Crustacean Research 2022 Vol.51: 47–54

 doi: 10.18353/crustacea.51.0_47

Morphological description and molecular barcoding of the aegathoid stage of *Nerocila japonica* Schioedte & Meinert, 1881 (Crustacea: Isopoda: Cymothoidae) infesting red seabream *Pagrus major* (Temminck & Schlegel, 1843)

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Abstract.— Here, the aegathoid stage of *Nerocila japonica* Schioedte & Meinert, 1881 is described based on a specimen collected from the Seto Inland Sea, Japan, and its identity is confirmed by both morphology and molecular barcoding. Cytochrome *c* oxidase subunit I and 16S rRNA sequences of the collected individual shared ≥99% similarity with the previous DNA records of *N. japonica*. In terms of morphology, the aegathoid stage of the specimen examined in the present study differed from that of *N. phaiopleura* Bleeker, 1857 by intermediate size of the eyes and setation on pereopods. This is the first record of *N. japonica* infesting the red seabream *Pagrus major* (Temminck & Schlegel, 1843).
LSID urn:lsid:zoobank.org:pub:A05E9736-E1CD-40C9-A1A1-7BAF2D7EEC84

Key words: Cymothoidae, DNA analyzed, fish parasite, juvenile, life history, morphological description, new host record

■ Introduction

Nerocila japonica Schioedte & Meinert, 1881 (Crustacea: Isopoda: Cymothoidae) is an ectoparasite infesting the body surface of fish. This isopod is known to infest 20 species of fish in 12 families under four orders as hosts (Kondo *et al*., 2021). Recent studies have explored the biology and ecology of *N. japonica* (Hashimoto, 2007; Yamauchi & Nagasawa, 2012; Nagasawa *et al*., 2018, 2019; Nagasawa & Kawai, 2019; Nagasawa & Tawa, 2019; Nagasawa, 2020; Nagasawa & Inoue, 2020; Kondo *et al*., 2021). To date, four juveniles of *N. japonica* have been collected in Japan (Yamauchi & Nagasawa, 2012; Nagasawa & Kawai, 2019; Nagasawa & Inoue, 2020). Of these, three were reported as the "aegathoid stage," and an illustration of the dorsal whole body, along with brief description, and a photograph, was provided by Yamauchi & Nagasawa (2012) and Nagasawa & Kawai (2019). However, detailed morphology, such as appendages, remains unknown. During our study on Japanese isopods, a specimen of *N. japonica* at the aegathoid stage was collected from the red seabream *Pagrus major* (Temminck & Schlegel, 1843). Herein, we describe the morphology and molecular barcoding of this specimen.

■ Material and Methods

The red seabream attached an isopod was captured at a line angling. Specimen of isopod was removed from host fish and served in 70% ethanol solution. Observations of morphological characters were made under a binocular microscope (Olympus X-II). Measurements and drawings were made with the aid of an Olympus BHB-Tr compound microscope equipped

Received: 11 Feb 2022. Accepted: 27 May 2022. Published online: 2 July 2022.

Table 1. Results of BLAST search based on cytochrome *c* oxidase subunit I (COI) and 16S rRNA sequence from cymothoid aegathoid stage infesting *Pagrus major*.

Target region	PCR fragment length (bp)	Identification results	E-value	Accession No.	Reference accession No.	Similarity (%)	Identity score	Max score	Query cover (%)
COI	606	*Nerocila japonica*	0.0	LC710516	LC159575	99.32	1106	1106	97
16S rRNA	441	*Nerocila japonica*	0.0	LC710517	LC159463	99.48	781	781	96

with a drawing tube. Body length of the isopod were measured from the tip of the cephalon to the posterior end of the pleotelson along the dorsal mid line. Other measurements and terminologies essentially follow those used by Bruce (1987) for the cymothoid. The scientific and common names of fishes follow those recommended by Motomura (2020) and Froese & Pauly (2021). The specimen examined in this study is deposited in the Seto Marine Biological Laboratory, Kyoto University (SMBL).

Total DNA was extracted from right pereopods 4–7 of the isopod specimen using TNES-urea buffer (Asahida *et al*., 1996) followed by standard phenol-chloroform methods. Partial cytochrome *c* oxidase subunit I (COI) and 16S rRNA genes were amplified by PCR according to Tomano *et al*. (2015) and Fujita *et al*. (2020), respectively. Dye-terminator methods were used to sequence PCR products with an ABI 3130xl Genetic Analyzer (Applied Biosystems, CA, USA); the sequences were deposited in GenBank (Table 1). BLAST searches were run on each sequence in NCBI. We established confidence values for identification with BLAST (≥99% similarity and an E-value = 0.0). In addition, we downloaded additional sequences from GenBank belonging to the genus *Nelrocila*. Sequences were aligned using MUSCLE (Edgar, 2004), implemented in MEGA 10 (Kumar *et al*., 2018), and trimmed. We generated a neighbor-joining tree (Saitou & Nei, 1987) using COI and 16S rRNA sequences. We included *Ceratothoa verrucosa* (Schioedte & Meinert, 1883) as an outgroup.

Results

Family Cymothoidae Leach, 1818
Genus *Nerocila* Leach, 1818
Aegathoid stage of *Nerocila japonica*
Schioedte & Meinert, 1881
(Figs. 2–4)

Material examined

Aegathoid stage (12.3 mm BL), SMBL-V0654, *ex*. body surface of *Pagrus major* (Temminck & Schlegel, 1843), collected from east off Iwaijima Island, Yamaguchi Prefecture, Iyo-Nada, western Seto Inland Sea, Japan (33°46.5′N 132°00′E), 29 October 2019, coll. A crew of the TR/V *Toyoshio-maru*.

Genetic information

For both partial COI and 16S rRNA gene sequences of the aegathoid specimen was ≥99% similar to those of *N. japonica*, respectively (Table 1). Our neighbor-joining tree of COI and 16S rRNA genes showed that sequences detected from the aegathoid specimen formed a well-supported single clade along with the sequence labeled as *N. japonica* (Fig. 1).

Description

Body elongated, lateral body margins slightly

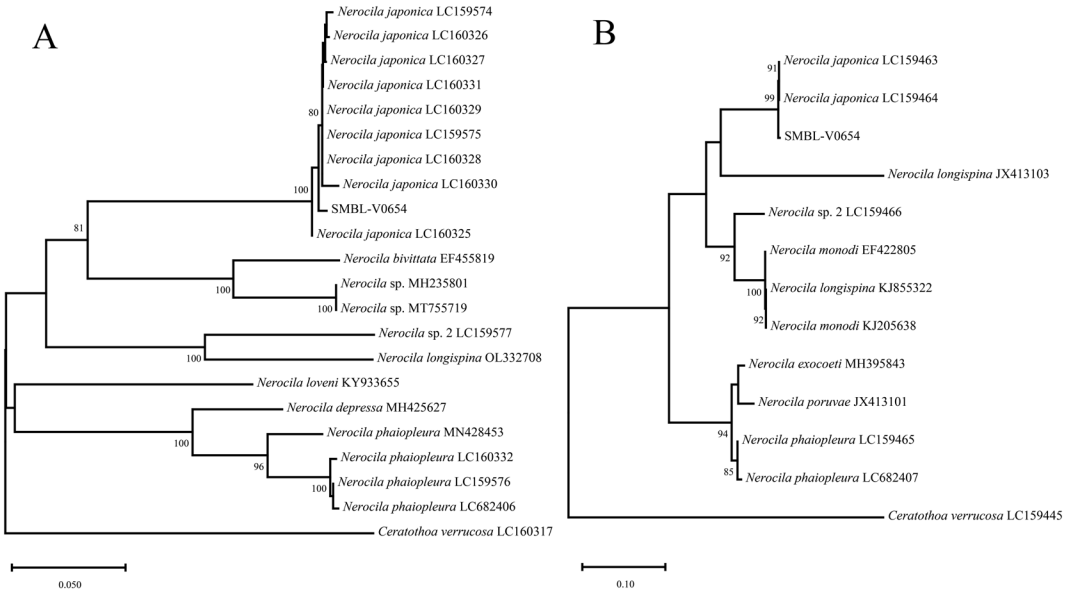

Fig. 1. Neighbor-joining trees based on (A) 578 bp of cytochrome *c* oxidase subunit I (COI) gene and (B) 367 bp of 16S rRNA gene, detected from the aegathoid specimen (SMBL-V0654) collected in this study along with sequences of other *Nerocila* downloaded from GenBank. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Bootstrap supports lower than 80% are not shown. The accession numbers were deposited in GenBank (COI: LC710516, 16S rRNA: LC710517).

curved, pereon and pleon not distinguishable, 2.8 times as long as wide, greatest width at pereonite 5; dorsal surface smooth.

Cephalon semi-ovoid, anterior margin produced a point, lacking rostrum. Eyes large; each made up of 6 rows of ommatidia in dorsal view, longest row with 10 ommatidia; covered 31% in dorsal surface of cephalon, posterior margins expanded; posterior margin of cephalon produced strongly and trilobed.

Pereonite 1 distinctly longer than other somites; pereonites 3 and 4 semi equal length, shortest; posterior margins of pereonites 5 to 7 concave. Posteroventral angles of coxae 2 and 3 rounded; those of coxae 4 to 7 produced, expanded toward posteriorly, not acute.

Pleon greatest width 0.8 times as wide as greatest body width; pleonite 1 shortest; pleonites 2 to 5 semi equal length; each of posteroventral angle acute, no wearing posterior somites.

Pleotelson triangular, 0.8 times as long as greatest width, posterior margin with marginal setae (missing partially).

Antennule bases separated; reaching beyond mid-point of pereonite 1; composed of 8 articles.

Antenna reaching beyond mid-point of pereonite 1; composed of 9 articles.

Pereopod 1 basis rectangular; ischium trapezoidal, 0.5 times as long as basis; merus, 0.4 times as long as ischium; carpus 1.4 times as long as merus, 1 robust seta on inferior margin; propodus elliptical, 1.2 times as long as combined lengths of merus and carpus, 3 tiny robust setae on palm; dactylus 1.5 times as long as propodus, recurved. Setations of pereopods 2 and 3 similar to pereopod 1. Pereopods 5–7 longer than pereopods 1–3. Pereopod 7, 5, 3, and 6 robust setae on each inferior margins of merus, carpus, and propodus, respectively.

Pleopods all lamellar, surface smooth. Pleopod 1 peduncle 1.8 times as wide as length, medial margin with 4 coupling hooks; endopod rectangular, 1.8 times as long as width; exopod

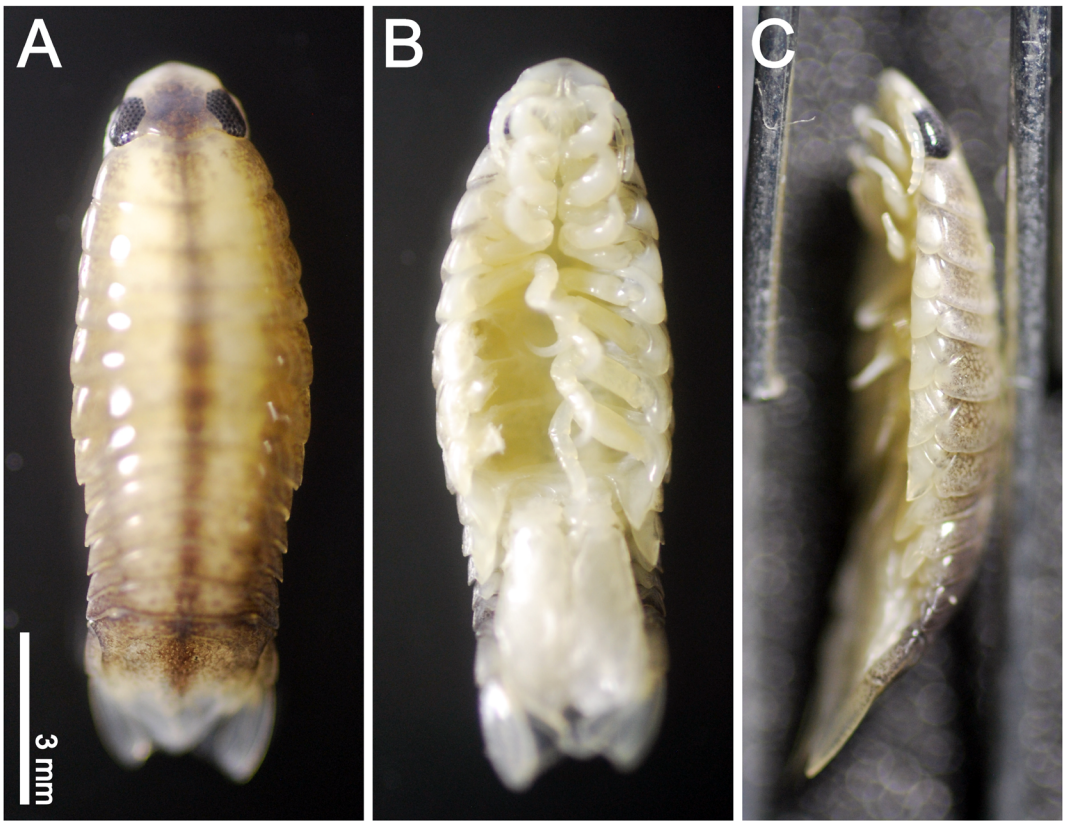

Fig. 2. Aegathoid stage of *Nerocila japonica* (12.3 mm BL), SMBL-V0654, *ex*. body surface of *Pagrus major*. A, dorsal view; B, ventral view; C, left lateral view.

trapezoidal, lateral margin almost straight, 1.5 times as long as width. Pleopod 2 similar to pleopod 1; lamellar gill attached; endopod with appendix masculina, subequal length of endopod.

Uropod peduncle triangular; endopod triangular, 1.9 times as long as greatest width, both lateral and medial margins with short marginal setae, 1 robust seta on apically; exopod longer than endopod, 3.3 times as long as greatest width, medial margins with short marginal setae, 4 robust setae on apically; each uropodal ramus extending to distal end of pleotelson.

Coloration

Ethanol-preserved specimen appeared pale yellow, with a dark brown longitudinal median band extending from the central dorsal surface pereon to the pleotelson. Eyes black. Coloration resembled the aegathoid stage of *Nerocila japonica* infesting the big-scaled redfin *Tribolodon hakonensis* (Günther, 1877) collected from the Hiroshima Bay in the Seto Inland Sea of Japan and photographed by Nagasawa & Kawai (2019).

Distribution

Pacific coast, Sea of Japan coast, Seto Inland Sea, Japan; China; and Malaysia. For details, see Nagasawa &Tawa (2019).

Ecological note

The specimen was collected from offshore water of western Seto Inland Sea, Japan. However, *Nerocila japonica* has been recorded in brackish water lakes and at river mouths; thus,

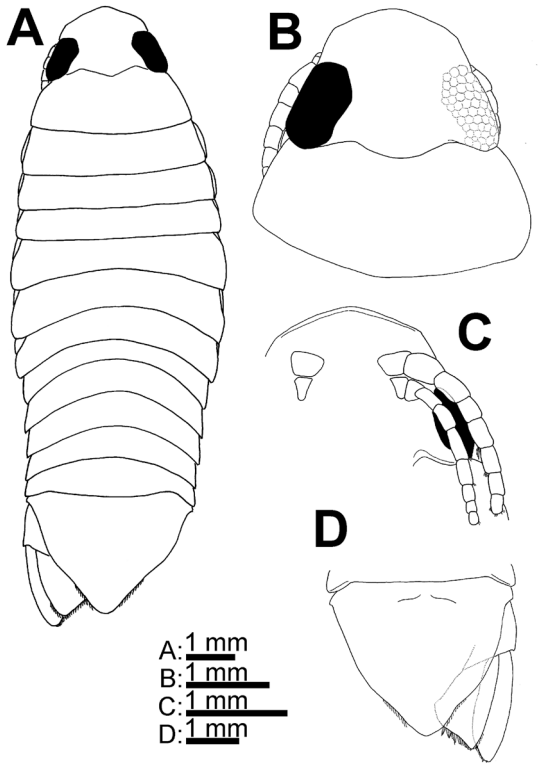

Fig. 3. Aegathoid stage of *Nerocila japonica* (12.3 mm BL), SMBL-V0654. A, whole body, dorsal view; B, cephalon and pereonite 1, dorsal view; C, frons and left antennae, ventral view; D, pleotelson and right uropod, dorsal view.

this species is considered to be low-salinity tolerant (Yamauchi & Nagasawa, 2012). The present finding represents the first record of *N. japonica* infesting *Pagrus major*.

Remarks

Based on the results of molecular analysis, the aegathoid specimen examined in the present study was identified as *Nerocila japonica. Nerocila japonica* was originally described based on a Japanese specimen (Schioedte & Meinert, 1881) and subsequently redescribed by Yamauchi & Nagasawa (2012) based on the holotype designated by Schioedte & Meinert (1881) and additional specimens from various localities in Japan. Mature females of *N. japonica* are characterized by the presence of protrusions: some acute protrusions on posterolateral angles of pereonites; expanded, some acute protrusions on coxae; and posteriorly directed protrusions, extending to pleonite 5, and protrusions on ventrolateral margins of pleonites 1 and 2. However, the examined aegathoid specimen of *N. japonica* differed from the mature female by the absence of these remarkable protrusions.

The general body outline of the present specimen was close to those in the previous photograph and illustration of the aegathoid stages of *Nerocila japonica* (Yamauchi & Nagasawa, 2012; Nagasawa & Kawai, 2019). However, the pointed anterior margin of cephalon in the examined aegathoid specimen differs from the round margin of cephalon in a specimen (10.5 mm BL) collected from the yellowfin goby *Acanthogobius flavimanus* (Temminck & Schlegel, 1845) off Kawajiri, Seto Inland Sea, Hiroshima Prefecture, Japan (Yamauchi & Nagasawa, 2012).

The genus *Nerocila* Leach, 1818 comprises 43 species worldwide (Boyko *et al*., 2008 onwards), and the following three species have been recorded in Japan (Yamauchi, 2016; Nagasawa & Isozaki, 2019): *N. phaiopleura* Bleeker, 1857, *N. trichiura* (Miers, 1878), and *N. japonica*. The aegathoid stage of *N. phaiopleura* was often collected (Bruce, 1987; Saito & Hayase, 2000; Saito *et al*., 2014; Saito & Ogawa, 2019). The aegathoid stage of *N. phaiopleura* reported by Saito & Ogawa (2019) was later reconfirmed its identity based on molecular analysis (COI: LC682406, 16S rRNA: LC682407). The overall elongated bodyline of the aegathoid stages of *N. japonica* and *N. phaiopleura* is similar. However, the aegathoid stage of *N. japonica* differs from that of *N. phaiopleura* by the size of eyes (intermediate size, 31% covered on the dorsal surface of the cephalon in *N. japonica vs.* large size, 67% covered in *N. phaiopleura*) and setation on pereopods 1–3 (3 minute robust setae on propodus in *N. japonica vs.* 4–5 remarkably large robust setae in *N. phaiopleura*) and pereopod 7

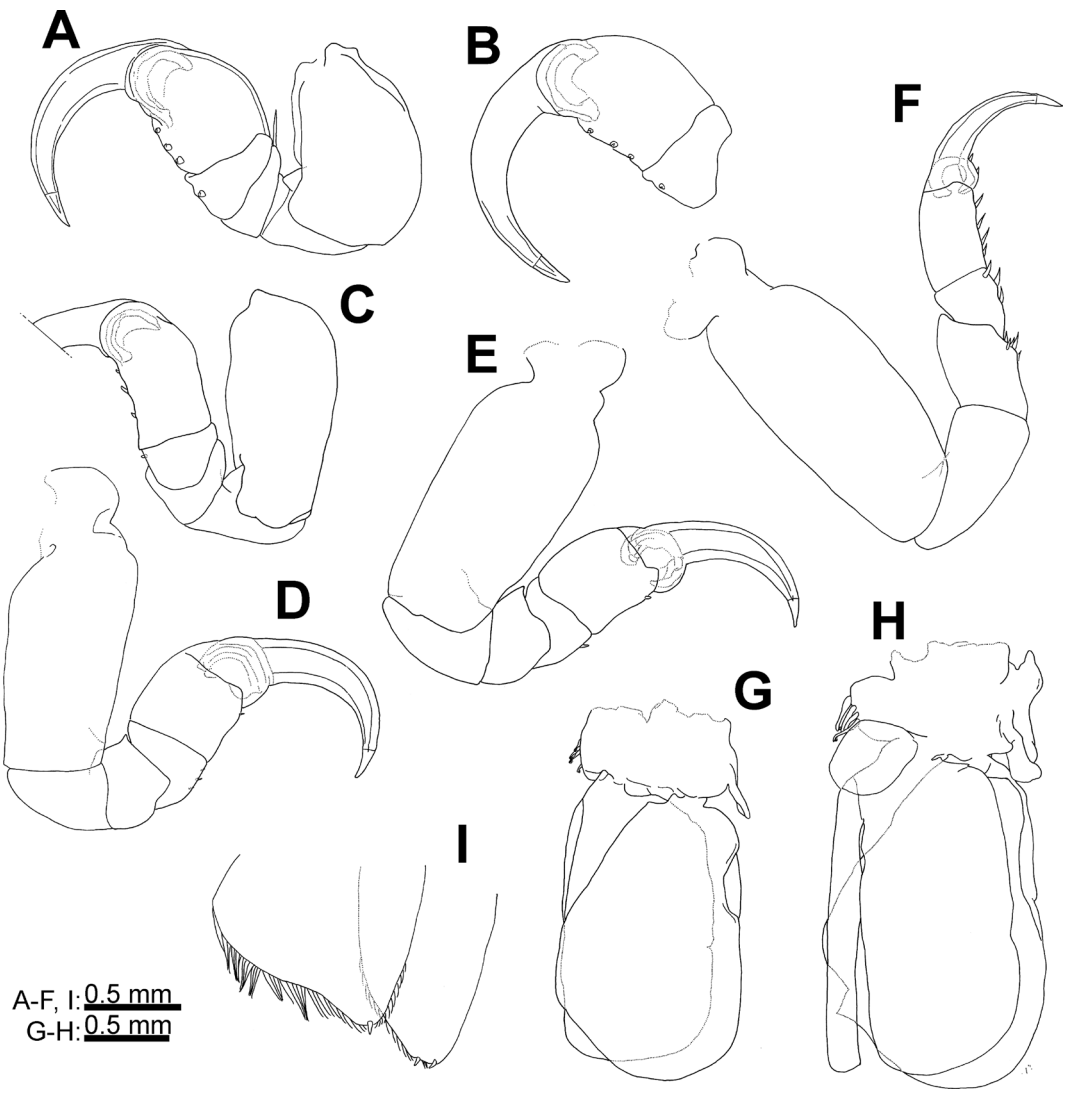

Fig. 4. Aegathoid stage of *Nerocila japonica* (12.3 mm BL), SMBL-V0654. A, left pereopod 1, lateral view; B, left pereopod 2, lateral view; C, left pereopod 3, lateral view; D, left pereopod 5, lateral view; E, left pereopod 6, lateral view; F, left pereopod 7, lateral view; G, left pleopod 1, ventral view; H, right pleopod 2, dorsal view; I, apex of right uropodal endopod and exopod, dorsal view.

(respectively 5, 3, and 6 robust setae on the inferior margins of the merus, carpus, and propodus in *N. japonica vs.* respectively 1, 2, and 5 robust setae in *N. phaiopleura*). The aegathoid stage of *N. trichiura* remains unknown.

■ Acknowledgements

We wish to express our gratitude to Dr. K. Wakabayashi (Hiroshima University), and the captain and crew of the TR/V *Toyoshio-maru* for providing specimen and valuable information. We would like to thank Editage (www.editage.com) for English language editing. Thanks are also extended to Dr. A. Asakura (Kyoto University), and the two anonymous reviewers for their review of the manuscript.

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