



Two new cryptic and sympatric species of the king crab parasite *Briarosaccus* (Cirripedia: Rhizocephala) in the North Pacific

CHRISTOPH NOEVER^{1*}, ANDREW OLSON² and HENRIK GLENNER^{1,3}

¹*Marine Biodiversity Group, Department of Biology, University of Bergen, Bergen, Norway*

²*Alaska Department of Fish and Game, Juneau, AK, USA*

³*CMEC, Natural History Museum, University of Copenhagen, Denmark*

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Rhizocephalan barnacles have been reported to parasitize a wide range of king crab species (Lithodidae). So far all these parasites have been assigned to a single species, *Briarosaccus callosus* Boschma, 1930, which is assumed to have a global distribution. Here we investigate *Briarosaccus* specimens from three different king crab hosts from the fjord systems of Southeastern Alaska: *Lithodes aequispinus* Benedict, 1895, *Paralithodes camtschaticus* (Tilesius, 1815), and *Paralithodes platypus* (Brandt, 1850). Using molecular markers and by morphological comparison we show that *Briarosaccus* specimens from these three commercial exploited king crabs are in fact morphologically distinct from *B. callosus*, and further represent two separate species which we describe. The two new species, *Briarosaccus auratum* n. sp. and *B. regalis* n. sp., are cryptic by morphological means and were identified as distinct species by the use of genetic markers (COI and 16S). They occur sympatrically, yet no overlap in king crab hosts occurs, with *B. auratum* n. sp. only found on *L. aequispinus*, and *B. regalis* n. sp. as parasite of the two *Paralithodes* hosts.

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INTRODUCTION

Parasitic barnacles (Rhizocephala) are highly adapted parasites of crustaceans. The adult barnacle has a strongly modified morphology and is lacking almost all traits normally found in arthropods. The female consists of a sac shaped reproductive body (the externa) visible on the outside of the host and a branched root like system (the interna) that infiltrates their host's body. Externa and interna are connected via a stalk through the cuticle of the host (Walker, 2001). Only a single species of Rhizocephala has been reported to parasitize king crabs: *Briarosaccus callosus*, first

described as a parasite of *Neolithodes agassizii* (Smith, 1882) from the Atlantic US coast (Boschma, 1930). This parasite is assumed to be globally distributed and has been reported from a wide range of different king crab species. In Southeastern Alaskan waters three species of king crabs have been commercially fished: the golden king crab (*Lithodes aequispinus*), the blue king crab (*Paralithodes platypus*), and the red king crab (*Paralithodes camtschaticus*). These three king crab species are hosts to rhizocephalan parasites, previously identified as *B. callosus* (Boschma, 1962; Haynes & Boschma, 1969; Sloan, 1984). This parasite grows an extensive system of green rootlets inside the crab, making infested king crabs unusable for marketing (Isaeva, Dolganov & Shukalyuk, 2005). Beside the direct commercial loss of parasitized crab individuals, the parasites have strong negative effects on their hosts,

*Corresponding author. Current address: Department of Biology, University of Bergen, P.O. Box 7803, 5020 Bergen, Norway. E-mail: christoph.noever@uib.no

including parasitic castration (Isaeva *et al.*, 2005; Shields, 2012). These effects, in combination with fishing pressure, and high prevalence levels of *Briarosaccus* (Hawkes, Meyers & Shirley, 1985a; Sloan, 1985), have lead to concerns about the parasite's impact on the stocks of highly valuable king crabs (Hawkes *et al.*, 1986b; Shukalyuk *et al.*, 2005).

The genus *Briarosaccus* currently contains only two species: *B. callosus*, reported as a parasite from various king crabs belonging to the genera *Glyptolithodes*, *Lithodes*, *Neolithodes*, *Paralithodes*, and *Paralomis* (Pohle, 1992; Guzman, Moreno & Moyano, 2002), and *B. tenellus* Boschma, 1970, which is found on the small hapalogastrid crab *Hapalogaster mertensii* Brandt, 1850. We describe two additional species of *Briarosaccus*, found on three king crab hosts in the fjord systems of Southeastern Alaska. Morphological comparison between the *Briarosaccus* specimens from Southeastern Alaska with the type specimen of *B. callosus* revealed that they are not conspecific. While the new species are morphological distinctly different from *B. callosus*, the two are indistinguishable by morphology and were identified as distinct species by their mitochondrial DNA and host specificity.

MATERIAL AND METHODS

MOLECULAR WORK

Briarosaccus specimens for molecular analyses were collected in the fjord systems of Southeastern Alaska. Parasitized hosts were sampled in 2010 and 2011, during king crab stock assessment surveys by the Alaska Department of Fish and Game and commercial king crab fisheries. King crabs were caught using conical crab pots and checked for *Briarosaccus* infections. 10 rhizocephalan parasites were sampled from *Lithodes aequispinus*, 19 specimens from *Paralithodes camtschaticus*, and two specimens from *Paralithodes platypus*. Host species and collection sites were recorded for each parasite specimen and tissue samples were taken from the externae and fixed individually in 95% ethanol.

Briarosaccus specimens from *P. camtschaticus* were sampled from the vicinity of Juneau (Favourite Channel, Auke Bay, Pt. Arden, and Stephens Passage), Gambier Bay, Pybus Bay, Port Houghten, Port Frederick, and northeast of Mitkof Island. Parasites from *P. platypus* were sampled from Holkham Bay and upper Lynn Canal near Haines. Parasites of *L. aequispinus* were sampled from Southern Lynn Canal, Mid- and Lower-Chatham Strait, and Holkham Bay.

Molecular work was conducted in the Biodiversity Laboratories, University of Bergen, Norway. Total genomic DNA was extracted using a Gene Mole automatic nucleic acid extractor from Mole Genetics

AS, Norway. Partial fragments of two mitochondrial genes, cytochrome *c* oxidase I (COI), and the 16S ribosomal RNA gene (16S), were amplified by PCR. For amplification of COI the primers H2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and L1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') (Folmer *et al.*, 1994), and for 16S the primers L12247L-16S (5'-TTAATYCAACATCGAGGTCRCAA-3') and H621-16S (5'-CYGTGCAAAGGTAGCATA-3') (Tsuchida, Lützen & Nishida, 2006) were used. PCR reactions were carried out with TaKaRa *Taq* in 25 µl reactions. For the COI gene, cycling started with an initial denaturation at 94 °C for 3 min, followed by 30 cycles of 30 s at 94 °C, 45 s at 48 °C, and 1 min at 72 °C, and finished with a final extension of 5 min at 72 °C. PCR conditions for 16S were identical, except that the annealing temperature was set to 50 °C. PCR products were checked for successful amplification on 1% agarose gels stained with GelRed. PCR products were purified and sequenced in both directions using each primer pair at MacroGen Inc. Contigs were assembled using Lasergene SeqMan Pro 8.1, and the sequences were aligned using eBios 1.5.1. The primer set amplified a 655-658bp long fragment for the COI gene, and a 340-341bp long fragment for the 16S gene. The COI alignment was trimmed down to a length of 636bp due to few not entirely readable sequences at the H2198 primer end. To exclude the presence of pseudogenes or gene duplicates, the individual sequence chromatogram files were checked for the presence of double peaks, and the COI alignment was translated into amino acids and checked for premature stop codons and frame shifts using BioEdit 7.2.3. Sequences are available on GenBank with the accession numbers KR812147 to KR812208.

EVOLUTIONARY ANALYSES

The genetic distances between the aligned sequences were calculated using MEGA 5.2 with the Kimura 2-parameter model for each of the two genes. Phylogenetic analyses, using neighbor joining, were conducted with the same software, running 1000 bootstrap replicates. The phylogenetic trees were visualized using the software Dendroscope 3.2.

MORPHOLOGY OF THE EXTERNA

For morphological analyses, 52 *Briarosaccus* specimens from *L. aequispinus* hosts were obtained from commercial fishermen in 2012, sampled from Clarence Strait and Mid-Chatham Strait. The externae were removed from their hosts, initially frozen at -20 °C, and fixed in 70% ethanol after thawing. Nine *Briarosaccus* specimens from *P. camtschaticus* hosts were sampled during a king crab stock assessment survey by the Alaska Department of Fish and Game

in 2012. These specimens were photographed alive and the externae were removed from their hosts and fixed in 70% ethanol. One *Briarosaccus* specimen from *P. camtschaticus* was kept attached to its host, fixed in 10% formalin, and transferred to 70% ethanol. All externae were examined for their gross morphology. 20 externae taken from *L. aequispinus* and six externae from *P. camtschaticus* hosts were dissected under a dissection microscope to investigate the morphology of the mantle cavity and visceral mass. Receptacles and vasa deferentia were dissected out of the surrounding visceral mass tissue of 14 *Briarosaccus* specimens from *L. aequispinus* and six specimens from *P. camtschaticus*.

The *Briarosaccus* specimens from Southeastern Alaska were compared with the morphology of *B. callosus* from its original description (Boschma, 1930), which is based on a single specimen from the Atlantic US coast. The type specimen of *B. callosus* is assumed to be lost, and only serial sections of part of the visceral mass remain at the Naturalis Biodiversity Center, Leiden, the Netherlands.

MORPHOLOGY OF THE INTERNA

To examine the extent and gross organization of the internal root system, three parasitized specimens of *P. camtschaticus* and seven specimens of *L. aequispinus* were narcotized by adding clove oil to their sea water filled container, and killed by dislodging the carapace from the body and cutting through the central nervous system. The strongly green coloured rootlets could easily be identified by the naked eye and their organization in the host's body was studied under low magnification and photographed for further analyses.

SCANNING ELECTRON MICROSCOPY (SEM)

Receptacles and vasa deferentia, as well as parts of the inner and outer mantle cuticle of parasites taken from *P. camtschaticus* and *L. aequispinus* were scanned using a Quanta FEG 450 SEM at 10 kV. Samples were dehydrated in ethanol, and critical point dried in CO₂. The dried samples were mounted on SEM stubs with carbon tape and sputter coated with gold / palladium.

COMPLEMENTARY DATA

The morphological descriptions of the two newly described *Briarosaccus* species are complemented with data from literature sources. Data taken or supplemented from the literature are indicated by the references.

SPECIMEN DEPOSITION

Type material of the two described species is deposited at the Yale Peabody Museum of Natural History,

Connecticut. Museum numbers are YPM 74280–74283.

RESULTS

MOLECULAR ANALYSES

Analyses of the mitochondrial gene fragments, COI and 16S, revealed that the *Briarosaccus* samples are divided into two distinct genetic groups. Both genes showed the same pattern, with one clade containing all *Briarosaccus* specimens taken from *Lithodes aequispinus* hosts, while the other clade contains all parasites from *Paralithodes camtschaticus* and *P. platypus* (Fig. 1). For the COI gene, the genetic differences calculated with the Kimura 2-parameter model ranged from 0 to 0.5% within, and 11.6 to 12.8% between the two groups. For the 16S gene the differences ranged from 0 to 0.6% within, and 4.3 to 4.6% between them. The translation of the trimmed COI gene fragment into amino acids resulted in an alignment of 212 amino acid positions. Amino acid differences within each group ranged from 0 to 1, and from 13 to 15 between the two groups. The entire clade consisting of parasites taken from the two *Paralithodes* host species is lacking three nucleotides, coding for a single amino acid, in the COI sequence. No double peaks were found in the individual sequences, and no stop codons or frameshifts were found in the COI amino acid alignment, indicating that no nuclear copies of mitochondrial genes (numts) or heteroplasmic genes were sequenced. Based on the molecular results, we conclude that specimens of *Briarosaccus* sampled from *L. aequispinus* and the two *Paralithodes* hosts represent two distinct species.

MORPHOLOGICAL DESCRIPTION

Comparisons of the externae from our sampled specimens with the original description of *Briarosaccus callosus* clearly showed that the specimens from Alaska are not conspecific with *B. callosus*, but should be regarded as new species (see Discussion).

No consistent morphological differences could be determined between specimens from the different hosts, thus the morphological description is combined for the two new *Briarosaccus* species. The parasite's externa is usually situated on the ventral side of the host's abdomen, where it is attached to the crab's integument via a short, hollow stalk, which connects the externa with the internal trophic root system. The overall shape of the parasite varies due to large size differences, and status of the reproductive cycle. The externa is bean shaped, elongated, and curved to various degrees, with the ventral outline being convex, and the dorsal side concave (Fig. 2a). *Briarosaccus* specimens of the different hosts have been reported with a similar

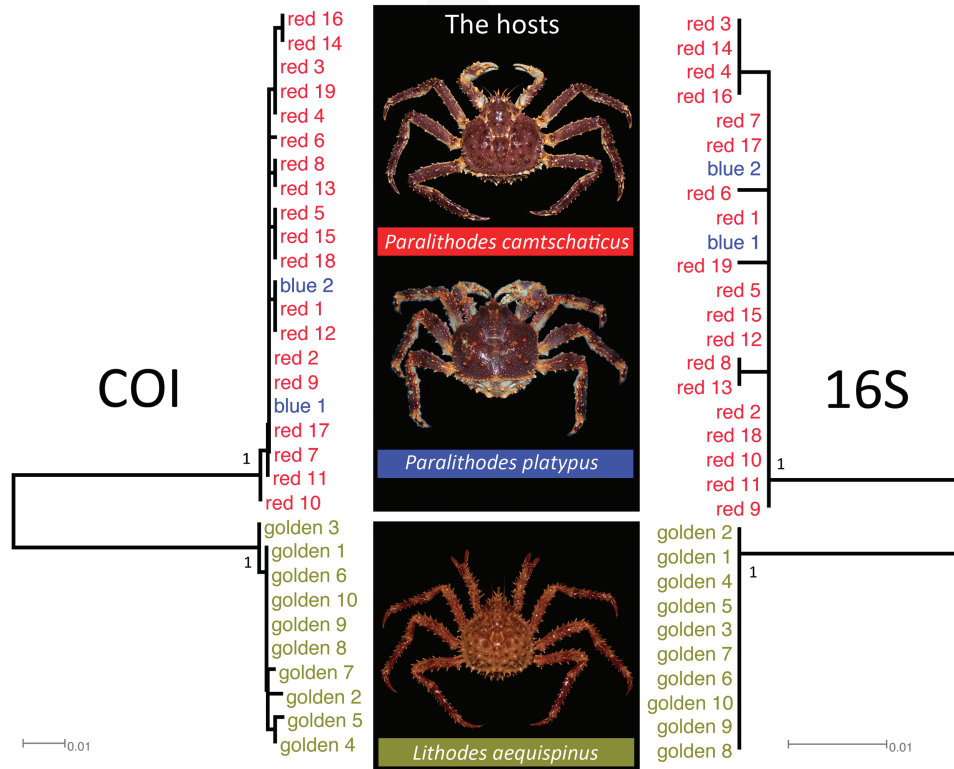


Figure 1. Unrooted phylogenetic trees of the COI and 16S genes from 31 *Briarosaccus* specimens, constructed using neighbor joining based on the Kimura 2-parameter model. Parasites taken from *P. camtschaticus* are named red 1 to 19, from *P. platypus* hosts are named blue 1 to 2, and from *L. aequispinus* hosts are named golden 1 to 10. The respective host king crabs to the parasite specimens of the two genetic clades are displayed in the black boxes between the trees. Numbers on the nodes indicate the bootstrap supports. The scale bars are indicating substitution sites.

size range, from 7 to 81 mm in anterior-posterior length, 2 to 39 mm in height, and 2 to 35 mm in width. The externa can be gravid from their smallest size on (Bower & Sloan, 1985; Hawkes *et al.*, 1985a).

The colour of the living externa varies from bright red in immature specimens to a pale orange in egg bearing parasites (Fig. 3) (Hawkes *et al.*, 1985a). The red colour is caused by the respiration pigment hemoglobin, which is rare in crustaceans (Shirley, Shirley & Meyers, 1986).

Usually the externa is attached in the middle of the soft ventral side of the host's abdomen, posterior to the narrow rigid cuticular bar of the fifth thoracic sternite (Bower & Sloan, 1985). The parasite's externa is usually orientated on its host with the mantle opening directed towards the left body side of the crab. The mantle opening is situated on the anterior pole, where it is erected on a short tube (Fig. 2a). The opening lacks a strongly developed sphincter muscle and is formed by a crenulated lip. The opposite, posterior end of the externa is broadly rounded. The short stalk is situated in the exact middle of the dorsal side, surrounded by a hard chitinous shield of dark

brown colour. The shield has numerous annuli, round marks, each resulting from a moulting cycle (Lützen, 1987).

In egg bearing specimens, the spacious mantle cavity, where the eggs are bred, takes up the main proportion of the externa. After the larvae broods are released the mantle cavity deflates, generating a much slimmer shaped externa.

The visceral mass, which contains the reproductive organs, is connected to the dorsal side of the mantle via a relatively thin mesentery, which is broadened in the region below the stalk. The mesentery runs from the mantle opening on the anterior end to the beginning of the rounded posterior end of the externa. The surface of the visceral mass is smooth, without lateral extensions. Its width is variable, due to large size variations of the parasite, and changes in the state of the ovaries due to the reproductive cycle, ranging from rounded to laterally flattened in cross section. At the anterior end the visceral mass is tapering, and protrudes into the mantle opening.

The paired receptacles, in which the male larvae settle, are situated in the dorsal part of the visceral

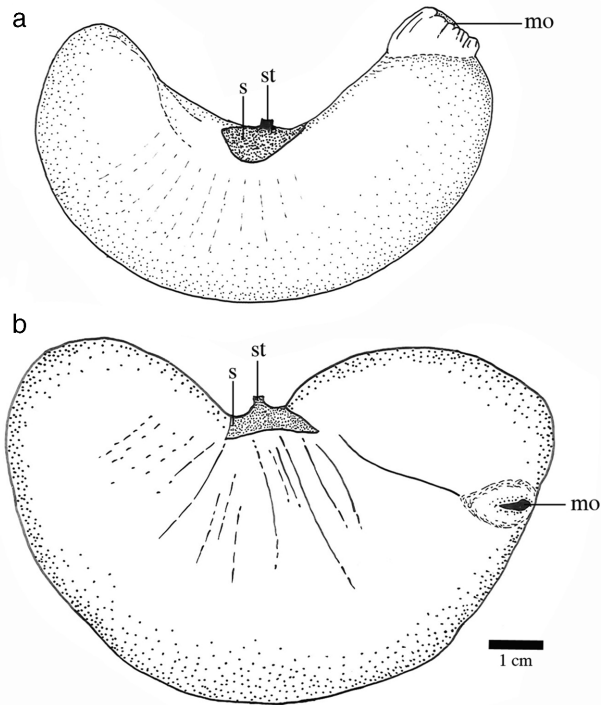


Figure 2. Morphological comparison between the herein described *Briarosaccus* species and the type specimen of *B. callosus*. Line drawings from the right side view of the externa. a) Large, ovigerous specimen of *B. regalis* n. sp., taken from *P. camtschaticus*. b) Type specimen of *B. callosus*, drawing modified from Boschma, 1930. Abbreviations: mo, mantle opening; s, cuticular shield; st, stalk. Scale bar indicating 1 cm.

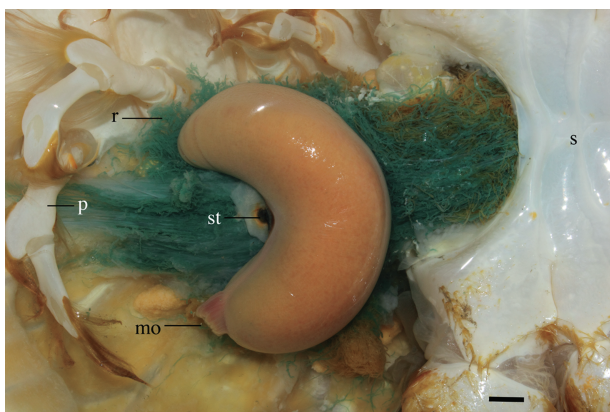


Figure 3. *In situ* photograph of an unfixed externa of *B. regalis* n. sp. on *P. camtschaticus*, showing the natural colouration. The soft ventral cuticle of the king crab's abdomen has been removed, displaying the green rootlets that are situated inside the crab's body. Abbreviations: mo, mantle opening; p, pleopod of the crab; r, green internal rootlets; s, sternum of the crab; st, stalk. Scale bar indicating 1 cm.

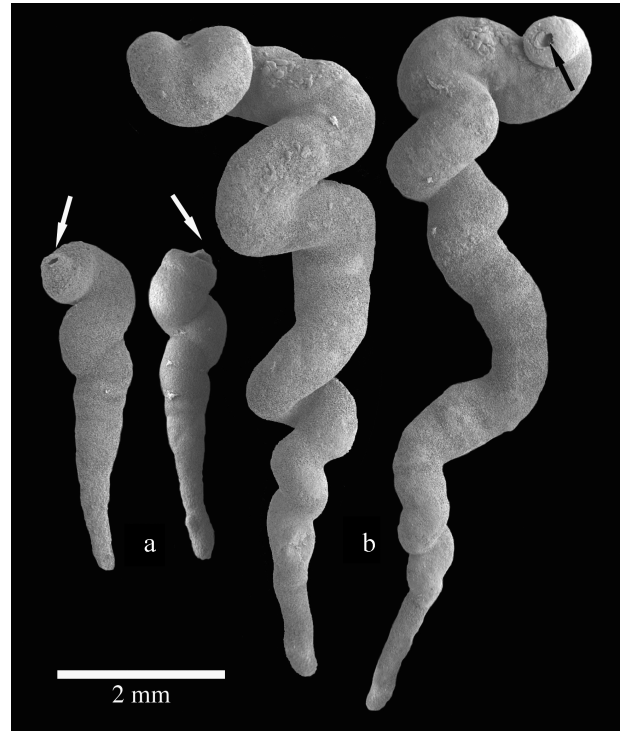


Figure 4. SEM photographs of two receptacle pairs from *B. regalis* n. sp. (a) from a 3.4 cm long externa (b) from (a) externa with an anterioposterior length of 8.5 cm. Arrows are indicating the connection sites to the vasa deferentia.

mass on each side of the stalk under the cuticular shield, parallel to the long axis of the externa. The receptacles are slightly broader towards the posterior end. They run as a relatively straight tube for a short distance in the anterior end, then irregularly meandering for most of their length (Fig. 4). Especially large externae display strongly coiled receptacles. The connection between the vas deferens and the posterior end of the receptacle is abrupt, with the vas deferens being much smaller in diameter than the connecting part of the receptacle (Fig. 4). The vasa deferentia run from each receptacle towards the lateral sides of the visceral mass, where they enter into the mantle cavity. The vasa deferentia have a highly irregular, wrinkled appearance and occasionally can even have short side branches that end blind.

The cuticular integument is thick, and slightly wrinkled on a macroscopic scale, which might be intensified during fixation. The inner mantle cuticle, facing the mantle cavity, has no lateral extensions. Under high magnification using SEM, irregular distributed fields of small papillae are visible on the outer mantle surface (Fig. 5a-c). Distributed over the inner surface are retinacula (Fig. 5g, h), groups of spindle shaped extensions of the surface, which are covered with barbs

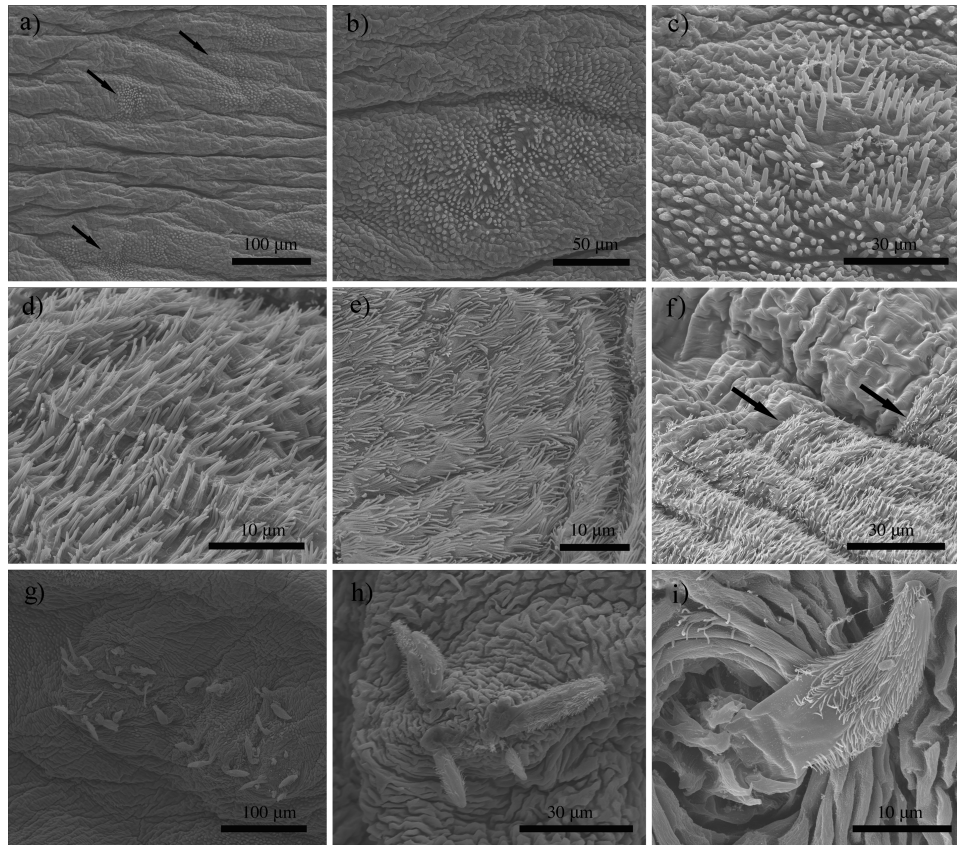


Figure 5. SEM photographs of cuticular ultrastructures of the externa. All images taken from specimens of *B. regalis* n. sp. (a) External surface of the mantle. Arrows are indicating fields of small papillae, which are irregularly distributed over the surface. (b, c) Details of papillae-patches on the external mantle surface. (d, e) Small hairs covering the inner mantle surface, close towards the mantle opening. (f) Outer edge of the mantle opening. Arrows are indicating the distinct break between the smooth outer and hairy inner mantle surface. (g, h) Groups of retinacula of the inner mantle surface. (i) Single retinaculum spindle.

directed downward to the base of the retinaculum spindle (Fig. 5i). Fine hairs are covering the inner cuticle surface (Fig. 5d, e), which are denser towards the mantle opening, and densely covering the inner part of the opening. There is a distinct change from the hair-free outer cuticle to the hairy inner surface on the edge of the mantle opening (Fig. 5f). These thin hair structures have previously been reported for other rhizocephalans (Rybakov & Høeg, 2002).

The trophic root system is coloured bright green when alive (Fig. 3), and can even be visible through the cuticle of the host's soft abdomen. The major part of the rootlets is situated in the abdomen of the crab, where they are found right below the host's cuticle, on top and partly interwoven with the host's hepatopancreas. The rootlets are also infiltrating the carapace region, and appear to be linked to the central nervous system. The muscular tissue of the host is mostly unaffected, however the rootlets can occasionally also penetrate into the muscular bases of the pereopods.

LARVAL MORPHOLOGY

The planktonic larval stages of the two new *Briarosaccus* species have been described by Hawkes, Meyers & Shirley (1985b). While the authors were unaware of the presence of two cryptic species in their samples, specimens taken from *P. platypus* and *L. aequispinus* were treated separately, but no differences were observed. The authors describe four naupliar stages and the cypris stage, which, like in other rhizocephalans, are all lecithotrophic. However, Walossek, Høeg & Shirley (1996) report five naupliar instars for *Briarosaccus tenellus* and note that the first instar might have been undetected in other species. The nauplii have no eye, and contain numerous lipid droplets. The second to fourth naupliar stages possess a large flotation collar around the body, as also known from other rhizocephalans of the families Peltogastridae and Lernaediscidae (Høeg, Møller & Rybakov, 2004). The sexes have profound size differences, with male larvae being larger than females (Hawkes *et al.*, 1985b).

SYSTEMATICS

Family: Peltogastridae Lilljeborg, 1859

Genus: *Briarosaccus* Boschma, 1930

Briarosaccus regalis N. SP.

Hosts: *Paralithodes camtschaticus* and *P. platypus*

Holotype: One externa in situ on its host, *P. camtschaticus*, Southeastern Alaska. Museum number YPM 74280. Paratype: One externa removed from its host, *P. camtschaticus*, Southeastern Alaska. Museum number YPM 74281.

Molecular reference data: GenBank accession numbers KR812178 to KR812198 for COI, and KR812157 to KR812177 for 16S

Etymology: The species is named due to its parasitism of the *Paralithodes* king crabs, which include the largest of the king crab species, *P. camtschaticus*.

Briarosaccus auratum N. SP.

Host: *Lithodes aequispinus*

Holotype: One externa removed from its host, *L. aequispinus*, Southeastern Alaska. Museum number YPM 74282. Paratype: One externa removed from its host, *L. aequispinus*, Southeastern Alaska. Museum number YPM 74383.

Molecular reference data: GenBank accession numbers KR812199 to KR812208 for COI, and KR812147 to KR812156 for 16S

Etymology: The species is named after its host *L. aequispinus*, which is known as the golden king crab.

DISCUSSION

NEW SPECIES OF *Briarosaccus*

Rhizocephalan parasites have been reported from a wide range of king crabs, including the three investigated species, *Lithodes aequispinus*, *Paralithodes camtschaticus*, and *Paralithodes platypus* (Hawkes *et al.*, 1986b). Until now all these records were assigned to a single species of Rhizocephala: *Briarosaccus callosus*. *B. callosus* was first described from the Atlantic US coast as a parasite of *Neolithodes agassizii* (Boschma, 1930). Several morphological characters of the investigated *Briarosaccus* specimens from the North Pacific are distinctly different in the type specimen of *B. callosus*, unambiguously showing that they are not conspecific.

With molecular markers of the mitochondrial genome we further show that the king crabs in Southeastern Alaska are parasitized by two distinct species of *Briarosaccus*, with each of the two parasite species having different host specificities. Two clearly separated genetic clades of *Briarosaccus* were independently obtained by analyses of the COI and 16S genes (Fig. 1). The underlying pattern for the two clades is very obvious: one

clade consists of all parasites from *L. aequispinus*, the golden king crab, while the other clade includes all parasites taken from the two *Paralithodes* species, the red king crab and the blue king crab. This reveals that instead of only one rhizocephalan parasite, there are in fact two species of *Briarosaccus* that parasitize king crabs in Southeastern Alaska, with each parasite having a different host specificity. The genetic separation between the two clades, which is supported by the distinct host specificities, justifies a separation into two formal species. Both genes display minimal intraspecific variation for the two new species, and an interspecific variation that is considerably higher. This clear separation between conspecific and congeneric distances is the key for successful species delimitation using molecular markers (Hebert *et al.*, 2004; Zemplak *et al.*, 2009), which has been shown to be widely applicable in Crustacea using COI (Costa *et al.*, 2007). Costa *et al.* (2007) report average intraspecific variations for crustaceans of 0.46%, which is in accordance with our data. No signs of numts or heteroplasmy could be detected in our data, which can cause potential pitfalls in studies using mitochondrial markers (Buhay, 2009; Gíslason *et al.*, 2013). A geographical explanation of the genetic pattern can be excluded since sampling sites of the different host species are located inside the same geographical area.

No consistent morphological difference between the two new Pacific *Briarosaccus* species could be found. Despite the lack of distinguishing characters by morphological means, delimitation between the two new species is yet possible by the use of molecular markers, as well as by their distinct host specificity. Unrecognized as cryptic, the two new *Briarosaccus* species have received considerable interest, as these parasites can have devastating effects on commercial valuable king crab populations (Hawkes *et al.*, 1985a; Hawkes, Meyers & Shirley, 1986a; Isaeva *et al.*, 2005). The possibility of separating between these two cryptic and sympatrically occurring king crab parasites, which previously were recognized as *B. callosus*, might have particular importance for practical applications in king crab fisheries management.

MORPHOLOGICAL DIFFERENCES TO *B. callosus*

The two new *Briarosaccus* species are clearly more closely related to each other than they are to *B. callosus* given the morphological differences to the former. While we could not determine morphological differences between *B. auratum* n. sp. and *B. regalis* n. sp., the morphology of the type specimen of *B. callosus* has a couple of striking differences:

- (1) In *B. callosus* the position of the mantle opening is not exactly situated on the anterior pole, but slightly shifted to the right side of the median plane.

In contrast to this, in the two new species the mantle opening is situated on the middle plane of the anterior end (Fig. 2).

- (2) The mantle opening in *B. callosus* is a narrow slit, on level with the mantle surface, and surrounded by a strong sphincter muscle. The mantle opening in the two new species is highly different, as it is situated erected on a short tube, formed by a wrinkled round wall, and is lacking a strong developed sphincter (Fig. 2).
- (3) In *B. callosus* the strong sphincter muscle is the only mechanism to regulate the closing and opening of the aperture, while in the two new species the anterior end of the visceral mass protrudes into the opening and serves as a plug like structure. In *B. callosus*, the visceral mass does not protrude into the opening and is unable to function as such a closing device.
- (4) The receptacles in the type specimen of *B. callosus* are described as straight tubes. In contrast to this, the receptacles in the two new species are strongly coiled towards the posterior end (Fig. 4). While Boschma analysed the structure of the receptacles by thin sectioning (Boschma, 1930), we investigated the receptacles by gross dissection, which gives a better and complete overview of their overall shape. However, since the *B. callosus* type specimen was of exceptionally large size, a strong coil shape, as found in the new species, would have been obvious even by thin sectioning.

CRYPTIC SPECIES IN *Briarosaccus*

Cryptic species are defined as a group of species previously identified as one (Bickford *et al.*, 2007). The two newly described species clearly fall into this definition. Material from both new *Briarosaccus* species was available to Boschma (Boschma, 1962; Haynes & Boschma, 1969), who described *B. callosus* and was at his time the authority on rhizocephalan taxonomy (Vervoort, 1977). Boschma, however, classified all rhizocephalans of king crabs as *B. callosus* (Boschma, 1970). This was largely due to the fact that his samples, which steadily increased over the years, showed high morphological variations, but from most hosts only very few specimens were available to him. Lacking modern species delimitation methods he did not find another solution than classifying all records as a single species. Following authors had little other choice than following Boschma's practice when new king crab hosts to rhizocephalan parasites were discovered (e.g. Arnaud & Do-Chi, 1977; Abelló & Macpherson, 1992; Pohle, 1992; Lützen, Glenner & Lörz, 2009; Pino *et al.*, 2010). Using genetic methods on these parasites for the first time, we show that even in one geographical region

multiple *Briarosaccus* species can co-occur. On a global scale a complex of cryptic *Briarosaccus* species can be expected, which will be covered in a following study. While this study shows that *Briarosaccus auratum* n. sp. and *B. regalis* n. sp. are clearly distinguishable from *B. callosus*, we could not determine consistent morphological differences between the two species. However, species delimitation by morphological means is not always sufficient, as speciation is not necessarily accompanied by morphological change (Bickford *et al.*, 2007), which is especially true for Rhizocephala considering the near absence of proper morphological characters in this taxon (Høeg, 1995). Cryptic species can contribute an important part of biodiversity (Bickford *et al.*, 2007; Nygren, 2014), and might be common among the Rhizocephala, as this study shows that morphology alone might underestimate the diversity of this group.

DISTRIBUTION RECORDS

While specimens for this study were obtained solely from Southeastern Alaska, both new *Briarosaccus* species are likely to have a much wider distribution range in the North Pacific. Reports of *Briarosaccus* from *L. aequispinus* and *P. camtschaticus* are ranging from British Columbia in the South (Sloan, 1985) northwards along the Pacific coast of Alaska (Haynes & Boschma, 1969; McMullen & Yoshihara, 1970; Hawkes *et al.*, 1986b), and the Sea of Okhotsk (Isaeva *et al.*, 2005; Shukalyuk *et al.*, 2005). On *L. aequispinus* *Briarosaccus* has additionally been reported from the Aleutian Islands (Blau & Pengilly, 1994), the Bering Sea (Boschma, 1962), and Japan (Watabe, 2007). On *P. platypus* *Briarosaccus* has been reported from Southeastern Alaska (Hawkes *et al.*, 1985a, 1986b), and Isaeva *et al.* (2005) mentioned a single observation from the Sea of Okhotsk.

As the present study indicates a strict host specificity of both new parasite species, we suppose that the three investigated king crabs are host to the same *Briarosaccus* species throughout their distribution range. The occurrence on their respective hosts should thus be sufficient for species identification, however confirmation using genetic markers will be preferential when investigating further host populations. The parasites prevalence can vary drastically between different regions, with prevalence reports on *P. platypus* ranging from under 1% in the Sea of Okhotsk (Isaeva *et al.*, 2005) to as high as 76% in Glacier Bay, Alaska (Hawkes *et al.*, 1985a). In Southeastern Alaska, parasitism levels of *P. camtschaticus* are highly variable between different locations, ranging from below 1% to 8.3% (Hawkes *et al.*, 1986b). The authors explain high local *Briarosaccus* prevalences by the silty clay sediments from associated glacial run-off in these areas. The turbid

waters might reduce the crab's effectiveness of gill cleaning by the fifth pereopods, enhancing the settlement success of parasite larvae. However, extreme prevalences are not necessarily representative only for small areas, as entire fjord systems can have highly parasitized king crab populations. Such found Sloan (1985) in the deep fjords of British Columbia, with a *Briarosaccus* prevalence of 41% in a large sample of 3800 golden king crabs.

SYMPATRIC OCCURRENCE

While the two *Briarosaccus* species in Southeastern Alaska are currently occurring sympatrically, it is uncertain if they also speciated in the same area. The Lithodidae most likely evolved from a pagurid hermit crab ancestor in the North Pacific (Cunningham, Blackstone & Buss, 1992; Bracken-Grisson *et al.*, 2013). The large king crab genera, especially *Paralomis*, *Lithodes*, and *Neolithodes*, did not solely remain in this area, but diversified on a global scale. The genus *Lithodes* developed as a deep-sea lineage (Hall & Thatje, 2009). As such, *L. aequispinus* originated geographically separated from the shallow water Lithodidae of the North Pacific coastline. Due to the formation of the deep-water fjord systems in the Northeastern Pacific after the last glaciation period, *L. aequispinus* could expand its distribution range by invading the newly formed deep-sea habitat inside the continental shelf. *L. aequispinus* tends to inhabit a deeper stratification range than *P. platypus* and *P. camtschaticus* (Somerton, 1981; Sloan, 1985). While still inhabiting different ecological niches due to depth preferences, *L. aequispinus* and the two investigated *Paralithodes* species are now found sympatrically in the fjord systems of the Northeastern Pacific. The strong host affinity of the two new *Briarosaccus* species indicates that the parasites co-speciated simultaneously with the divergence of the genera *Lithodes* and *Paralithodes*. When *Briarosaccus* followed the speciation of its host king crabs, it became specialized to the different host physiologies, and as a result, each of the two species became restricted to their respective *Paralithodes* or *Lithodes* hosts. Our molecular data indicate that *B. auratum* n. sp. is not able to infest the hosts of *B. regalis* n. sp. and *vice versa*.

It has been questioned before if *Briarosaccus* specimens from the different host king crabs in the Northeast Pacific represent in fact only a single species (Hawkes *et al.*, 1985b), and the authors noted its potential importance for fisheries management strategies. Investigating the planktonic larval stages, they found no morphological differences between the larval stages of parasites from *Lithodes* and *Paralithodes* hosts and concluded that they most likely represent the same species. However, this study did not use SEM and might

have missed important morphological fine structural differences (Glenner *et al.*, 1989).

MULTIPLE INFECTIONS

Briarosaccus occasionally occurs with multiple externae on a single host and parasitism with up to five externae has been observed (Sloan, 1984; Hawkes *et al.*, 1985a). Certain rhizocephalan genera (e.g. *Polyascus*, *Peltogasterella*, or *Cyphosaccus*) can form multiple externae by budding from a single parasite specimen (Glenner, Lützen & Takahashi, 2003). In *Briarosaccus*, however, the rate of multiple infections is lower than expected by chance and usually only occurs in heavily parasitized host populations (Sloan, 1984). This indicates infestations of the crab by multiple female *Briarosaccus* larvae in the case of multiple externae, as it has been shown for the rhizocephalan *Sacculina carcini* Thompson, 1836 using the mitochondrial control region (Rees & Glenner, 2014).

FURTHER *Briarosaccus* RECORDS IN THE NORTH PACIFIC

Besides the three investigated king crab species, *Briarosaccus* has been reported from a number of other hosts in the North Pacific. *Lithodes couesi* Benedict, 1895 has been reported as host to *Briarosaccus* from the Bering Sea (Boschma, 1970) and the Gulf of Alaska (Somerton, 1981). From Japan *Briarosaccus* has been reported on *Paralomis hystrix* (De Haan, 1849), *Paralomis japonicus* Balss, 1911, and *Paralomis multispina* (Benedict, 1895), of which *P. multispina* was observed with an exceptional infestation prevalence of 98.6% in 10,875 king crab specimens from the Tokyo Submarine Canyon (Watabe, 2007). *P. multispina* and a further *Paralomis* host, *P. verrilli* (Benedict, 1895), have been recorded with *Briarosaccus* in the Sea of Okhotsk (Poltev, 2008). Further south, on the Californian coast, *Briarosaccus* has been reported on *Paralithodes californiensis* (Benedict, 1895) and *Paralithodes rathbuni* (Benedict, 1895) (Cadien & Martin, 1999).

The two new *Briarosaccus* species show strict host specificities in this study, and seem to be restricted to one or a few closely related king crabs. Therefore, it is questionable if the *Briarosaccus* records on other king crabs in the North Pacific can be attributed to the described species or represents further yet unrecognized ones. We assume that the second option is more likely, especially for the records on the *Paralomis* hosts, as well as the observations on the two further *Paralithodes* hosts from the Californian coast, since this genus appears to be paraphyletic (Snow, 2010). No *Briarosaccus* material from these hosts was

available for this study, thus the species status of their associated parasites remains unknown.

EFFECTS ON THE HOSTS AND IMPLICATIONS TO KING CRAB FISHERIES

Host populations of both parasite species, *B. auratum* n. sp. and *B. regalis* n. sp., have been reported with extremely high infestation rates, with up to 76% of the king crabs being parasitized (Sloan, 1985; Hawkes *et al.*, 1986b). Parasitic infestation with *Briarosaccus* has profound effects for its hosts, as the parasite causes sterilization (McMullen & Yoshihara, 1970; Hawkes *et al.*, 1985a; Sparks & Morado, 1986; Isaeva *et al.*, 2005), induces effects on host hemolymph (Shirley *et al.*, 1986), reduces growth and body condition (Sloan, 1985; Hawkes *et al.*, 1986a), induces feminization of male hosts (Sloan, 1984; Isaeva *et al.*, 2005; Shukalyuk *et al.*, 2005), and changes in behaviour (Sloan, 1984). The interna of *Briarosaccus* penetrates several organs, in particular the nervous system, which presumably has a profound effect on neuroendocrine controls (Sparks & Morado, 1986).

Briarosaccus is a continuous breeder, and at least 33 reproduction cycles may occur in an average sized parasite (Bower & Sloan, 1985), with up to 500 000 larvae being released in one spawning event (Hawkes *et al.*, 1985b). The high reproductive potential of the parasite, given their enormous size for Rhizocephala, might be a major factor why under certain circumstances king crab populations can reach extreme infestation rates (Hawkes *et al.*, 1985a). High parasitism rates of king crab populations causes concern for fishery management, since the partial sterility of a population combined with fishing pressure might easily cause a decline in population size and yield (Hawkes *et al.*, 1986b; Shukalyuk *et al.*, 2005). Misidentification of economically important species in cryptic complexes can have serious negative consequences in fisheries management (Bickford *et al.*, 2007). The recognition of cryptic species of rhizocephalan parasites on commercially important king crab hosts should therefore be of interest for fisheries management. For example, this may explain frequently observed differences of *Briarosaccus* prevalence on different king crab host species in the same area (Sloan, 1985; Hawkes *et al.*, 1986b; Isaeva *et al.*, 2005).

USE OF MOLECULAR METHODS IN RHIZOCEPHALA

Some previous studies showed the efficacy of genetic markers for species delimitation and population studies in Cirripedia (e.g. York, Blacket & Appleton, 2008; Pinou *et al.*, 2013), and especially in the morphological poor Rhizocephala (e.g. Murphy & Goggin, 2000; Rees & Glenner, 2014), which are largely deficient of diagnostic characters.

The mitochondrial COI gene confirmed the rhizocephalan *S. carcini* as a single species with a wide range of hosts in its European distribution range, with genetic variations under 1% (Gurney, Grewe & Thresher, 2006). Tsuchida *et al.* (2006) showed that COI can be used as a species delimiting tool in three sympatric species of the rhizocephalan genus *Sacculina*, which are difficult to distinguish by morphology. They observed maximal variations under 1% for each of the species, and a much larger genetic differences between them, ranging from 30 to 45%. Kruse, Hare & Hines (2012) reported a more complicated genetic relationship for the rhizocephalan *Loxothylacus panopaei* (Gissler, 1884) on different hosts from the US East Coast. Also in this study the host association was largely responsible for genetic variations. However, this pattern was not entirely consistent, and mitochondrial and nuclear markers gave different signals. The authors explained their results with distinct genetic lineages of uncertain taxonomic status, and mitochondrial cross-overs. A following study did not find further evidence for the inconsistent pattern in the COI gene (O'Shaughnessy, Freshwater & Burge, 2014), and we suggest that this pattern should be further investigated since the possibility that the data had been affected by gene duplications and PCR contamination problems should also be considered.

The current study further highlights the advances in the use of molecular markers for the study of the morphological poor Rhizocephala, in particular their use in delimitating species boundaries and investigating host specificities.

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