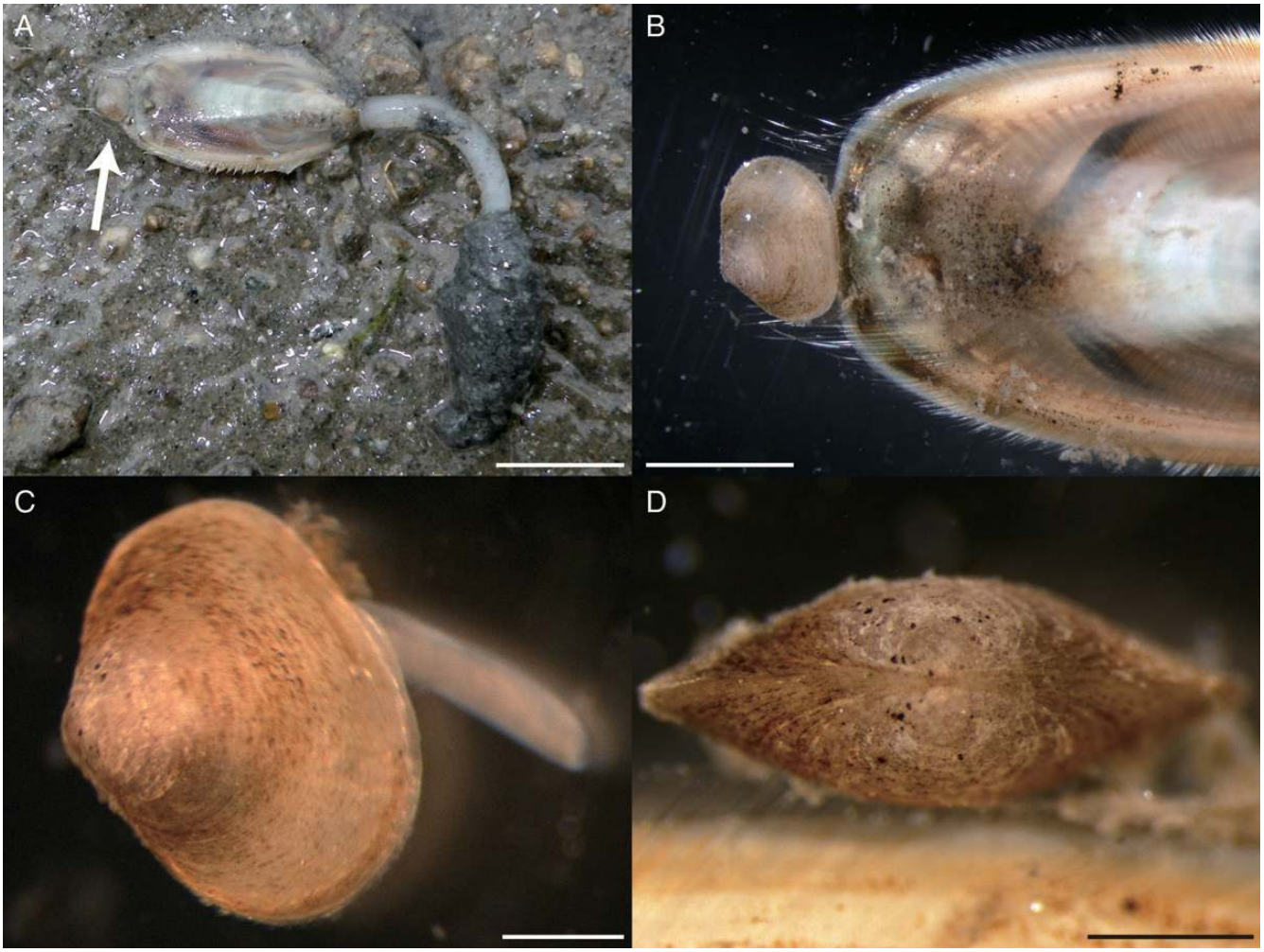

Figure 3. *Koreamya setouchiensis* n. sp. (holotype, NMST-Mo 78584) and its host *Lingula anatina*. **A, B.** *K. setouchiensis* attached to the shell of *L. anatina*. **C.** A creeping *K. setouchiensis* using its muscular foot. **D.** Dorsal view of *K. setouchiensis*. Scale bars: **A** = 1 cm; **B** = 3 mm; **C, D** = 1 mm. (Photographs: R. Goto.)

and 9.5 mm, respectively. Paratype 1 (Fig. 5): NSMT-Mo 78585. Shell length = 2.3 mm, shell height = 1.8 mm. The specimen was collected from *L. anatina* on a subtidal muddy sandy flat (ca. 2 m depth) of Maajiro, Yawatahama, Ehime, Japan (Fig. 1) on 11 September 2011. Soft tissue was used for the molecular phylogenetic analysis (Table 1). The host size was not recorded. Paratype 2: NSMT-Mo 78586. Shell length = 1.7 mm, shell height = 1.5 mm. The specimen was collected from *L. anatina* on an intertidal muddy sand flat of Nigata, Kure, Hirosima, Japan (Fig. 1) on 26 May 2009. The shell was partly broken when the bivalve was removed from the host. Shell length and width of the host *L. anatina* were 21.0 mm and 9.5 mm, respectively. Paratype 3: NSMT-Mo 78587. Shell length = 2.7 mm, shell height = 2.2 mm. The specimen was collected from *L. anatina* on an intertidal muddy sand flat of Gogoshima Island, Matsuyama, Ehime, Japan (Fig. 1) on 23 May 2012. Soft tissue was used for the molecular phylogenetic analysis (Table 1). Shell length and width of the host *L. anatina* were 15.4 mm and 7.2 mm, respectively.

Etymology: The new species is named after the Seto Inland Sea, where the type locality (Hakatajima Island) is situated.

Shell (Figs 3–6): Shell small (maximum 3.5 mm), ovate, thin but dorsally inflated, equivalve, inequilateral. Umbo slightly prominent; beak prosogyrate, located slightly posterior to mid-length of shell; anterior end rounded; posterior end subtruncate; anterodorsal margin nearly straight; posterodorsal margin nearly straight, sometimes slightly incurved posterior to umbo; anterodorsal margin slightly longer than posterodorsal margin; ventral margin rounded. Shell colour brownish white to white. Shell sculpture of many close-set commarginal growth lines and evenly spaced radial ribs; radial ribs indistinct to distinct (Figs 4A, C, 5A, C). Periostracum ornamented with numerous lamellae (Fig. 6). Prodissoconch (Fig. 5G, H): that of holotype heavily corroded; paratype 1 has indistinct prodissoconch I (134 × 104 μm; SL × SH) and distinct prodissoconch II (410 × 353 μm; SL × SH) with faint growth lines. Hinge (Figs 4E, F, 5E, F) of right shell valve has short but distinct teeth, one anterior and one posterior, diverging approximately 120°; length of the anterior and posterior tooth nearly equal; resilium on right valve located immediately below umbo and between anterior and posterior tooth, with a rounded to trapezoid lithodesma. Hinge of left valve has elongated but indistinct lamella-like teeth, one anterior and one posterior, diverging approximately 130°; anterior tooth slightly longer than posterior tooth; resilium on left valve located immediately below umbo, between anterior and posterior tooth, slightly smaller than that on right valve. External ligament absent.

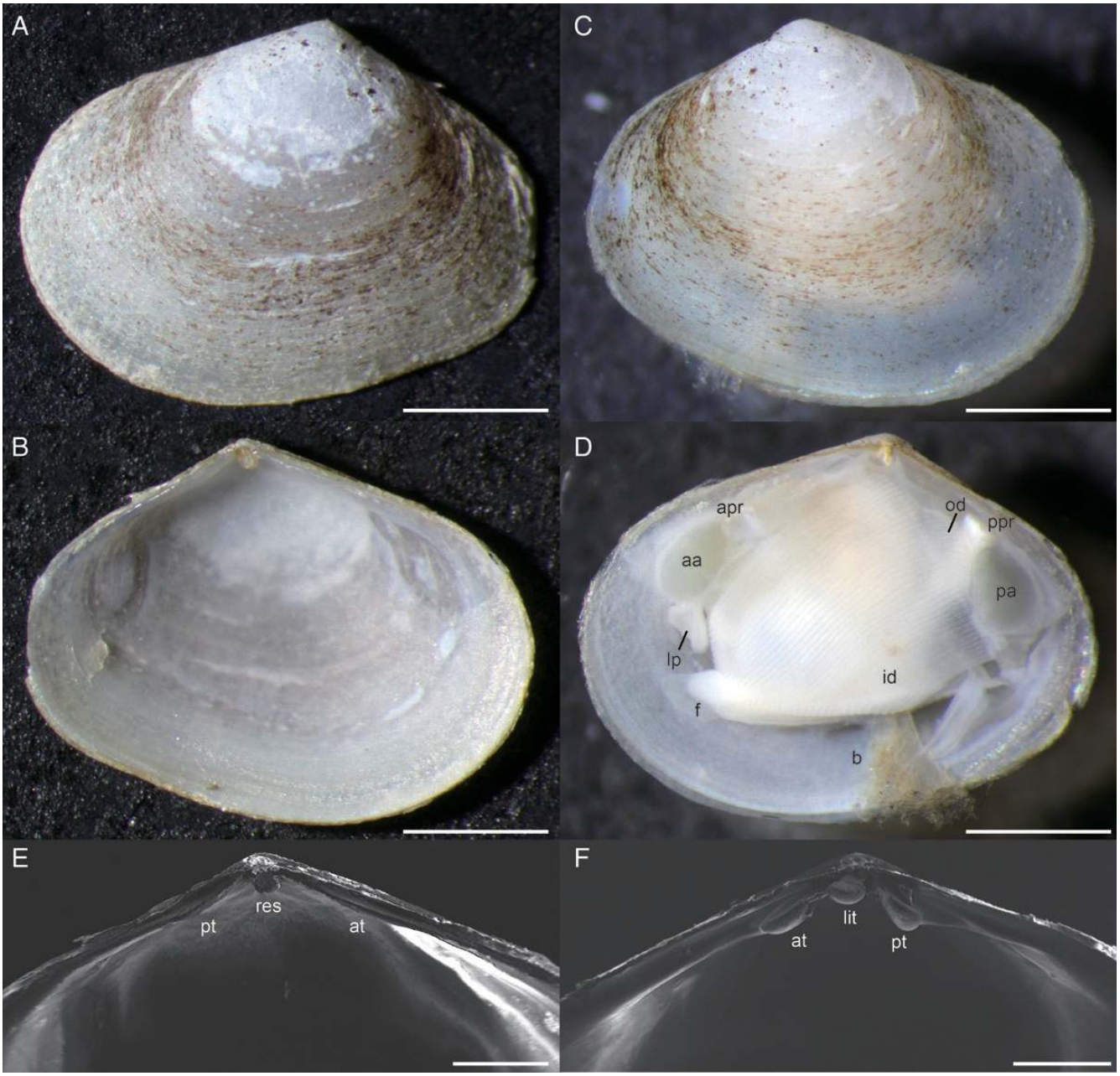

Figure 4. *Koreamya setouchiensis* n. sp. (holotype, NMST-Mo 78584). **A, B.** External and internal view of left valve. **C, D.** External and internal view of right valve. **E, F.** Hinge structure of left and right valves. Abbreviations: aa, anterior adductor muscle; apr, adductor pedal retractor muscle; at, anterior tooth; b, byssal threads; f, foot; id, inner demibranch; lp, labial palp; lit, lithodesma; od, outer demibranch; pa, posterior adductor muscle; ppr, posterior pedal retractor muscle; pt, posterior tooth; res, resilium. Scale bars: **A–D** = 1 mm; **E, F** = 400 μm; **G** = 100 μm. (Photographs: R. Goto.)

Soft parts (Fig. 4D): Mantle not reflected, with no tentacles. Both anterior and posterior adductor muscles ovate, subequal and located in relatively dorsal position. Both anterior and posterior pedal retractors dorsal to adductors. Ctenidia: gill axis nearly vertical; flat, consisting of inner and outer demibranch; reduced outer demibranch about one tenth the length of the inner and of only one descending lamella; inner demibranch of both asceding and descending lamellae; ctenidia joined anteriorly to inner and outer labial palps. Labial palps relatively large, leaf-shaped. Foot slender, of moderate size, with a small heel; byssal glands located just in front of heel, with fine, transparent byssal threads.

Distribution (Fig. 1)*:* Seto Inland Sea and Uwa Sea, Japan.

Habitat (Fig. 2A)*:* Lower intertidal to subtidal zone of muddy sand flats.

Host association (Fig. 3A, B): Host *L. anatina* Lamarck, 1801 (Lingulidae, Brachiopoda). The bivalve was attached to the anterior end of the shell valves of *L. anatina* by byssal threads, with its lateral side nearly horizontal to the host shell surface (Fig. 3A, B). Of the four specimens observed, two were attached to the ventral valve of *Lingula*; however, no data were recorded regarding

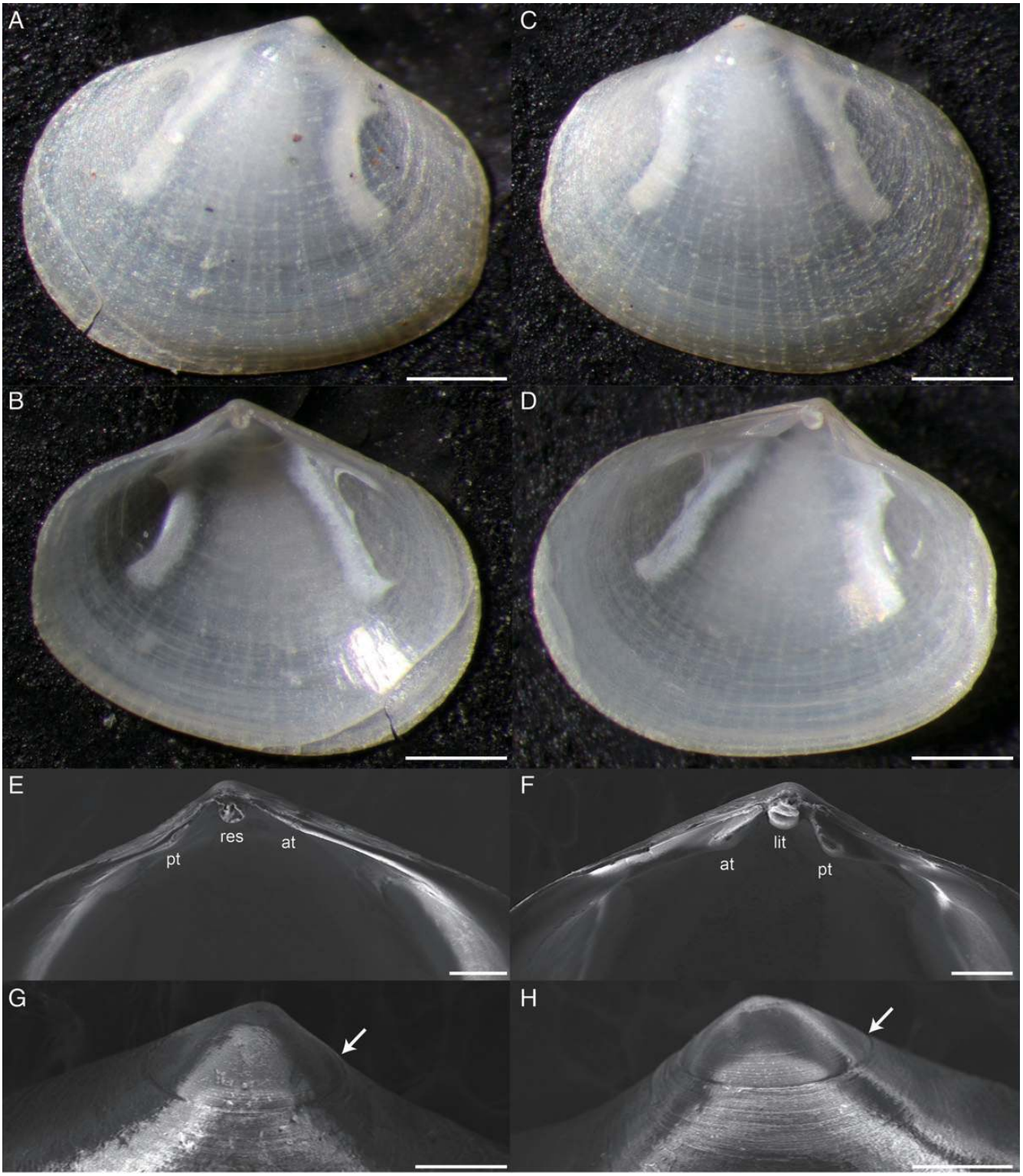

Figure 5. *Koreamya setouchiensis* n. sp. (paratype 1, NMST-Mo 74585). **A, B.** External and internal view of left valve. **C, D.** External and internal view of right valve. **E, F.** Hinge structure of left and right valves. **G, H.** Prodissoconch of left and right valve. White arrows indicate prodissoconch II. Abbreviations: at, anterior tooth; pt, posterior tooth; lit, lithodesma; res, resilium. Scale bars: **A–D** = 0.5 mm; **B–H** = 200 μm. (Photographs: R. Goto.)

whether the other two specimens were attached to the ventral or dorsal valve. The number of bivalves per host was always one ($N = 4$). Once the bivalves were removed from their hosts in the aquaria, they actively moved using their foot (Fig. 3C).

Remarks: The new species shares several morphological and anatomical characteristics with *K. arcuata*, such as a hinge structure, the presence of a lithodesma, the shell surface ornamented with numerous lamellae, evenly spaced radial ribs and a highly

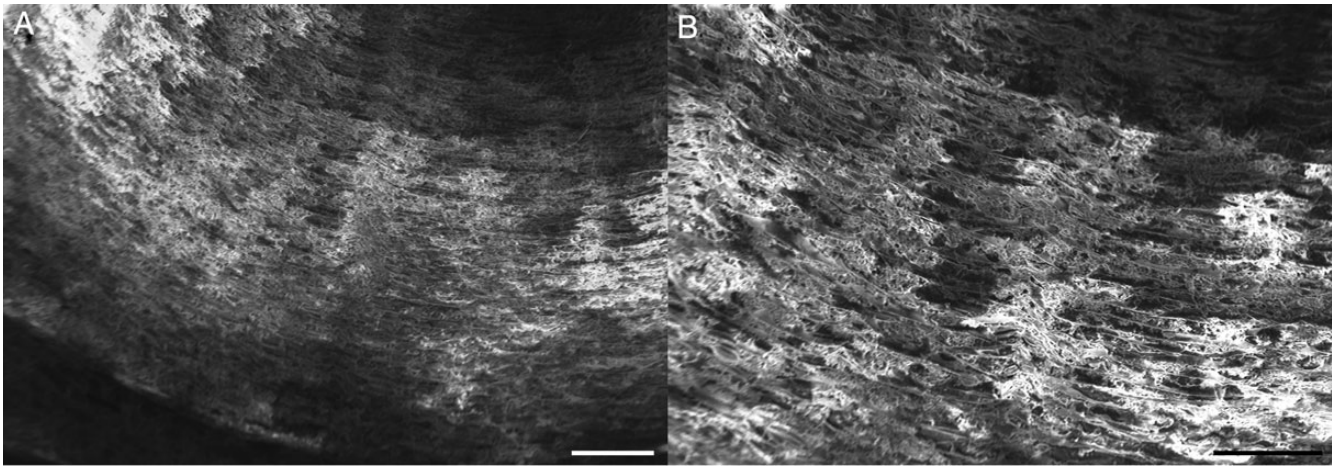

Figure 6. SEM images of the shell of *Koreamya setouchiensis* n. sp. (holotype, NMST-Mo 78584). **A, B.** Periostracum ornamented by numerous lamellae. Scale bars = 100 μm. (Photographs: R. Goto.)

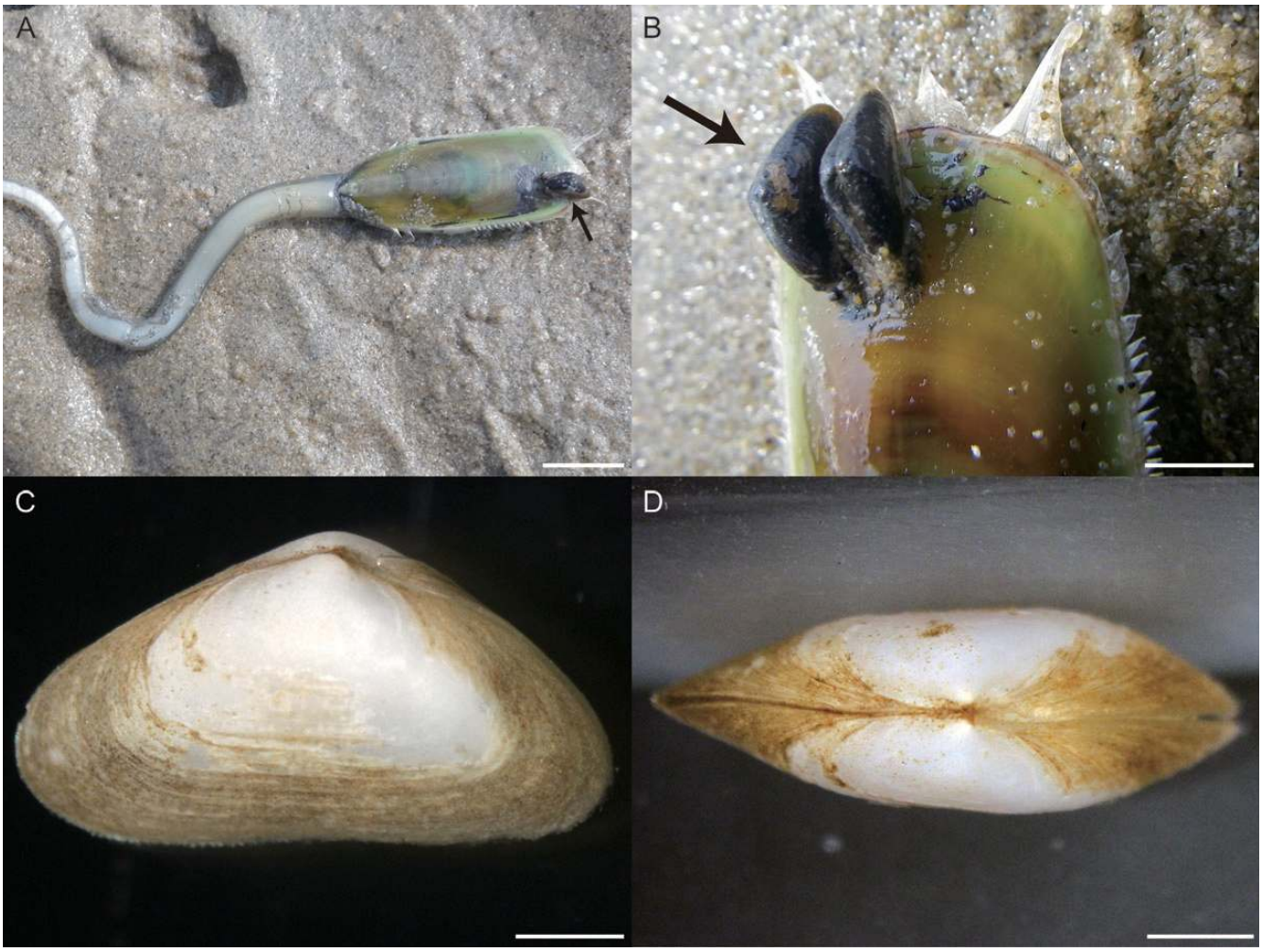

Figure 7. *Koreamya arcuata* and its host *Lingula anatina*, from Seondo-ri, Biin-myeon, Seocheon-gun, Chungcheongnam-do, South Korea. **A, B.** *K. arcuata* attached to shell of *L. anatina*. **C.** Lateral view of *K. arcuata*. **D.** Dorsal view of *K. arcuata*. Scar bars: **A** = 1 cm; **B** = 3 mm; **C, D** = 1 mm. (Photographs: **A, B**, S. Sato; **C, D**, R. Goto.)

reduced outer demibranch (Figs 3–6; Lützen, Hong & Yamashita, 2009; Sato *et al*., 2011). In addition, both bivalves are ecologically very similar in having a symbiotic association with *Lingula* (Figs 3, 7). Thus, we place the new species in the genus *Koreamya*, which consequently includes two species, *K. setouchiensis* and *K. arcuata*. These two species are clearly

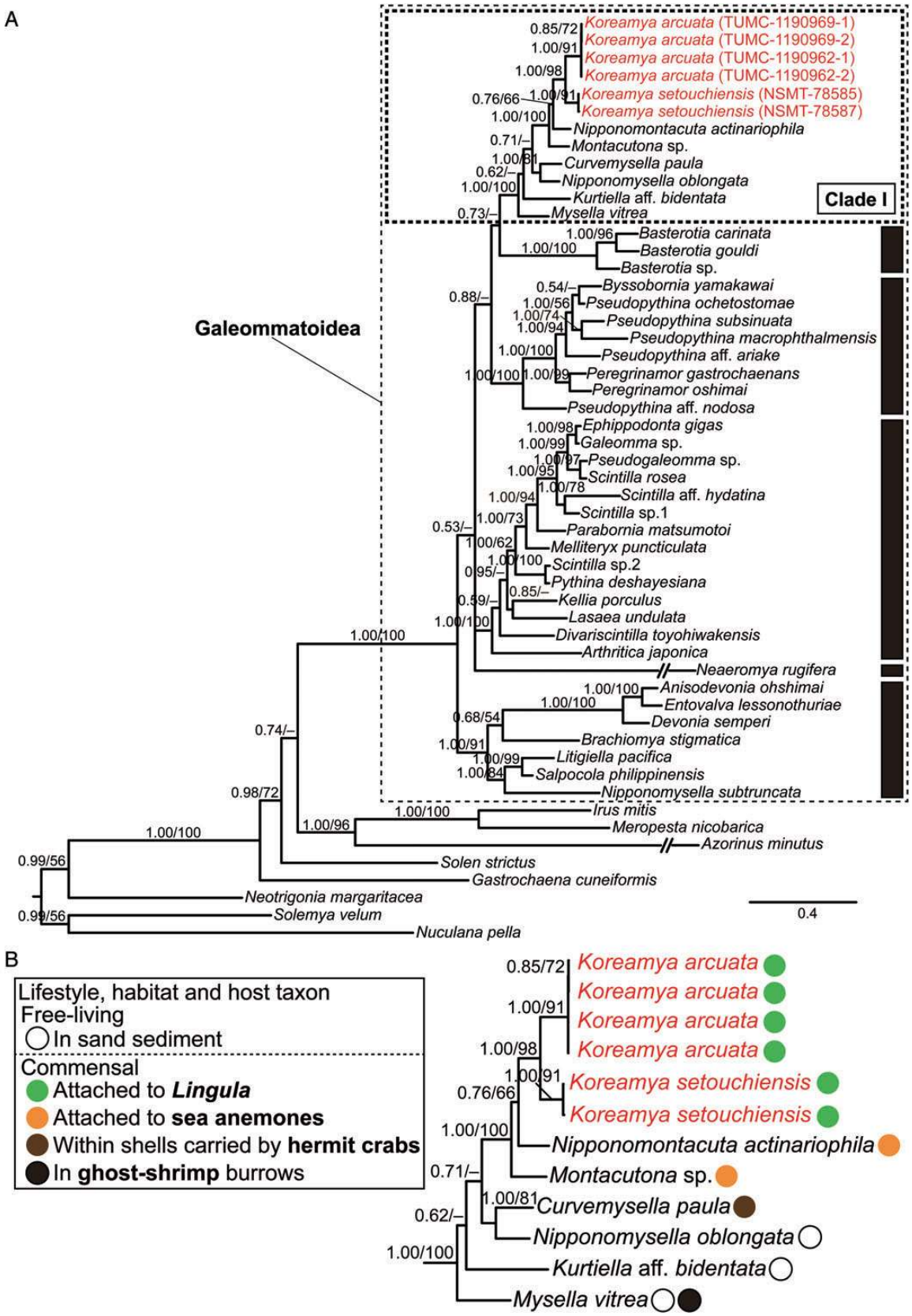

Figure 8. Bayesian tree of Galeommatoidea, including *Koreamya setouchiensis* n. sp. and *K. arcuata*, based on the combined dataset of 18S, 28S, H3 and COI genes. Numbers above branches indicate Bayesian posterior probabilities followed by maximum likelihood bootstrap support values. **A.** Entire tree. Vertical black bars indicate the five major clades except Clade I. **B.** Partial tree showing Clade I, with symbols indicating lifestyle, habitat and host taxon. The samples of which sequences were newly obtained in this study, are shown in red. The information on the lifestyle, habitat and host taxon is based on the data of the present and previous studies (Kerr & Corfield, 1998; Taylor *et al*., 2007; Sato *et al*., 2011; Goto *et al*., 2012; J.D. Taylor, personal communication).

distinguished by several shell characteristics. The shell outline of *K. setouchiensis* is ovate (Figs 3–5), while that of *K. arcuata* is elongated and triangular (Fig. 7). The ventral margin of the former is rounded (Figs 3–5), while that of the latter is straight or slightly incurved (Fig. 7C; Savazzi, 2001; Lützen, Hong & Yamashita, 2009; Sato *et al.*, 2011). *Koreamya setouchiensis* is smaller and thinner than *K. arcuata*. The shell length of *K. setouchiensis* is up to 3.5 mm (holotype), but that of *K. arcuata* often exceeds 7.0 mm (Lützen, Hong & Yamashita, 2009; Sato *et al.*, 2011).

Radial ribs are indistinct in the holotype, but distinct in paratype 1 (Figs 4, 5). In addition, the shell outline is slightly different between holotype and paratype 1 (Figs 4, 5). Similar intraspecific morphological variation was also reported in *K. arcuata* (Sato *et al.*, 2011). The shell length of the holotype is about 1.5 times larger than that of paratype 1, perhaps due to a difference in the developmental stage.

PHYLOGENETIC ANALYSIS

The Bayesian and ML analyses based on the combined dataset (18S + 28S + H3 + COI) recovered six major clades in Galeommatoidea (Fig. 8A), in accordance with Goto *et al.* (2012). *Koremaya arcuata* and *K. setouchiensis* were grouped into one of the major clades (Clade I in Fig. 8A) and each recovered as monophyletic [*K. arcuata*: Bayesian posterior probability (BPP) = 1.00, ML bootstrap percentage (MBP) = 91; *K. setouchiensis*: BPP = 1.00, MBP = 91] (Fig. 8). A sister-group relationship between *K. arcuata* and *K. setouchiensis* was highly supported (BPP = 1.00, MBP = 98; Fig. 8). The *Koreamya* clade formed a monophyletic group with the anemone-associated galeommatoideans (*Montacutona* sp. and *Nipponomontacuta actinariophila* Yamamoto & Habe 1961) (BPP = 1.00, MBP = 100; Fig. 8B). Within this group, the sister-group relationship of *Koreamya* and *N. actinariophila* was weakly supported (BPP = 0.76, MBP = 66; Fig. 8B). On the other hand, *K. arcuata* and *Curvemysella paula* were separated into different clades (Fig. 8B), although these two have previously been considered conspecific (Morton & Scott, 1989).

DISCUSSION

Evolution of symbiotic association with Lingula

Our phylogenetic analyses indicate that the symbiotic associations with *Lingula* in *Koremaya arcuata* and *K. setouchiensis* have a single evolutionary origin (Fig. 8). Furthermore, the *Koreamya* clade formed a monophyletic group with the anemone-associated galeommatoideans (*Montacutona* sp. and *Nipponomontacuta actinariophila*) (Fig. 8B), suggesting that that host switching may have occurred between sea anemones and *Lingula*. Species of *Koreamya*, *Montacutona* and *Nipponomontacuta* share several morphological characteristics (e.g. hinge teeth structure, the presence of a lithodesma and a reduced outer demibranch) (this study; Yamamoto & Habe, 1961; Morton, 1980; Lützen, Hong & Yamashita, 2009; R. Goto, unpublished). In addition, they are attached to the host body directly by byssal threads (this study; Yamamoto & Habe, 1961; Morton, 1980; Savazzi, 2001; Lützen, Hong & Yamashita, 2009; Goto *et al.*, 2012). These morphological and ecological similarities also support the possibility of host switching between sea anemones and *Lingula*. According to the topology (Fig. 8B), host switching appears to have occurred from sea anemones to *Lingula*. However, since the support for the clade of *Koreamya* and *Nipponomontacuta* is low (Fig. 8B), additional genetic data and taxon sampling are needed to confirm the direction of host switching.

Taxonomic implications

We tentatively assigned the new species *K. setouchiensis* to the family Montacutidae, referring to the classification by Lützen, Hong & Yamashita (2009). However, it is widely recognized that the family-level classification of Galeommatoidea is confused and requires revision (Ponder, 1998; Bieler, Carter & Coan, 2010). In fact, a recent molecular phylogeny suggested that Montacutidae are polyphyletic (Goto *et al.*, 2012).

The close relationship between *Montacutona* and *K. arcuata* was first suggested based on several morphological characteristics (e.g. the presence of a lithodesma, a reduced outer demibranch and a particular type of sperm receptacle) (Lützen, Hong & Yamashita, 2009). However, they are distinguished by their shell morphology; the shell of *Montacutona* is rounded to ovate (Morton, 1980), while that of *K. arcuata* is elongated and triangular with a slightly incurved ventral margin (Lützen, Hong & Yamashita, 2009; Fig. 7). Based on this morphological difference, the genus *Koreamya* was recently established (Lützen, Hong & Yamashita, 2009). Nevertheless, the new species *K. setouchiensis* has an ovate shell (Figs 3–5), which is relatively similar to that of *Montacutona*. Thus, the inclusion of this species in *Koreamya* suggests that the morphological definition of this genus should be revised (i.e. shell is ovate or elongated and triangular).

The genus *Nipponomontacuta* includes the only one species, *N. actinariophila*. This species formed a sister-group relationship with *Koreamya* in the phylogenetic tree although the support for this relationship was low (Fig. 8). *Nipponomontacuta actinariophila* has an ovate shell with hinge teeth similar to those of *Montacutona* and *Koreamya* (R. Goto, unpublished). In addition, this species has a lithodesma and a highly reduced demibranch like *Koreamya* and *Montacutona* (R. Goto, unpublished). However, this species has orange to reddish shells, which are clearly different from those of *Montacutona* and *Koreamya*.

Koreamya arcuata has been considered identical to *C. paula*, because both have anteroposteriorly elongated, dorsoventrally compressed shells with slightly incurved ventral margins (Morton & Scott, 1989). Our molecular phylogeny clearly shows that *C. paula* and *K. arcuata* are separated into different clades, suggesting that unusually elongated shells have evolved at least twice in Clade I (Fig. 8). The shell shape of *C. paula* is suggested to be a morphological adaptation to symbiotic life in the empty snail shells occupied by hermit crabs (Goto *et al.*, 2007). Thus, the apparently similar shaped shells of *K. arcuata* and *C. paula* are probably the consequence of convergent adaptation to symbiotic life on convex substrata.

Host associations

Koreamya setouchiensis always attaches to the anterior end of *Lingula* shells (Fig. 3A, B), probably to utilize the water currents created by the host for filter feeding. *Lingula anatina* has three holes on the anterior end of the mantle for filter feeding, one central exhalant hole and two inhalant holes on either side of the central hole. Since the holotype of *K. setouchiensis* was attached to the central part of the anterior end of the host (Fig. 3A, B), this bivalve species may prefer to use the exhalant current created by the host, although further observation is needed to confirm this. It may also feed on mucus secreted by the host. Savazzi (2001) suggested that *K. arcuata* collects food particles from the mantle of the host *Lingula* using the adhesive foot. However, such behaviour was not observed in *K. setouchiensis* in this study. Considering that *K. setouchiensis* is a filter feeder and does not damage the host by feeding, the association between *K. setouchiensis* and its host is probably commensalism, as was suggested to be the case in *K. arcuata* (Savazzi, 2001; Lützen, Hong & Yamashita, 2009).

Differential adaptation to symbiotic life with Lingula

The genus *Koreamya* comprises two species, *K. setouchiensis* and *K. arcuata*. These are clearly different in shell appearance; *K. setouchiensis* has a laterally flattened, ovate shell (Figs 3–5), while *K. arcuata* has a dorsoventrally compressed, anteroposteriorly elongated-triangular shell (Fig. 7C, D; Lützen, Hong & Yamashita, 2009; Sato *et al*., 2011). These morphological differences likely correspond to the difference in posture on the hosts; *K. setouchiensis* is attached to the host with its lateral side parallel to the host shell surface (Fig. 3A, B), whereas *K. arcuata* is attached to the host with its lateral side nearly perpendicular to the host shell surface (Fig. 7B, C; Lützen, Hong & Yamashita, 2009; Sato *et al*., 2011). The morphology and posture of *K. setouchiensis* are thought to be adaptive because they reduce friction when *Lingula* shells withdraw into their burrows, which they do frequently. In contrast, the posture of *K. arcuata* in the narrow interspace between the host shell and burrow wall results in morphological constraint as the bivalves cannot grow dorsoventrally, but only laterally and anteroposteriorly. Thus, the morphological differences of *K. setouchiensis* and *K. arcuata* are probably consequences of differential specializations to symbiotic life with *Lingula*.

Distribution and host

Koreamya arcuata has a wide distribution from South Korea to the Philippines, India and Australia (Fernando & Fernando, 1983; Savazzi, 1991, 2001; Hong, Yamashita & Sato, 2007; Lützen, Hong & Yamashita, 2009; Sato *et al*., 2011). In contrast, *K. setouchiensis* is only known in and around the Seto Inland Sea, Japan (Fig. 1a–d), although it is possible that it has a wider geographic distribution. The hosts of *K. arcuata* include *L. anatina*, *L. adamsi* and *L. translucida* Dall, 1920 (Fernando & Fernando, 1983; Savazzi, 1991, 2001; Lützen, Hong & Yamashita, 2009; Sato *et al*., 2011), while we identified the host of *K. setouchiensis* as *L. anatina*. However, the shell appearance of *L. anatina* in South Korea (the host of *K. arcuata*) and that of *L. anatina* in the Seto Inland Sea (the host of *K. setouchiensis*) differ slightly; the latter has smaller, thinner and more yellowish shells than the former and, in addition, the shell width of the former is constant toward the anterior end, while that of the latter becomes narrower towards the anterior end (Figs 3A, B, 7A, B). A previous study suggested that *L. anatina* from around Japan is genetically separated into several groups, which could be separate species (Endo, Ozawa & Kojima, 2001). Thus, further morphological and molecular assessments on *L. anatina* are required to identify precisely the hosts of *K. setouchiensis* and *K. arcuata*.

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