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A *Neoheterocotyle* Species First Found in Japan

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Abstract

During a study of the monogeneans of *Rhinobatos hynnicephalus* Richardson, 1846 (Rhinopristiformes: Rhinobatidae) collected from the central Seto Inland Sea, Hiroshima Prefecture and the southern Sea of Japan, Fukuoka Prefecture, one undescribed species of Neoheterocotyle was found. In the present paper, the new species is described with a molecular information. This is the first report of a *Neoheterocotyle* species from Japan. The new species are most similar in morphology to Nepenthes fragilis and N. rhinoceros. They have two pairs of dorsal parascleral sclera, but they differ in that the penis is curved proximally and the accessory teeth have a blade-like process.

Keywords

Rhinobatos hynnicephalus; Monocotylidae; Elasmobranchii; Japan.

Introduction

Currently, 16 species, 11 genera in four families of monogenean (Platyhelminthes) have been reported from 14 species of chondrichthyans in Japan, but there is the possibility to obtain more monogenean species because of the rich chondrichthyan fauna of Japan. Species of *Neoheterocotyle* Hargis, 1955 (Monogenea: Monocotylidae) are gill parasites of elasmobranchs and have been reported from India, China, Australia, Tunisia, and South Africa. Most monocotylids have a haptor with one central and eight peripheral loculi separated by septa, and characteristics of *Neoheterocotyle* are the presence of a single sinuous or zig-zagged sclerotized ridge on all septa and two or three pairs of accessory sclerites on the dorsal surface of the haptor. During a study of the monogeneans of *Rhinobatos hynnicephalus* Richardson, 1846 (Rhinopristiformes: Rhinobatidae) collected from the central Seto Inland Sea, Hiroshima Prefecture and the southern Sea of Japan, Fukuoka Prefecture, one undescribed species of *Neoheterocotyle* was found. In the present paper, the new species is described with a molecular information. This is the first report of a *Neoheterocotyle* species from Japan (Boudaya and Neifar, 2016; Brunansk et al., 2017; Brunanska and Poddubnaya, 2017).

Materials and Methods

Four specimens of *Rhinobatos hynnicephalus* caught by commercial trawl fishing were examined: two were individually collected from the central Seto Inland Sea off Ōsaki-kami-jima island (34°14′ N, 132°48′ E), Hiroshima Prefecture, on 25 July 2014 and 24 September 2015, and the other two were collected from the southern Sea of Japan off Tsuyazaki Port (33°47′N, 130°24′E), Fukuoka Prefecture, on 3 July 2016. The rays were brought alive to the laboratory, identified based on Hatooka *et al*., and examined for parasites under a dissecting microscope (Chero et al., 2016; Irigoitia et al., 2016; Kritsky et al., 2017).

Monogeneans from the gills were collected using fine needles and forceps and placed on slides with a drop of water under a coverslip. Some monogeneans were fixed in modified picrate glycerin for observation of sclerotized structures under strong coverslip pressure, whereas others were fixed in 5% formalin or acetic acid-formalin-alcohol and stained in Heidenhain's iron hematoxylin or alum carmine under slightly coverslip pressure. For molecular analysis, some specimens were preserved in 99% ethanol. All specimens for morphological analysis were dehydrated through a graded ethanol series, cleared in xylene, and mounted in Canada balsam (Kritsky et al., 2017; Poddubnaya et al., 2016; Vaughan et al., 2016).

Drawings were made with the aid of a drawing tube fitted on an Olympus BX51 light microscope (Olympus). The method of measuring sclerotized structures is presented in Figure 1. The penis was measured on images taken by an Olympus DP20 microscope digital camera on an Olympus BX51 light microscope at a magnification of ×1,000 using ImageJ software (version 1.48i). A photograph of the ejaculatory bulb was taken using a CANON EOS Kiss X2 digital camera (Canon) fitted on an Olympus BX60 microscope. Measurements, in micrometers, are expressed as the range followed in parentheses by the mean and the number (n) of specimens examined. The type-specimens were deposited in the Platyhelminthes collection of the National Museum of Nature and Science (NSMT-Pl), Tsukuba City, Ibaraki Prefecture, Japan.

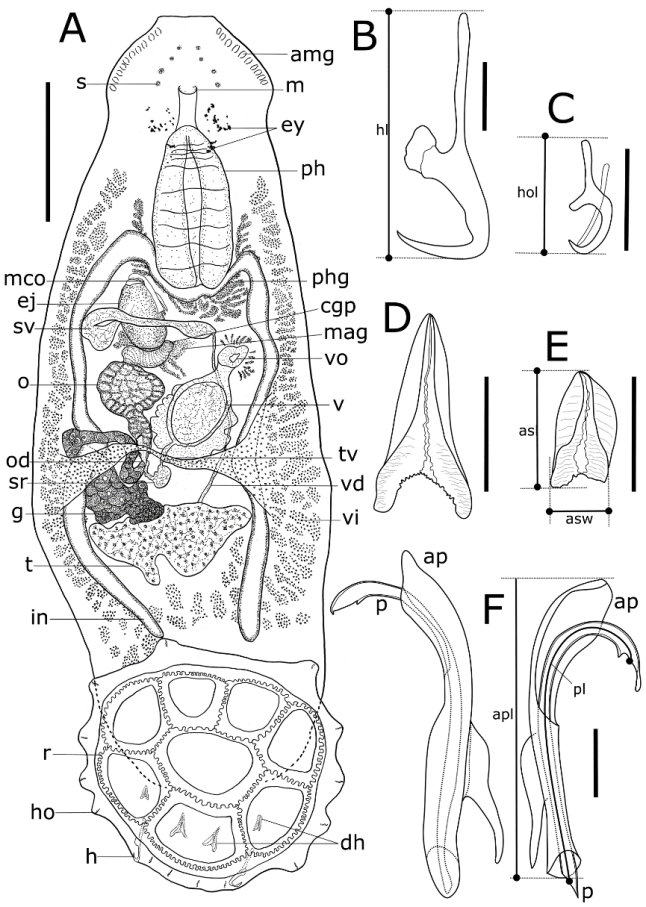

Figure 1: *Neoheterocotyle quadrispinata* n. sp. Holotype (NSMT-Pl 6381) for A, paratypes (NSMT-Pl 6383) for B–F. A, whole mount (ventral view); B, hamulus; C, hooklet; D, sclerotized haptoral accessory sclerite associated with posterior loculus; E, sclerotized haptoral accessory sclerite associated with posterolateral loculus; F, male copulatory organs (from two specimens, left: ventral view, right: dorsal view). Scale bars: A, 200 µm; B, D–F; 20 µm, C, 10 µm. Abbreviations for morphological characters: ap, accessory piece; amg, anteromedian gland; cgp, common genital pole; dh, dorsal haptoral accessory structure; ej, ejaculatory bulb; ey, eyespots; g, germarium; h, hamulus; ho, hooklet; in, intestine; m, mouth; mag, male accessory gland; mco, male copulatory organ; o, oötype; od, oviduct; p, penis; ph, pharynx; phg, pharyngeal glands; r, sinuous ridge; s, anterior ventral sac; sr, seminal receptacle; sv, seminal vesicle; t, testis; tv, transverse vitelline duct; v, vagina; vd, vas deferens; vi, vitellaria; vo, vaginal opening. Abbreviations for measurements of hard parts: apl, accessory piece length; asl, haptoral accessory sclerite length; asw, haptoral accessory sclerite base width; hl, hamulus length; hol, hooklet length; pl, penis length.

DNA was extracted from two specimens collected fromoff Ō saki-kami-jima island using the DNeasy blood andtissue kit (Qiagen) in accordance with the manufacturer ' sinstructions. Partial fragments of 28S rRNA gene were amplifiedusing the polymerase chain reaction (PCR) with theprimer pair C1 (*5 '-ACC CGC TGA ATT TAA GCA T-3 '*) and D2 (*5 '-TGG TCC GTG TTT CAA GAC-3 '*), and partial fragments of the mitochondrial cytochrome*c* oxidase gene subunit 1 (CO1) were amplified using theprimer pair JB3 (*5 '-TTT TTT GGG CAT CCT GAG GTTTAT-3 '*) and CO1-R trema (*5 '-CAA CAA ATC ATG ATGCAA AAG G-3 '*). A total of 25 µL PCR reaction consisted of 1 µL of *DNA* template, 1×ExTaq Buffer (TaKaRa), 0.2 mmol/L of each dNTP,1 µmol/L of each primer, and 2.5 units of TaKaRa Ex Taq DNAPolymerase (TaKaRa). PCR was carried out with the followingprotocol: 94°C for 30 sec followed by 35 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 2 min, and 10 min offinal hold at 72°C. PCR products were purified using NucleoSpinGel and PCR Clean-up kit (Macherey-Nagel) andsequenced with a 3130X Genetic Analyzer (Applied Biosys tems) with the same primers that generated the PCR products.

The partial 28S *rDNA* (858 bp) and CO1 (890 bp) sequences from the two specimens were identical, submitted to the *DNA* Data Bank of Japan Centre (DDBJ) and were compared with the available sequences for species of mono- geneans in GenBank using BLAST software. In addition, two sequences of *Neoheterocotyle rhinobatidis* registered in GenBank (AF026107 and AF348361) were compared to each other using BLAST.

Monocotylidae Taschenberg, 1879 [Tamban-chū-ka] Heterocotylinae Chisholm, Wheeler, and Beverley-Burton, 1995 [New Japanese name: Iban-chū-a-ka]

Neoheterocotyle Hargis, 1955 [New Japanese name: Toge-iban-chū-zoku] *Neoheterocotyle quadrispinata* n. sp. [New Japanese name: Yotsu-toge-iban-chū] (Figure 1, Figure 2).

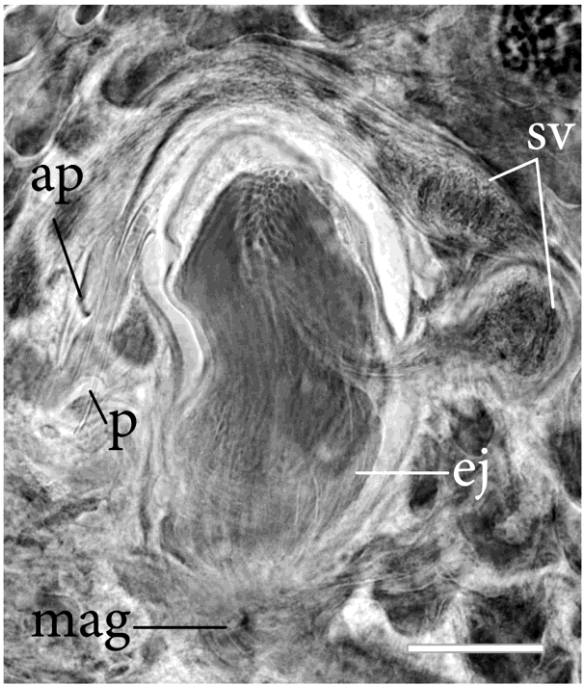

Figure 2: Light micrograph of ejaculatory bulb of *Neoheterocotyle quadrispinata* n. sp. (NSMT-Pl 6382, dorsal view). Scale bar: 30 µm. Abbreviations: ap, accessory piece; ej, ejaculatory bulb; p, penis; mag, male accessory gland; sv, seminal vesicle.

Holotype. Adult (NSMT-Pl 6381) collected from off Ōsaki-kami-jima island on 24 September 2015.

Paratypes. Fourteen specimens (NSMT-Pl 6382, 6383) from off Ōsaki-kami-jima island on 25 July 2014; fifteen specimens (NSMT-Pl 6384) from off Tsuyazaki Port on 3 July 2016.

Description. Body (Figure 1A) including haptor 625-1475 (996; *n*=11) long, 218-480 (349, *n*=11) wide at level of germarium. Head organs ten pairs (*n*=10), located along anterior margin of head. Four pairs of anterior ventral sac present. Eyespots dispersed over dorsal body surface between mouth and pharynx. Mouth ventral, subterminal. Pharynx muscular, spherical to oval, 99-237 (175, *n*=11) long, 55-132 (85, *n*=11) wide. Esophagus not present, bifurcate intestine extending to end of body along each body margin. Pharyngeal glands present on either side of posterior part of pharynx.

Haptor elliptical, length 239-337 (296, *n*=11), width 269-379 (316, *n*=11), ventral surface of haptor with 1 central and 7 peripheral loculi. Pair of hamuli (Figure 1B), length 55-69 (63, *n*=25). Single sinuous ridge present on ventral surface of all septa. Fourteen hooklets (Figure 1C), length 10-12 (11, *n*=25), located in marginal valve as illustrated (Figure 1A). Two pairs of fang-shaped, haptoral accessory sclerites of unequal size present on dorsal surface of haptor. Larger pair (Figure 1D) length 28-44 (37, *n*=25), base width 15-27 (21, *n*=25), located on dorsal surface of posterior loculus. Smaller pair (Figure 1E) length 18-33 (24, *n*=25), base width 9-20 (14, *n*=25), dorsal to each posterolateral loculi. Larger pair length to smaller pair length ratio, 1: 0.55-0.78; base ratio, 1: 0.56-0.88.

Testis single, with sinuous folds, posterior to germarium, 58-210 (161, *n*=10) long, 104-229 (156, *n*=10) wide. Vas deferens arising from anterior portion of testis, extending anteriorly and dorsal to vagina. Seminal vesicle located ventral over ejaculatory bulb, connected to right side of ejaculatory bulb (Figure 2). Ejaculatory bulb (Figure 2) muscular, oval, length 60-102 (79, *n*=11), width 35-68 (48, *n*=11). Male accessory glands enter posterior part of ejaculatory bulb. Sclerotized male copulatory organ consisting of penis and accessory piece (Figure 1F). Penis curved proximally, length 88-113 (98, *n*=27), with accessory piece associated with most of entire length. Accessory piece slightly curved, with blade-shaped process on outside in distal, 77-101 (87, *n*=27) long.

Germarium dextral to body mid-region, elongate, wrap- ping around right intestine. Oötype length 68-149 (112, *n*=9), opening ventrally at unarmed common genital pore located lower left of ejaculatory bulb. Vaginal pore opening on left side of ventral body surface at level of base of ejaculatory bulb. Unsclerotized muscular vagina, 94-244 (150, *n*=9) long, 48-127 (87, *n*=9) wide, expanding middle part, connecting seminal receptacle through thin tube. Seminal receptacle 23-35 (29, *n*=7) long, 25-45 (30, *n*=7) wide. Vitellarium approximately co-extensive with intestine. Transverse vitelline duct lying at level of base of oötype.

Type host. *Rhinobatos hynnicephalus* (Rhinopristiformes: Rhinobatidae).

Type locality. Off Ōsaki-kami-jima island, Ōsaki-kami-jima town, Hiroshima Prefecture, in the Seto Inland Sea (34°14′N, 132°48′E).

Other locality. Off Tsuyazaki Port, Fukutsu city,

Fukuoka Prefecture, in Genkainada, south Sea of Japan (33°47′N, 130°24′E).

Site of infection. Gill filaments.

Etymology. The species name *quadrispinata* is from *quadri* (Latin), four, and *spinata* (Latin), spiny, referring to the two pairs of the haptoral accessory sclerites.

Japanese name. Ijima translated Heterocotylea as "Iban-a-moku" (a-moku means suborder), and the name is used for the new Japanese names "Iban-chū-a-ka" and "Toge-iban-chū-zoku" ("chū", "a-ka", and "zoku" mean worms, a subfamily, and a genus, respectively). The part of the new Japanese generic name, "toge" refers to the haptoral accessory sclerites, and the part of the new Japanese name of the species is "yotsu-toge" which refers to the two pairs of the haptoral accessory sclerites.

Sequence data. The newly generated sequence of the 28S *rDNA* (858 bp) and CO1 (890 bp) were submitted to DDBJ (accession nos. LC428038 and LC469716, respectively).

Molecular data comparison. BLAST searches of the sequences did not have any identical hit. The closest hits of newly generated sequence of the 28S *rDNA* are *Troglocephalus rhinobatidis* Young, 1967 (AF348364 and AF026110, 90.2% and 89.9% similarity with 99% and 98% coverage, respectively), *Neoheterocotyle rhinobatidis* (AF026107 and AF348361, 89.8% and 89.2% similarity with 98% and 99% coverage, respectively), *Neoheterocotyle rhynchobatis* (AF348363, 89.2% similarity with 99% coverage), and *Neoheterocotyle rhinobatis* (AF348362, 88.1% similarity with 99% coverage). The closest hits of newly generated sequence of the CO1 are Benedenia cf. seriolae FAS-2013 (KC633872-633877, 76.6-78.7% similarity with 45-59% coverage). Two registered sequences of Neoheterocotyle rhinobatidis (AF026107 and AF348361) show 92.7% similarity with 100% coverage.

Discussion

Comparisons with 28S *rDNA* sequences on GenBank suggested that *N. quadrispinata* n. sp. shows similarity with *Troglocephalus rhinobatidis*, but the new species is readily separated from *T. rhinobatidis* by the composition of haptoral structures. As with the phylogenetic analysis by Chisholm *et al*., *Neoheterocotyle* species are paraphyletic, including *Troglocephalus rhinobatidis*. However, *T. rhinobatidis* clearly differs from *Neoheterocotyle* species morphologically, and Chisholm *et al*. regarded the genus *Troglocephalus* Young, 1967 as *incertae sedis*. It is necessary to redefine based on the morphology and more phylogenetic analyses, including more *Neoheterocotyle* and *Troglocephalus* species with the other DNA regions, *e.g.*, CO1 sequence. In addition, two sequences of 28S ribosomal *DNA* of *Neoheterocotyle rhinobatidis* (AF026107 and AF348361) did not match (92.7% similarity). Each analyzed specimen was obtained from the same host, *Glaucostegus typus* (as *Rhinobatos typus*), and locality, Heron Island in Australia. It is considered the existence of a cryptic species or misidentification, and the molecular data of *N. rhinobatidis* should be re-analyzed based on new materials.

Neoheterocotyle species are widely distributed with their hosts (Rhinobatidae, Rhynchobatidae, and Pristidae), and *N. nagibinae* and *N. rhinobatis* have a wide distribution in the Indo-West Pacific with *Rhynchobatus djiddensis* and *Rhinobatos granulatus* Cuvier, 1829. *Rhinobatos hynni-cephalus* found in the coastal waters of Far East Asia from South Japan to South China, and if *N. quadrispinata* n. sp. has strict host-specificity, the new species shows a similar distribution range with its host. In addition, *R. granulatus* also occur in Japanese waters, and there is the possibility to obtain *N. rhinobatis* from the fish. More studies are needed to clarify the diversity and host range of *Neoheterocotyle* in Japan (Kume et al., 2009) (Kume et al., 2009).

Rhinobatos hynnicephalus harbor two species of *Neo-heterocotyle*, *N. forficata* from Yellow Sea in China and *N. quadrispinata* n. sp. from Seto Inland Sea and Sea of Japan in Japan, but in this study, no specimen of *N. forficata* was collected from *R. hynni-cephalus* examined in this study. It is generally considered that plural *Neoheterocotyle* species occur on a single host in the same locality, as is the case for the monogenean fauna of *Rhynchobatus djiddensis* and *Glaucostegus typus* in Heron Island. Only four individuals of Japanese *R. hynnicephalus* were examined in this study, and studies of more host specimens from wider areas will clarify the difference or unity in monogenean fauna of the hosts between Chinese and Japanese waters.

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