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Authors: Goto, Ryutaro, Ishikawa, Hiroshi, and Hamamura, Yoichi

Source: Zoological Science, 33(4) : 441-447

Published By: Zoological Society of Japan

URL: <https://doi.org/10.2108/zs160009>

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Morphology, Biology, and Phylogenetic Position of the Bivalve *Platomysia rugata* (Heterodonta: Galeommatoidea), a Commensal with the Sipunculan Worm *Sipunculus nudus*

Ryutaro Goto^{1,2,3*}, Hiroshi Ishikawa⁴, and Yoichi Hamamura⁵

¹Museum of Zoology and Department of Ecology and Evolutionary Biology, University of Michigan, 1109 Geddes Avenue, Ann Arbor, MI 48109-1079, USA

²Atmosphere and Ocean Research Institute, University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8564, Japan

³Graduate School of Human and Environmental Studies, Kyoto University, Yoshida-Nihonmatsu-cho, Sakyo, Kyoto 606-8501, Japan

⁴965-1 Kawachi-ko, Uwajima, Ehime 798-0075, Japan

⁵14-16 Yakeyama-Hibarigaoka-cho, Kure, Hiroshima 737-0901, Japan

The bivalve superfamily Galeommatoidea is characterized by its symbiotic associations with other marine invertebrates. However, for many galeommatoideans, the host species remains unknown. *Platomysia* (Galeommatoidea) is a monotypic genus including a single species *P. rugata*, which is distinguished from other galeommatoideans in having distinct and evenly spaced commarginal ribs on its shell surface. This species was described based on a single right valve shell collected in Nanao Bay, Japan Sea, by Habe in 1951 and has been known only from Japanese waters. However, the biology of living animals has never been reported. We found that this species lives in the burrows of the sipunculan worm *Sipunculus nudus* in mud flats in the Seto Inland Sea, Japan. We investigated its host association and described its shell morphology and anatomy. In addition, we performed a phylogenetic analysis using two nuclear (18S and 28S ribosomal RNA) genes to determine its phylogenetic position in Galeommatoidea. The result suggests that this species belongs to the clade of commensal bivalves together with *Pseudopythina*, *Byssobornia*, and *Pergrinamor*. *Platomysia rugata* and other two groups of sipunculan-associated galeommatoideans were not monophyletic, suggesting that association with sipunculans occurred at least three times in the galeommatoid evolution.

Key words: Annelida, burrow, commensalism, Galeommatoidea, mud flat, *Platomysia*, Seto Inland Sea, Sipuncula, *Sipunculus*, symbiosis

INTRODUCTION

The bivalve superfamily Galeommatoidea (Heterodonta) exhibits extremely high diversity in intertidal zones (Bouchet et al., 2002; Paulay, 2003; Lützen and Nielsen, 2005). As a distinct ecological characteristic, many members of this superfamily have symbiotic associations with other invertebrates in sediment bottom (Boss, 1965; Morton and Scott, 1989; Li et al., 2012; Goto et al., 2012). Most live in the burrows or on the body surface of host animals, whereas some live inside the host body (Boss, 1965; Kato, 1998; Morton and Scott, 1989; Goto et al., 2012). The known host taxa of galeommatoideans are highly diverse, belonging to Porifera, Cnidaria, Annelida, Mollusca, Brachiopoda, Bryozoa, Arthro-

poda, and Echinodermata (Boss, 1965; Morton and Scott, 1989; Goto et al., 2012). However, hosts remain unknown in many galeommatoideans.

Sipunculans are marine worms, which were previously assigned to their own phylum, but are now recognized as members of Annelida (Struck et al., 2007). Most sipunculans burrow in bottom sediments, whereas some groups live in the empty shell or narrow spaces in hard substrata (Stephen and Edmonds, 1972). They are often utilized as hosts by commensal galeommatoideans (Boss, 1965; Morton and Scott, 1989). To date, at least 14 species in 11 genera (*Barrimysia*, *Epilepton*, *Fronsella*, *Jousseaumella*, *Litigiella*, *Mioerycina*, *Montacuta*, *Mysella*, *Nipponomysella*, *Pseudopythina*, and *Salpocola*) of galeommatoideans have been reported to have commensal associations with sipunculans (Bourne, 1906; Habe, 1958; Boss, 1965; Gage, 1979; Morton and Scott, 1989; Lützen and Kosuge, 2006; Jespersen et al., 2007; Lützen et al., 2008; Goto et al., 2012). Most live attached to burrow wall or body surface of sipunculans,

* Corresponding author. Tel. : +1-734-276-7523;
Fax : +1-734-763-4080;
E-mail: gotoryutaro@gmail.com

doi:10.2108/zs160009

whereas some species attach to gastropod shells occupied by sipunculans. Recent molecular phylogenetic analysis suggested that sipunculan-associated galeommatoideans are separated into two different lineages (Goto et al., 2012).

Platommisia is a monotypic genus including a single species *P. rugata*. The genus is distinguished from other galeommatoideans in having distinct and evenly spaced commarginal ribs on its shell surface (Habe, 1951). This species was described based on a single right valve collected in Nanao Bay, Toyama, Japan and is known only from Japanese waters (Habe, 1951; Fukuda, 2012). However, the biology of living animals has never been reported. The information on shell and anatomical characteristics and phylogenetic position of *P. rugata* remains poorly known (Huber, 2015).

In the present study, we found that *P. rugata* lives in the burrows of the sipunculan worm *Sipunculus nudus* (Annelida: Sipunculidae) in mud flats in the Seto Inland Sea, Japan. We described its host association, shell morphology, and anatomy. In addition, we performed a molecular phylogenetic analysis using the two nuclear (18S and 28S ribosomal RNA) genes to understand the phylogenetic position of this species in the superfamily Galeommatoidea.

MATERIALS AND METHODS

Sample collection and observations

We have investigated the diversity of commensal galeommatoideans in mud flats in Hakatajima, Ehime Prefecture and Takehara, Hiroshima Prefecture, Japan since 2009 by digging up various invertebrates' burrows (Goto et al., 2011, 2012, 2014). *Sipunculus nudus* (Sipunculidae) is one of the major burrowing invertebrates in these study sites. This sipunculan lives in temporal burrows, which are horizontal, not branched, and weakly lined with mucus, in bottom sediments. We collected *P. rugata* from the burrows of *S. nudus* in the middle or lower intertidal zone of mud flats at spring low tide during 2011 and 2012. The bivalves were preserved in 70–100% ethanol and brought back to the laboratory. The detailed observations of shell morphology and anatomy were performed under a dissecting microscope. We used a specimen collected from Takehara for molecular analysis (Table 1).

DNA extraction, PCR, and sequencing

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 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 supernatant (700 μl) was

Table 1. GenBank accession numbers of the specimens used in this study. The sequences obtained in this study are shown in bold with asterisk.

Superfamily	Family	Species	18S rRNA	28S rRNA
Galeommatoidea	Galeommatidae	<i>Divariscintilla toyohiwakensis</i>	AB714745	AB714788
		<i>Ehippodonta gigas</i>	AB714746	AB714789
		<i>Galeomma</i> sp.	AB714747	AB714790
		<i>Pseudogaleomma</i> sp.	AB714748	AB714791
		<i>Scintilla rosea</i>	AB714749	AB714792
		<i>Scintilla</i> aff. <i>hydatina</i>	AB714750	AB714793
		<i>Scintilla</i> sp.1	AB714751	AB714794
		<i>Scintilla</i> sp.2	AB714752	AB714795
	Lasaeidae	<i>Anisodevonia ohshimai</i>	AB714754	AB714797
		<i>Arthritica japonica</i>	AB714755	AB714798
		<i>Brachiomya stigmatica</i>	AB714753	AB714796
		<i>Byssobornia yamakawai</i>	AB714756	AB714799
		<i>Curvemysella paula</i>	AB714757	AB714800
		<i>Devonia semperi</i>	AB714758	AB714801
		<i>Entovalva lessonothuriae</i>	AB714759	AB714802
		<i>Kellia porculus</i>	AB714760	AB714803
		<i>Koreamya arcuata</i>	AB907557	AB907563
		<i>Koreamya setoensis</i>	AB907562	AB907568
		<i>Lasaea undulata</i>	AB714761	AB714804
		<i>Litigiella pacifica</i>	AB714762	AB714805
		<i>Melliteryx puncticulata</i>	AB714763	AB714806
		<i>Montacutona</i> sp.	AB714764	AB714807
		<i>Mysella</i> aff. <i>bidentata</i>	AB714765	AB714808
		<i>Neaeromya rugifera</i>	AB714766	AB714809
		<i>Nipponomontacuta actinariophila</i>	AB714767	AB714810
		<i>Nipponomysella oblongata</i>	AB714768	AB714811
		<i>Nipponomysella subtruncata</i>	AB714769	AB714812
		<i>Paraborniola matsumotoi</i>	AB714770	AB714813
		<i>Peregrinamor gastrochaenans</i>	AB714771	AB714814
		<i>Peregrinamor ohshimai</i>	AB714772	AB714815
		<i>Platommisia rugata</i>	LC126833*	LC126832*
		<i>Pseudopythina ochetostomae</i>	AB714773	AB714816
		<i>Pseudopythina subsinuata</i>	AB714774	AB714817
		<i>Pseudopythina macrophthalmensis</i>	AB714775	AB714818
		<i>Pseudopythina</i> aff. <i>ariake</i>	AB714776	AB714819
		<i>Pseudopythina</i> aff. <i>nodosa</i>	AB714777	AB714820
		<i>Pythina deshayesiana</i>	AB714778	AB714821
		<i>Salpocola philippinensis</i>	AB714779	AB714822
Outgroups	Basterotiidae	<i>Basterotia carinata</i>	AB714780	AB714823
		<i>Basterotia gouldi</i>	AB714781	AB714824
		<i>Basterotia</i> sp.	AB714782	AB714825
		<i>Azorinus minutus</i>	AB714783	AB714826
		<i>Gastrochaena cuneiformis</i>	AB714784	AB714827
	Splecurtidae	<i>Irus mitis</i>	AB714785	AB714828
	Gastrochaenidae	<i>Meropesta nicobarica</i>	AB714786	AB714829
	Veneridae	<i>Solen strictus</i>	AB714787	AB714830
	Solenidae	<i>Solemya velum</i>	AF120524	AY145421
	Solemyidae	<i>Nuculana pella</i>	AY070111	AJ307553
	Nuculanidae	<i>Neotrigonia margaritacea</i>	AF411690	DQ279963
	Trigoniidae			

* Sequences obtained for this study are marked with an asterisk.

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- μ l TE buffer.

We sequenced fragments of the 18S and 28S genes. Polymerase chain reactions (PCRs) were used to amplify ~1700 bp of 18S and ~1000 bp of 28S. Amplifications were performed in 20- μ l mixtures consisting of 0.4 μ l of forward and reverse primers (20 μ M each; Table 2), 2.0 μ l of ExTaq buffer, 1.6 μ l of dNTPs (2.5 μ M each), 0.1 μ l of ExTaq polymerase (TaKaRa, Otsu, Japan), and 15.1 μ l 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 (50–55°C), 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 2) and a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA); the reaction products were electrophoresed on an ABI 3130 sequencer (Applied Biosystems). The obtained sequences were deposited in the DDBJ/EMBL/GenBank databases with accession numbers LC126832–LC126833 (Table 1).

Phylogenetic analyses

In addition to the sequences of *P. rugata* obtained in this study, we also collected sequence data of other galeommatoidaeans and outgroups from GenBank (Table 1). Sequences of the 18S and 28S genes were aligned using the Muscle program (Edgar, 2004) with default settings in the software Seaview (Galtier et al., 1996; Gouy et al., 2010). We employed Gblocks v0.91b (Castresana, 2000; Talavera and Castresana, 2007) to eliminate the ambiguously aligned regions in 18S and 28S alignments. Phylogenetic trees were constructed using the Bayesian and maximum likelihood (ML) methods. Bayesian analyses were performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) with substitution models chosen by Kakusan 4 (Tanabe, 2011). In the combined data set, substitution parameters were estimated separately for each gene partition (18S: SYM Gamma, 28S: GTR Gamma). 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 1000 generations. Using the 'stoprule' option, analyses were continued until ASDSF dropped below

Table 2. Primers used in this study.

Primer	Direction	Sequence 5'–3'	References
18S rRNA			
PCR amplification and sequencing			
G01	Forward	CACCTGGTTGATCCTGCCAG	Saunders and Kraft (1994)
G03	Forward	GTCTGGTGCCAGCAGCCGCGG	Saunders and Kraft (1994)
G07	Reverse	AGCTTGATCCTTCTGCAGGTTACCTAC	Saunders and Kraft (1994)
G08	Reverse	GAACGGCCATGCACCACCACC	Saunders and Kraft (1994)
Sequencing			
1155F	Forward	CTGAAACTTAAAGGAATTGACGG	Wollscheid and Wägele (1999)
18d	Forward	CACACCGCCCGTCGCTACTACCGATTG	Hillis and Dixon (1991)
18Sop	Reverse	GCTCCCTCTCCGGAATCGAACCC	Hoso et al. (2010)
28S rRNA			
PCR amplification and sequencing			
D1	Forward	ACCCSCTGAAYTTAAGCAT	Colgan et al. (2003)
D3	Reverse	GACGATCGATTTCACGTCA	Vonnemann et al. (2005)
Sequencing			
D2F	Forward	CCCGTCTTGAAACACGGACCAAGG	Vonnemann et al. (2005)
C2R	Reverse	ACTCTCTCTTCAAAGTTCTTTTC	Dayrat et al. (2001)

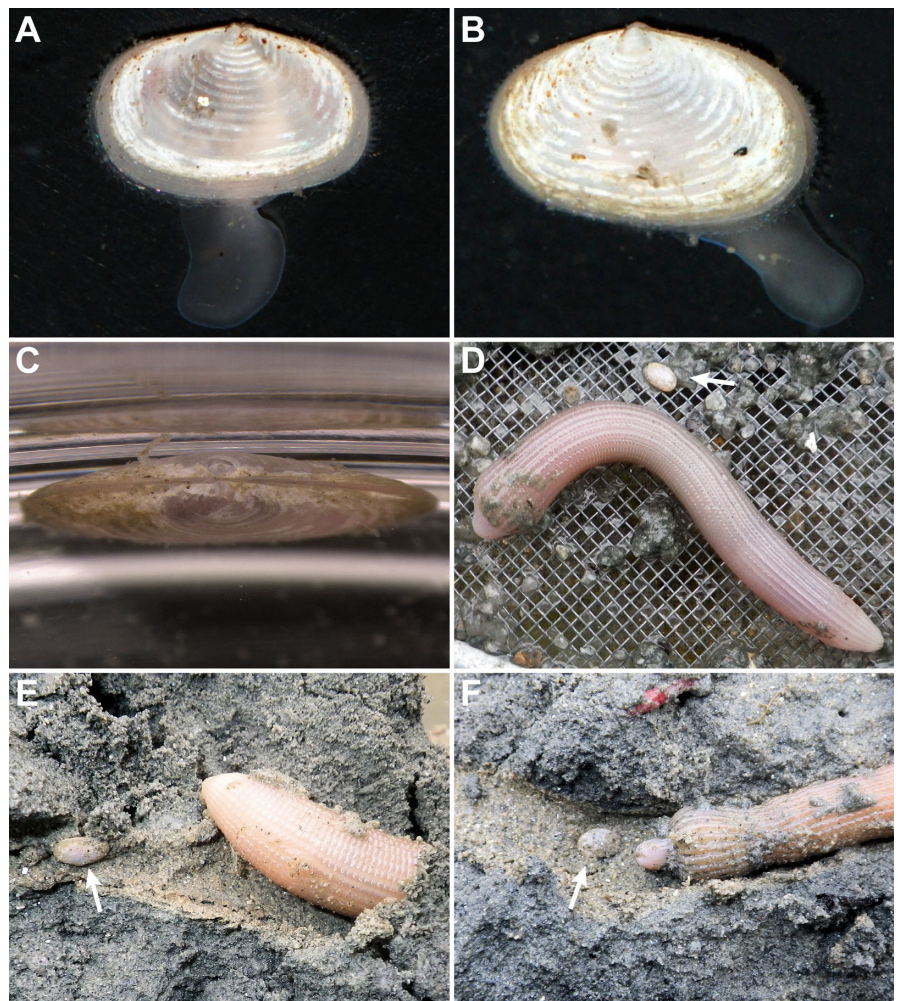


Fig. 1. *Platomyia rugata* and its host sipunculan *Sipunculus nudus*. (A) Left side of *P. rugata*. (B) Right side of *P. rugata*. (C) Dorsal view of *P. rugata*. (A–C) are different individuals. (D) *Platomyia rugata* and its host *S. nudus*. (E, F) *Platomyia rugata* in the burrows of *S. nudus*. Each arrow indicates *P. rugata*.

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. Maximum likelihood analyses were performed using RAxML (Stamatakis, 2006) as implemented in raxmlGUI 1.31 (Silvestro and Michalak, 2012). The robustness of the ML tree was evaluated by 1000 bootstrap replications. Datasets were partitioned by gene and the GTRGAMMA model was implemented.

RESULTS

Host association

We surveyed approximately 200 and 45 burrows of *S. nudus* in Hakatajima and Takehara, respectively, resulting that eight and three specimens of *P. rugata* were collected in each study site. All the bivalves were found as a solitary in the burrows of *S. nudus* (Fig. 1D–F). The occurrence rate of *P. rugata* in *S. nudus* burrows was ~4 and ~6.7% in Hakatajima and Takehara, respectively. The bivalves were attached to the burrow-wall surface mainly near the host body (Fig. 1E, F), whereas those directly attaching to the hosts were not found. We observed the bivalve behavior in an aquaria with its host for a while. The bivalve hid under the host body, but did not attach to the host. The other sipunculan *Siphonosoma cumanense* commonly inhabits mud flats in these study sites (Goto et al., 2011). We surveyed at least 20 burrows of *S. cumanense* in each study site. However, *P. rugata* was not collected from the *S. cumanense* burrows. We also surveyed the burrows of echinurans (*Ikedosome gogoshimense* and *Arhynchite hayaoi*), holothurians (*Protankyra bidentata*), and echinoids (*Echinocardium cordatum*), but did not find any *P. rugata*.

Shell morphology and anatomy

Shell: The shell is very thin and fragile, its shape elongate-ovate and equivalve and nearly equilateral with beak in the middle (Figs. 1, 2). The anterior is slightly more expanded than posterior. Shell is covered by whitish, thin periostracum (Figs. 1, 2). Sculpture is prominent, consisting of distinct and evenly spaced commarginal ribs and close-set concentric striae at shell ventral margin. Radial ribs are absent. The pallial line is entire. Hinge of each valve consists of a single stout cardinal tooth in front of the umbo and well-developed oblique internal ligament posterior to the umbo (Fig. 2F, G).

Anatomy: A single large inner demibranch is present

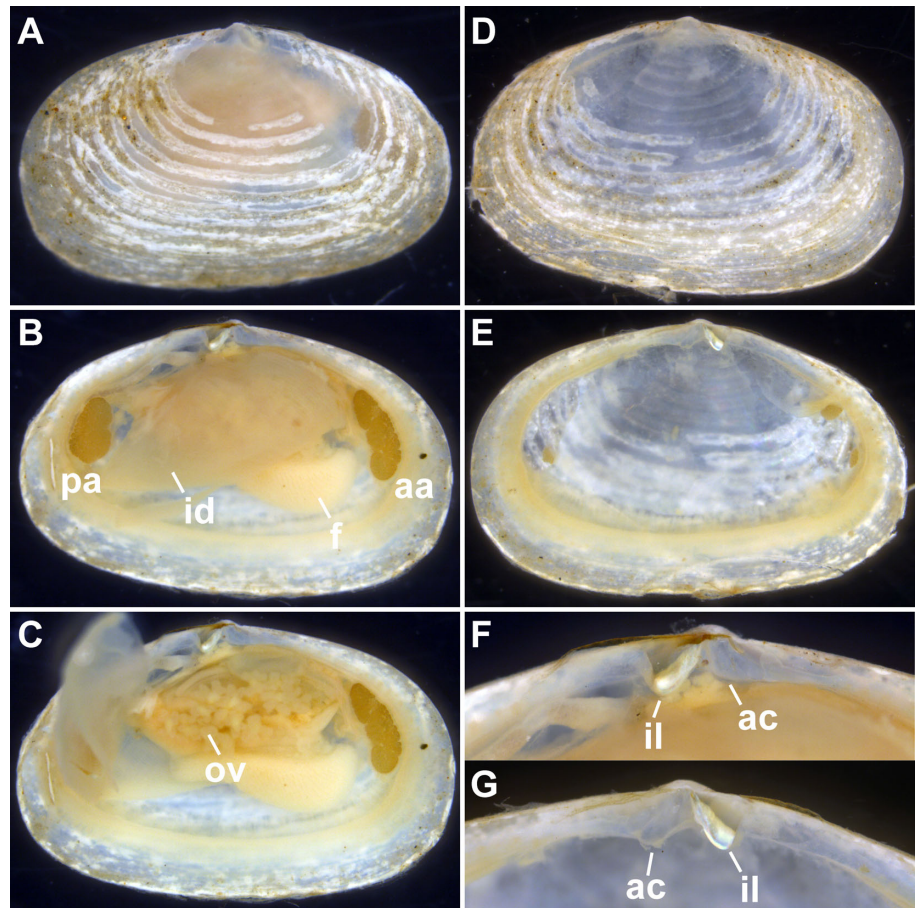


Fig. 2. *Platymysia rugata*. Left valve (A–C). Right valve (D, E). The hinge structure of left valve (F) and right valve (G). Shell length = 5.5 mm. Abbreviations: aa, anterior adductor muscle; ac, anterior cardinal tooth; f, foot; id, inner demibranch; il, internal ligament; ov, ovary; pa, posterior adductor muscle.

and covers the frequently branched ovary (Fig. 2B, C). Small labial palps are attached to the anterior end of the demibranch. Anterior and posterior adductor muscles are ovate, anterior is more elongate and larger than posterior (Fig. 2B). Foot is moderate-sized, tongue-shaped, with a small, prominent heel and a large rounded toe (Fig. 1A, B). The mantle edges narrowly extend beyond the margin of the shell and bear numerous regularly arranged very short papillae (Fig. 1A, B). No prominent tentacles were observed.

Phylogenetic position

To determine the phylogenetic position of *P. rugata* in Galeommatoidea, we performed molecular phylogenetic analyses using 41 galeommatoideans and eight outgroup species (including five heterodont and three non-heterodont bivalve species) (Table 1). In the resulting tree (Fig. 3), *P. rugata* was monophyletic with *Pseudopythina*, *Peregrinamor*, and *Byssobornia* (Bayesian posterior probability = 1.00, Bootstrap support value = 100). Sipunculan-associated species was not recovered as monophyletic (Fig. 3). *Platymysia rugata* is relatively closely related with another sipunculan-associated species *Pseudopythina* aff. *nodosa* (Fig. 3), although they were not monophyletic (Fig. 3).

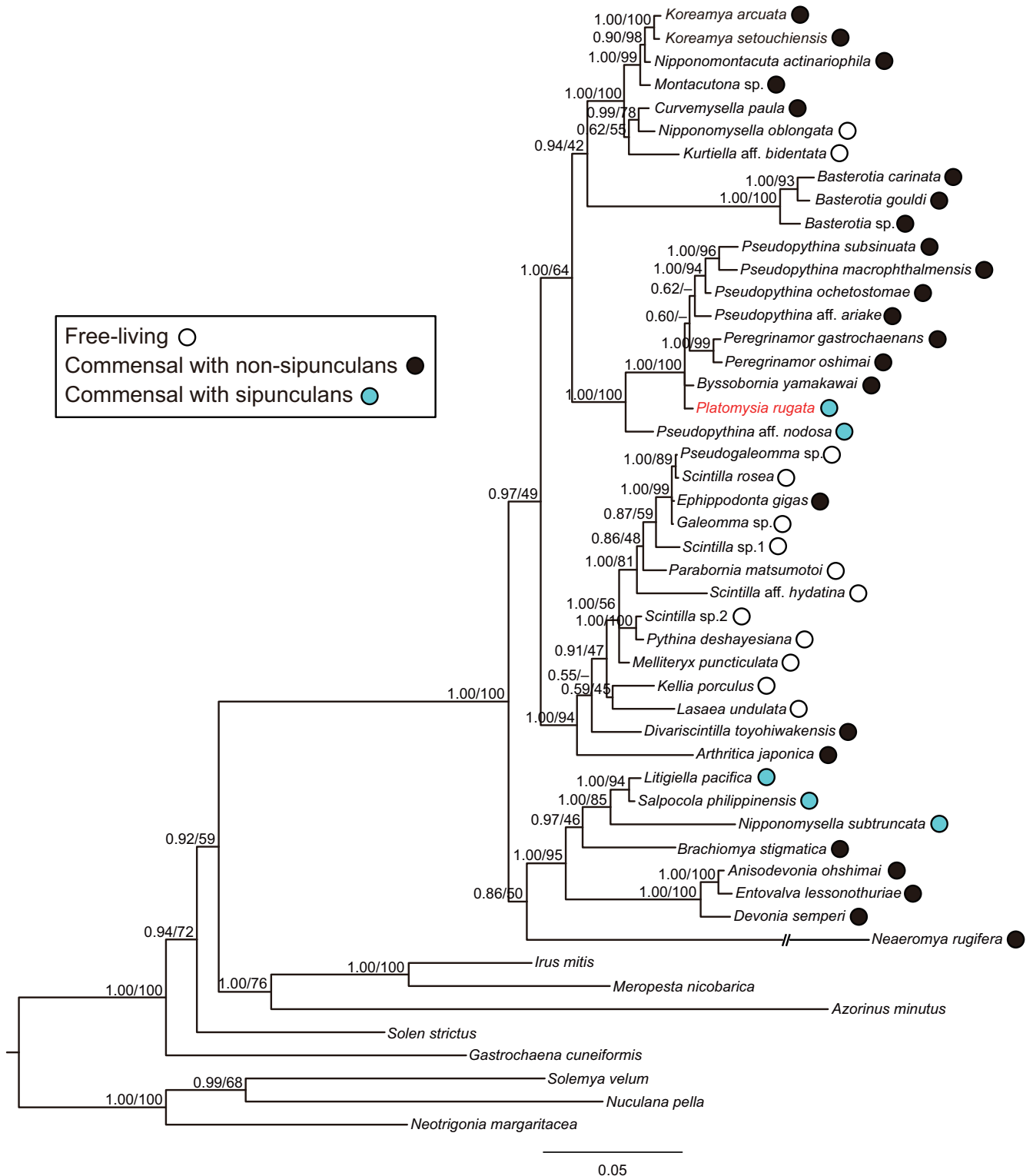


Fig. 3. Phylogenetic position of *Platomysia rugata* within Galeommatoidea. The Bayesian tree was reconstructed based on combined sequence data (18S + 28S). Numbers above branches indicate Bayesian posterior probabilities followed by supporting maximum likelihood bootstrap values. White circles indicate free-living species; black circles, commensal species associated with non-sipunculan hosts; blue circles, commensal species associated with sipunculan hosts. Information on host taxa is based on Goto et al. (2012) and Goto et al. (2014).

DISCUSSION

We found that *P. rugata* is a commensal, living in the

burrow of the sipunculan *S. nudus*. This bivalve probably feeds on plankton or small organic particles in the water currents caused by host sipunculans, using the host burrows as

a shelter from predators. *Platommisia rugata* was always attached to the host burrow wall and has never been found from the host body surface in this study. Such a host-use pattern is different from those of other galeommatoideans associated with *S. nudus*; *Pseudopythina nodosa* and *P. aff. nodosa* are attached to the host body surface (Morton and Scott, 1989; Goto et al., 2012), *Salpocola philippinensis* and *Fronsella ohshimai* are attached to the posterior end of the host (Habe, 1951, 1958; Lützen et al., 2008), and *Litigiella pacifica* is attached to both host body surface and burrow wall (Lützen and Kosuge, 2006). Such niche partitioning in *S. nudus*-associated galeommatoideans may be the result of adaptations that reduce the interspecific competition for the host. On the other hand, recent molecular phylogeny showed that *S. nudus* includes not a few cryptic species (Kawauchi and Giribet, 2013). Thus, it is also possible that difference of host-use mode among *S. nudus*-associated galeommatoideans is related with specialization to different host species. Except for *P. rugata*, four galeommatoideans are associated with *S. nudus* in Japan: *L. pacifica*, *P. aff. nodosa*, *S. philippinensis*, and *F. ohshimai* (Habe, 1958; Lützen and Kosuge, 2006; Lützen et al., 2008; Goto et al., 2012). *Platommisia rugata* has been known from temperate coasts of Japan (Habe, 1951; Fukuda, 2012), whereas *L. pacifica*, *P. aff. nodosa*, and *S. philippinensis* are distributed in subtropical to tropical coasts of Japan (mainly the Ryukyu Islands) (Lützen and Kosuge, 2006; Lützen et al., 2008; Goto et al., 2012; Goto pers. obs.). Thus, the distribution of *P. rugata* does not overlap those of the latter three species. The holotype of *F. ohshimai* was collected with *S. nudus* in Amakusa, Nagasaki Prefecture, Japan (Habe, 1958). However, no living animals have been recorded since the first description.

Habe (1977) suggested that, although *P. rugata* resembles thracids, it belongs to Galeommatoidea because it has no pallial sinus. Contrary to this, Huber (2015) doubted its assignment in Galeommatoidea and perceived it as a juvenile of another Japanese heterodont species, e.g., *Lutrophora*, or even *Panomya* or *Panopea*. However, our phylogenetic analyses suggest that *P. rugata* definitely belongs to Galeommatoidea (Fig. 3). Our analyses also suggest that sipunculan-associated galeommatoideans are not monophyletic (Fig. 3). *Platommisia rugata* is relatively closely related with other sipunculan-associated species *P. aff. nodosa* (Fig. 3). However, whether evolutionary origin of their symbiotic associations with sipunculans is same or not remains unclear. On the other hand, *P. rugata* belongs to the clade of *Pseudopythina*, *Peregrinamor*, and *Byssobornia* (Fig. 3). All of the members of this clade are commensally associated with burrowing invertebrates (Morton and Scott, 1989; Goto and Kato, 2012). Some are attached to the host body surface (Kato and Itani, 1995), whereas the others are attached to the host burrow-wall surface (Morton, 1972; Morton and Scott, 1989; Goto and Kato, 2012). In this clade, the host taxa are highly diverse, including mantis shrimps (*P. subsinuata*), crabs (*P. macrophthalmensis*), echiurans (*P. ochetostomae* and *B. yamakawai*), sipunculans (*P. rugata*), and holothurians (*P. aff. ariake*) (Morton and Scott, 1989; Goto and Kato, 2012; Goto et al., 2012; this study). This suggests that host switching across different animal groups have occurred frequently in this clade.

Habe (1951) described *P. rugata* based only on a single right valve shell. In this study, we described the hinge structure of both of right and left valves, each of which has one anterior cardinal and one oblique internal ligament posterior to the umbo (Fig. 2F, G). This hinge structure is basically similar to those of *Pseudopythina* described in Morton and Scott (1989). In addition, this species has a single demi-branch like *Pseudopythina* (Jespersen et al., 2009). Thus, close relatedness of this species with *Pseudopythina* is supported by both the shell morphology and anatomical characteristics.

Platommisia rugata is a new example of commensal galeommatoidean from the Seto Inland Sea. Until now, various commensal galeommatoideans have been reported from around this area, including *Arthritica japonica*, *Divariscintilla toyohiwakensis*, *Koreamya setouchiensis*, *Nipponomysella subtruncata*, *Basterotia gouldi*, *Curvemysella paula*, *Peregrinamor oshimai*, *Pseudopythina subsinuata*, and *Pseudopythina aff. ariakensis* (Kato and Itani, 1995; Lützen and Takahashi, 2003; Goto et al., 2007, 2011, 2012, 2014; Yamashita et al., 2011). Each of these galeommatoideans uses a different burrowing invertebrate as a host. Thus, the diversity of symbiotic galeommatoideans in the Seto Inland Sea is probably a function of a rich burrowing invertebrate fauna in mud flats.

ACKNOWLEDGMENTS

We wish to express our sincere gratitude to S. Kojima (University of Tokyo), Y. Kano (University of Tokyo), and D. Ó Foighil (University of Michigan) for hosting the first author as a research fellow for this study and permission to use the facilities in the laboratory. We also thank T. Lee (University of Michigan) for helping the first author to set up the laboratory facilities to observe specimens. This study was partially supported by grants to RG from the Japan Society for the Promotion of Science (JSPS) Research Fellowship for Young Scientists and the JSPS Postdoctoral Fellowships for Research Abroad.

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(Received January 9, 2016 / Accepted March 15, 2016)