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Author(s)	Kakui, Keiichi; Sekiguchi, Shogo
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Colonial Entoproct Epibiotic on a Sea Spider

Keiichi Kakui^{1*} and Shogo Sekiguchi²

¹Department of Biological Sciences, Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan

²Department of Natural History Sciences, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan

We report an entoproct epibiotic on the surface of a sea spider (Pycnogonida). The pycnogonid was identified as *Nymphon* sp. (Nymphonidae). The entoproct was colonial, with three zooids, and was identified both morphologically and by a molecular phylogeny as *Barentsia* sp. (Barentsiidae). The largest zooid had eight tentacles and was about 0.7 mm long, smaller than for most colonial entoproct species. We determined partial sequences for the 18S rRNA and 28S rRNA genes from the entoproct. In an 18S-based maximum likelihood tree (1507 characters), the entoproct was the sister taxon to *Barentsia gracilis*.

Key words: benthos, epibiont, Kamptozoa, phylogeny, Pycnogonida, sessile

INTRODUCTION

Entoprocta is a phylum of small-sized, sessile or slow-moving invertebrates. About 200 species have been reported worldwide, 150 of which are solitary and the rest colonial (Borisanova and Schepetov, 2023). Although most entoprocts are marine, a few species inhabit fresh or brackish water. The majority of solitary entoprocts are commensals on other invertebrates. They can depart from the substratum, and a few may also be able to swim (Wasson, 2002). Colonial species are sessile, attaching to various abiotic or biotic substrata (e.g., stones, algae, mollusk shells, arthropods), and are generally believed not to show selectivity in their substratum preference (Borisanova, 2018).

Pycnogonids, or sea spiders, are almost exclusively marine invertebrates (cf. Kakui and Fujita, 2023). Except for some commensal or parasitic species, most of ca. 1400 species are free living (Arnaud and Bamber, 1987; Bamber et al., 2024). As they are slow-moving epibenthic animals with a hard exoskeleton, they provide attachment or egg-laying sites for many other organisms, including algae, diatoms, foraminiferans, ciliophorans, sponges, hydrozoans, bryozoans, brachiopods, polychaetes, leeches, gastropods, barnacles, and tunicates (Khan and Paul, 1995; Wambreuse et al., 2021). Some mobile epibionts (nemerteans, nematodes, mites, and isopods) have also been reported associated with them (Wambreuse et al., 2021).

In 2024, we found an entoproct colony on the surface of a sea spider in Japan. To our knowledge, there are only a few reports of entoprocts epibiotic on sea spiders (e.g., Marfenin and Belorustseva, 2006: p. 240). Here we describe the gross morphology of the entoproct, present partial nucleotide sequences for its nuclear 18S ribosomal RNA (18S) and 28S ribosomal RNA (28S) genes, and infer the species' phylogenetic position in Entoprocta based on 18S data.

MATERIALS AND METHODS

The sea spider with the attached entoproct was collected from a brackish estuary, Akkeshi-ko (43.047654° 144.857062°), at a depth of about 8 m on 23 June 2024, by means of a scallop dredge launched from the R/V *Misago-maru* (Hokkaido University, Japan). The sea spider was photographed alive on 25 June and fixed in 80% ethanol on 28 June. The entoproct colony comprising three zooids was detached from the fixed sea spider. One zooid was preserved in 99% ethanol; the others were observed with an Olympus BX53 light microscope and then preserved in 99% ethanol.

DNA was extracted from the three zooids by using a NucleoSpin Tissue XS Kit (Macherey–Nagel, Germany). For the 18S gene, primers SR1 and SR12 (Nakayama et al., 1996) were used for amplification, and primers 18S-b3F, 18S-a4R, 18S-b5F, 18S-a6R, and 18S-b8F (Kakui et al., 2011, 2021; Kakui and Shimada, 2017; Kakui and Hiruta, 2022) for cycle sequencing. For the 28S gene, primers U178 and L1642 (Lockyer et al., 2003) were used for amplification, and primers U178, 300F, 300R, 900F, U1148 (Lockyer et al., 2003) for cycle sequencing. PCR amplification conditions for 18S and 28S with KOD ONE PCR Master Mix (Toyobo, Japan) were 45 cycles of 98°C for 10 s, 60°C for 5 s, and 68°C for 10 s. All nucleotide sequences were determined with a BigDye Terminator Kit ver. 3.1 and a 3730 DNA Analyzer (Life Technologies, USA). Fragments were concatenated by using MEGA7 (Kumar et al., 2016). The sequences we determined were deposited in the International Nucleotide Sequence Database (INSD) through the DNA Data Bank of Japan under accession numbers LC830819 (18S) and LC830820 (28S).

The 18S dataset for phylogenetic analysis included the one sequence we determined, and 20 entoproct sequences and an out-group sequence from the INSD (Mackey et al., 1996; Littlewood et al., 1998; Fuchs et al., 2009, 2010; Rundell and Leander, 2012; Hartikainen et al., 2013; Kajihara et al., 2015; Borisanova et al., 2015, 2018; Borisanova and Schepetov, 2023). The sequences were aligned by using the online version of MAFFT ver. 7 (Katoh and Standley, 2013; Katoh et al., 2019) with the “Auto” strategy (“L-INS-i” selected; Katoh et al., 2005) and then trimmed with MEGA7 to match the shortest length among them. Alignment-ambiguous sites were removed with Gblocks ver. 0.91b (Castresana, 2000) in NGPhylogeny.fr (Lemoine et al., 2019) and the “relaxed”

* Corresponding author. E-mail: kakui@eis.hokudai.ac.jp
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parameters described in Talavera and Castresana (2007). The aligned dataset contained 1507 characters. Methods for selecting the optimal substitution model (GTR+F+I+G4), the maximum likelihood (ML) analysis, estimation of clade support (analyses of 1000 pseudoreplicates for both Shimodaira–Hasegawa-like approximate likelihood ratio tests [SH-aLRT] and ultrafast bootstraps [UFBoot]), and drawing the tree were as described by Shimada et al. (2023).

RESULTS AND DISCUSSION

The sea spider bearing the entoproct (Fig. 1A) had an ocular tubercle posterior to the neck, uniarticulate chelifores, denticulate chelae, 5-articulate palps, and ovigers with a terminal claw, and four paired legs, and lacked cement gland pores. We thus identified it as a female of *Nymphon* sp. (Nymphonidae). Its trunk length (from the base of the chelifore to the posterior end of the fourth lateral process) was 3.62 mm. Along with the entoproct, several tunicates, hydroids, and ciliophorans were attached to the sea spider.

One entoproct colony with three zooids was found on the dorsal surface of trunk segment 3 of the sea spider (Fig. 1). As each zooid sprouted from a creeping stolon and had a stalk with a thick muscular node and a thin, stiff peduncle (*Barentsia*-type zooid), and the colony lacked *Pedicellina*-type zooids, we identified the entoproct as *Barentsia* sp. (Barentsiidae) (Borisanova and Potanina, 2016). We could not rule out that larger colonies might also contain *Pedicellina*-type zooids, i.e., that the ectoproct was a species of *Pseudopedicellina*. The largest zooid in the colony had eight tentacles and was about 0.7 mm long (Fig. 2), smaller than for the majority of colonial entoproct species (average lengths around several millimeters; Borisanova, 2018). In an 18S tree (Fig. 3), *Barentsia* sp. was the sister taxon to *B. gracilis*, with high nodal support (SH-aLRT/UFBoot, 97.5%/96%). The p-distance in the aligned region between *Barentsia* sp. and *B. gracilis* was 0.2%.

Colonial entoprocts are generally thought to lack substrate preference (Borisanova, 2018). *Loxokalypus socialis*, however, is epibiotic on a certain polychaetous annelid species, and some other species are found more frequently on certain substrata (e.g., “*B. [Barentsia] conferta* is found on algae more often than other barentsiids are.”; Wasson, 1997: p. 30). Because colonial entoprocts are sessile, the type of substratum and site of attachment can potentially affect their survival rate. This raises the question whether single entoproct species reported from various substrata might represent several substrate-specific cryptic species. It is unclear whether the sea spider is the sole substrate

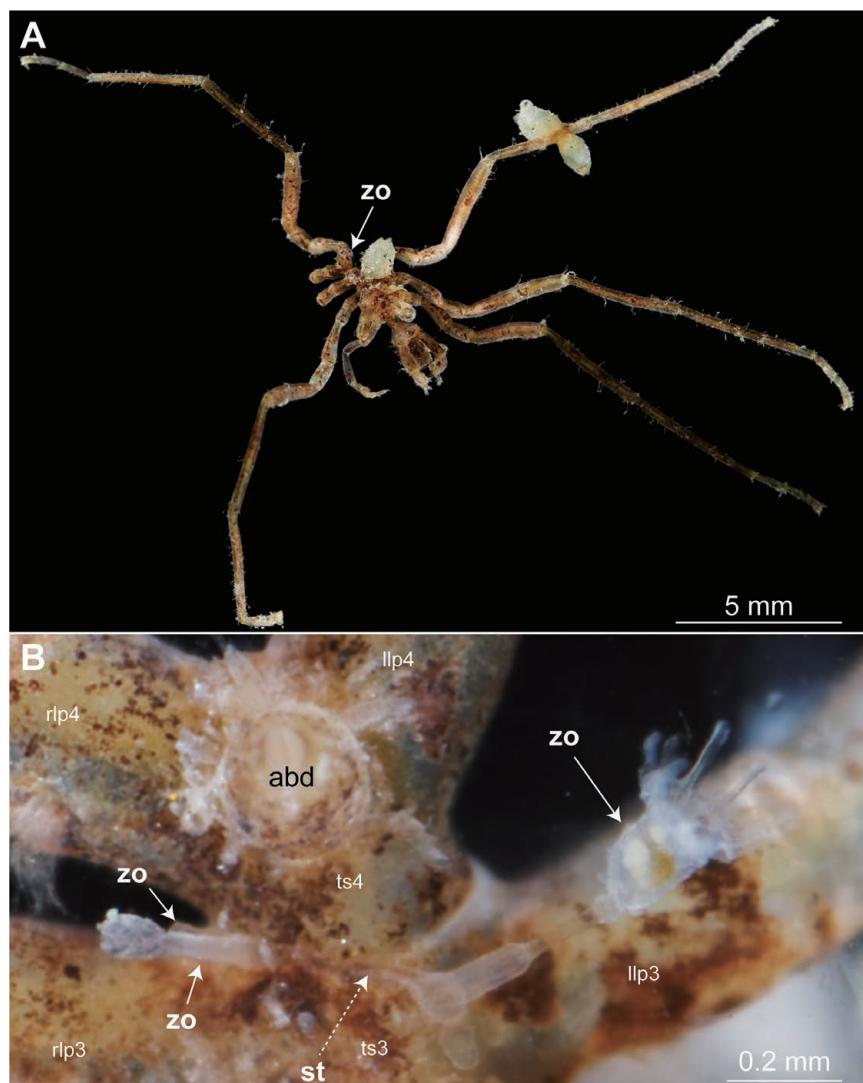


Fig. 1. Female *Nymphon* sp. with the entoproct *Barentsia* sp. attached; living animals photographed on 25 June 2024. **(A)** Habitus of *Nymphon* sp., dorsal view. **(B)** Posterior portion of *Nymphon* sp., dorsal view. Abbreviations: abd, abdomen; llp3, 4, left lateral processes 3, 4; rlp3, 4, right lateral processes 3, 4; st, sterile segment; ts3, 4, trunk segments 3, 4; zo, zooid.



Fig. 2. Two zooids of *Barentsia* sp. arising from a stolon (one zooid lacking its calyx; the loss occurred sometime between 25 June and 28 June); ethanol fixed specimen (fixed on 28 June 2024).

utilized by our entoproct species. If it is restricted to one species of sea spider, or sea spiders in general, our entoproct is likely undescribed. Among about 50 colonial species, 18S sequences are currently publicly available for only

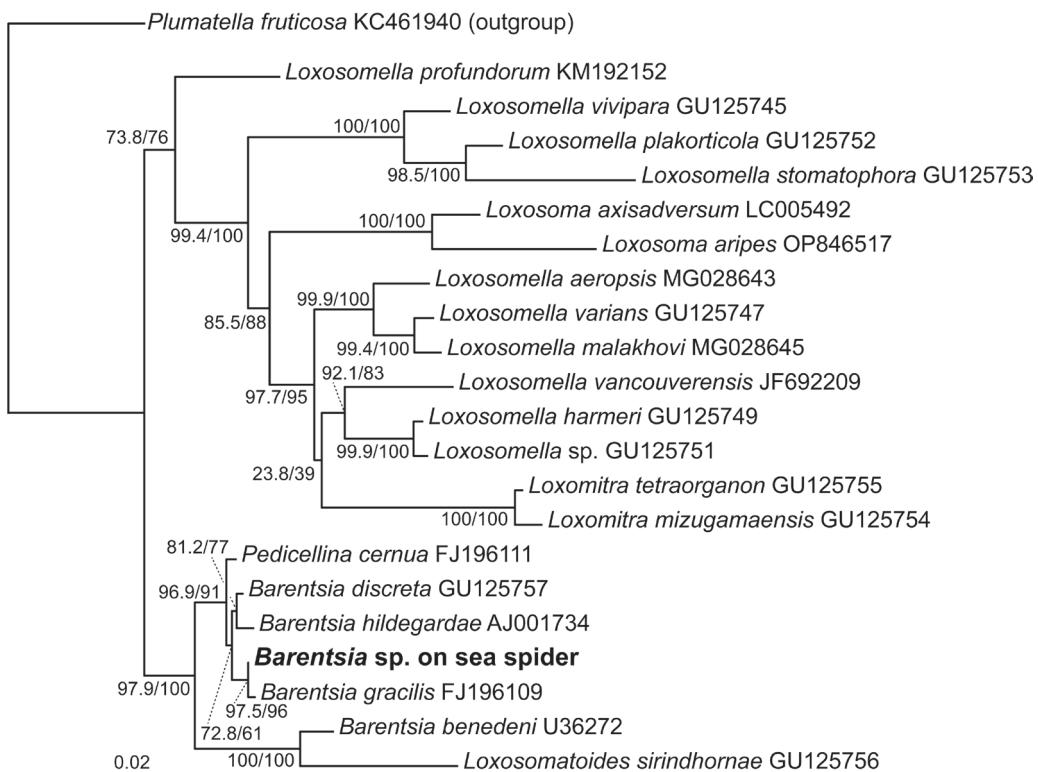


Fig. 3. ML tree for entoprocts based on 18S sequences (1507 characters). Numbers near nodes are SH-aLRT/UFBoot values as percentages; the scale below the tree indicates branch length in substitutions per site.

six. Additional molecular data from colonial entoprocts on various substrate types may reveal higher species diversity and unexpected relationships between entoprocts and their substrata.

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COMPETING INTERESTS

We declare no competing interests.

AUTHOR CONTRIBUTIONS

KK conceived and designed the study, observed the morphology of the entoproct, and conducted the molecular analysis. SS observed the morphology of the sea spider. KK and SS collected the sea spider, wrote the manuscript, and read and approved the final draft.

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