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First record of *Scutariella japonica* (Platyhelminthes: Rhabdocoela) from Hokkaido, Japan, and notes on its host shrimp *Neocaridina* sp. aff. *davidi* (Decapoda: Caridea: Atyidae)

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Abstract

We report the first occurrence record of the freshwater ectoparasitic platyhelminth *Scutariella japonica* (Matjašič, 1990) from Hokkaido, Japan. The flatworms were collected from the surface of exoskeleton and the inside of branchial chamber of the atyid shrimp *Neocaridina* sp. aff. *davidi*. Phylogenetic analysis based on the 18S + 28S concatenated dataset of rhabdocoel flatworms retrieved the *Scutariella* clade, and our specimens identified with *Scutariella japonica* were genetically distinct from those referred to *S. sinensis* Chen, Feng, Lin, Lu & Wang, 2018. Phylogenetic analyses using COI showed that the host shrimp specimens from Sapporo were clustered with *N. koreana* identified by Shih et al. (2017) and placed close to *N. palmata* (Shen, 1948) and *N. davidi* (Bouvier, 1904). Our specimens did not agree with the original description of *N. koreana* Kubo, 1938, particularly in the shape of the endopod of the male pleopod 1. The identity of the present host shrimp specimens could not be established satisfactorily, and thus they were referred to *N.* sp. aff. *davidi* for the time being.

Key words: anthropogenic introduction; DNA barcode; freshwater; Scutariellidae; Temnocephalida; Temnocephalidae

Introduction

Scutariella japonica (Matjašič, 1990) is one of the five species of *Scutariella* in Temnocephalidae, known as an ectoparasite on freshwater shrimps in Atyidae (Chen et al. 2018; van Steenkiste et al. 2021). In Japan, it has been reported from *Paratya compressa* (De Haan, 1884), *Paratya improvisa* Kemp, 1917, and *Neocaridina denticulata* (De Haan, 1844), collected from various localities in Honshu and Kyushu Islands of the mainland (Kawakatsu et al. 2007). Outside of Japan, Ohtaka et al. (2012, 2015) recorded this species from southeast China and Taiwan; however, Chen et al. (2018) suggested that *S. japonica* recorded by Ohtaka et al. (2012) from China might actually represent *S. sinensis* Chen, Feng, Lin, Lu & Wang, 2018, described from Guangdong Province, China. Recently, Maciaszek et al. (2021) reported the invasion of *S. japonica* into Poland, Europe, which is likely due to intentional or unintentional releases of infected ornamental shrimp *Neocaridina davidi* (Bouvier, 1904). Used as fishing bait and ornamental shrimp, *N. davidi* have spread across the world (Englund and Cai 1999; Niwa 2010; Klotz et al. 2013; Jabłońska et al. 2018; Weiperth et al. 2019).

Here we report the first occurrence record of *S. japonica* from Hokkaido, Japan, along with the record of the host shrimp, representing the genus *Neocaridina* Kubo, 1938. The identity of the parasitic flatworm was assessed by genetic analyses with partial nucleotide sequences of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene and the nuclear 18S rRNA (18S) and

28S rRNA (28S) genes along with morphology. Species of *Neocaridina* was already recorded from Hokkaido as "*Neocaridina denticulata sinensis*?" by Saito and Okamoto (2008). The genetic analyses using COI revealed that the shrimp specimens examined in this study are identical with *Neocaridina koreana* (Kubo, 1938) identified by Shih et al. (2017), but the morphological comparison does not support the identity. The identity of the host shrimp could not be satisfactorily established because of the taxonomic problems relating to *N. davidi* and allied taxa (cf. Klotz et al. 2013; Nishino 2017; Fuke et al. 2021), and thus we refer our specimens to *Neocaridina* sp. aff. *davidi*. We provide diagnostic features of our host shrimp for future study.

Materials and Methods

Two shrimps parasitized by *Scutariella japonica* were collected from Yasuharu River, Sapporo, Hokkaido (43°06'55.2"N 141°18'53.4"E) by using a hand net (mesh opening = 3 mm) on 24 June 2021. Additional specimens of the host shrimp species used for morphological examination were collected from the same location on 12 July 2021. Parasitic flatworms were anesthetized with menthol, and flatworms were detached from hosts by forceps. Anesthetized shrimps were fixed and preserved in 99 % ethanol. *Scutariella japonica* individuals for morphological observation were put under a coverslip, anesthetized with 5 % ethanol, fixed in Bouin's fluid or ethanol, and preserved in 70 % ethanol; the others for DNA extraction were fixed and preserved in 99 % ethanol. The former was transferred into a roughly 1:9 mixture of glycerin and 70 % ethanol and placed in a thermostatic chamber at 40°C for 4 h, after which they were mounted on glass slides in glycerin and observed with an Olympus BX53 microscope. The illustrations of *Neocaridina* shrimp were prepared with the aid of a drawing tube mounted on a LEICA MZ8 stereomicroscope. The carapace length (cl), as an indication of specimen size, was measured from the orbital margin to the midpoint of the posterodorsal margin of carapace. Specimens examined in this study are deposited in the Invertebrate Collection of the Hokkaido University Museum, Sapporo, Japan (ICHUM) and in the Natural History Museum and Institute, Chiba, Japan. COI from one specimen of

Table 1. List of PCR and cycle sequencing (CS) primers used in this study.

Gene	Primer	Sequence	Reaction	Source
COI (host)	LCO1490	GGTCAACAAATCATAAAGATATTGG	PCR, CS	Folmer et al. (1994)
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	PCR, CS	Folmer et al. (1994)
COI (parasite)	425F	GGNGCTAGNTCNATWTTAGGRGC	PCR, CS	Hoyal Cuthill et al. (2016)
	new 1200R	CCCATTGAWAMNACATAATGAAAATG	PCR, CS	Hoyal Cuthill et al. (2016)
18S	SR1	TACCTGGTTGATCCTGCCAG	PCR	Nakayama et al. (1996)
	SR12	CCTTCCGCAGGTTCACCTAC	PCR	Nakayama et al. (1996)
	SR3	AGGCTCCCTGTCCGGAATC	CS	Nakayama et al. (1996)
	18S-b3F	CCTGAGAAACGGCTACCACAT	CS	Kakui and Shimada (2017)
	18S-b4F	TGCGGTTAAAAAGCTCGTAGTTG	CS	Kakui et al. (2011)
	18S-b4R	TCCAACTACGAGCTTTTTAACC	CS	Kakui et al. (2011)
	18S-b5F	GATCGAAGGCGATYAGATACC	CS	Kakui et al. (2021)
	18S-b6F	CCTGCGGCTTAATTTGACTC	CS	Kakui et al. (2011)
	18S-a6R	AACGGCCATGCACCAC	CS	Kakui et al. (2011)
	18S-b8F	GGTCTGTGATGCCCTTAGATG	CS	Kakui et al. (2011)
28S	U178	GCACCCGCTGAAYTTAAG	PCR, CS	Lockyer et al. (2003)
	300F	CAAGTACCGTGAGGGAAAGTTG	CS	Lockyer et al. (2003)
	300R	CAACTTTCCCTCACGGTACTTG	CS	Lockyer et al. (2003)
	900F	CCGTCTTGAAACACGGACCAAG	CS	Lockyer et al. (2003)
	U1148	GACCCGAAAGATGGTGAA	CS	Lockyer et al. (2003)
	L1642	CCAGCGCCATCCATTTTCA	PCR, CS	Lockyer et al. (2003)

Table 2. Rhabdocoels included in the phylogenetic analysis based on 18S + 28S sequences.

Higher taxon	Species	INSD accession number 18S	28S	Source
Limnotyphloplanida				
Dalyelliidae	*Castrella truncata*	KC529439.1	KC529570.1	van Steenkiste et al. (2013)
	Gieysztoria knipovici	KC529463.1	KC529594.1	van Steenkiste et al. (2013)
	Dalyellia viridis	KC529444.1	KC529575.1	van Steenkiste et al. (2013)
	Pseudodalyellia alabamensis	KC529440.1	KC529571.1	van Steenkiste et al. (2013)
	Microdalyellia fusca	KC529453.1	KC529584.1	van Steenkiste et al. (2013)
Jenseniidae	*Jensenia angulata*	-	KC529568.1	van Steenkiste et al. (2013)
	Halammovortex sp.	KC529437.1	KC529567.1	van Steenkiste et al. (2013)
	Grappleria corona	MW052803.1	MW052802.1	van Steenkiste et al. (2021)
Temnocephalidae	*Scutariella sinensis*	MF773690.1	MF773687.1	Chen et al. (2018)
	Scutariella japonica	LC664090	LC664092	This study
	Didymorchis sp.	AY157182.1	AY157163.1	Lockyer et al. (2003)
	Decadidymus sp.	MZ457909.1	-	unpublished
	Decadidymus sp.	MG345101.1	MG345102.1	unpublished
	Diceratocephala boschmai	KC517073.1	-	Ngamniyom et al. (2014)
	Diceratocephala boschmai	MZ475304.1	-	unpublished
	Temnocephala sp.	AJ012520.1	-	Littlewood et al. (1999)
	Temnosewellia fasciata	-*	KC869888.1	Laumer and Giribet (2014)
	Temnosewellia minor	AY157183.1	AY157164.1	Lockyer et al. (2003)
	Craspedella pedum	MN073837.1	-	Ngamniyom (2020)
Typhloplanidae	*Typhloplana viridata*	KC529484.1	KC529615.1	van Steenkiste et al. (2013)
	Acrochordonoposthia conica	KC529487.1	KC529617.1	van Steenkiste et al. (2013)
	Opistomum arsenii	KC529491.1	KC529620.1	van Steenkiste et al. (2013)
	Phaenocora foliacea	KC529492.1	KC529621.1	van Steenkiste et al. (2013)
	Olisthanella truncula	KC529494.1	KC529623.1	van Steenkiste et al. (2013)
	Dochmiotrema limicola	KC529495.1	KC529624.1	van Steenkiste et al. (2013)
	Rhynchomesostoma rostratum	KC529499.1	KC529625.1	van Steenkiste et al. (2013)
	Mesostoma thamagae	AY775760.1	-	Willems et al. (2006)
Thalassotyphloplanida (outgroup)				
Kytorhynchidae	Kytorhynchidae sp. 1	KC529401.1	KC529527.1	van Steenkiste et al. (2013)

*KC869834.1 was not used as it contains long gaps not found in other sequences.

Neocaridina shrimp from Chiba City (CBM-ZC 14978) was also sequenced in this study.

Total DNA was extracted from the whole body of two *S. japonica* individuals and egg or appendage of three shrimp specimens (two females and one male) from Yasuharu River by using a NucleoSpin Tissue XS Kit (Macherey-Nagel, Germany). Primers used for PCR and sequencing are listed in Table 1. PCR amplification conditions for the COI with TaKaRa Ex Taq DNA polymerase (TaKaRa Bio, Japan) were 94°C for 1 min; 35 cycles of 98°C for 10 s, 50°C for 30 s, and 72°C for 50 s; and 72°C for 2 min. Conditions for 18S and 28S amplification with KOD FX Neo (Toyobo, Japan) were 94°C for 1 min; 45 cycles of 98°C for 10 s, 65°C (18S) or 60°C (28S) for 30 s, and 68°C for 75 s (18S) or 45 s (28S); and 68°C for 3 min. All nucleotide sequences were determined by direct sequencing with a Big Dye Terminator Kit ver. 3.1 and a 3730 DNA Analyzer (Life Technologies, USA). The genomic DNA extraction, COI amplification and sequencing for one specimen from Chiba City followed Komai et al. (2021). Fragments were concatenated by using MEGA7 (Kumar et al. 2016). Kimura 2-parameter (K2P) (Kimura 1980) distances between the aligned sequences were calculated with MEGA7.

The 18S + 28S dataset for phylogenetic analysis

of flatworms comprised of the sequences we determined and homologous sequences from another 26 limnotyphloplanidans (18S or 28S sequence was not available in several species) and one outgroup taxon (a thalassotyphloplanidan) (Table 2). Methods for alignment (1745 aligned positions for 18S; 1680 for 28S), selection of the optimal substitution model (GTR + I + G for both genes), maximum likelihood (ML) analysis, and drawing of the tree were as described by Kakui (2022).

The COI dataset for phylogenetic analyses of taxa of *Neocaridina* comprised of four sequences we determined and 133 sequences attributed to *N. davidi* and allied taxa registered in the GenBank database; one sequence of *Caridina multidentata* Stimpson, 1860 is also included as outgroup (Supplementary Table S1; Shih and Cai 2007; Fujita et al. 2011; Yu et al. 2014; Shih et al. 2017; Wang et al. 2018; Park et al. 2019; Han et al. 2019; Chen et al. 2020; Fuke et al. 2021; Zhou et al. 2021; Nagai and Imai 2021). This dataset was aligned by using MUSCLE (Edgar 2004); the aligned sequences were trimmed to the shortest length among the sequences, from which several one-nucleotide insertions caused by sequencing errors were removed in MEGA7 (376 positions in the aligned dataset). Optimal substitution models determined for different codons under the corrected AIC (Akaike information criterion) option in ModelFinder (Kalyaanamoorthy et al. 2017) were TIM+F+G4, HKY+F+I, and TIM2+F+G4 for the first, second, and third codons, respectively. A partitioned ML analysis was conducted in IQ-TREE ver. 2.1.2 (Minh et al. 2020); nodal support values were obtained from an ultrafast bootstrap analysis of 1000 pseudoreplicates under the "bnni" option (Hoang et al. 2018). The ML tree was drawn with FigTree v1.4.4 (Rambaut 2021).

The sequences we determined were deposited in the International Nucleotide Sequence Database (INSD) through the DNA Data Bank of Japan (DDBJ), under the accession numbers LC664090–LC664099. The aligned datasets used for phylogeny reconstructions were presented as Supplementary Files S1 and S2.

Results

Scutariella japonica (Matjašič, 1990)

(Fig. 1)

Material examined. ICHUM 8269, 13 hermaphroditic specimens (including two used for DNA extraction), Yasuharu River, Sapporo, Hokkaido, 24 June 2021, parasitic on *Neocaridina* sp. aff. *davidi* (see below), collected by K. Kakui.

Diagnosis. *Scutariella* flatworms ectoparasitic on *Caridina*, *Neocaridina*, and *Paratya* shrimps. Paired black eyes present, located dorsal to pharynx. Sucker U-shaped. Vitelline glands distributed along intestine. Ovary located ventral to intestine.

Remarks. Individuals of *Scutariella japonica* were found on the exoskeleton and in the branchial cavities of host shrimps (Fig. 1). Several individuals may have been lost during field sampling, but at least 13 individuals were recovered from two fresh host shrimps.

The two 18S sequences from *S. japonica* (1779 bp) we sequenced were identical, as were the two 28S (1548 bp) and the two COI (649 bp, encoding 216 amino acids) sequences. The ML tree for 18S + 28S (Fig. 2) retrieved a clade composed of *S. japonica* and *S. sinensis* with 100% bootstrap support. The K2P distances between *S. japonica* and *S. sinensis* were 2.0 % (18S) and 8.6 % (28S). Two 280-bp 28S sequences of *S. japonica* were available in INSD (INSD accession numbers

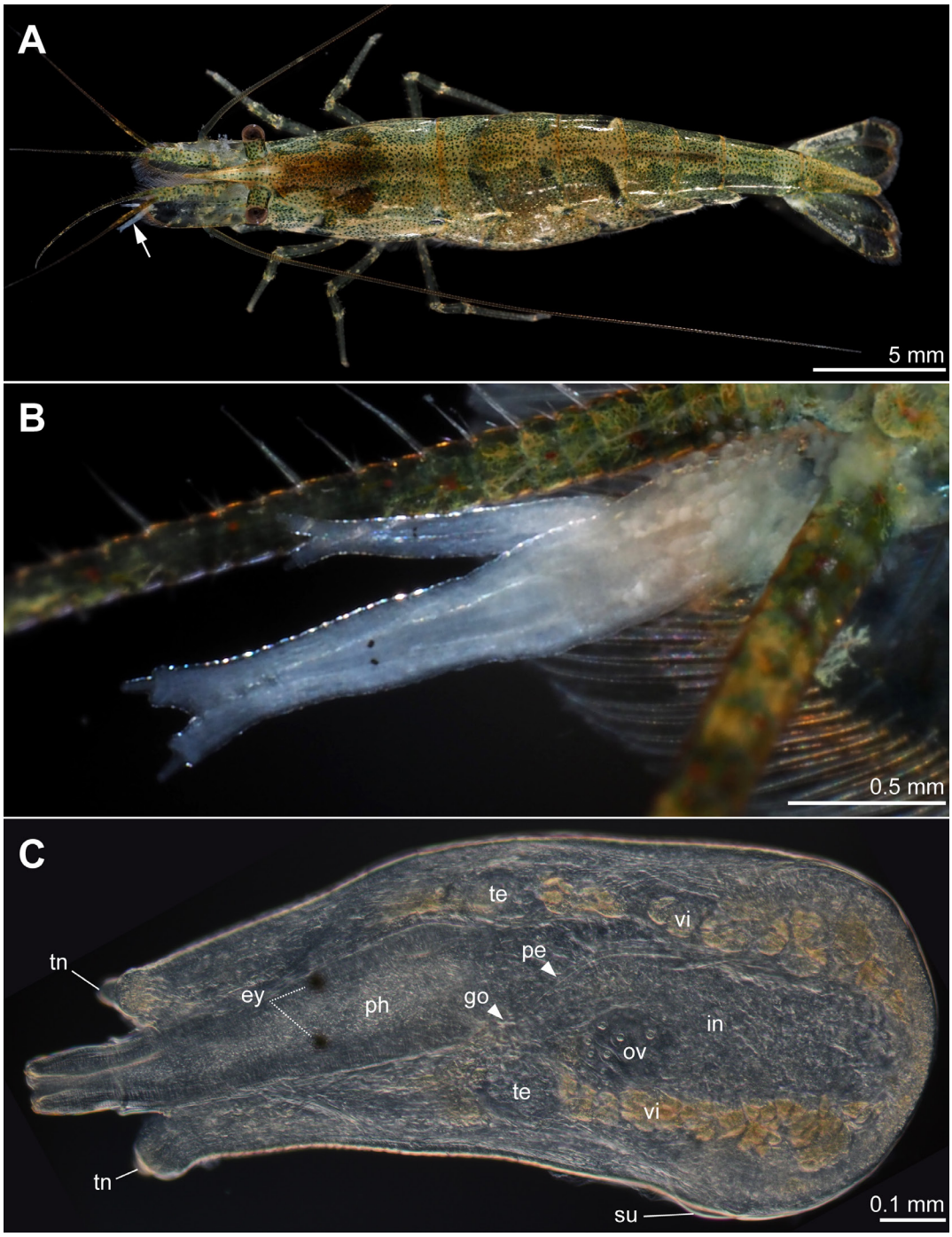

Fig. 1. *Scutariella japonica* (Matjašič, 1990), parasitic on *Neocaridina* sp. aff. *davidi*. A, B, living animals on host shrimp (arrow in A indicating *S. japonica* on host shrimp); C, ethanol fixed *S. japonica*, ventral view, Nomarsky optical image. Abbreviations: ey, eye; go, gonopore; in, intestine; ov, ovary; pe, penis; ph, pharynx; su, sucker; te, testis; tn, tentacle; vi, vitelline gland.

MW581586 and MW581587; unpublished); however, considering that the K2P distances between our and these two sequences (265 positions in the aligned dataset) were 28.6–30.4 %, greater than intergeneric distances (e.g., 25.5 % between our *S. japonica* and *Didymorchis* sp.), misidentification, contamination, or sequencing errors may have been occurred during the

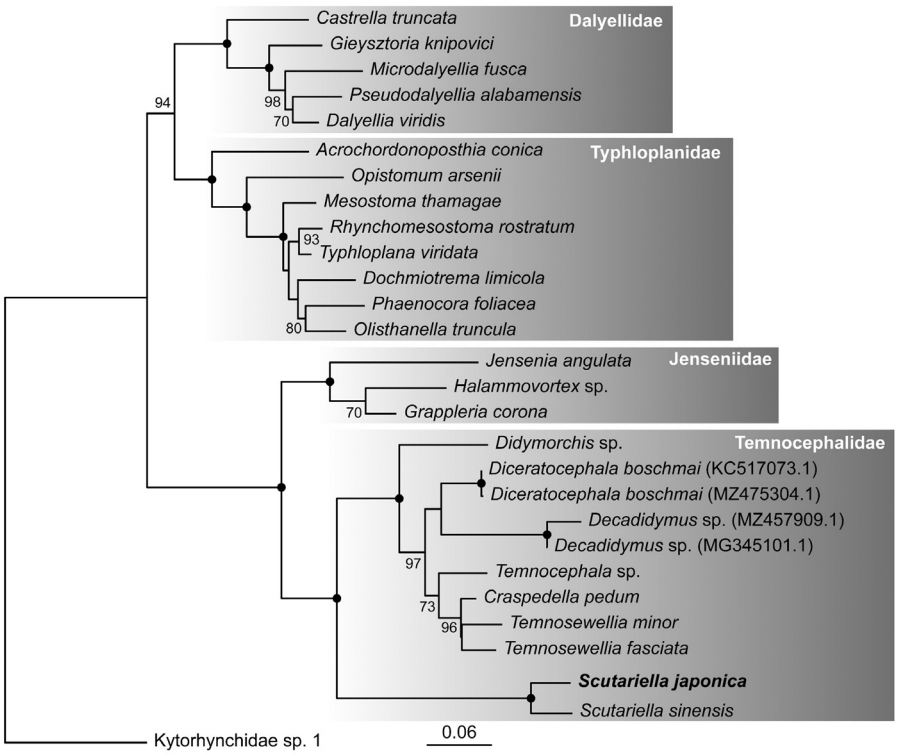

Fig. 2. ML tree constructed from 18S + 28S sequences (3425 positions), including *Scutariella japonica* (Matjašič, 1990). Values near nodes are bootstrap values ≥ 70 %; black circles indicate 100 % bootstrap support.

determination of these two sequences. The temnocephalid COI sequence in the INSD most similar to our sequence, determined by BLAST searches (Altschul et al. 1990), was from *Temnosewellia fax* Sewell, Cannon & Blair, 2006 (INSD accession number KX095350.1; identity score 80.25 %, query cover 84 %; Hoyal Cuthill et al. 2016). To date, no other COI sequences from species of *Scutariella* have been deposited in the public database (DDBJ 2022).

In spite of the record of *Neocaridina* shrimp from Hokkaido (Saito and Okamoto 2008), there have been no records of the parasitic *Scutariella japonica*. Although we could not rule out the possibility that this parasitic species had been overlooked or never been introduced so far, that Yasuharu River keeps its warm condition in winter due to the inflow of warm advanced treated wastewater may have served an opportunity for *S. japonica* to colonize water bodies in Hokkaido (see Discussion section).

Distribution. Japan (Honshu, Kyushu, and Hokkaido); Taiwan; China (but doubted by Chen et al. 2018).

Neocaridina sp. aff. *davidi*

(Figs. 1, 3, 4)

Material examined. Hokkaido. CBM-ZC 16692, 1 immature male (cl 6.1 mm), Yasuharu River, Sapporo, 24 June 2021, collected by K. Kakui, hand net, DNA voucher (accession number LC664099); CBM-ZC 16693, 1 ovigerous female (cl 6.2 mm), same data, DNA voucher (accession number LC664098); ICHUM 8275, 1 ovigerous female (cl 6.3 mm), same data, DNA voucher (accession number LC664097); CBM-ZC 16694, 9

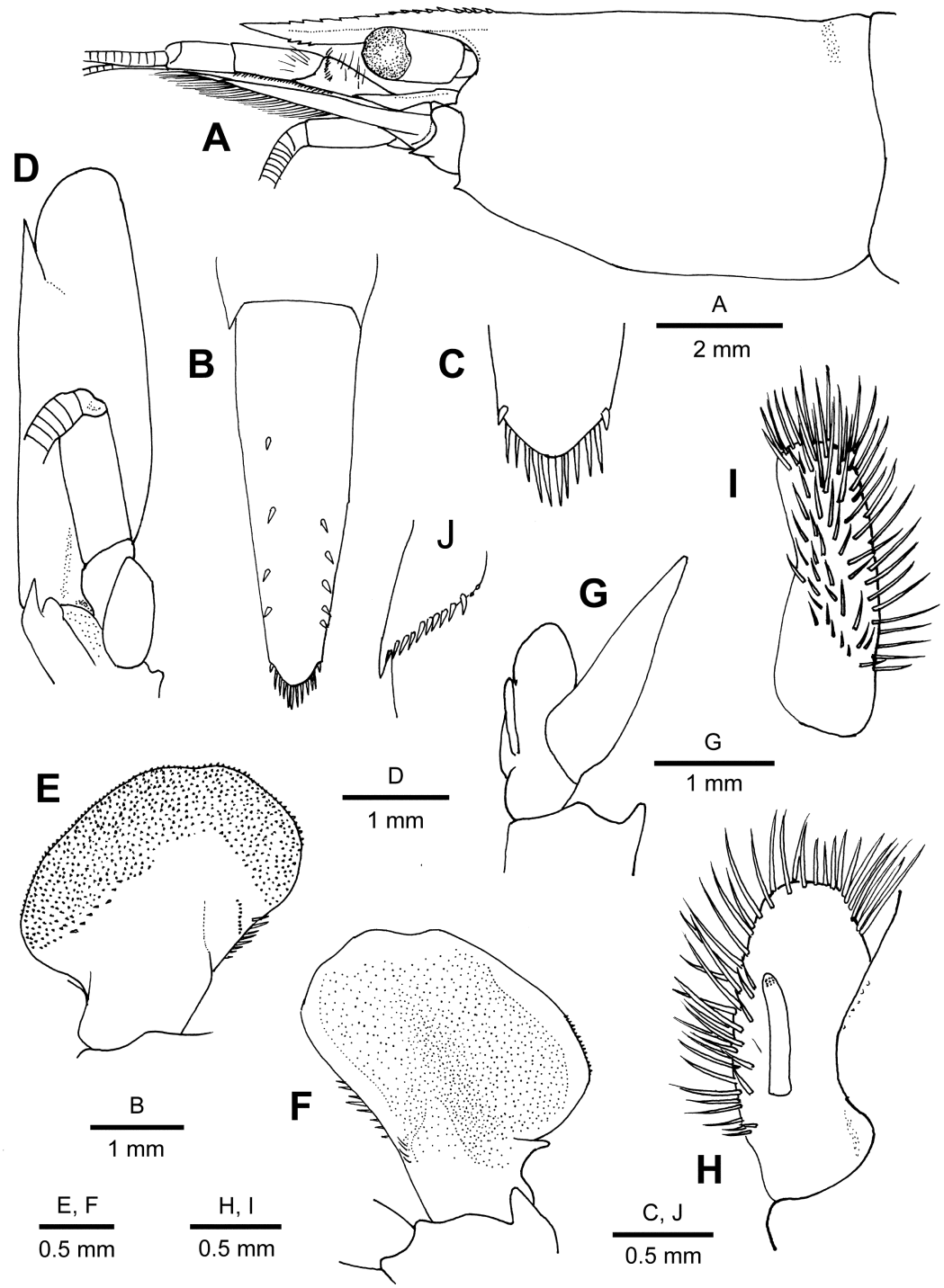

Fig. 3. *Neocaridina* sp. aff. *davidi*, male (cl 6.1 mm), CBM-ZC 16692. A, carapace and cephalic appendages, left lateral view; B, telson, dorsal view; C, posterior margin of telson, dorsal view; D, right antenna, ventral view (setae omitted); E, endopod of left pleopod 1, anterior view; F, same, posterior view; G, endopod of left pleopod 2, ventral view (spiniform setae on appendix masculina, and all setae omitted); H, appendices interna and masculina of left pleopod 2 endopod, mesial view; I, appendix masculina of left pleopod 2 endopod, dorsal view; J, diaeresis on exopod of left uropod.

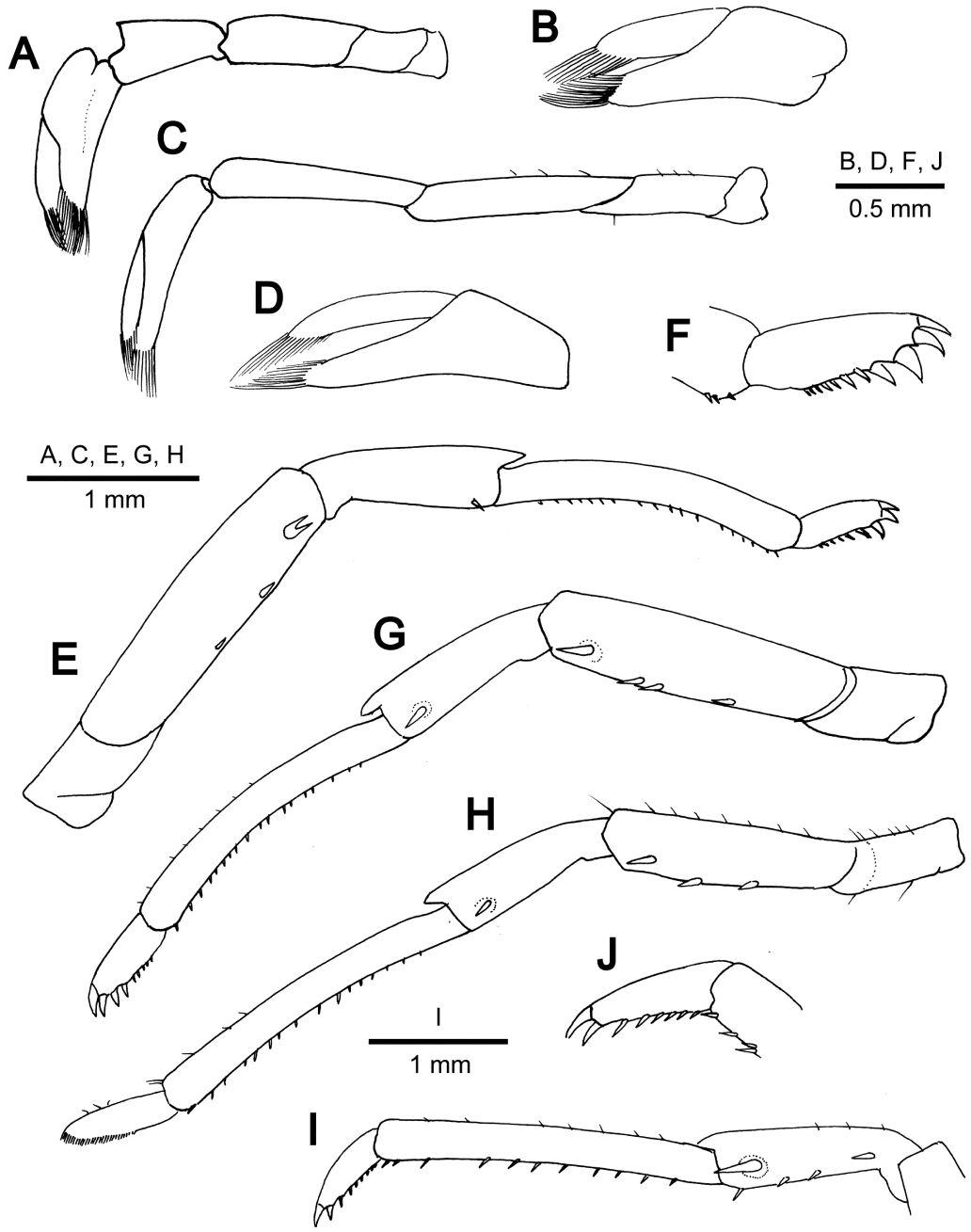

Fig. 4. *Neocaridina* sp. aff. *davidi*. A–H, male (cl 6.1 mm), CBM-ZC 16692; I, J, female (cl 6.7 mm), CBM-ZC 16694. A, left pereopod 1, lateral view; B, same, chela, extensor view; C, left pereopod 2, lateral view; D, same, chela, extensor view; E, right pereopod 3, lateral view; F, same, dactylus, lateral view; G, left pereopod 4, lateral view; H, left pereopod 5, lateral view; I, dactylus and propodus of left pereopod 3, lateral view; J, dactylus of left pereopod 3, lateral view.

mature males (cl 5.3–6.5 mm), 4 females (cl 5.8–7.4 mm), 3 ovigerous female (cl 6.2–6.7 mm), same locality, 12 July 2021, collected by K. Kakui, hand net.

Chiba Prefecture. CBM-ZC 14978, 1 female (cl 7.7 mm), Kaneoya, Wakaba Ward, Chiba City, 9 September 2014, collected by R. Kuranishi, dip net, DNA voucher (accession number LC664096).

Diagnosis. A small shrimp species, body size up to 35 mm. Rostrum (Fig. 3A) slender, reaching mid-length of article 2 of antennular peduncle to falling slightly short of distal end of antennular peduncle, 0.5–0.6 times as long as carapace, unarmed near tip; dorsal margin armed with 13–22 teeth, including 2–3 postorbital; ventral margin with 4–7 teeth (rostral teeth generally less conspicuous in males than in females). Carapace (Fig. 3A) without supraorbital spine; antennal spine moderately small; pterygostomial angle with tiny spine in both male and female. Telson (Fig. 3B) with 4–6 dorsolateral spiniform setae on either side; posterior margin convex, with 6 pairs of spiniform setae (lateral-most pair shortest) (Fig. 3C). Antennal scaphocerite (Fig. 3D) 3.3 times as long as wide; distolateral spine falling far short of rounded distal lamella. Pereopods without exopods. Pereopod 1 (Fig. 4A, B) carpus with moderately deep excavation distally; dactylus 1.4 times as long as palm. Pereopod 2 (Fig. 4C, D) dactylus 1.2 times as long as palm. Pereopods 3 and 4 exhibiting marked sexual dimorphism: propodi of male (Fig. 4E, G) curved, those of female almost straight (Fig. 4I); dactyli in male bearing stronger and more erect accessory spiniform setae on flexor margin than in female (cf. Fig. 4F and Fig. 4J). Endopod of male pleopod 1 (Fig. 3E, F) strongly, asymmetrically expanded, 1.1–1.2 times as long as broad in fully mature individuals. Appendix masculina on male pleopod 2 (Fig. 3G–I) thick, bean-shaped, with numerous spiniform setae of various length on mesial face, extending onto broadly rounded terminus; appendix interna reaching to distal 0.3 of appendix masculina. Uropodal exopod diaeresis (Fig. 3J) with row of 10–12 spiniform setae. Egg size approximately 0.8 × 1.1 mm in eyed-stage.

Color in life. Body and appendages generally greenish brown semi-transparent; corneas dark brown (Fig. 1A).

Remarks. The presence of non-indigenous species of *Neocaridina* was recognized in Japan in 2004, and *Neocaridina* shrimps are now widely spread, ranging from Hokkaido to the Ryukyu Islands (e.g., Nishino and Niwa 2004; Niwa 2010; Fujita et al. 2011; Nishino 2017; Fuke et al. 2021; Nagai and Imai 2021). Niwa (2010, 2017) suggested a possibility that more than one species of *Neocaridina* were introduced from China and Korea to Japan. Klotz et al. (2013) identified specimens from Germany with *N. davidi* in comparison with the type material and assumed that *N. heteropoda heteropooda* Liang, 2002 and *N. denticulata sinensis* (Kemp, 1918) were junior synonyms of *N. davidi*, although the authors did not reach the final conclusion. Mitsugi et al. (2017) applied the name *N. davidi* to specimens from Tateyama, Boso Peninsula, central Japan, placing *N. heteropoda heteropoda* and *N. denticulata sinensis* under the synonymy of *N. davidi*. Shih et al. (2017) also treated *N. davidi* as a senior synonym of those two taxa. The taxonomic actions have been followed by several subsequent workers (Jabłońska et al. 2018; Han et al. 2019; Toyota et al. 2019; Onuki 2021). As summarized by Fuke et al. (2021), however, the taxonomy of *Neocaridina davidi* and related taxa (cf. Shih et al. 2017) is not fully settled.

Our ML tree using COI reflects the unsettled state of the taxonomy of *Neocaridina*. The specimens from Sapporo are clustered with *Neocaridina koreana* of Shih et al. (2017) with full ultrafast bootstrap support and low genetic divergence (0.0–1.6 % K2P distances within the clade), along with other individuals collected from Shimane and Hyogo Prefectures, Japan, that were originally identified as *N. denticulata denticulata* (De Haan, 1844) (cf. Fujita et al. 2011). Nagai and Imai (2021) attributed this clade to *Neocaridina* sp. 1. The clade including the specimens from Sapporo

is sister to *N. palmata* (Shen, 1948) with K2P distance 5.6–6.7 %. The clade consisting of specimens attributed to *N. davidi* Types I and II sensu Nagai and Imai (2021) is the sister to the clade consisting of the clade including the specimens from Sapporo and *N. palmata*. K2P distances between the clade including the specimens from Sapporo and the clade consisting of *N. davidi* are 4.4–7.1 %, suggesting that the clades are specifically distinct.

It should be noted that the present specimens from Sapporo are clearly different morphologically from *N. koreana* in the proportionally broader pleopod 1 endopod (Fig. 3E, F versus Kubo 1938: fig. 12A–D; Liang 2004: fig. 46k, l). Dr. H.-T. Shih kindly informed us that the voucher specimen he identified with *N. koreana* (cf. Shih et al. 2017) might not represent the true *N. koreana* (personal communication on 10 November 2021). *Neocaridina palmata* differs from the present specimens from Sapporo in the more erect accessory spiniform setae of dactyli of the male pereopods 3 and 4 (Shen 1948: pl. 2, fig. e; Liang 2004: fig. 51k versus Fig. 4F), the less expanded, more symmetrical endopod of the male pleopod 1 (Shen 1948: pl. 2, fig. g; Liang 2004: fig. 51o–q versus Fig. 3E, F) and the better developed appendix interna of the male pleopod 2, which reaches the distal 0.2 of the appendix masculina (Shen 1948: pl. 2, fig. i; Liang 2004: fig. 51r versus Fig. 3H). When compared with the descriptions of *N. davidi* (cf. Liang 2004, as *Neocaridina heteropoda heteropoda*; Klotz et al. 2013), no clear morphological difference could be detected

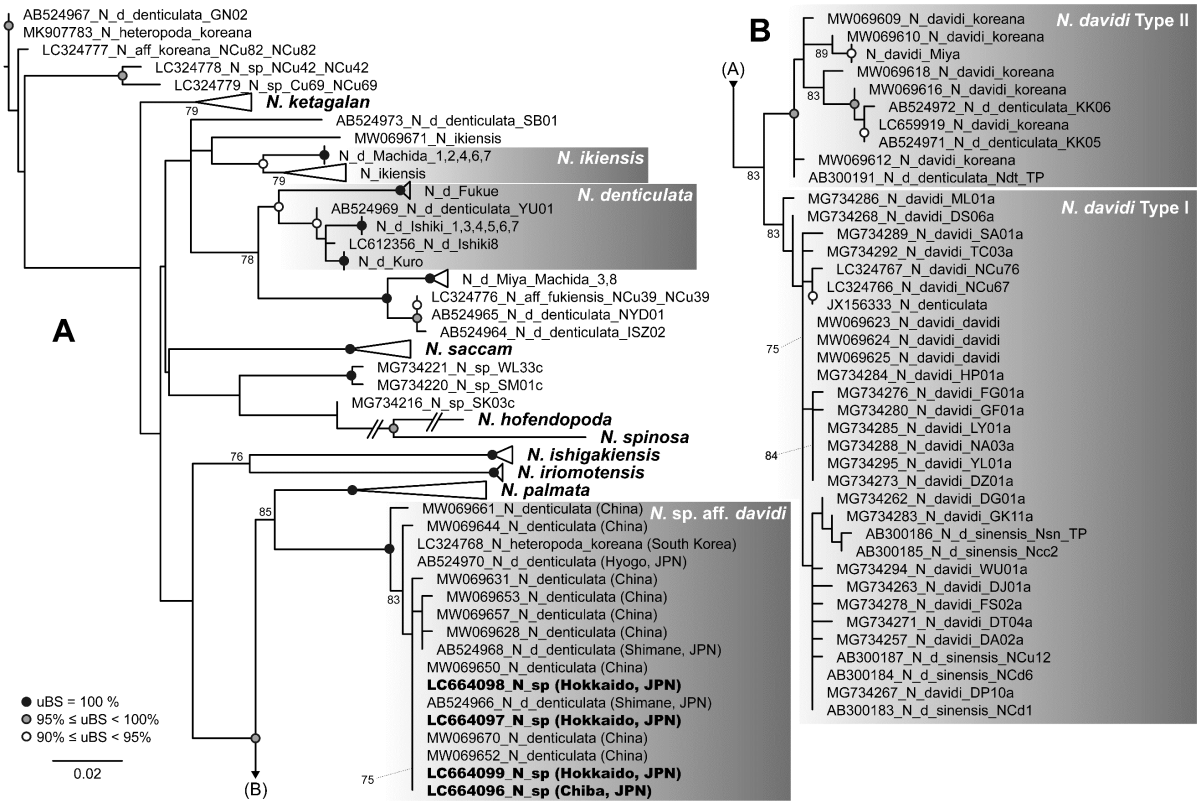

Fig. 5. ML tree for COI sequences (376 positions), including our *Neocaridina* sequences. A, basal part of tree; B, terminal part of tree. Numbers near nodes are ultrafast bootstrap values ≥ 75 %. Outgroup taxon is not shown. uBS, ultrafast bootstrap support.

between our specimens and those previous descriptions. Unfortunately, during this study, we could not examine specimens certainly referable to the *N. davidi* clade (Fig. 5). In order to fully establish the identity of the present specimens, a thorough revision of *N. davidi* and allied taxa, based on integrative approach, is strongly recommended. Such a revisionary study, however, requires examination of material from outside Japan, and is far beyond the scope of this study. We thus refrained from identifying our specimens at the species level for the time being and refer them to as *Neocaridina* sp. aff. *davidi*.

During this study, we have tried to sequence COI from specimens of *Neocaridina* preserved in the collection of CBM, although not very successful. COI sequencing from one specimen from Chiba City, Chiba Prefecture, was successful: the specimen is clustered with the specimens from Sapporo, documenting the presence of *N.* sp. aff. *davidi* in Kanto District, central Japan.

Discussion

This is the first occurrence record of *Scutariella japonica* from Hokkaido. As no native host-candidate shrimp species (e.g., *Caridina*, *Neocaridina*, and *Paratya* shrimps) are distributed in Hokkaido (Komai et al. 1992; Hayashi 2007), *S. japonica* must be an invasive species in Hokkaido.

Identification of specimens of *Neocaridina* based only on morphology is sometimes very difficult at present. Comparison of specimens, for which sequence data on DNA marker(s) is available, would be strongly advisable for assessment of diagnostic characters for species recognition. Unfortunately, we could not make decision on the specific identity of the host shrimp specimens examined in this study, but we hope that the data presented in this study would be useful for future studies.

In Hokkaido, Saito and Okamoto (2008) first reported the occurrence of *Neocaridina* species, provisionally identified as "*Neocaridina denticulata sinensis*?" from Toyohira River, Sapporo, of which voucher specimens had been collected in 2003–2006, and noted that *Neocaridina* shrimps had been already found in several rivers in Sapporo since 1998. As *Neocaridina* shrimps are popular animals in aquarium trades and as fishing bait (cf. Niwa 2010), the occurrence in Hokkaido must be anthropogenic introduction. Species of *Neocaridina* are primarily inhabitants of warm temperate to subtropical areas in East Asia (Cai 1996; Liang 2004; Shih et al. 2007), but the presence of warm wastewater or warm drainage might enable *Neocaridina* shrimps to survive and expand their distribution even in cool temperate regions, as documented by records from European countries (Klotz et al. 2013; Jabłońska et al. 2018; Weiperth et al. 2019). The invasion of *Neocaridina* sp. aff. *davidi* in Yasuharu River may be a very recent event; it is because no *Neocaridina* species were found during a faunal survey at the river made in 2011 (Maeda and Yoshida 2012). Due to the inflow of warm advanced treated wastewater, Yasuharu River is warmer than other rivers in Sapporo, e.g., average water temperature in winter was 13.3 °C in Yasuharu River whereas less than 4 °C in several other rivers (Maeda and Yoshida 2012), which may enable *Neocaridina* shrimps to survive in the river.

Impact of *Neocaridina* shrimp and the parasitic flatworm *Scutariella japonica* to the native fauna and environments in Hokkaido remain unknown at present, but measures to prevent distribution expansion of the host shrimp will be required.

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