On a New Species of Parasitic Barnacle (Crustacea: Rhizocephala), *Sacculina shiinoi* sp. nov., Parasitizing Japanese Mud Shrimps *Upogebia* spp. (Decapoda: Thalassinidea: Upogebiidae), Including a Description of a Novel Morphological Structure in the Rhizocephala

Author(s): Jørgen Lützen , Gyo Itani , Åse Jespersen , Jae-Sang Hong , David Rees and Henrik Glenner

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On a New Species of Parasitic Barnacle (Crustacea: Rhizocephala), *Sacculina shiinoi* sp. nov., Parasitizing Japanese Mud Shrimps *Upogebia* spp. (Decapoda: Thalassinidea: Upogebiidae), Including a Description of a Novel Morphological Structure in the Rhizocephala

Jørgen Lützen[1], Gyo Itani[2], Åse Jespersen[1], Jae-Sang Hong[3], David Rees[4], and Henrik Glenner[4,5]*

[1]*Marine Biology Section, Biological Department, Copenhagen University, Universitetsparken 4, DK2100, Copenhagen Ø, Denmark*
[2]*Laboratory of Marine Symbiotic Biology, Faculty of Education, Kochi University, 2-5-1 Akebono, Kochi 780-8520, Japan*
[3]*Department of Ocean Science College of Natural Sciences, Inha University, Incheon 22212, Republic of Korea*
[4]*Marine Biodiversity, Department of Biology, University of Bergen, Thormøhlensgate 53a, Box 7800 N-5020, Bergen, Norway*
[5]*CMEC, Natural History Museum, University of Copenhagen, Denmark. Universitetsparken 4, DK2100, Copenhagen Ø, Denmark*

The rhizocephalan *Sacculina shiinoi* sp. nov. parasitizes three species of *Upogebia* in Japan. It is described morphologically and compared with another *Upogebia* parasite, *Sacculina upogebiae* Shiino, 1943 from Japan and Korea. These two species are the only sacculinids that parasitize mud shrimps. DNA analyses clearly show the two species to be separate and not closely related. The cuticle differs in being provided with close-set, branched, and spiny excrescences in *S. shiinoi*, while it lacks excrescences, but forms small scales in *S. upogebiae*. In *S. upogebiae*, the bulbous sperm-producing part and the narrow receptacle duct are separated by a compartmentalized mid portion, which is missing in *S. shiinoi*. A ridge, having a thickened, fluffy cuticle with a U-shaped course, passes across the visceral mass between the two receptacle openings in *S. shiinoi*. Such a structure has never been described in other rhizocephalans, and its function is uncertain.

Key words: *Sacculina shiinoi*, *Sacculina upogebiae*, *Upogebia*, Rhizocephala, DNA analysis

INTRODUCTION

Rhizocephala is a small group of highly modified parasites of other crustaceans, mainly decapod crustaceans (e.g., shrimps and crabs). Although they are phylogenetically closely related to thoracican barnacles, the adult organism is morphologically extremely reduced and bears no resemblance to conventional suspension-feeding barnacles. The adult female parasite is situated ventrally on the abdomen of the host, at a protected position in which gravid, adult female decapods usually carry their eggs. The parasite consists of an external reproductive organ, the externa, and inside the host, a root-like structure, the interna. The smooth sac-like externa is clad with a delicate cuticle, which covers a mantle and a mantle cavity, which contains the egg mass of the parasite, and the visceral mass with the ovary. Also located in the visceral mass is a pair of hollow receptacles, in which a pair of diminutive dwarf males is harbored. The dwarf males are modified larvae, which, nourished by the female parasite, produce the semen necessary to fertilize the female-laid eggs in the mantle cavity. The externa is connected to the interna via a stalk that penetrates the integument of the host. These root-like structures are interwoven with the viscera of the host, from which they extract nutrition through an epicuticular epithelium.

Currently about 280 species of rhizocephalans are known, which are classified into the two groups Akentrogonida and Kentrogonida, based on the presence/absence of a particular infectious larval stage, the kentrogon. The kentrogonid family Sacculinidae currently contains seven genera and ca. 125 species that are parasitic on brachyuran crabs, with only two known exceptions: *Sacculina anceps* Boschma, 1931 and *S. flacca* Boschma, 1931, which are are parasitic on the anomurans *Albunea symnista* (Linnaeus, 1758) and *Galathea* sp.; and *S. upogebiae* Shiino, 1943, which is a parasite of the thalassinidean

* Corresponding author. Tel. : +47-55584526;
Fax : +47-55584400;
E-mail: henrik.glenner@uib.no
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Upogebia major (de Haan, 1841). From the description of *S. upogebiae* by Shiino (1943), there can be little doubt about its correct familial affiliation, which will be confirmed in the present study, in which we investigate new *S. upogebiae* material from two specimens of *Upogebia major* collected in Korea.

When additional parasites from three other species of *Upogebia* in Japanese waters became available, we took the opportunity to examine them in more detail. Unexpectedly, they differed in so many respects from *S. upogebiae* that we had to establish a new species. In addition, we discovered a novel morphological structure within the externa (reproductive body) that is either exclusive to the new species or, if it occurs in other rhizocephalans, has been overlooked until now.

The familial status of the Sacculinidae is unsettled, as the family is clearly diphyletic and in need of revision (Glenner and Hebsgaard, 2006). Naturally, the clade containing *S. carcini* Thompson, 1836, the type species of the type genus, represents the true Sacculinidae, whereas it is uncertain at present by which higher-taxon name the other clade should be referred to. We present morphological and molecular evidence that the present two rhizocephalan species parasitizing *Upogebia* spp. not only represent different species, but also that each belongs to its own "sacculinid" clade. Because the "family" needs a thorough revision, which is far from the scope of the present study, we have provisionally used the genus name *Sacculina* for both species in question, well aware that future studies will likely result in the transfer of the two species to other, existing or new, genera.

MATERIALS AND METHODS

Sampling

Mud shrimps were dug at tidal flats in Korea (Boryeong, Chungcheongnam-Do) and Japan (Shikoku, Hiroshima and Kagawa Prefecture). The infected shrimps were fixed in formalin or ethanol with the rhizocephalan externae undetached. Eight externae of the new species were procured from seven individuals of mud shrimps (six *U. yokoyai*, one *U. issaeffi*, and one *U. sakaii*) in Japan. Three specimens of *S. upogebiae*, each represented by an externa on a host were found on *U. major* from in Korea. The material was deposited at the Natural History Museum of Denmark (University of Copenhagen) and provided with serial numbers (ZMUC-CRU-4759-4771), except for the material of *U. sakaii* and the attached externa, which were identified but not deposited.

Morphological observation

Two externae of the new species (4.5–5.0 mm in length, both from *U. yokoyai*) were selected for microanatomical examination. The mid portion of the receptacles of one specimen of *S. upogebiae* was dissected out. Specimens to be sectioned were embedded in araldite and cut on a Leica Ultracut ultramicrotome into 2-μm-thick serial sections that were stained with toluidine blue. One of the externae of the new species was cut parallel to the axis connecting the mantle opening and the stalk, the other one more obliquely to that axis. The receptacles of *S. upogebiae* were cut in transverse section. Scanning electron microscopy (SEM) was used to study the surface of the external cuticle of the mantle. The SEMicrographs were taken with a JEOL JSM-840 microscope. , In the Results section, the material examined is referred to using museum voucher numbers.

Molecular techniques

DNA extraction. Total genomic DNA was extracted from ~1 mm^3 of tissue from the mantle of individual externae using the Qiagen DNeasy Blood & Tissue Kit following the Qiagen DNeasy Protocol for Animal Tissues 07/2006.

Gene amplification. DNA fragments from two nuclear and one mitochondrial genes were amplified and sequenced using the primers indicated in Table 1. Nearly complete coverage (~1800 bp) of the nuclear 18S rDNA was achieved together with a ~700-bp fragment of the nuclear 28S rDNA. For the mitochondrial 16S rDNA, two primer pairs were utilized yielding approximately 500 bp and 360 bp, respectively, for *S. shiinoi* and *S. upogebiae*.

All PCR reactions were carried out using a Bio-Rad C1000 Thermal Cycler in 25 μl volumes containing 1-μl DNA extract, 2.5-μl 10× PCR buffer, 1.2-μl dNTP mixture (2.5 μM each), 1 μl of each 10-μM primer, and 0.75 U of Takara polymerase. Conditions for all amplifications were as follows: 94°C for 5 min, 35 cycles of 30 s at 94°C, 1 min at 52°C, and 1 min at 72°C, then 7 min at 72°C. All PCR products were visualized on 1% agarose gels and stored at 4°C prior to purification and sequencing. PCR products were cleaned by the addition of 0.1 μl (1 U) exonuclease I, 1 μl (1 U) of Shrimp Alkaline Phosphatase and 0.9 μl of ddH_2O to 8 μl of PCR product. This was followed by incubation at 37°C for 30 min and deactivation of the enzymes at 85°C for 15 min. Sequence reactions were performed using the BigDye v. 3.1 Cycle Sequencing kit (Applied Biosystems, Inc., Norwalk, CT, USA) with the same primers used for initial PCR amplification. Both strands of all PCR products were sequenced using an ABI 3730 capillary sequencer. Sequence data for all samples have been submitted to GenBank under the accession numbers KF539757–KF539762.

Sequence analyses. All PCR products were sequenced for both the sense and anti-sense strands in order to improve accuracy and aligned using Clustal W (Thompson et al., 1994) implemented in eBioX v. 1.5.1 (www.ebioinformatics.org) with characters equally weighted and using default parameters. Additional data for outgroup and ingroup taxa were taken from GenBank; accession numbers for all taxa included in alignments and phylogenetic analyses are shown in Table 2. Following minor improvements by eye (performed in Mesquite v. 2.6; Maddison and Maddison, 2009), duplicates were made of alignments for each gene prior to further analyses. One duplicate of the data set was analyzed without further manipulation and the other was run through Gblocks (implemented in Seaview: Gouy et al., 2010; Castresana, 2000) to remove hypervariable and potentially problematic regions in the alignment. Gblocks criteria

Table 1. Primer details for gene amplification (16S, 18S, 28S rDNA); primer directions are indicated in parentheses by F (forward) and R (reverse).

Gene	Primer	Sequence (5′ to 3′)	Reference
18S	329 (F)	TAATGATCCTTCCGCAGGTT	Spears et al. 1992
	a- (R)	CAGCMGCCGCGGTAATWC	Spears et al. 1992
	345+ (F)	GCATCGTTTAHGGTT	Spears et al. 1992
	UnivF15 (R)	CTGCCAGTAGTCATATGC	Frischer et al. 2002
28S	1274 (F)	GACCCGTCTTGAAACACGGA	Nunn et al. 1996
	FF (R)	GGTGAGTTGTTACACACTCCTTAG	modified from Hillis and Dixon 1991
16S	16Sar-L (F)	CGCCTGTTTATCAAAAACAT	Palumbi 1991
	16Sbr-H (R)	CCGGTCTGAACTCAGATCACGT	Palumbi 1991
	H621 (F)	CYGTGCAAAGGTAGCATA	Tsuchida et al. 2006
	L12247L (R)	TTAATYCAACATCGAGGTCRC	Tsuchida et al. 2006

Table 2. GenBank accession numbers for the species (other than *S. upogebiae* and *S. shiinoi*) included in the phylogenetic analyses. Genes sequenced for the present study are indicated by an asterisk.

Taxon	16S	18S	28S
Clistosaccus paguri	–	GU190697	GU190709
Heterosaccus californicus	AY520756	AY520657	AY520623
Heterosaccus dollfusi	FJ481949	EU082413	EU082333
Heterosaccus lunatus	FJ481947	EU082414	EU082334
Ibla quadrivalvis	AY520755	AY520655	AY520621
Lepas anatifera	FJ906773	GU993670	GU993603
Loxothylacus panopaei	FJ481956	AY265364	–
Loxothylacus texanus	–	L26517	–
Peltogaster paguri	unpublished	EU082415	EU082335
Poecilasma kaempferi	–	EU082410	EU082329
Polyascus gregaria	JN616263	AY265363	GU190705
Polyascus plana	FJ481954	AY265368	GU190698
Polyascus polygenea	–	AY265362	GU190704
Pottsia serenei	–	DQ826567	GU190702
Sacculina carcini	FJ481957	AY265366	AY520622
Sacculina confragosa	–	AY265361	GU190706
Sacculina leptodiae	FJ481952	AY265365	–
Sacculina oblonga	FJ481953	AY265367	GU190699
Sacculina sinensis	–	AY265360	GU190707
Sylon hippolytes	–	DQ826564	GU190700
Thompsonia littoralis	–	DQ826573	–

used for this were for a less stringent selection, allowing for gaps within blocks and smaller final blocks. The two treatments (Gblocks and 'non-Gblocks') for each gene alignment were then concatenated in Seaview (Gouy et al., 2010) before final phylogenetic analyses. Following alignment with Clustal W, lengths of individual gene data sets were 2278 bp for 28S, 1969 bp for 18S, and 569 bp for 16S; the concatenated non-Gblocks data set was 4816 bp. Following removal of the more variable and problematic regions of the alignment with Gblocks, the data sets were 1652 bp, 668 bp, and 411 bp for 18S, 28S, and 16S respectively. The concatenated Gblocks data set was 2731 bp.

Phylogenetic analyses. Ribosomal DNA sequences from three thoracican and 20 rhizocephalan barnacle species were obtained from GenBank (Table 2) to provide phylogenetic framework for the taxonomic placement of *S. upogebiae* and the new species.

Two different methods for determining phylogenies were performed in this study: Bayesian inference (BI) and maximum likelihood (ML). ML analyses were performed using Seaview v. 4.4.1 (Gouy et al., 2010), with 500 bootstrap pseudo-replicates to determine node support. BI was performed using MrBayes v. 3.2.2 (Ronquist and Huelsenbeck, 2003). Metropolis-coupled Markov chains Monte Carlo were run for 20 million generations in two simultaneous runs, each with three heated chains and one cold chain. Monitoring the fluctuating value of the likelihood and comparing standard deviation of the split frequencies, validated convergence of the analyses. jModelTest v. 0.1.1 (Posada, 2008) were used to select the best-fit model of nucleotide substitution for the Baysian analyses, and the likelihood scores from each model were compared using Akaike information criterion. The general time reversible model with rate heterogeneity and invariable sites (GTR + G + I) was selected as the best-fit model of nucleotide substitution for all the molecular data set, except the Gblocked 28S gene data set where the GTR + G model was found best-fit. Therefore, the GTR + G + I model was applied to the combined Gblocked analysis while the unconstrained data set was partitioned prior to the MrBayes analyses using two models: GTR + G (28S) and GTR + G + I (18S + 16S). Topologies were sampled every 2000 generations and the first 2500 trees (25%) were discarded as 'burn in'. The posterior probabilities for individual clades obtained from the two separate runs were compared for congruence and summarized on a majority-rule consensus tree.

RESULTS

Taxonomy

Sacculina shiinoi sp. nov.
(Figs. 1, 2A, B, 3, 4A–C, 5A)

Diagnosis. External cuticle with numerous, close-set, spiny excrescences. Complete dorsal mesentery membranous, ventral mesentery absent. Colleteric glands with relatively few, but wide, blind tubes. Seminiferous part represented by two, well-separated receptacles spheriform to oviform; receptacle ducts long, straight, and narrow. Receptacles placed between ovary and base of stalk. U-shaped ridge lined by tall, split-up cuticle running across posterior part of visceral mass from one receptacle opening to the other.

Material examined. *Holotype*: ZMUC-CRU-4767, in alcohol, intact specimen, 10.4 × 7.4 mm, attached to *U. yokoyai*, collected on a tidal flat, Doki River, Kagawa Prefecture, Shikoku, Japan, 12 March 2013.

Paratypes. The following six specimens were examined: ZMUC-CRU-4764, on the same host as holotype; ZMUC-CRU-4759, in alcohol, intact specimen, externa (8.2 × 4.8 mm) on *U. yokoyai*, collected at Susaki Bay, Kochi Prefecture, Shikoku, Japan, 30 June 2010 (Fig. 2A); ZMUC-CRU-4760, in alcohol, a scar on *U. yokoyai*, collected in a tidal flat at Uranouchi Inlet, Kochi Prefecture, Shikoku, Japan, 15 June 2011; ZMUC-CRU-4762, in alcohol, partly intact specimen on *U. yokoyai*, collected in Susaki Bay, Kochi Prefecture, Shikoku, Japan, June 2008, ZMUC-CRU-4763, on *U. yokoyai* collected at a tidal flat in Uranouchi Inlet, Kochi Prefecture, Shikoku, Japan, in May 2012; ZMUC-CRU-4769, serial sections made from ZMUC-CRU-4763; ZMUC-CRU-4761, serial sections, from *U. issaeffi* collected at a tidal flat, Kurahashi Island, Hiroshima Prefecture, Japan, in July 2012; ZMUC-CRU-4770, serial sections made from ZMUC-CRU-4761, 52 slides and SEM.

Etymology. The specific name is a noun in the genitive case, in honor of the late Sueo M. Shiino (1908–1978), a distinguished Japanese zoologist and expert on crustacean fish parasites and Rhizocephala (Ooishi, 1979).

Description. All externae were found attached near the median line on the ventral surface of the first or second abdominal segment of the host shrimp. The cephalothorax lengths of the hosts (CL) were 6.5, 12.0, 13.5,14.0, and 14.0 mm (*U. yokoyai*), 7.5 mm (*U. issaeffi*), and 8.6 mm (*U. sakaii*), respectively.

The externa is bean-shaped or more or less triangular, with the dorsal and ventral halves being bluntly rounded posteriorly (Fig. 1). The opening is slightly raised above the surface and may be displaced at one side. In one specimen either the dorsal or ventral half was inflated and hump-shaped. The mantle surface is quite smooth, with only a few wrinkles. The stalk is short with a broad base towards the host and issues from the middle of the externa. Width (= dorso-ventral distance) and height (= distance from stalk to the anterior-most margin) measured 8.2 mm wide (ZMUC-

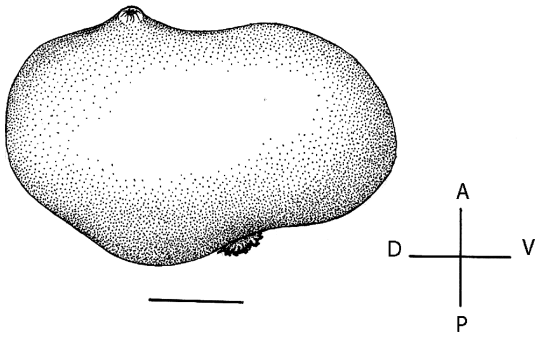

Fig. 1. *Sacculina shiinoi* sp. nov., holotype (ZMUC-CRU-4767), seen from left side. A, D, P, and V denote anatomical directions (anterior, dorsal, posterior, ventral). Mantle opening upwards, stalk downwards. Scale represents 5 mm.

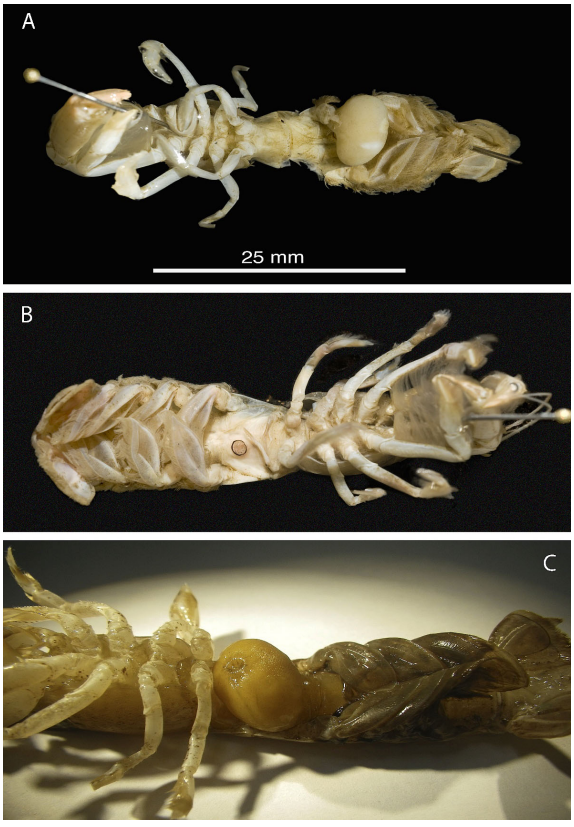

Fig. 2. **(A)** *Sacculina shiinoi* sp. nov., paratype (ZMUC-CRU-4759), an ovigerous externa on the abdomen of *Upogebia yokoyai*, CL 14.0 mm; Suzaki, Kochi, Japan. **(B)** *Sacculina shiinoi* sp. nov., paratype (ZMUC-CRU-4760), a scar, probably from lost externa of *S. shiinoi* sp. nov. on the abdomen of *U. yokoyai,* CL 14.5 mm; Uranouchi Bay, Kochi, Japan. **(C)** *Sacculina upogebiae* Shiino, 1943, non-type specimen (ZMUC-CRU-4765), on the abdomen of *Upogebia major*; Apeojang, Incheon, Republic of Korea. Scale bar valid for all figures.

CRU-4759), scarred crab (ZMUC-CRU-4760), 4.5 × 3.2 mm (ZMUC-CRU-4762), 4.5 × 3.5 mm (ZMUC-CRU-4761), 5.0 × 3.3 mm (ZMUC-CRU-4763), not measured (ZMUC-CRU-4764) in the six voucher specimens, and 10.4 × 7.4 mm in the largest externa (ZMUC-CRU-4767). A thin mesentery connects the visceral mass to the dorsal mantle and ranges from near the minute mantle aperture to the region of the stalk. A ventral mesentery is missing. In an evidently fairly large specimen that had been lost, the stalk had left a distinct circular and heavily cuticularized blackish scar upon the host skin (Fig. 2B). At least four of the externae were ovigerous and had the mantle cavity full of ova that seemed to be newly laid in three, while it contained advanced embryos in a fourth. The exterior mantle cuticle is provided with numerous minute and close-set excrescences. Each consists of an oblong conical stem, up to 25 μm long and beset with many short but stout and pointed bristles which emerge more or less perpendicular to the stem (Figs. 3A, 5A).

The surface of the lower part of the visceral mass forms a distinct U-shaped ridge, the two branches of which start at the margin of each of the two receptacle openings (Fig. 3E). From there, each of them runs in a more or less ventral direction to unite at the ventral margin of the visceral mass (Fig. 3D). For most of their length the ridges are raised 100 μm above the surface and are lined by a 20–25-μm high epithelium composed of tall, slender cells in stark contrast to the otherwise 2–3-μm high squamous epithelium lining the visceral mass (Fig. 3B, C). The epithelium of the ridges produces an up to 18-μm-thick cuticle, the surface of which looks fluffy as it splits into many hairy processes. At the receptacle opening, the ridges flatten out and the specialized cuticle lowers, continuing into the cuticle of the openings (Fig. 3E). The cuticle on the ridge was higher in the externa (ZMUC-CRU-4763 and ZMUC-CRU-4769 [serial sections made from the former]) containing advanced embryos than in another one with new laid ova (ZMUC-CRU-4761 and ZMUC-CRU-4770 [serial sections made from the former]). This indicates that it is being replaced immediately at the molt of the internal mantle cuticle, but also that it possibly increases further in height until the moment the larvae are released.

The paired colleteric glands, which produce the spongy ovisac enveloping the laid eggs (Delage, 1884; Lange, 2002), are placed superficially on each side of the visceral mass, closer to the mantle aperture than to the stalk. Each gland is disc-shaped and consists of a moderate number of blind and slightly divided tubules, which issue from the gland's center, or atrium (Fig. 4A). In the two sections (ZMUC-CRU-4770 and ZMUC-CRU-4769) the glands measure ca. 650 μm in diameter. Each of the tubule cells produces an individual cone-shaped secretion just as in *Sacculina carcini* (Lange, 2002).

The paired receptacles are placed in the tissue of the visceral mass, which separates the ovary from the base of the stalk. The receptacles are well separated from each other throughout and never fuse. The structure of each receptacle is very simple. The terminal sperm-producing part is a spherical to oviform body, 250 × 150 μm in largest and smallest diameter, and surrounded by a very thin muscle coating (Fig. 4C). Within this muscle layer there is a massive tissue consisting of a multitude of rounded cells with solid walls. The peripheral part of this spherical body consists of a thick wall consisting of a multitude of rounded cells with solid walls that form a massive not-layered tissue. Centrally the tissue gives way to a cavity in which spermatogenesis takes place. The cavity is connected to the mantle

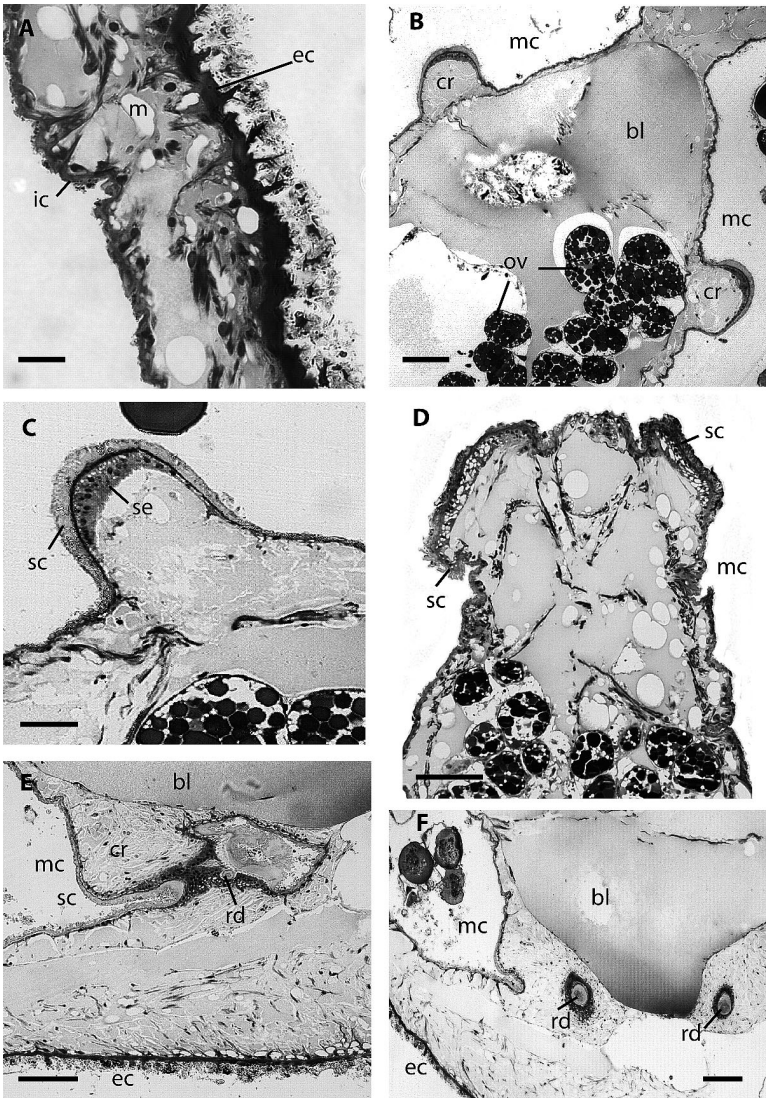

Fig. 3. *Sacculina shiinoi* sp. nov., paratype (ZMUC-CRU-4770). **(A)** Transverse section through mantle. **(B–D).** Horizontal sections through visceral mass. **(E)** Horizontal section through opening of receptacle duct. **(F)** Horizontal section through both receptacle ducts. Abbreviations: bl, blood lacuna; cr, cuticularized ridge; ec, external mantle cuticle with excrescences; ic, internal mantle cuticle; m, mantle; mc, mantle cavity; ov, ovary; rd, receptacle duct; sc, specialized cuticle; se, specialized epithelium. 2-μm thick araldite sections stained with toluidine blue. Scale bars: 25 μm **(A)**, 100 μm **(B, D–F)**, and 50 μm **(C)**.

cavity via a narrow, almost straight, receptacle duct ca. 450 μm in length and 50 μm in diameter. The duct is plugged with a solid cuticle substance (Figs. 3F, 4B). This plug is presumably removed when the interior mantle cuticle is cast in connection with the moulting of the mantle cavity cuticle following the release of the larvae. The openings of the two ducts into the mantle cavity are fairly wide and placed rather far from each other. One of the receptacles in one of the sectioned specimens (ZMUC-CRU-4761 and ZMUC-CRU-4770 [serial sections made from the former]) was sterile, as it did not produce spermatozoa. It was smaller and more compact than the other, fertile one (ZMUC-CRU-4763 and ZMUC-CRU-4769 [serial sections made from the former]), as it consisted mostly of the naked female wall cells surrounding an extremely small central cavity.

Remarks. The morphology of the *Sacculina shiinoi* fits well to the genus description of *Sacculina*: The externa is laterally compressed and the mantle opening is more or less opposite the stalk. The colleteric glands are situated centrally in the lateral surfaces of the visceral mass and provided with a moderate number of branched tubes. The receptacles are placed posteriorly in the basal region of the stalk. However, the phylogenetic analyses of the DNA sequencing data places, with high support values, *S. shiinoi* basal to two monophyletic clades consisting of three *Polyascus* species in one clade and four south east Asian occurring Sacculinids in another (see Fig. 6). The phylogenetic position of the two other *Sacculina* species, *S. carcini* and *S. upogebiae*, in yet another part of the phylogenetic tree, among species of the genera *Loxothylacus* and *Heterosaccus*, clearly demonstrates that the genus *Sacculina* is polyphyletic and that there is a need for a revision of the entire family Sacculinidae. Since such a revision is not the focus of the present study and would require the inclusion of a substantial number of other species from the family, we have decided to provisionally use the genus name *Sacculina*, although this is likely to change in the future.

Sacculina upogebiae Shiino, 1943
(Figs. 2C, 4D, 5B)

Material examined.

Paratypes:. ZMUC-CRU-4765, in alcohol, single externa, 11.0 × 8.0 mm, on *U. major*, collected in Apeojang, Seonjae-do, Korea, date unknown; ZMUC-CRU-4766, in formalin, single externa, 12.0 × 9.0 mm, on *U. major* (Fig. 2C), Apeojang, Seonjae-do, Korea, date unknown. ZMUC-CRU-4771, in formalin, single externa, 12.0 × 9.0 mm, on *U. major* collected at Jugyo tidal flats, Boryeong City, South Korea, on 16 April 2014.

Description. The three specimens have the same general shape as described by Shiino (1943, fig. 18A). SEM (ZMUC-CRU-4766) shows the external mantle cuticle of all specimens to be smooth without excrescences and, as noted by Shiino (1943), that the surface is divided into minute interdigitating scales with a star-shaped contour, each measuring 9–12 μm (Fig. 5B).

Each of the two receptacles consists of three portions. A terminal sperm-producing part is of conventional histological structure and thus similar to that in *S. shiinoi*. A middle portion consists a thickened whitish body, which is in direct continuation with the terminal sperm-producing part and of nearly the same diameter and wall thickness. It differs from the sperm-producing part, however, in that the lumen is not simple, but split up into many irregularly-shaped compartments (Fig. 4D). In contrast to the terminal part, the interior layer of cells lining the lumen seems to be covered by an extremely thin cuticle, while in the terminal part these cells

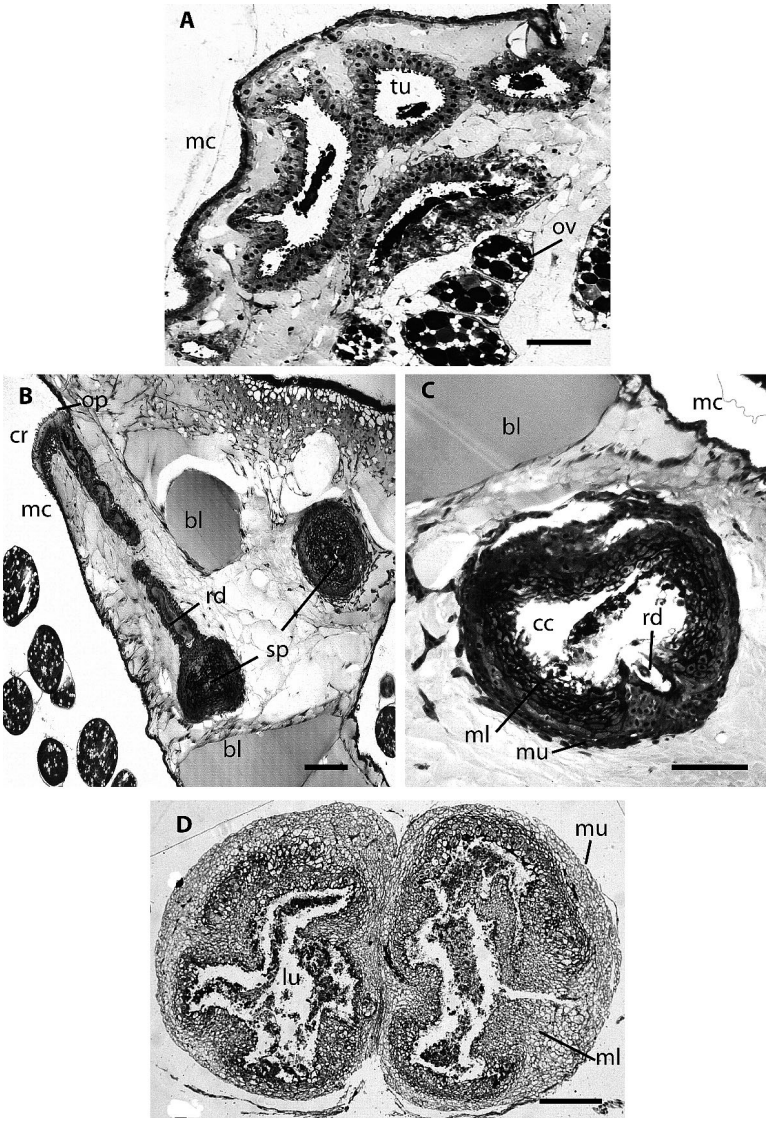

Fig. 4. **(A–C)** *Sacculina shiinoi* sp. nov., paratype (ZMUC-CRU-4769): **(A)** Transverse section through colleteric gland near its opening into the mantle cavity; **(B)** section through paired receptacles, one of them cut longitudinally; **(C)** section through sperm-producing part of a receptacle (sp) showing the receptacle duct (rd) opening into it (op). cc, central cavity with spermatogenesis; ml, multilayered wall; mu, muscle cells. **(D)** *Sacculina upogebiae* Shiino, 1943. (ZMUC-CRU-4768), transverse section of mid portion of the paired receptacles, compartmentalized lumen (lu) filled with sperm cells and cellular debris. bl blood sinus; cr cuticular ridge; mc, mantle cavity; ov ovary; tu tubule. 2-μm thick araldite sections stained with toluidine blue. Scale bars represent 100 μm **(A–B)**, 50 μm **(C)** and 200 μm **(D)**.

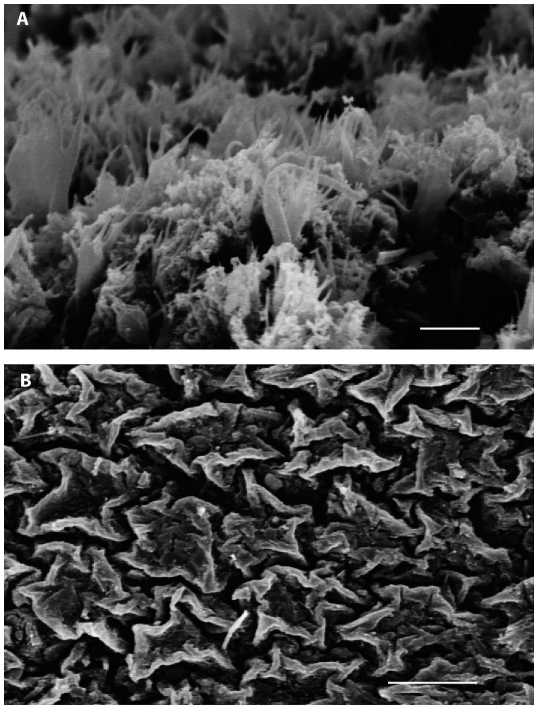

Fig. 5. SEM showing cuticle structure. **(A)** *Sacculina shiinoi* sp. nov., paratype (ZMUC-CRU-4761). **(B)** *Sacculina upogebiae* Shiino, 1943, non-type (ZMUC-CRU-4766). Scale bars represent 5 μm **(A)** and 10 μm **(B)**.

are naked. The mid portions of the two receptacles are in close contact with each other, whereas their lumina remain separate. They contain mature sperm cells and cellular debris of questionable origin. Whereas a mid portion as described above is entirely absent in *S. shiinoi*, the distal part of each receptacle is a narrow, cuticle-filled duct similar to that of *S. shiinoi*.

Molecular phylogeny

Sacculina upogebiae was nested within a well-supported clade of sacculinids comprised of *S. carcini* as well as species belonging to *Heterosaccus* and *Loxothylacus*, with *S. carcini* being the sister to all the other members (Figs. 6, 7). We regard this clade as Sacculinidae s. str., because *S. carcini* is the type species of the genus *Sacculina*. Because of its phylogenetic position, it would be natural to include *S. upogebiae* in the genus *Heterosasccus*. However, we hesitate to do so because species of *Heterosaccus* have a very reduced dorsal mesentery while that of *S. upogebiae* is very well developed.

With maximum support values, all the phylogenetic analyses indicated *S. shiinoi* sp. nov. as a sister taxon to a clade of "sacculinids" consisting of two subclades: 1) three species of *Polyascus* and 2) four species relegated to, but actually not true members of, *Sacculina*, i.e., *S. sinensis*, *S. leptodiae*, *S. oblona*, and *S. confragosa* (Figs. 6, 7).

Distribution

Sacculina shiinoi sp. nov. is known from Seto Inland Sea (Kurahashi Island and Doki River tidal flat) and Pacific side of Shikoku (Susaki Bay and Uranouchi Inlet), both Japan. The infected hosts are *Upogebia yokoyai*, *U. issaeffi*, and *U. sakaii*. *Sacculina upogebiae* parasitizes exclusively *Upogebia major*; Shiino (1943) recorded it from Tanabe Bay and Hiroshima, both Honshu, and Hakata Bay, Kyushu, all Japan, but the present paper has extended its distribution to the Republic of Korea (Apeojang, Seonjae-Do, Incheon). The record of *S. upogebiae* from Tanabe Bay by Shiino (1943) may be questionable, since at least at present, *U. yokoyai*, is the dominant species of *Upogebia* in that

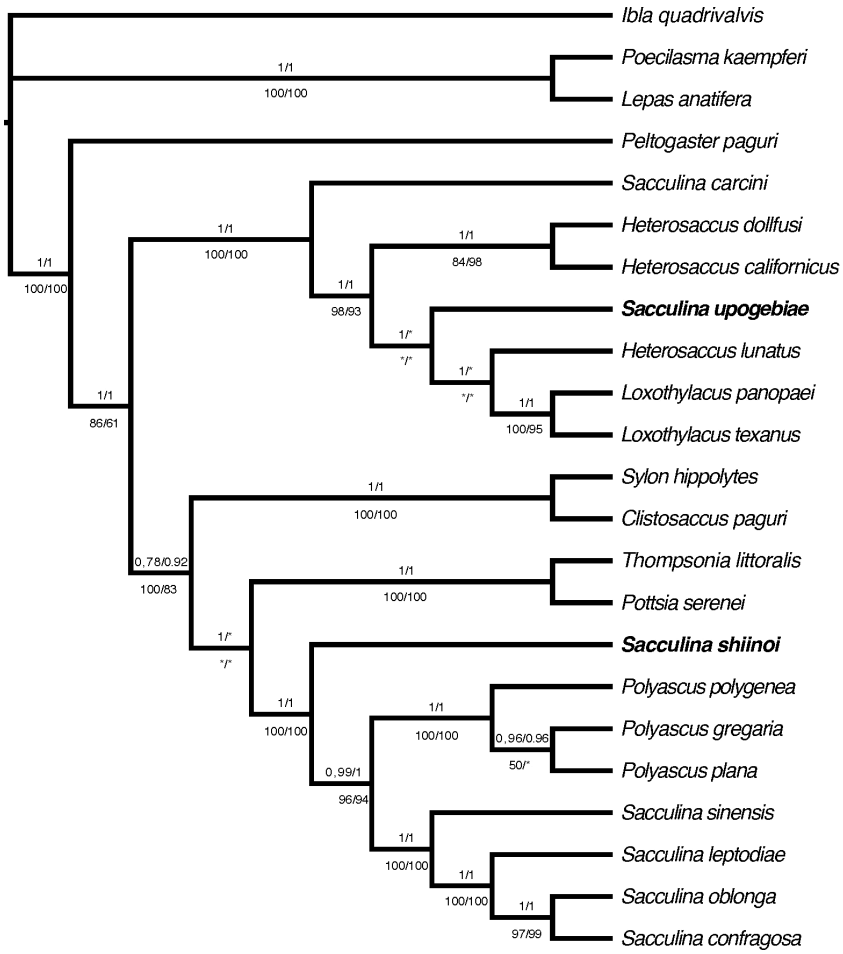

Fig. 6. Tree topology based on the Bayesian analyses of the complete combined dataset of 16S, 18S, and 28S rDNA. The mud shrimp parasitizing sacculinids in bold. Node support numbers represent the posterior probabilities of the Bayesian analysis of the complete dataset (left to the slash) and the dataset with divergent and ambiguous aligned DNA sequences eliminated by the Gblocks algorithm (right to the slash). The values below the branch bars show the bootstrap values of parsimony analyses on the complete and Gblock reduced dataset respectively. Asterisks indicate analyses suggesting alternative or collapsed nodes. Note that we have not committed ourselves to the generic affiliations of *S. upogebiae* and *S shiinoi* and that the name Sacculina is solely provisional.

location. In both *S. upogebiae* and *S. shiinoi*, a solitary parasite is the rule, but in some cases two, or even four per a host (*S. upogebiae*), may be present. Nothing is known about the prevalences of either species.

All species of the host mud shrimps are inhabitants of tidal flats in the littoral zone and live in burrows which they dig into the muddy bottom. *Upogebia yokoyai* and *U. issaeffi* live in Y-shaped burrows which, when inhabited by a large shrimp, may extend to a depth of more than one meter. The burrows of smaller specimens, to which most of the infected specimens belong, are usually connected to those of larger ones (Kinoshita and Itani, 2005; Kinoshita et al., 2010). The burrows also serve as a habitat for other smaller animals of the tidal flat.

DISCUSSION

Externae of species in Sacculinidae possess a minimum of structures, tissues, and organs, almost all of which are engaged in the processes of reproduction. The ridges lined by a specialized cuticle that we have discovered in *S. shiinoi* sp. nov. are a novel structure which has never been described before in any sacculinid (or non-sacculinid) rhizocephalan. It may represent a unique character peculiar to the present species, but on the other hand, it could also be present, but remained overlooked, in some other sacculinids. Even though the course and the histological structure of these ridges are clear, it is not evident which purpose they serve. Their association with the receptacle openings suggests that they might be connected with reproduction. That they should help guiding the sperm to the ovisac, however, is not likely since the ovisac occupies the entire mantle cavity and can be penetrated by sperm cells easily everywhere along its surface. The most likely function of the ridge is that—just as the retinacula—it may assist in holding and retaining the eggs and embryos within the mantle cavity. Retinacula are minute cuticular structures arising from the inner mantle wall in many sacculinids (and peltogastrids). The variation in their structure has been extensively studied by Rybakov and Høeg (2002). Lange (2002) described how the ovisac is produced by the colleteric glands in *Sacculina carcini*, and how it comes to surround the fertilized ova in the mantle cavity. Two days after oviposition the thin ovisac disintegrates. The developing eggs are kept together as a coherent mass because the egg membranes of adjacent ova glue them together. The egg mass or early embryos keep their position in the mantle cavity because the membranes of peripheral ova become attached to the spiny tips of numerous retinacula, emerging from the interior mantle cuticle as illustrated by Lange (2002, figs. 28, 29). The cohesion of the egg mass is probably important as otherwise some of the ova or embryos would be lost when the seawater is expelled during respiratory ventilation by the externa. Retinacula are sometimes absent from species of *Sacculina* (Rybakov and Høeg, 2002) and may have been replaced by other structures. The rugged cuticular surface of the U-shaped ridge in *S. shiinoi* may represent just such a structure.

In the simplest case, each of the paired receptacles in Sacculinidae consists of only two parts. Deeply embedded in the visceral mass of the externa is a bulbous massive wall of rounded cells, which surround a central cavity in which spermatogenesis takes place. This proximal part of the receptacle is connected with the mantle cavity by means of a shorter or longer narrow duct that is normally plugged by solid cuticle. At the moment of oviposition, the plug is removed in continuation with the cuticle of the mantle cavity, allowing the sperm to be released. *Sacculina shiinoi* sp.

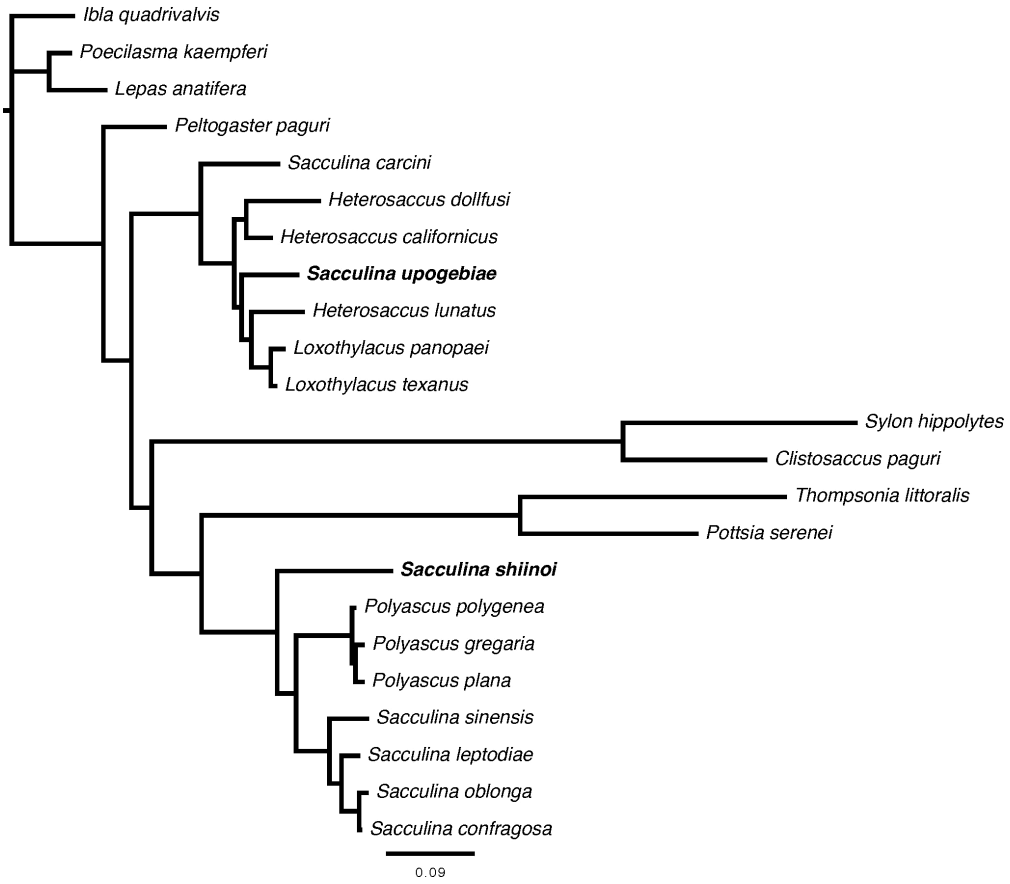

Fig. 7. Phylogram based on the Bayesian Inference analysis depicted in Fig. 6 showing branch lengths. The mud shrimp parasitizing sacculinids in bold.

nov. represents such a simple receptacle. In most species of *Sacculina*, however, a third region is inserted between the other two, the wall of which is often septate or heavily folded. The receptacles of *S. upogebiae* belong to this type, as was also noted and illustrated by Shiino (1943, fig. 18B–D, F). Furthermore, while the two receptacles of *S. shiinoi* are widely separate throughout, in *S. upogebiae* their mid portions are nearly fused. Thus, the structure of the receptacles affords no evidence of a relationship between the two species parasitizing mud shrimps. It should be added that in Europe and New Zealand, another rhizocephalan genus, *Parthenopaea* Kossmann, 1874, represented by two species, parasitize thalassinideans of the genera *Callianassa*, *Pestaina*, and *Vulcanocalliax* (Øksnebjerg, 2000; Lützen et al., 2009) and that the colonial rhizocephalan *Polysaccus* Høeg and Lützen, 1993, with two species are parasites on European and Japanese species of *Pestaina* and *Callianassa* (Lützen and Takahashi, 1996). However, both *Parthenopaea* and *Polysaccus* are non-sacculinids, as they belong to the families Peltogastridae and Polysaccidae.

The result of the analyses of the molecular data clearly shows that *S. upogebiae* and the new species, *S. shiinoi*, although both parasitize species of *Upogebia* and found within a fairly limited geographical region, are unrelated (Fig. 6). The phylogenetic analyses provide strong support for *S. shiinoi* to be closely related to a Southeast Asian assembly of species, consisting of the monophyletic genus *Polyascus* and a monophyletic group of "sacculinids": *S. oblonga*, *S. leptodiae*, *S. sinensis*, and *S. confragosa*. *Sacculina shiinoi* is placed basal to a clade comprised of these four species as well as the species in *Polyascus* included in the present analysis (Fig. 6). The very simple structure of the receptacles in *S. shiinoi* is shared with that of the species of *Polyascus*, while they are more complicatedly build in the other four species.

Sacculina upogebiae is robustly clustered within a diverse, but monophyletic clade of sacculinids, with *S. carcini* as a basal sister taxon. The clade is divided into two branches; *S. upogebiae* is linked basally to a branch consisting of two *Loxothylacus* species and *Heterosaccus lunatus*; the other branch consists of two *Heterosaccus* species (Fig. 6). Because the mesentery of *S. upogebiae* is complete and the mantle opening very simple, while in species of *Heterosaccus* the mantle opening is surrounded by a distinct circular lip and the mesentery incomplete, we have refrained from placing it in this genus and left the problem of its generic affiliation to future studies.

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