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Sympatric Two-species Infestation by Rhizocephalan Barnacle Parasites in the Spider Crab *Pugettia* aff. *ferox* Ohtsuchi & Kawamura, 2019 from Peter the Great Bay (Northwestern Sea of Japan)

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Sympatric two-species infestation by rhizocephalan parasites in the spider crab *Pugettia* aff. *ferox* Ohtsuchi & Kawamura, 2019 (Brachyura: Epialtidae) was investigated in the Vostok Bay (Peter the Great Bay, northwestern Sea of Japan). Morphological and molecular analyses showed that this crab was infested simultaneously by *Sacculina pugettiae* Shiino, 1943 and *Parasacculina pilosella* (Van Kampen et Boschma, 1925) (Cirripedia: Rhizocephala). *Sacculina pugettiae* was found in the northwestern Sea of Japan for the first time. The two rhizocephalan species are clearly distinguishable by the morphology of their external cuticles, the shape and position of their receptacles, and the structure of their colleteric glands. Retinacula are present in the mature externae of both species. Molecular analysis showed that these rhizocephalans are unrelated, although both species parasitize *Pugettia* aff. *ferox* and are sympatric. *Sacculina pilosella* should be placed in the genus *Parasacculina* Høeg & Glenner, 2019, belonging to the family Polyascidae Høeg & Glenner, 2019. The intensity of infestation reached two externae in *P. pilosella* and three externa in *S. pugettiae* per host. A simultaneous settlement of two rhizocephalans on the same crab specimen was shown for the first time. The intensity of the two-species multiple infestations reached four externae per host. Externae with developing embryos occurred from June to September in *P. pilosella* and July to September in *S. pugettiae*, at water temperatures of 15–24°C, indicating that the reproductive periods of these species are confined to the summer months in the investigated locality.

Key words: Rhizocephala, *Parasacculina pilosella*, *Sacculina pugettiae*, Morphology, Multiple infestation.

BACKGROUND

The rhizocephalan barnacles (Crustacea: Cirripedia) are extremely simplified parasites that infest mostly decapods and some other crustaceans. The rhizocephalan female consists of two functional parts: an external reproductive body (the externa) connected through a stalk to an internal system of trophic rootlets (the interna). The externa contains an ovary, receptacles with dwarf males reduced to the spermatogenic cells, and a mantle cavity with developing embryos (Høeg et al. 2014). Since the number of morphological characters of the externa is very limited, molecular analysis is required to correctly identify rhizocephalan species.

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Parasacculina pilosella (Rhizocephala: Polyascidae) was first described by Van Kampen and Boschma (1925) in Sumatra (Indonesia) on *Quadrella coronata* Dana, 1852 (Brachyura: Trapeziidae) and in Java on *Ozius tuberculosus* H. Milne Edwards, 1834 (Brachyura: Oziidae) and *Eriphia sebana* (Shaw & Nodder 1803) (Brachyura: Eriphiidae). Later, this species was also found in Seto (Honshu, Japan) on *Q. coronata*, *Menaethius monoceros* (Latreille, 1825) and *Pugettia quadridens* (De Haan, 1839) (Brachyura: Epialtidae) (Shiino 1943). *Sacculina pugettiae* (Rhizocephala: Sacculinidae) was described by Shiino (1943) in Seto (Honshu, Japan) on *P. quadridens*. Later, it was found in Samani (Hokkaido, Japan); peculiarities of this species in northern Japan were described by Boschma (1960). Shiino (1943) noted that these two parasites are easily distinguished from each other by the morphology of their external cuticle and the position of the receptacles, which are within the visceral mass in *S. pugettiae* and outside in *P. pilosella*. Thus, short morphological descriptions of both species are available, but the present study is the first to conduct any molecular analysis on either species.

In Russian waters, *P. pilosella* was found on *P. quadridens* in 1997. The larvae of this rhizocephalan were reared under laboratory conditions. It was shown that the development of *P. pilosella* comprises five naupliar stages, as in most rhizocephalans (Korn and Rybakov 2001). Later, the muscular system in the interna of *P. pilosella* was visualized (Miroliubov et al. 2019) and specialized rootlets used to interact with the host's nervous system were described (Lianguzova et al. 2021). In 2019, we found a second rhizocephalan parasite of *P. quadridens*, tentatively identified as *S. pugettiae*.

Until now, it was believed that the spider crab recorded frequently in northeast Asian waters – including Japan, Korea, northern China, Hong Kong, and far-eastern Russia – was *P. quadridens* (De Haan, 1839) (Vinogradov 1950; Sakai 1976; Fuseya and Watanabe 1993). However, a detailed morphological investigation of Pacific *Pugettia* species showed that all specimens of *P. quadridens* from northeastern Japan – as well as from the Korean Peninsula, northern China and Russian waters – were most probably actually *Pugettia ferox* Ohtsuchi & Kawamura, 2019. Ohtsuchi and Kawamura (2019) did not present the molecular data on this new species from its type locality. Our material from Russia was identified by Dr. Ohtsuchi as *P. ferox* based on morphological characters (personal communication). Comparative molecular investigation of *Pugettia* from Peter the Great Bay using partial sequences of two mitochondrial loci (16S rDNA and COI) showed that these specimens differ from the typical Japanese *P. quadridens*. However, until a molecular analysis of *P. ferox* in the type locality is made, we identified them as *Pugettia* aff. *ferox*.

The aim of this investigation was to identify both rhizocephalans from Russian waters using morphological and molecular methods and obtain preliminary data on spider crab infestations in the investigated locality.

MATERIALS AND METHODS

Sampling

Specimens of *Pugettia* aff. *ferox* infested by *Parasacculina pilosella* and *Sacculina pugettiae* (Fig. 1) were collected by SCUBA diving at a depth of 1.5–3 m in Vostok Bay (Peter the Great Bay, Sea of Japan). Crabs were sampled once a month from May to September 2019. All material was fixed in 95% ethanol. One male and one female of *Pugettia* aff. *ferox* with the rhizocephalan externae undetached were deposited into the Museum of the A. V. Zhirmunsky National Scientific Center of Marine Biology, Vladivostok, Russia (MIMB, catalogue numbers 40810 and 40811).

Morphological investigation of the externa

The species identification of rhizocephalans was carried out by the shape and position of receptacles. This character can be seen on the living or fixed adults; the virginal stage was investigated using SEM.

In both species, we measured the width of each detached parasite externa (the greatest dorsoventral distance), then recorded their developmental stage and the position on the abdominal segments. The following stages were identified: virginal externa (white, without spermatogenic cells in the receptacles), immature externa (yellow, without larvae in the mantle cavity), mature 1 (yellow, embryos in the mantle cavity without eyes), mature 2 (light brown, embryos in the mantle cavity with eyes). The carapace width (including lateral spines) of the host crabs (males and females) was also measured.

The mantle cuticles from numerous externae of both species were fixed in 70% ethanol, dehydrated in an ethanol series and acetone, critically point dried in CO_2, and sputtered with chromium. The SEM micrographs were taken with a Zeiss Sigma 300 VP microscope.

Three externae of each species were detached from the host crabs and fixed in Bouin solution, dehydrated through a gradient ethanol-xylene series and embedded in paraffin. Transverse and longitudinal sections, 6 μm

thick, were stained with Ehrlich hematoxylin, examined with a Carl Zeiss Axio Imager Z.2 light microscope furnished with a digital camera.

The data on the dynamics of water temperature were obtained from a hydrometeorological station at the Vostok Marine Biological Station (A.V. Zhirmunsky National Scientific Center of Marine Biology FEB RAS).

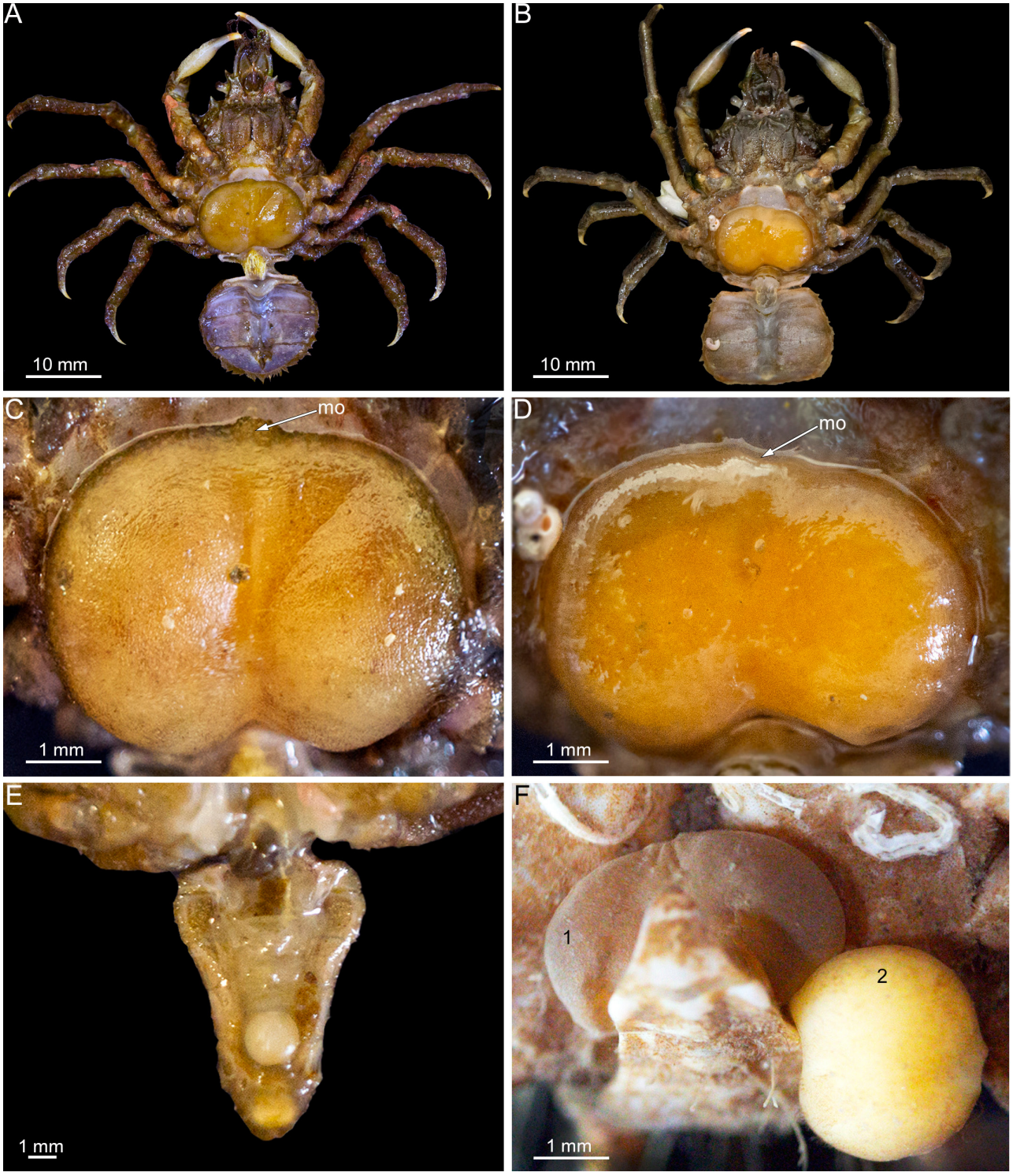

Fig. 1. The host crab, *Pugettia* aff. *ferox*, infested by *Parasacculina pilosella* (A, C), *Sacculina pugettiae* (B, D, E) and both rhizocephalans (F). 1, *P. pilosella*, 2, *S. pugettiae*, mo, mantle opening.

Molecular investigation of the externa

Live externae of both rhizocephalans were fixed in 95% ethanol. Voucher specimens were deposited into the Museum of the A.V. Zhirmunsky National Scientific Center of Marine Biology FEB RAS (MIMB, catalogue numbers 40795–40809).

Total DNA was extracted from a piece of ovarian tissue using a CTAB extraction method (Dawson et al. 1998). Fragments of the mitochondrial large-subunit ribosomal RNA (16S rRNA) and cytochrome *c* oxidase subunit I (*COI*) genes were amplified and sequenced using the universal invertebrate primer pairs: 16SL3-Ven (5'-GCAAYGAGAGTTGTRCTAAGGT AGC-3') (Kappner and Bieler 2006) and 16SRHTB (5'-ACGCCGGTTTGAACTCAGATC-3') (Kocher et al. 1989) for 16S rDNA; LCO1490(F) (5'-GGTCAA CAAATCATAAAGATATTGG-3') and HCO2198(R) (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer at al. 1994) for *COI*. We also used: 18S-5' (F) (5'-CTGGTTGATYCTGCCAGT-3') and 5R (5'-CTTGGCAAATGCTTTCGC-3') (Giribet et al. 1996) for fragments of nuclear markers 18S rDNA; LSU5 (F) (5'-TAGGTCGACCCGCTGAAYTTAAG CA-3') and LSU3 (5'-TCC TGA GGG AAA CTT CGG-3') for 28S rDNA.

PCR amplification was performed with a ScreenMix kit (Evrogen) and cycling parameters according the manufacturer's protocol. The annealing temperatures were 42°C for *COI*, 52°C for 16S rDNA, and 60°C for 18S and 28S rDNA. Amplification products were applied as templates for sequencing, using the same primers as for PCR and BrilliantDye™ Terminator Cycle Sequencing kit v3.1 (NimaGen) according to the manufacturer's protocol. Sequencing reaction products were purified by ethanol precipitation and analyzed on an ABI-3500 Genetic Analyzer (Applied Biosystems). Sequences were verified by forward and reverse comparisons.

The contigs were obtained and edited using ChromasPro v. 1.7.6 (http://www.technelysium.com.au/chromas.html). A BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to check new sequences against the database for possible contamination and sequence artifacts. All sequences determined in the present study were deposited deposited into GenBank (NCBI, http://www.ncbi.nlm.nih.gov/) under the accession numbers MW418446–MW418458 (16S rDNA), MW418428–MW418436 (18S rDNA), MW418439–MW418444 (28S rDNA), and MW401796, MW401797 (*COI*). Sequences of *Peltogaster paguri* (Peltogastridae) were selected as the outgroup. Sequences were aligned using MUSCLE (Edgar 2004) implemented in the MEGA X program (Kumar et al. 2018). The quality of alignment was checked visually. The models of nucleotide substitution for trees were selected using jModelTest v. 2.1.4 (Software Foundation, Inc., Boston, MA) (Darriba et al. 2012). The TN+I+G model were selected for all genes separately under the Akaike information criterion. To construct BI-trees, MrBayes 3.2.6 was used (Huelsenbeck and Ronquist 2001), implemented in CIPRES Science Gateway (http://www.phylo.org/) (Miller et al. 2010), for the Bayesian analysis of 10,000,000 generations, with four parallel chains and sample frequencies set to 500, in two separate runs. Based on the convergence of likelihood scores, 25% of the sampled trees were discarded as burn-in. The uncorrected pairwise genetic distances (*p*-distances) for these species were calculated using MEGA X.

The manuscript provides the tables and phylogenetic trees for only 16S and 18S rRNA genes. The rest of the data are available as supplemental materials.

RESULTS

Morphological identification of two rhizocephalan species

Superorder Rhizocephala Müller, 1862
Family Sacculinidae Lilljeborg, 1861, amended by Høeg et al. (2020)
Genus *Sacculina* Thompson, 1836

Sacculina pugettiae Shiino, 1943: 23–24, fig. 16.
Sacculina pugettiae – Boschma 1960: 19–24, figs. 1–5.

Family Polyascidae Høeg & Glenner, 2019 in Høeg et al. (2020)
Genus *Parasacculina* Høeg & Glenner, 2019 in Høeg et al. (2020)

Parasacculina pilosella (Van Kampen et Boschma, 1925) comb. nov.: 24–27, figs. 14, 15.
Sacculina pilosella – Shiino 1943: 11–12, figs. 1E, 7; Korn and Rybakov 2001: 177–179; Miroliubov et al. 2019: 48–56, fig. 3; Lianguzova et al. 2021: 101009.

Host: Carapace width of the males of *Pugettia* aff. *ferox* infested by rhizocephalans ranged from 14.0 to 31.0 mm, females – from 9.9 to 25.0 mm.

Bathymetrical range: In Vostok Bay (Peter the Great Bay, Sea of Japan), crabs infested by both rhizocephalans were found at a depth of 1.5–3 m.

Location on the host: The position of the externae of both rhizocephalans was not connected with specific abdominal segment of the host. Parasites were found on

1, 2, 3, 4, 5, 6 segments and also on the borders between 2 and 3, 3 and 4, 4 and 5 segments. Most crabs had only one rhizocephalan externa.

External morphology: *Parasacculina pilosella* and *Sacculina pugettiae* were externally very similar (Fig. 1C, D). The width of the externae varied from 1.1 to 13.5 mm in *P. pilosella* and from 1.0 to 13.7 mm in *S. pugettiae*. The virginal externae of both species were white (Fig. 1E), immature externae – yellow (Fig. 1C, D), mature externae – yellow (embryos without eyes) or light brown (embryos with eyes) (Fig. 1F). The mature externae of both species had prominent dorsoventral wrinkles. The external cuticle varied from 22 to 40 μm thick.

In *P. pilosella*, the external cuticle was covered by numerous hyaline spines 28–38 μm length united in groups with a common base. This character is visible on the SEM photos (Fig. 2A, D) as well as on the histological sections of the externa (Fig. 3A). In *S. pugettiae*, we found two types of the external surface. The external cuticle of about 2 thirds of the investigated specimens was smooth, without spines and excrescences, but often covered with the epibionts (Fig. 2B), the cuticle of about 1 third of specimens was divided into small star-shaped areas with a diameter of 5–8 μm (Fig. 2C). The cuticle of three found specimens was two-layered: smooth cuticle was folded back, revealing a star-shaped surface (Fig. 2E).

The receptacles of the mature externae in both rhizocephalans were easily detached. In *P. pilosella*, isolated receptacles were globular with the cavity (lumen) inside (Fig. 4A). Their diameter was of 300–800 μm. The spermatogenic cells were placed in the central part of the receptacle. Each receptacle was connected to a folded receptacle duct by a short probably chitinous tubule (Fig. 4C, E). In *S. pugettiae*, the receptacles presented the elongate tubes, directed dorsoventrally, of 1200–1700 μm length and with a diameter of 200–600 μm (Fig. 4B). They were placed closely together but always clearly separated. The spermatogenic cells were found in the narrower dorsal part of the receptacle (Fig. 4D, F). The receptacle ducts were slightly flattened, of 200–300 μm width. In *P. pilosella*, the receptacles were located outside of the visceral mass in the basal region of the stalk (Fig. 4C, E), whereas in *S. pugettiae*, they were placed within the visceral mass (Fig. 4D, F).

In *P. pilosella*, the colleteric glands were weakly branched from the atrium (central part of the gland) attaining 16 tubes (canals), arranged in one layer (Fig. 3C, E). In *S. pugettiae*, they were highly branched exceeding 33 tubes, arranged in several layers (Fig. 3D, F). The maximum number of tubes was located in the central part of the externa. Their diameter was 40–90 μm in *P. pilosella* and 30–80 μm in *S. pugettiae*.

Retinacula: In both rhizocephalans, the internal cuticle had a wrinkled surface. In the mature externae of *P. pilosella*, the internal cuticle was covered with ridges spirally twisted and ended with short finger-like processes (Fig. 2F). We have not found the retinacula in the virginal externa of *P. pilosella* (4.4 mm width). In three virginal externae of *Sacculina pugettiae* (3–3.5 mm width), the retinacula were also not found. The internal cuticle of the fourth virginal specimen (2.3 mm width) was covered with numerous undeveloped flattened retinacula of 5 μm in diameter (Fig. 2G). In the mature externa of *P. pilosella* (10.5 mm width), rare solitary barbed spindles (9 μm length) placed in the shallow depressions were noted in the region of the stalk (Fig. 2H, I). In the mature externae of *S. pugettiae* (6.5–9.3 mm width), numerous retinacula presented the groups of 4–5 barbed spindles (6–8 μm length) at a common base placed in the shallow depressions (Fig. 2J, K). The retinacula of both species were covered with a layer of secretion and with numerous bacteria.

Molecular identification of two rhizocephalan species

The investigated samples of rhizocephalans were relegated into two clades – Sacculinidae for *Sacculina pugettiae* and Polyascidae for *Parasacculina pilosella* (pp = 1 for all markers) – confirming their status as different and not closely related species.

Molecular data showed that all rhizocephalans implemented into the analysis form two monophyletic clades with high posterior probability (pp = 1 for all markers). These clades correspond to the families Sacculinidae and Polyascidae (Figs. 5, 6). However, for the family Sacculinidae, branch topologies within this clade are not identical for each gene. The specimens in the family Polyascidae form three groups of sequences (pp = 1). The first consists of *Polyascus* species and the second consists of *Parasacculina* species. The third group contains *P. shiinoi* (for 16S rDNA) and *P. shiinoi* + *P. bicuspidata* (for 18S rDNA), which are basal to other Polyascidae on the trees presented (Figs. 5, 6). The comparison of pairwise genetic distances indicated stronger differences between species of the families Sacculinidae and Polyascidae (Tables 1, 2).

Preliminary investigation of the multiple infestation of *Pugettia* aff. *ferox* by rhizocephalans

The preliminary data on the infestation of the spider crab *Pugettia* aff. *ferox* in Vostok Bay showed

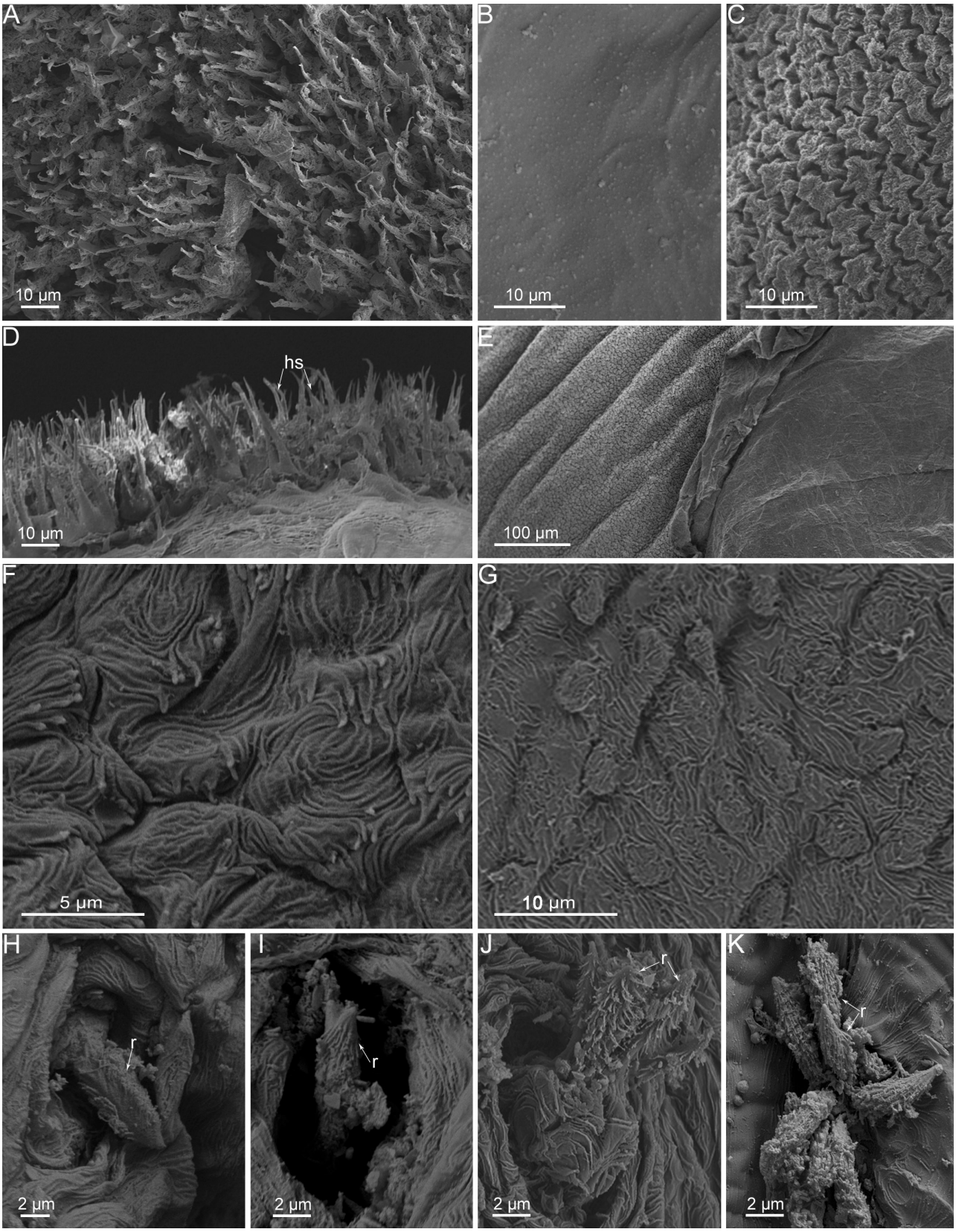

Fig. 2. SEM showing external cuticle (A–E), internal cuticle (F, G) and retinacula (H–K) of *Parasacculina pilosella* (A, D, F, H, I) and *Sacculina pugettiae* (B, C, E, G, J, K). hs, hyaline spines of external cuticle; r, retinacula.

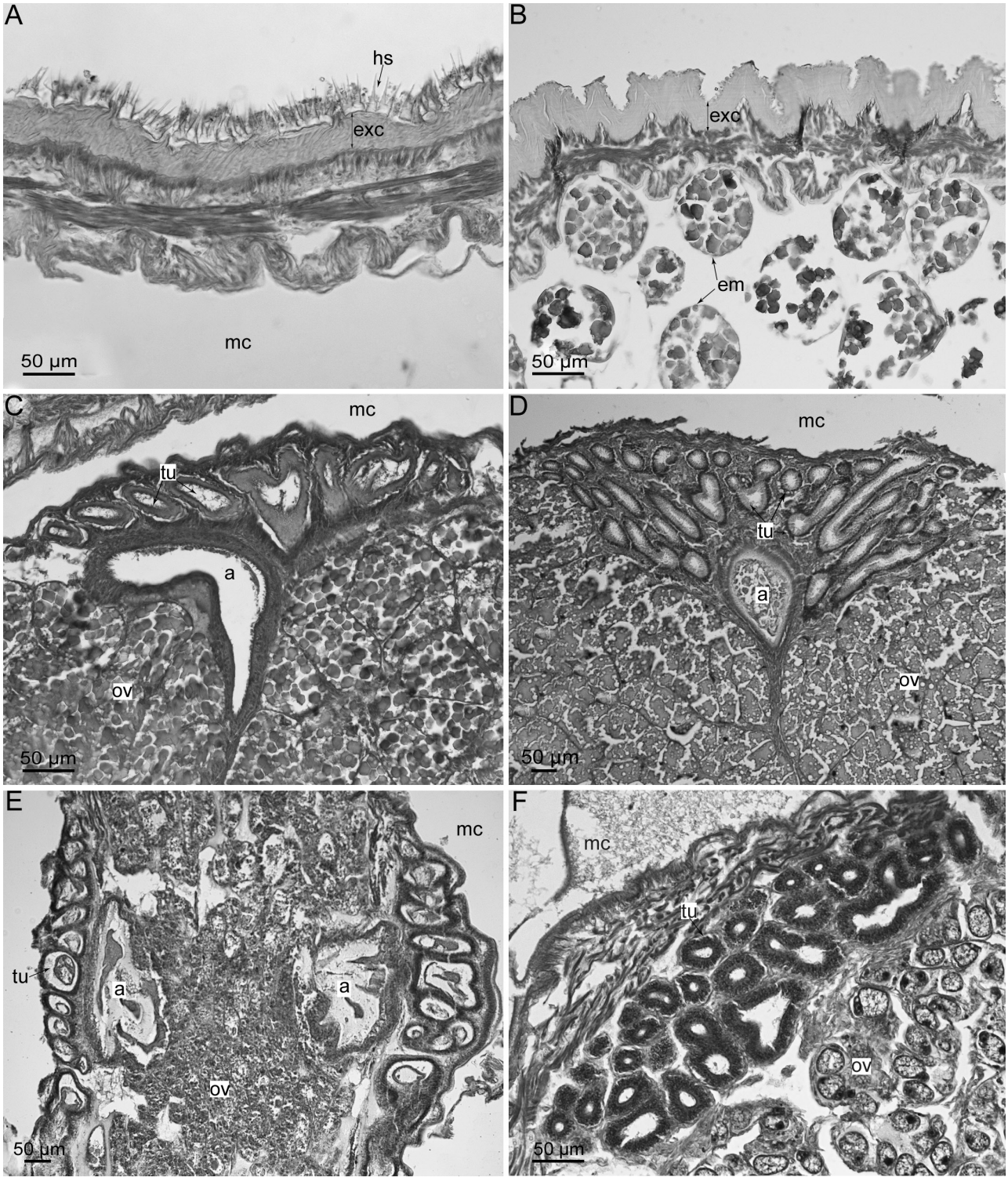

Fig. 3. Mantle (A, B) and colleteric glands (C–F) of *Parasacculina pilosella* (A, C, E) and *Sacculina pugettiae* (B, D, F). C, D, transverse sections; E, F, longitudinal sections. a, atrium of colleteric gland; em, embryos; exc, external cuticle; hs, hyaline spines, mc, mantle cavity; ov, ovary; tu, tubes of colleteric gland.

that *Sacculina pugettiae* occurred more often than *Parasacculina pilosella* (Table 3). Over seven months, we found 86 specimens of *Pugettia* aff. *ferox* infested by rhizocephalans. Among them, 56 specimens (65.1%) possessed the externae of *S. pugettiae*, 14 (16.3%) – the externae of *P. pilosella*, and 16 specimens (18.6%) were infested by both rhizocephalans simultaneously. 80.3% of crabs with *S. pugettiae* had one externa, 12.5% – two externae and 7.2% – three externae of the parasite. All crabs with *P. pilosella* had only one externa. Moreover, each of 10 crabs possessed two externae of different species, five crabs – three externae, but one crab – four externae (two of *S. pugettiae* and two of *P. pilosella*). Thus, the intensity of infestation reached two externae

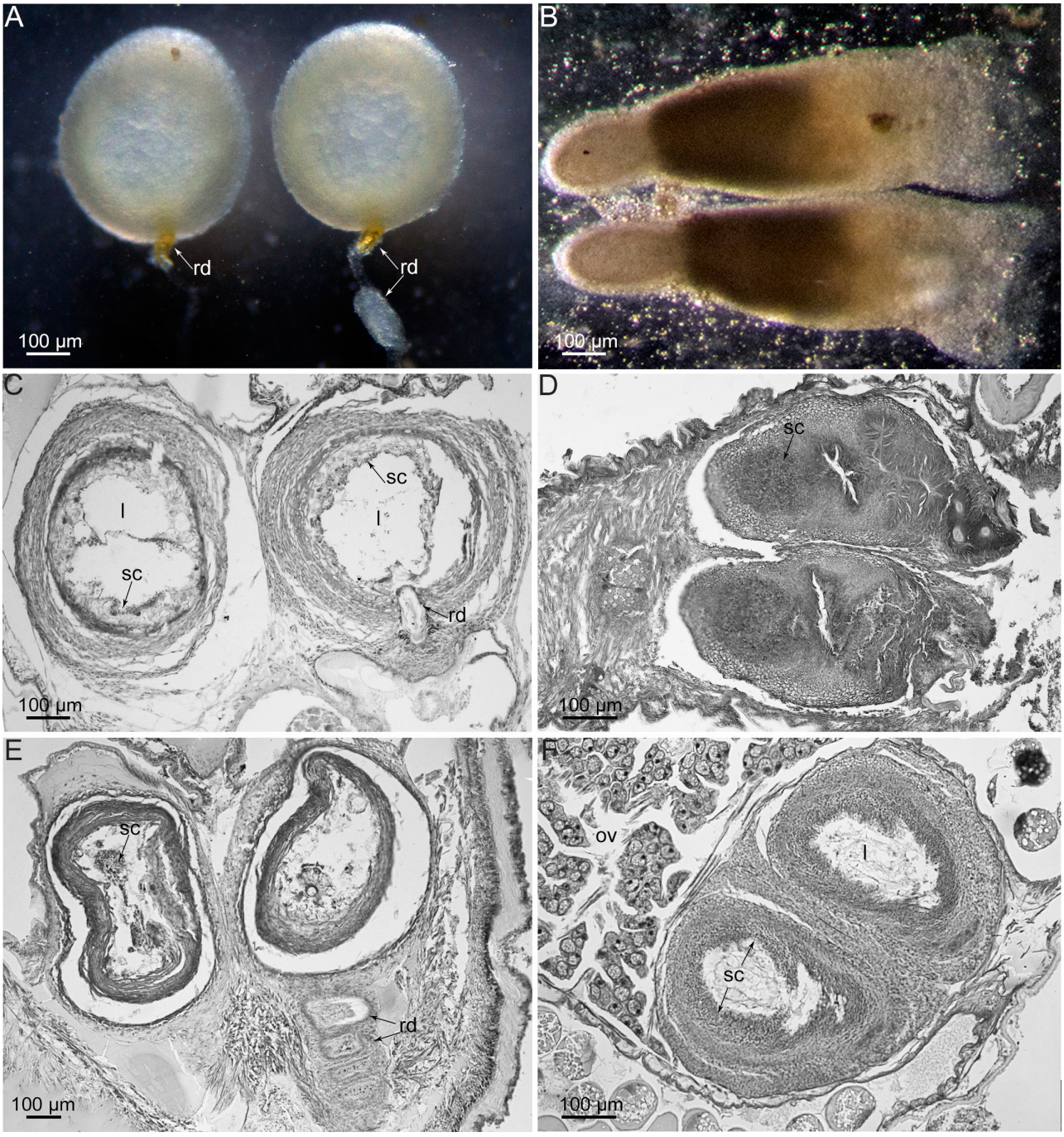

Fig. 4. Receptacles of *Parasacculina pilosella* (A, C, E) and *Sacculina pugettiae* (B, D, F). A, B, light microscopy; C, D, longitudinal sections; E, F, transverse sections. l, lumen; ov, ovary; rd, receptacle duct; sc, spermatogenic cells.

per host in *P. pilosella* and three externae per host in *S. pugettiae*. The intensity of two-species multiple infestations reached four externae per host. *Pugettia* females were infested more often than males.

The virginal externae were found on host crabs from May to August in *P. pilosella*, and from May to September in *S. pugettiae*, gradually decreasing in number (Fig. 7). The externae of *P. pilosella* with developing embryos appeared in June, at a temperature of 14.6 ± 2.0°C; ovigerous externae of *S. pugettiae* appeared in July, at a temperature of 18.2 ± 1.7°C. Both ovigerous parasites occurred until to September. The immature externae were noted from May to September. In spring and early summer, the "old" immature

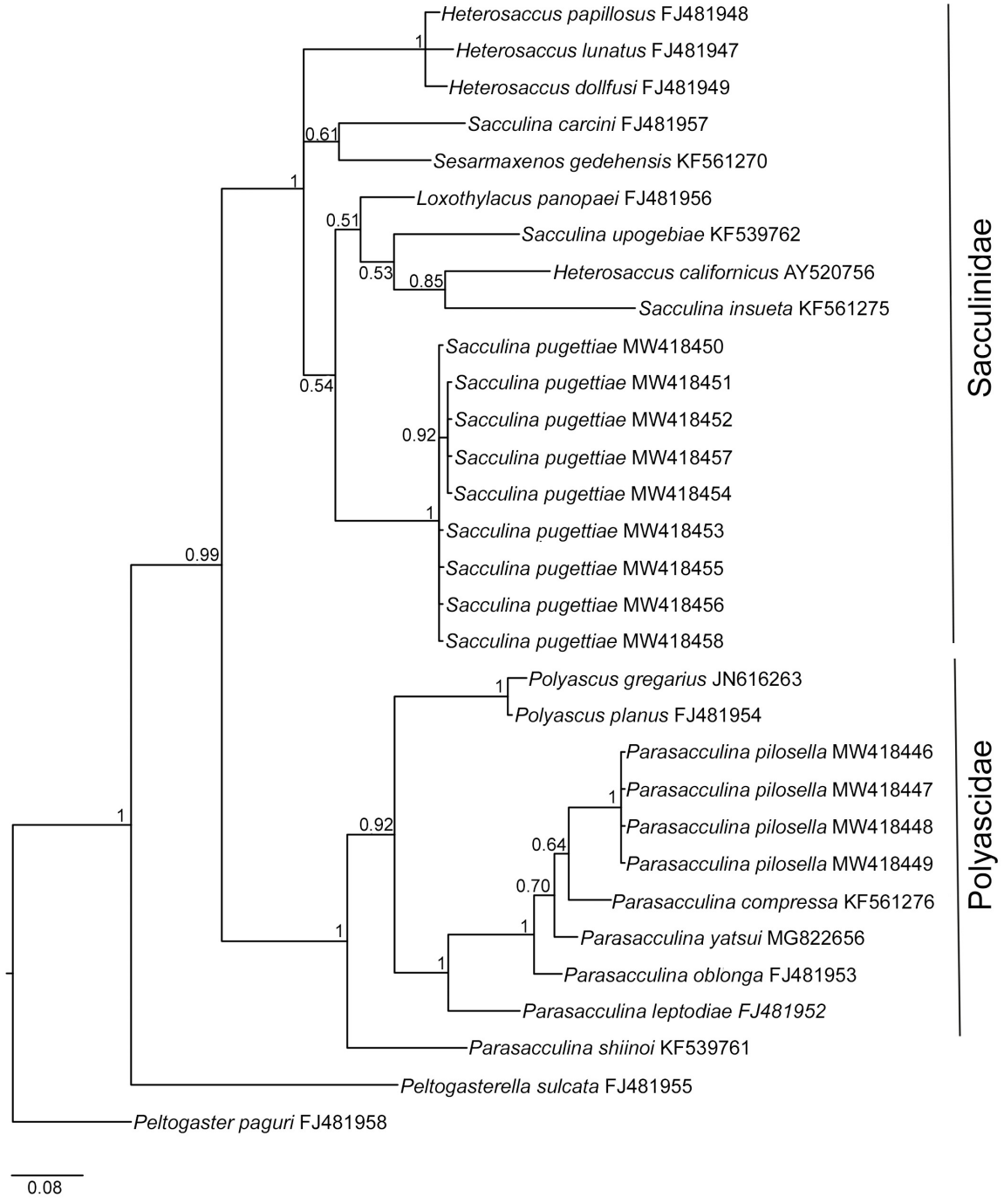

Fig. 5. Bayesian inference analysis of 16S rDNA sequences for the Sacculinidae and Polyascidae. Numerals above or below the branches are Bayesian posterior probabilities.

externae with a thickened mantle, probably retained from the preceding reproductive season, were observed. During the research period no one crab with a scar was found.

DISCUSSION

Comparative morphology of two parasites

The main morphological features of the two rhizocephalans that infested the spider crab *Pugettia* aff. *ferox* in Russian waters match all the characters of the genus *Sacculina* summarized by Høeg and Lützen (1985), Øksnebjerg (2000), and Høeg et al. (2020). The cordiform externa was laterally compressed. The mantle opening was situated on the anterior margin more or less opposite the stalk. Thin dorsal mesentery extended from the stalk to the mantle opening. The colleteric glands with a number of branched tubes were situated in the central part of the lateral surfaces of the visceral mass. The receptacles were presented as tubes, directed dorsoventrally in the visceral mass in *S. pugettiae*, whereas they were globular and in the basal region of the stalk in *P. pilosella*.

The detailed morphological assessment of our material revealed that the two rhizocephalans were similar to the species *Parasacculina pilosella* and *S. pugettiae* described by Shiino (1943) and Boschma (1960) from Japanese waters. At the same time, our

Table 1. 16S rDNA uncorrected genetic distances between species of the families Sacculinidae and Polyascidae. Above the diagonal is the SD

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	*Heterosaccus californicus* AY520756		0.0248	0.0254	0.0252	0.0218	0.0287	0.0294	0.0290	0.0292	0.0248	0.0258	0.0286	0.0269	0.0280	0.0287	0.0279	0.0254	0.0290	0.0235
2	*Heterosaccus dollfusi* FJ481949	0.1704		0.0121	0.0107	0.0235	0.0275	0.0302	0.0276	0.0272	0.0266	0.0258	0.0277	0.0277	0.0291	0.0277	0.0276	0.0264	0.0274	0.0234
3	*Heterosaccus lunatus* FJ481947	0.1749	0.0359		0.0105	0.0226	0.0280	0.0300	0.0275	0.0272	0.0261	0.0258	0.0277	0.0273	0.0285	0.0280	0.0283	0.0262	0.0277	0.0245
4	*Heterosaccus papillosus* FJ481948	0.1749	0.0269	0.0269		0.0228	0.0273	0.0300	0.0269	0.0266	0.0261	0.0255	0.0276	0.0274	0.0290	0.0276	0.0278	0.0266	0.0273	0.0244
5	*Loxothylacus panopaei* FJ481956	0.1300	0.1435	0.1345	0.1390		0.0272	0.0298	0.0273	0.0269	0.0207	0.0222	0.0267	0.0254	0.0276	0.0271	0.0271	0.0221	0.0271	0.0220
6	*Parasacculina pilosella* MW418446	0.2691	0.2735	0.2735	0.2691	0.2556		0.0293	0.0254	0.0245	0.0286	0.0290	0.0152	0.0295	0.0221	0.0157	0.0241	0.0288	0.0152	0.0298
7	*Peltogasterella sulcata* FJ481955	0.2691	0.3094	0.3049	0.3049	0.2915	0.3094		0.0300	0.0298	0.0306	0.0306	0.0291	0.0302	0.0282	0.0291	0.0275	0.0304	0.0293	0.0309
8	*Polyascus gregarius* JN616263	0.2870	0.2691	0.2691	0.2601	0.2511	0.1839	0.3004		0.0075	0.0286	0.0287	0.0250	0.0301	0.0249	0.0250	0.0263	0.0276	0.0250	0.0288
9	*Polyascus planus* FJ481954	0.2825	0.2556	0.2556	0.2466	0.2422	0.1704	0.2960	0.0135		0.0285	0.0284	0.0244	0.0299	0.0243	0.0243	0.0255	0.0274	0.0244	0.0284
10	*Sacculina pugettiae* MW418450	0.2018	0.2063	0.1973	0.1973	0.1211	0.2960	0.3229	0.2780	0.2735		0.0242	0.0279	0.0274	0.0292	0.0292	0.0284	0.0261	0.0283	0.0239
11	*Sacculina carcini* FJ481957	0.1928	0.1973	0.1973	0.1973	0.1345	0.2780	0.3049	0.2870	0.2870	0.1614		0.0285	0.0274	0.0285	0.0285	0.0289	0.0270	0.0293	0.0226
12	*Parasacculina compressa* KF561276	0.2780	0.2646	0.2601	0.2646	0.2242	0.0583	0.3049	0.1839	0.1749	0.2691	0.2646		0.0295	0.0206	0.0144	0.0253	0.0281	0.0133	0.0292
13	*Sacculina insueta* KF561275	0.1973	0.2108	0.2063	0.2063	0.1883	0.3094	0.3408	0.3004	0.2915	0.2377	0.2287	0.3184		0.0298	0.0298	0.0286	0.0258	0.0301	0.0266
14	*Parasacculina leptodiae* FJ481952	0.2691	0.2870	0.2825	0.2870	0.2511	0.1300	0.2870	0.1659	0.1570	0.2915	0.2825	0.1211	0.3274		0.0204	0.0237	0.0281	0.0213	0.0288
15	*Parasacculina oblonga* FJ481953	0.2735	0.2691	0.2691	0.2646	0.2377	0.0583	0.3049	0.1749	0.1659	0.2915	0.2735	0.0538	0.3229	0.1076		0.0239	0.0288	0.0133	0.0299
16	*Parasacculina shiinoi* KF539761	0.2511	0.2466	0.2556	0.2466	0.2511	0.1928	0.2691	0.1973	0.1839	0.2870	0.3004	0.2063	0.2915	0.1614	0.1794		0.0282	0.0252	0.0289
17	*Sacculina upogebiae* KF539762	0.1794	0.2018	0.1973	0.2063	0.1300	0.2646	0.3139	0.2511	0.2422	0.1973	0.2108	0.2422	0.1883	0.2691	0.2601	0.2735		0.0287	0.0262
18	*Parasacculina yatsui* MG822656	0.2825	0.2646	0.2601	0.2601	0.2332	0.0583	0.3139	0.1883	0.1794	0.2735	0.2735	0.0448	0.3274	0.1256	0.0448	0.1928	0.2556		0.0297
19	*Sesarmaxenos gedehensis* KF561270	0.1525	0.1570	0.1659	0.1659	0.1345	0.2915	0.2915	0.2646	0.2511	0.1614	0.1480	0.2780	0.2152	0.2825	0.2870	0.2691	0.1973	0.2870	

Table 2. 18S rDNA uncorrected genetic distances between species of the families Sacculinidae and Polyascidae. Above the diagonal is the SD

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	*Parasacculina pilosella* MW418429		0.00967	0.00957	0.00996	0.00992	0.01079	0.01010	0.01019	0.00984	0.00987	0.00896	0.00750	0.00713	0.00696	0.00952	0.00621	0.00367	0.00188	0.00213
2	*Sacculina pugettiae* MW418434	0.09662		0.00453	0.00454	0.00499	0.00719	0.00482	0.00664	0.00643	0.00558	0.01063	0.00977	0.00950	0.00948	0.01060	0.01001	0.00981	0.00960	0.00970
3	*Sesarmaxenos gedehensis* KF561256	0.09895	0.01979		0.00508	0.00433	0.00712	0.00467	0.00660	0.00643	0.00557	0.01050	0.00990	0.00967	0.00967	0.01075	0.01000	0.00963	0.00948	0.00956
4	*Heterosaccus californicus* AY520657	0.09546	0.01979	0.02095		0.00553	0.00723	0.00575	0.00667	0.00629	0.00600	0.01028	0.00999	0.00967	0.00967	0.01051	0.01021	0.01011	0.00992	0.01003
5	*Sacculina insueta* KF561258	0.10244	0.02445	0.01863	0.02910		0.00730	0.00499	0.00692	0.00688	0.00619	0.01065	0.00991	0.00970	0.00972	0.01077	0.01017	0.01009	0.00988	0.00981
6	*Heterosaccus dollfusi* EU082413	0.12340	0.05239	0.05355	0.05122	0.05937		0.00772	0.00842	0.00781	0.00811	0.01099	0.01094	0.01071	0.01076	0.01117	0.01092	0.01098	0.01093	0.01096
7	*Sacculina carcini* AY265366	0.10943	0.02328	0.02328	0.03143	0.02910	0.06403		0.00671	0.00662	0.00605	0.01105	0.01009	0.00984	0.00987	0.01085	0.01035	0.01024	0.01002	0.01006
8	*Heterosaccus lunatus* EU082414	0.10827	0.04191	0.04191	0.04307	0.04889	0.06752	0.04424		0.00706	0.00519	0.01066	0.01019	0.00986	0.01000	0.01079	0.01037	0.01036	0.01006	0.01010
9	*Sacculina upogebiae* KF539758	0.10477	0.04424	0.03958	0.03958	0.04773	0.06636	0.04773	0.05006		0.00628	0.01040	0.01029	0.00990	0.00995	0.01044	0.01020	0.00997	0.00972	0.00977
10	*Loxothylacus panopaei* AY265364	0.10477	0.02910	0.02794	0.03260	0.03725	0.05704	0.03609	0.02678	0.03725		0.01057	0.01027	0.00994	0.00995	0.01108	0.01031	0.01002	0.00979	0.00983
11	*Parasacculina bicuspidata* KF561260	0.07683	0.11059	0.11409	0.10827	0.11292	0.12689	0.12456	0.11874	0.11292	0.11758		0.00953	0.00941	0.00933	0.00993	0.00886	0.00906	0.00875	0.00887
12	*Polyascus planus* AY265368	0.05821	0.10361	0.10943	0.10012	0.10594	0.12573	0.11525	0.11176	0.11525	0.11641	0.08964		0.00231	0.00320	0.00955	0.00761	0.00787	0.00766	0.00774
13	*Polyascus gregarius* AY265363	0.05239	0.09895	0.10477	0.09546	0.10128	0.12224	0.11059	0.10594	0.10943	0.11059	0.08615	0.00582		0.00204	0.00924	0.00766	0.00757	0.00726	0.00734
14	*Polyascus polygeneus* AY265362	0.05122	0.09895	0.10477	0.09779	0.10128	0.12456	0.11176	0.10827	0.11176	0.11059	0.08615	0.00931	0.00349		0.00933	0.00756	0.00743	0.00705	0.00715
15	*Parasacculina shiinoi* KF539757	0.08615	0.11758	0.12224	0.11292	0.11758	0.13271	0.13038	0.12573	0.12340	0.12922	0.09895	0.08382	0.08033	0.08149		0.00934	0.00963	0.00950	0.00942
16	*Parasacculina sinensis* AY265360	0.03725	0.10710	0.10943	0.10361	0.10594	0.12689	0.11991	0.12107	0.11641	0.11525	0.08265	0.06636	0.06286	0.06054	0.09080		0.00630	0.00610	0.00590
17	*Parasacculina leptodiae* AY265365	0.01281	0.09779	0.10128	0.09779	0.10477	0.12689	0.11176	0.10943	0.10710	0.10710	0.08265	0.06403	0.05821	0.05704	0.08847	0.03958		0.00350	0.00334
18	*Parasacculina yatsui* MG604305	0.00349	0.09546	0.09779	0.09430	0.10128	0.12340	0.10827	0.10710	0.10361	0.10361	0.07567	0.05937	0.05355	0.05239	0.08382	0.03492	0.01164		0.00112
19	*Parasacculina oblonga* AY265367	0.00466	0.09662	0.09895	0.09546	0.10012	0.12456	0.10943	0.10827	0.10477	0.10477	0.07683	0.06054	0.05471	0.05355	0.08265	0.03376	0.01048	0.00116	

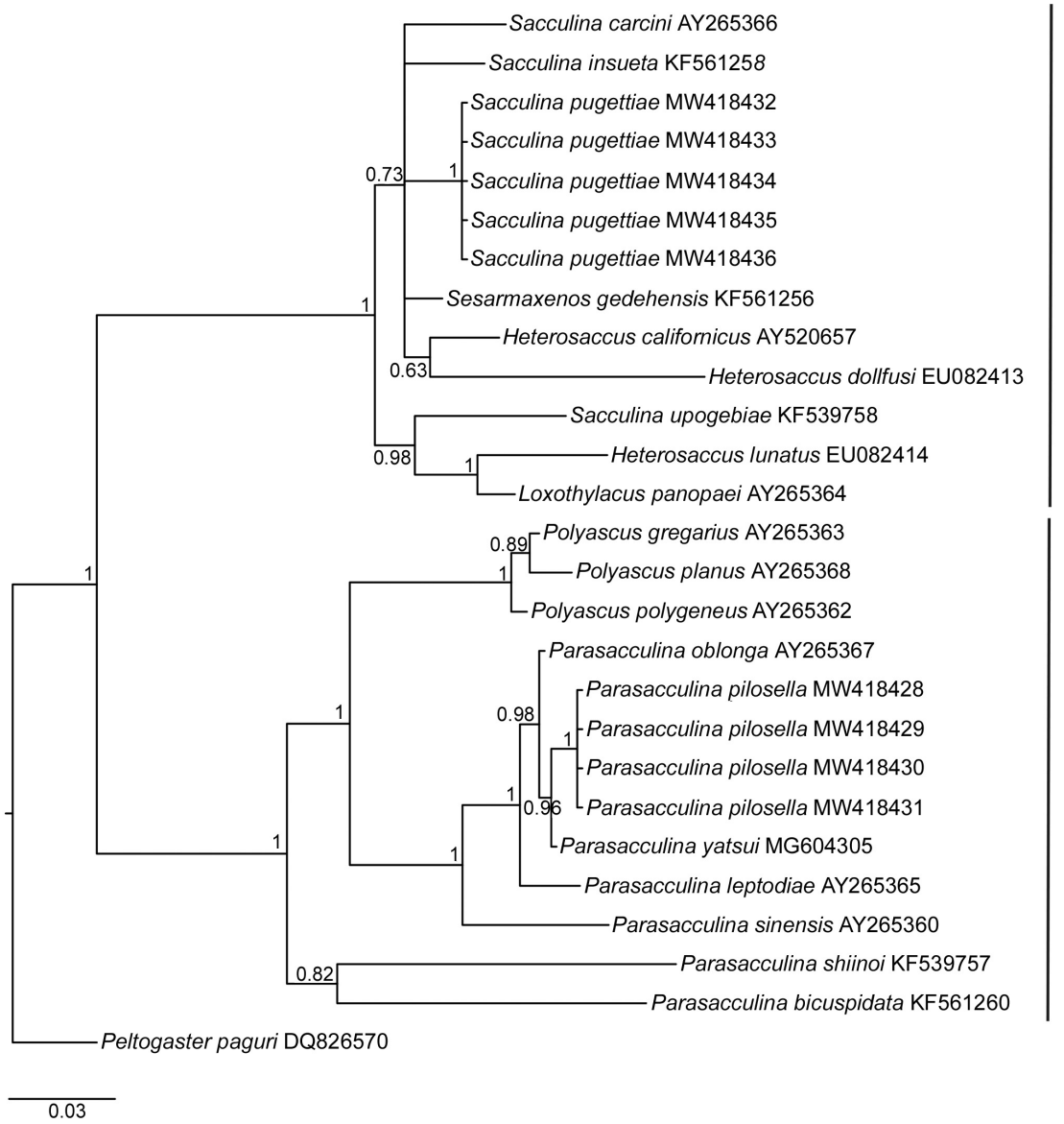

Fig. 6. Bayesian inference analysis of 18S rDNA sequences for the Sacculinidae and Polyascidae. Numerals above or below the branches are Bayesian posterior probabilities.

Table 3. Intensity of infestation of *Pugettia* aff. *ferox* from northwestern Sea of Japan by *Parasacculina pilosella* and *Sacculina pugettiae*

The number of externae per host	*P. pilosella*		*S. pugettiae*		*P. pilosella* + *S. pugettiae*	
	♂	♀	♂	♀	♂	♀
1	7	7	15	30		
2			3	4	4	6
3				4	1	4
4						1

exemplars showed some differences from the type specimens.

The external cuticle of *P. pilosella* was considerably thicker in our material (22–40 µm) than that described by Shiino (1943) (8–20 µm). The shape of the lumen of the receptacle duct depended on the direction of the sections. The lumen was oval in the Japanese species (Shiino 1943; Boschma 1960) and on our longitudinal sections, but compressed and had an irregular shape on our transverse sections.

The number of tubes of the colleteric gland in *S. pugettiae* reached 50 in Seto and 56 in Samani (Shiino 1943; Boschma 1960), whereas it did not exceed 32 in our material. This character may depend on the size of the externa. The tubes of the colleteric glands were at some distance from margins of the visceral mass in Japanese *S. pugettiae* (Shiino 1943; Boschma 1960). However, this feature also depended on the direction of the histological sections. In our material, the tubes were placed at some distance from the margins of the visceral mass on longitudinal sections, but remained close to the margins on transverse sections.

Shiino (1943) described "small areas having sinuous contour" on the external cuticle of *S. pugettiae*, whereas Boschma (1960) found only a smooth external cuticle. Using SEM, we found both variants and identified the external cuticle of *S. pugettiae* to be two-layered. The star-shaped external cuticle was covered by a thin cuticular layer that was easily damaged. The smooth thin cuticle was always dirty and covered by numerous bacteria, while the star-shaped layer was considerably cleaner and free from bacterial contamination. The presence of the two-layer cuticle can also reflect a molting process. A two-layer external cuticle was also found in *Sacculina nigra* Shiino, 1943 and in some unusual specimens of *S. pinnotherae* Shiino, 1943. In the latter species, the outer layer was thinner than the inner layer (Shiino 1943). Numerous layers were also described in the external cuticle of *S. nectocarcini* Gurney, Rybakov, Høeg & Kuris, 2006 (Gurney et al. 2006).

Sympatric species *P. pilosella* and *S. pugettiae*

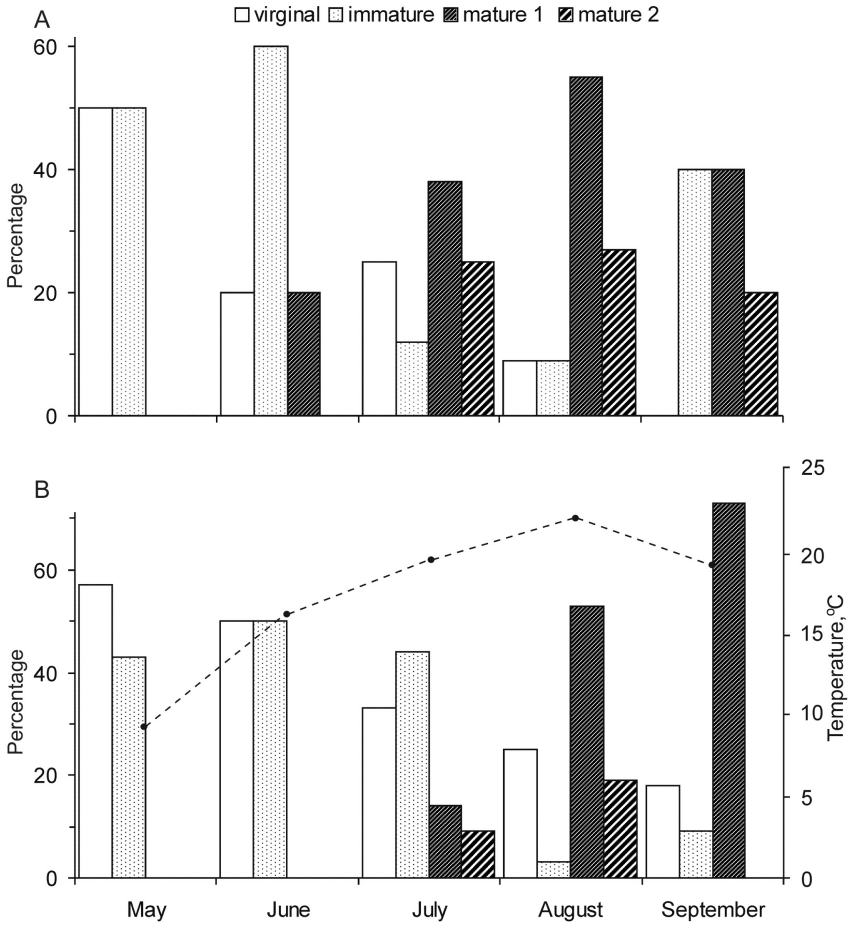

Fig. 7. Relative proportion (%) of the different developmental stages of *Parasacculina pilosella* (A) and *Sacculina pugettiae* (B). Dashed line in (B) represents the monthly average of the water temperatures at 1.5 m depth.

were externally similar, but very distinct in a number of anatomical characters, namely in the morphology of the external cuticle, shape and position of the receptacles and the structure of the colleteric glands. These characters were often used to distinguish sympatric rhizocephalans. *Sacculina upogebiae* Shiino 1943 and *Parasacculina shiinoi* (Lützen, Itani, Jespersen, Hong, Rees & Glenner, 2016) infest burrowing shrimps in Japan and Korea. *Sacculina upogebiae* has a smooth external cuticle, tubular receptacles placed within the visceral mass and highly branched colleteric glands, whereas *P. shiinoi* has a cuticle with spiny excrescences, globular receptacles located outside of the visceral mass and weakly branched oviducal glands (Shiino 1943; Lützen et al. 2016). The morphology of the external cuticle and the structures of the receptacles and the colleteric glands were used to identify three sympatric rhizocephalan species – *Sacculina confragosa* Boschma, 1933, *S. imberbis* Shiino, 1943 and *Parasacculina yatsui* (Boschma, 1936) – infesting *Pachygrapsus crassipes* Randall, 1840 crab host in eastern Japan (Tsuchida et al. 2006), and two sympatric species – *P. yatsui* and *S. confragosa* – infesting *Hemigrapsus sanguineus* (De Haan, 1835) crab host in northwestern Japan (Kobayashi et al. 2018). In *S. imberbis*, the cuticle is smooth, in *S. confragosa*, it is smooth with many winding lines, and in *P. yatsui*, it has spiny excrescences (Shiino 1943; Tsuchida et al. 2006; Kobayashi et al. 2018). In *P. yatsui*, the receptacles are outside of the visceral mass and the colleteric glands are weakly branched, whereas in *S. confragosa*, the receptacles are located in the visceral mass and the colleteric glands are highly branched (Kobayashi et al. 2018).

The morphological analysis of rhizocephalans that infested *Pugettia* aff. *ferox* in Russian waters of the Sea of Japan revealed that *P. pilosella* was more similar to *P. yatsui*, *P. shiinoi*, *P. oblonga*, *P. leptodiae*, and *P. sinensis*, whereas *S. pugettiae* shared some characters with *S. upogebiae* and *S. confragosa* (see Lützen and Yamaguchi 1999; Chan et al. 2005; Tsuchida et al. 2006; Lützen et al. 2016; Kobayashi et al. 2018).

Retinacula

The internal cuticle of the externa of both rhizocephalans was covered with ridges, and in *Parasacculina pilosella* they were spirally twisted and ended with finger-like processes. Similar surfaces were found in *Peltogasterella sulcata* (Lilljeborg, 1859), *P. gracilis* (Boschma, 1927), *Peltogaster paguri* Rathke, 1842 (Rybakov and Høeg 2002) and *P. reticulata* Shino, 1943 (Korn et al. 2020a).

The externae of sacculinids have different types of retinacula (Rybakov and Høeg 2002). *Sacculina carcini* Thompson, 1836 possesses typical lamp brushes or lamp brushes and balloon-like retinacula on the common base. More interesting structures were found in *S. triangularis* Anderson, 1862. This species has conical tubercles scattered at regular intervals over the internal mantle cuticle. There is a slit-like or rounded "crater" at the tip of each tubercle, with a mass of secretion and sometimes with a pore (Rybakov and Høeg 2002). In *S. nectocarcini* Gurney, Rybakov, Høeg & Kuris, 2006, the internal cuticle bears a few scattered large retinacula consisting of a cylindrical basal part and 11–25 barbed spindles. The retinaculum can sometimes be seen as groups of smooth spindles located at the bottom of the oval depression in the cuticle (Gurney et al. 2006).

In *S. pugettiae*, Shiino (1943) and Boschma (1960) described the retinacula as groups of spindles on the common base. We observed the same picture in the mature externae of this species. There were 4–5 spindles in specimens from Russian waters and from Seto (Shiino 1943), but 7–12 in specimens from Samani (Boschma 1960). In the virginal externae, retinacula were absent or not completely developed and presented small balloon-like structures. In *P. pilosella*, Shiino (1943) did not find retinacula; however, Van Kampen and Boschma (1925) noted the presence of solitary barbed spindles only in the largest specimens. We found no retinacula in the virginal externae of *P. pilosella*, but noted rare solitary barbed spindles in the mature externae.

Thus, the present data on two rhizocephalans confirmed our observations of *Peltogaster reticulata* and *Lernaeodiscus rybakovi* (Korn et al. 20020a b): retinacula are probably present in the mature externae of all rhizocephalans; retinacula as well as the externa itself pass through different stages of development, and their structures may transform.

Molecular phylogeny

New molecular data have led to significant changes in the traditional taxonomy of rhizocephalan barnacles (Glenner et al. 2003 2008 2010; Glenner and Hebsgaard 2006; Pérez-Losada et al. 2008; Lützen et al. 2016; Waiho et al. 2017; Høeg et al. 2019 2020). The subdivision of Rhizocephala into Kentrogonida and Akentrogonida was abandoned because both suborders are polyphyletic. The polyphyletic family Lernaeodiscidae was also abandoned. Molecular analysis confirmed the monophyly of the genus *Lernaeodicus*, which was transferred to the family Peltogastridae. The new family Peltogasterellidae Høeg & Glenner, 2019 comprised peltogastrid species with colonial externae. The polyphyletic and species-rich family Sacculinidae was divided into a redefined Sacculinidae and a new family Polyascidae

Høeg & Glenner, 2019 (Høeg et al. 2020). The amended Sacculinidae now includes three sacculinid species – *Sacculina carcini*, *S. upogebiae* and *S. insueta* – plus species in the genera *Heterosaccus*, *Loxothylacus*, *Ptychascus*, and *Sesarmaxenos*. The new family Polyascidae comprises the monophyletic genus *Polyascus* and five species formerly placed in *Sacculina* that, however, do not belong to the redefined Sacculinidae based on molecular data but in fact form a new genus *Parasacculina* Høeg & Glenner, 2019. The genetic data for the remained 167 species are not yet available and these species are included in Sacculinidae by default.

Our molecular analysis showed that *Parasacculina pilosella* and *S. pugettiae* are not related, although both parasitize *Pugettia* aff. *ferox* and are sympatric. *Sacculina pugettiae* is clustered within the monophyletic clade of Sacculinidae, whereas the other parasite is nested in the genus *Parasacculina*, belonging to the family Polyascidae Høeg & Glenner, 2019, and thus should be named *Parasacculina pilosella*. Although the genus *Parasacculina* is erected based only on molecular data, species in this genus share some common characters. The external cuticle of all species belonging now to this genus is covered by numerous hyaline spines. Most species in the genus *Parasacculina* also have globular receptacles outside the visceral mass and weakly branched colleteric glands.

Infestation

Multi-species infestation of a single host species is not rare; however, different rhizocephalans are rarely recognized sympatrically. In 2006, three species – *Sacculina confragosa*, *S. imberbis* and *P. yatsui* – were found to parasitize a single host crab, *Pachygrapsus crassipes*, in a restricted locality. However, the externa of only one species of parasite was found on each of 35 infested crabs (Tsuchida et al. 2006). In the present study, we found not only sympatric infestation of the spider crab *Pugettia* aff. *ferox* by two rhizocephalans, but also a simultaneous settlement of both parasites on one host specimen. To the best of our knowledge, this is the first finding of multi-species infestation of a single crab specimen. The intensity of this two-species multiple infestation was as high as four externae per host. Further investigations of the internae of these rhizocephalans and the peculiarities of interaction between two parasites will be interesting. The population structure of *Pugettia* aff. *ferox* in the northwestern Sea of Japan and the prevalence of infestation of this crab by two rhizocephalans should also be studied further.

Peter the Great Bay (the northwestern Sea of Japan) is characterized by significant fluctuations in water temperature throughout the year, reaching as low as -1.9°C in winter, and the presence of ice cover in December–March. The reproductive season of many invertebrates in this area coincides with the summer—the most favorable time for their embryonic development, larval release and settlement (Kornienko et al. 2017; Korn et al. 2018). The reproductive period of the rhizocephalan *Polyascus polygeneus* (Lützen & Takahashi, 1997), which parasitizes the intertidal crab *Hemigrapsus sanguineus* (De Haan, 1835), is also confined to the summer months (Korn et al. 2004). The mature externae of *P. pilosella* and *S. pugettiae* with developing embryos in the mantle cavity occur during summer and produce multiple larval generations per reproductive season. Since these rhizocephalans reproduce almost simultaneously and parasitize the same host crab, competition between them is inevitable.

CONCLUSIONS

In Russian waters of the Sea of Japan, the spider crab *Pugettia* aff. *ferox* is simultaneously infested by two rhizocephalans, *Parasacculina pilosella* and *Sacculina pugettiae*. These species differ well by the morphology of the external cuticle, the shape and position of the receptacles, and the structure of the colleteric glands. Molecular analysis showed that these rhizocephalans are unrelated and should be placed in different genera and families. The reproductive periods of two parasites are confined to the summer months in the investigated locality, and competition between them is inevitable.

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Authors' contributions: OMK made the histology, wrote the manuscript; DDG made all illustrations including SEM; SNS made the molecular analysis; NIS sampled the material. All authors approved the final manuscript.

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Availability of data and materials: DNA sequences generated in the study were deposited into the National

Center for Biotechnology Information (NCBI). Voucher specimens were deposited into the Museum of the A. V. Zhirmunsky National Scientific Center of Marine Biology, Vladivostok, Russia (MIMB).

Consent for publication: All the authors consent to the publication of this manuscript.

Ethics approval consent to participate: All applicable international, national, and institutional guidelines for use of animals were followed by the authors.

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Supplementary materials

Fig. S1. Bayesian inference analysis of 28S sequences for the Sacculinidae and Polyascidae. Numerals above or below the branches are Bayesian posterior probabilities. (download)

Fig. S2. Bayesian inference analysis of *COI* sequences for the Sacculinidae and Polyascidae. Numerals above or below the branches are Bayesian posterior probabilities. (download)

Table S1. 28S uncorrected genetic distances between species of the families Sacculinidae and Polyascidae. Above the diagonal is the SD. (download)

Table S2. *COI* uncorrected genetic distances between species of the families Sacculinidae and Polyascidae. Above the diagonal is the SD. (download)