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Temnosewellia aff. *vietnamensis* (Platyhelminthes: Rhabdocoela: Temnocephalidae) associated with freshwater crabs from Kagoshima, southern Japan, with review of records of the genus from East to South Asian countries

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Abstract

Temnocephalids are ectosymbionts of various freshwater animals. A species tentatively identified as *Temnosewellia* aff. *vietnamensis* (Platyhelminthes: Rhabdocoela: Temnocephalidae) is reported based on materials collected from the body surface of the freshwater crabs *Eriocheir japonica* (Brachyura: Varunidae) and *Geothelphusa exigua* (Potamidae) in Kagoshima, southern Japan. The temnocephalid is characterized as follows: the cirrus composed of a cone-shaped shaft and a cylindrical introvert 42–77 µm long; the introvert covered with approximately 30 vertical rows of fine sharp spines; the four seminal receptacles; and a long, curved oviduct with vaginal gland; a pair of gland cells (Haswell's cells) present anterior to the excretory ampullae. Bayesian inference trees using partial nuclear 28S rDNA (28S) and mitochondrial cytochrome c oxidase subunit I (COI) genes supported that the specimens collected from both crab species are conspecific but these also showed the geographical variations among them on both 28S and COI. The previous records of the genus *Temnosewellia* in East to South Asian countries are assembled and shown on the map (fig.7, this paper).

Introduction

Members of the family Temnocephalidae *sensu* Van Steenkiste *et al.* (2021) (Platyhelminthes), which was traditionally regarded as Temnocephalida, are ectosymbionts associated with various animals, mainly with crustaceans in freshwaters (Joffe *et al.*, 1998). Species of the genus *Temnosewellia* Damborenea & Cannon, 2001, which is currently recognized as the largest genus of the family, have been recorded from Australia and South East Asian countries (e.g. Cannon, 1993; Cannon & Sewell, 2001; Sewell *et al.*, 2006). Currently 52 valid species of the genus are recognized in total (Tyler *et al.*, 2006–2022), 50 species of which are naturally distributed in Australia (see Haswell, 1888, 1893, 1900; Hickman, 1967; Williams, 1980; Cannon, 1993; Cannon & Sewell, 2001; Sewell *et al.*, 2006). Of those 50 species, only *Temnosewellia rouxi* (Merton, 1913) was also found in Indonesia. While both *Temnosewellia minor* (Haswell, 1888) and *Temnosewellia chaeropsis* have also been reported from outside of Australia (i.e. the former from Japan, Germany, Italy, and South Africa and the latter from South Africa, respectively), those are considered to have been imported (e.g. Mitchell & Kock, 1988; Avenant-Oldewage, 1993; Cannon & Sewell, 2001; Tavakol *et al.*, 2021; Vecchioni *et al.*, 2021). On the other hand, *Temnosewellia semperi* (Weber, 1889) and *Temnosewellia vietnamensis* Damborenea & Brusa, 2009 have only been reported from East to South Asian countries (e.g. Semper, 1872; Weber, 1889; Damborenea & Brusa, 2009).

Although the species considered to be *Temnosewellia* have been found from freshwater crabs in various localities from southern Japan, all of those records have lacked the descriptions of diagnostic characters, except *T. minor* found from the crayfish *Cherax tenuimanus* (Smith, 1912) (Parastacidae) which was introduced from western Australia to Kagoshima (Oki *et al.*, 1995).

In this study, a species of *Temnosewellia* that is probably native is recorded based on individuals and eggs attaching on the freshwater crabs *Eriocheir japonica* (De Haan, 1835) (Varunidae) and *Geothelphusa exigua* Suzuki & Tsuda, 1994 (Potamidae) collected from the Osumi Peninsula, Kagoshima, southern Japan. Phylogenetic analyses are conducted based on partial sequences of two genes.

Materials and methods

Crabs were caught from the upper to middle reaches of Oda River (31°15′N, 131°3′E), a tributary of Kimotsuki River (31°18′N, 131°0′E), and Kushira River (31°31′N, 130°47′E), Osumi Peninsula, Kagoshima, southern Japan. Temnocephalids were carefully removed from the body surface of the crabs, using forceps and preserved in 70% ethanol under coverslip pressure to observe both external and internal morphology. The specimens attached on the slide grasses were stained in Heidenhain's iron–haematoxylin, dehydrated through a graded ethanol series (70 to 99%) for one to three days, cleared in xylene for three days, and mounted in Canada balsam. Several whole individuals and eggs were fixed in 10% formalin in hot distilled water. Before observation of the penial stylet (cirrus), a fixed specimen was soaked in lactophenol during 24 h, and then dissected using sharpened stainless needles. Drawings were made with the aid of a drawing tube mounted on a compound microscope (Olympus BX53). For observation using a scanning electron microscope (SEM) (Hitachi TM3000), fixed whole or dissected individuals and eggs were dehydrated through a graded ethanol series and *t*-butanol, freeze-dried, sputter coated with gold, and examined.

Using five specimens of one of the caligids fixed in 80% ethanol, DNA was extracted and sequenced for two genes, cytochrome c oxidase subunit I (COI) in mitochondria and 28S rDNA in nucleus. These methods are based on those we have published in the past (Tosuji *et al.*, 2019). The primer sets used were 425F (5′-GGNGCTAGNTCNATWTTAGGRGC-3′) + new 1200R (5′-CCCATTGAWAMNACATAATGAAAATG-3′) for COI, and Ltem180 (50-GAAGTTCGCACGATTGCGG-30) + Ltem1000R (50-CACAAGCATAGTTCACC-30) for 28S, according to Hoyal Cuthill *et al.* (2016). The data have been submitted to the DNA Data Bank of Japan (DDBJ) database under accession numbers LC651441 and LC651443–LC651454. Using the nucleotide sequence data of these specimens with other temnocephalids obtained from GenBank (table 1), Bayesian inference (BI) trees were obtained. The sequences were aligned using the DNA alignment software MUSCLE ver. 3.8.31 (Edgar, 2004). MrBayes v. 3.1.6 (Ronquist *et al.*, 2012) was used to obtain BI tree on the basis of the substitution model GTR + G + I. As a result, 2,000,000 generations [Average Standard Deviation of Split Frequencies (ASDSF) = 0.005009] for COI and 500,000 generations (ASDSF = 0.006170) for 28S were obtained. The first 25% of the samples were discarded as burn-in samples.

The taxonomy of temnocephalids follows Tyler *et al.* (2006–2022), and the terminology basically follows Cannon (1993) and Sewell *et al.* (2006). Measurements were reported in mm or μm as range followed by mean, and standard deviation in parentheses. Materials are deposited in the Platyhelminthes collection of the National Museum of Nature and Science, Tsukuba (NSMT), Japan, and the Kagoshima University Museum (KAUM), Kagoshima, Japan. Materials used by Okawachi *et al.* (2013) deposited in the Ryukyu University Museum, Fujukan (RUMF) were examined.

Results

Genus *Temnosewellia* Damborenea & Cannon, 2001

Japanese name: Yadori-itsu-tsuno-mushi-zoku

Temnosewellia* aff. *vietnamensis Damborenea & Brusa, 2009

Syn. *Temnosewellia* sp.: Okawachi *et al.*, 2013: 76–79, fig. 1c–h.

New Japanese name: Yamataro-yadori-tsuno-mushi

(Figures 1–5)

Materials examined

One specimen (NSMT-Pl 6494), one specimen (NSMT-Pl 6494), one specimen (NSMT-Pl 6494), two specimens (NSMT-Pl 6494) and one specimen (NSMT-Pl 6494) in whole mount slide, *ex E. japonica* (De Haan, 1835) (Brachyura: Varunidae) from middle reaches of Oda River (31°15′N, 131°3′E), Kimotsuki, Kagoshima, Japan, 4 November 2018; one specimen (NSMT-Pl 6495), one specimen (NSMT-Pl 6495), one specimen (NSMT-Pl 6495), one specimen (NSMT-Pl 6495), one specimen (NSMT-Pl 6495), one specimen (NSMT-Pl 6495), and one specimen (NSMT-Pl 6495) in whole mount slides, three specimens (NSMT-Pl 6495) in 10% formalin in distilled water, 65 specimens (NSMT-Pl 6495) in 99.5% ethanol, and 14 eggs (NSMT-Pl 6495) in 10% formalin in distilled water, *ex E. japonica* from middle reaches of Oda River (31°15′N, 131°3′E), Kimotsuki, Kagoshima, Japan, 20 September 2017; two specimens (NSMT-Pl 6496) in whole mount slides, five specimens (NSMT-Pl 6496) in 10% formalin in distilled water, 12 specimens (NSMT-Pl 6496) in 99.5% ethanol, and 13 eggs (NSMT-Pl 6496) in 10% formalin in distilled water, *ex E. japonica* from middle reaches of Oda River (31°15′N, 131°3′E), Kimotsuki, Kagoshima, Japan, 9 August 2021; three specimens (KAUM–PT–1) in 10% formalin in distilled water, collection data same as that of (NSMT-Pl 6496); nine specimens, prepared for observation by SEM, collection data same as that of (NSMT-Pl 6495); four specimens, prepared for observation by SEM, collection data same as that of (NSMT-Pl 6496); two specimens (NSMT-Pl 6497), two specimens (NSMT-Pl 6497), two specimens (NSMT-Pl 6497) in whole mount slides, four specimens (NSMT-Pl 6497) in 10% formalin in distilled water, six specimens (NSMT-Pl 6497) in 99.5% ethanol, and nine eggs (NSMT-Pl 6497) in 10% formalin in distilled water, *ex Geothelphusa exigua* Suzuki & Tsuda, 1994 (Potamidae) from upper reaches of a tributary of Kimotsuki River (31°18′N, 131°0′E), Kimotsuki, Kagoshima, Japan, 9 August 2021; 2 specimens (KAUM–PT–2) in 10% formalin in distilled water, collection data same as that of (NSMT-Pl 6497); two specimens, prepared for observation by SEM, collection data same as that of (NSMT-Pl 6497); five specimens (NSMT-Pl 6498) in 99.5% ethanol, *ex G. exigua* from upper reaches of Kushira River (31°31′N, 130°47′E), the Takakuma Experimental Forest, Kagoshima University, Tarumizu, Kagoshima, Japan, 20 October 2021; five specimens (RUMF-ZF-00006) in 2.5% lutaraldehyde in distilled water, *ex G. exigua* from upperstream of the Ogawa River, Osumi Peninsula, Kagoshima, Japan, 8 November 2012; five specimens (RUMF-ZF-00007) in 2.5% glutaraldehyde in distilled water, *ex E. japonica* from upperstream of Hitotsutani River, Osumi Peninsula, Kagoshima, Japan, 8 November 2012; and five specimens (RUMF-ZF-00008) in 2.5% glutaraldehyde in distilled water, *ex E. japonica* from upperstream of Honjo River, Osumi Peninsula, Kagoshima, Japan, October 2012.

Molecular sequence

The sequences of the following accession numbers of DDBJ: LC651446 and LC651447 (28S), LC651452–LC651454 (COI) were obtained from specimens collected from Oda River. LC651441 and LC651443–LC651445 (28S) and LC651448–LC651451 (COI) were obtained from specimens collected from another locality, a tributary of Kimotsuki River.

Table 1. List of temnocephalids using the phylogenetic analysis in this paper with GenBank accession numbers for 28S rDNA (28S) and cytochrome c oxidase subunit I (COI).

Taxa	28S	COI	Localities	References
Diceratocephala boschmai	MW443038	–	Eastern Australia	Tavakol *et al.* (2021)
	MW443039	–	Eastern Australia	Tavakol *et al.* (2021)
	MW443040	–	South Africa	Tavakol *et al.* (2021)
	–	MZ128776	New Guinea Island, Indonesia	Ložek *et al.* (2021)
Temnocephala sp.	AJ228802	–	–	Littlewood *et al.* (1998)
	–	AJ405989 AJ405990	–	Telford *et al.* (2000)
Temnosewellia acicularis	KX095259	KX095328	Eastern Australia	Hoyal Cuthill *et al.* (2016)
Temnosewellia alba	KX095260	KX095329	Eastern Australia	Hoyal Cuthill *et al.* (2016)
	KX095261	KX095330	Eastern Australia	Hoyal Cuthill *et al.* (2016)
Temnosewellia albata	KX095262	KX095331	Eastern Australia	Hoyal Cuthill *et al.* (2016)
Temnosewellia aphyodes	KX095263	KX095332	Eastern Australia	Hoyal Cuthill *et al.* (2016)
Temnosewellia apiculus	KX095264	KX095333	Eastern Australia	Hoyal Cuthill *et al.* (2016)
Temnosewellia arga	KX095265	KX095335	Eastern Australia	Hoyal Cuthill *et al.* (2016)
Temnosewellia aspinosa	KX095266	KX095336	Eastern Australia	Hoyal Cuthill *et al.* (2016)
Temnosewellia aspra	KX095267	KX095334	Eastern Australia	Hoyal Cuthill *et al.* (2016)
Temnosewellia bacrioniculus	KX095268	KX095337	Eastern Australia	Hoyal Cuthill *et al.* (2016)
	KX095269	KX095339	Eastern Australia	Hoyal Cuthill *et al.* (2016)
	KX095270	KX095338	Eastern Australia	Hoyal Cuthill *et al.* (2016)
Temnosewellia batiola	KX095271	KX095340	Eastern Australia	Hoyal Cuthill *et al.* (2016)
Temnosewellia belone	KX095272	KX095341	Eastern Australia	Hoyal Cuthill *et al.* (2016)
Temnosewellia comythus	KX095273	KX095342	Eastern Australia	Hoyal Cuthill *et al.* (2016)
	KX095274	KX095343	Eastern Australia	Hoyal Cuthill *et al.* (2016)
Temnosewellia coughrani	KX095275	KX095344	Eastern Australia	Hoyal Cuthill *et al.* (2016)
	KX095276	KX095345	Eastern Australia	Hoyal Cuthill *et al.* (2016)
Temnosewellia dendyi	KX095258	KX095327	Eastern Australia	Hoyal Cuthill *et al.* (2016)
Temnosewellia fasciata	KX095277	KX095346	Eastern Australia	Hoyal Cuthill *et al.* (2016)
	KX095278	KX095347	Eastern Australia	Hoyal Cuthill *et al.* (2016)
	KX095279	KX095348	Eastern Australia	Hoyal Cuthill *et al.* (2016)
	KX095280	KX095349	Eastern Australia	Hoyal Cuthill *et al.* (2016)
	KC869888	–	–	Laumer & Giribet (2014)
Temnosewellia fax	KX095281	KX095350	Eastern Australia	Hoyal Cuthill *et al.* (2016)
	KX095282	KX095351	Eastern Australia	Hoyal Cuthill *et al.* (2016)
Temnosewellia flammula	KX095283	KX095352	Eastern Australia	Hoyal Cuthill *et al.* (2016)
Temnosewellia gingrina	KX095284	KX095353	Eastern Australia	Hoyal Cuthill *et al.* (2016)
	KX095285	KX095354	Eastern Australia	Hoyal Cuthill *et al.* (2016)
	KX095286	KX095355	Eastern Australia	Hoyal Cuthill *et al.* (2016)
	KX095287	KX095356	Eastern Australia	Hoyal Cuthill *et al.* (2016)
Temnosewellia gracilis	KX095288	KX095357	Eastern Australia	Hoyal Cuthill *et al.* (2016)
Temnosewellia keras	KX095289	KX095360	Eastern Australia	Hoyal Cuthill *et al.* (2016)
Temnosewellia maculata	KX095292	KX095361	Eastern Australia	Hoyal Cuthill *et al.* (2016)
Temnosewellia minima	KX095293	KX095363	Eastern Australia	Hoyal Cuthill *et al.* (2016)
Temnosewellia minor	KX095257	KX095326	Eastern Australia	Hoyal Cuthill *et al.* (2016)

(*Continued*)

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Table 1. (*Continued.*)

Taxa	28S	COI	Localities	References
	AY157164	–	Eastern Australia	Lockyer *et al.* (2003)
	MW314801	–	Italy	Vecchioni *et al.* (2021)
Temnosewellia muscalingulata	KX095290	KX095358	Eastern Australia	Hoyal Cuthill *et al.* (2016)
	KX095291	KX095359	Eastern Australia	Hoyal Cuthill *et al.* (2016)
Temnosewellia unguiculus	KX095294	KX095362	Eastern Australia	Hoyal Cuthill *et al.* (2016)
Temnosewellia aff. *vietnamensis*	LC651441	LC651449	A tributary of Kimotsuki River, Kagoshima, Japan	This paper
	LC651443	LC651448	A tributary of Kimotsuki River, Kagoshima, Japan	This paper
	LC651444	LC651451	A tributary of Kimotsuki River, Kagoshima, Japan	This paper
	LC651445	LC651450	A tributary of Kimotsuki River, Kagoshima, Japan	This paper
	LC651446	LC651454	Oda River, Kagoshima, Japan	This paper
	LC651447	LC651453	Oda River, Kagoshima, Japan	This paper
	–	LC651452	Oda River, Kagoshima, Japan	This paper
Temnosewellia sp.	–	MK421405	Thailand	Ngamniyom *et al.* (2019)
	–	MK421406	Thailand	Ngamniyom *et al.* (2019)

Description

General external characters: body (figs 1c–f, 2a and 3a, b) oval, dorsoventrally compressed bearing five tentacles, fresh colour white or brownish white, 1.21–5.56 (2.83 ± 0.97) mm long (*n* = 22) not including tentacles and 0.93–3.18 (1.75 ± 0.62) mm wide (*n* = 22); and tentacle elongate, gradually narrower to tip, 223–1154 (438 ± 195) 231–517 (336 ± 86) μm, length of tentacle/body ratio 0.10–0.29 (0.16 ± 0.04) (*n* = 21). Black pigments confined to fresh and fixed eyes (see fig. 1e, f). Posterior adhesive disc (figs 2a and 3b–d) pedunculate; disc 415–1331 (726 ± 222) μm in diameter (*n* = 22); and stalk 212–685 (397 ± 103) μm in diameter (*n* = 22). Epidermis thin, smooth; cilia entirely absent.

Syncytial plates: post-tentacular syncytium (fig. 3f, g) saddle shaped with constriction at adjacent areas of excretory pore. Horizontal bar-shaped syncytium situated posterior to post-tentacular syncytium (fig. 3f, h, i).

Alimentary system: mouth opening (figs 2a and 3c) situated posterior to level of eyes. Pharynx (fig. 2a) wider than long, 149–972 (417 ± 200) 302–1084 (596 ± 187) μm (*n* = 22), bearing large sphincter. Intestine (fig. 2a) saccular, rectangular with septa, 0.50–1.65 (0.97 ± 0.36) 0.87–2.70 (1.52 ± 0.57) mm (*n* = 21).

Excretory system: pair of excretory pores situated on dorsolateral surface of body syncytium not on post-tentacular syncytium (fig. 3f, g). Paired excretory ampullae (fig. 2a) represented by simple sac; major excretory duct conspicuous on dorsal surface.

Glands: numerous rhabdite glands (fig. 2a) forming bunches developed in lateral fields of body from posterior to excretory ampullae to posterior testis. Pair of Haswell's cells (fig. 2a) situated anterior to eyes. Clusters of disc glands (fig. 2a) surrounding basement of posterior sucker.

Reproductive system of female: gonopore (figs 2a, b and 3c, d) situated mid ventral, posterior third of body. Genital atrium (fig. 2b) commodious, elongate, connecting to oviduct via vagina. Vagina 18–92 (50 ± 22) μm long (*n* = 16), muscular with small sac-like vaginal gland. Oviduct long, curved, constricted at middle. Ovary elliptical, longer than wide, 38–172 (87 ± 33) 26–107 (53 ± 19) μm (*n* = 20). Four spherical seminal receptacles present. Vitellaria developed on dorsal and ventral surfaces of intestine: length and width as in intestine (fig. 2a). Vesicula resorbens elliptic or mushroom shape.

Reproductive system of male: pair of anterior testes ellipsoid (fig. 2a), 88–806 (334 ± 185) μm long (*n* = 22), situated in lateral side of intestine, connecting to posterior testes via vasa deferentia. Pair of posterior testes (fig. 2a) globular, 108–992 (361 ± 206) μm long (*n* = 22), longer than anterior testes, situated posterior to intestine, connecting to seminal vesicle via vasa deferentia with swollen pyriform proximal parts; seminal vesicle slender, 68–220 (119 ± 40) (*n* = 20) 12–68 (24 ± 13) μm (*n* = 19). Ejaculatory sac (fig. 2b) spherical, 21–97 (50 ± 24) 13–69 (34 ± 16) μm (*n* = 21). Prostate (fig. 2b) spherical, 18–71 (39 ± 16; 30) 13–65 (32 ± 14) μm (*n* = 21), separated by constriction from cirrus. Cirrus (figs 2b, c, 4a and 5a) 137–426 (311 ± 88) μm long, 13–68 (51 ± 18) μm wide (*n* = 21); shaft cone-shaped, straight or slightly curved; introvert (figs 2b–d, 4b and 5a–c) cylindrical, 42–85 (65 ± 11) μm long (*n* = 21), with distal opening not oblique, eversible distal region covered with approximately 30 vertical rows of fine sharp spines connecting to unspined distal region via thick ring.

Egg: these (figs 1g–j, 2e, f and 5d, e) longer than wide, 570–866 (746 ± 99) 338–423 (394 ± 28) μm (*n* = 7), bean-shaped, fresh colour yellow to pale yellow, directly attached to host without peduncle. Polar filament short (figs 1h, j, 2e, f and 5d), situated on middle of operculum; opercular plates forming ring (fig. 5d, e).

Attachment site

Specimens attached on body surface of hosts. Eggs attached on lateral sides of carapaces and on meri of walking legs 2 to 4 (fig. 1a–d, g–j).

Remarks

Damborenea & Cannon (2001) reviewed the species of the genus *Temnocephala* Blanchard, 1849 recorded from South to Central

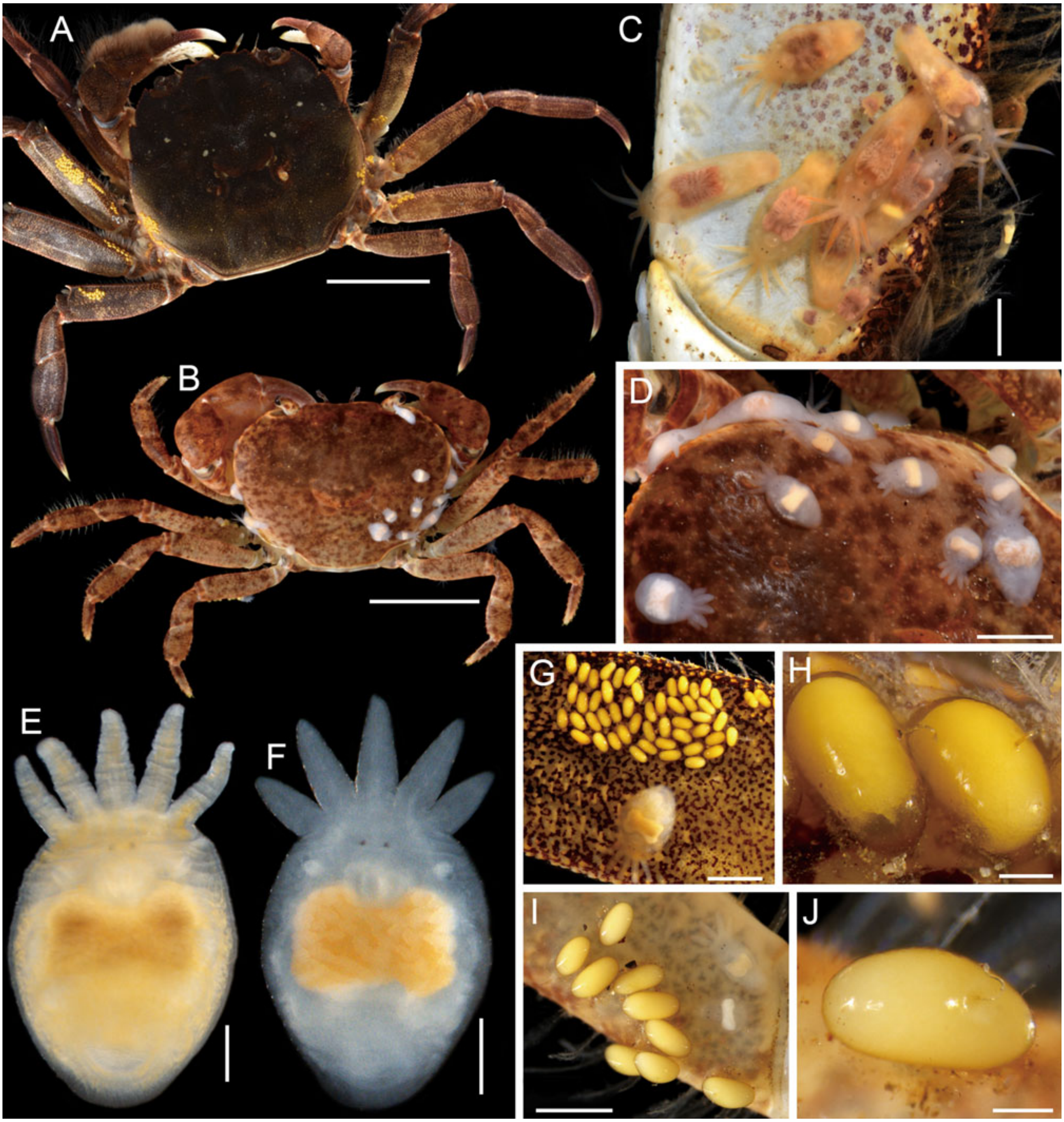

Fig. 1. Live specimens of *Temnosewellia* aff. *vietnamensis* from host crabs, (a, c, e, g, h) specimens or eggs attached on *Eriocheir japonica* (De Haan, 1835) from Oda River, (b, d, f, i, j) specimens and eggs attached on *Geothelphusa exigua* Suzuki & Tsuda, 1994 from a tributary of Kimotsuki River. (a) crab with eggs of *T.* aff. *vietnamensis* on legs; (b) crab with specimens of *T.* aff. *vietnamensis* on carapace; (c, d) specimens on hosts; (e, f) habitus of specimens, dorsal view; and (g–j) specimens and eggs on meri of legs of crabs. Scale bars: 20 mm (a); 5 mm (b); 2 mm (c, d, g); 500 μm (e, f); 200 μm (h, j); and 1 mm (i).

America and defined several synapomorphic characters. In their review, the genus *Temnosewellia* Damborenea & Cannon, 2001 was established based on *T. minor*, which was originally described from Australia, to accommodate 15 species of *Temnocephala* from Australia and Southeast Asia. *Temnosewellia* clearly differs from *Temnocephala* by having the single saddle-shaped post-tentacular syncytium without excretory pores (vs. a pair of ellipsoid post-tentacular syncytia with an excretory pore) (see Damborenea & Cannon, 2001). Subsequently, Cannon & Sewell (2001) reviewed *Temnosewellia* inhabiting the crayfish genus, *Cherax* Erichson, 1846 (Parastacidae), from Australia and described four species of the genus. Sewell *et al.* (2006) added 31 species in their review of *Temnosewellia* from another Australian crayfish genus, *Euastacus* Clark, 1936 (Parastacidae). So far, a total of 52 valid species are recognized in the genus with *Temnosewellia vietnamensis* recently added from Vietnam (Tyler *et al.*, 2006–2020; Damborenea & Brusa, 2009). Since the specimens collected in this study are closely related to *T. vietnamensis*, the species is tentatively identified as *T.* aff. *vietnamensis*. Of those congeners, *T.* aff. *vietnamensis* share a cirrus composed of a cone-shaped shaft and cylindrical introvert

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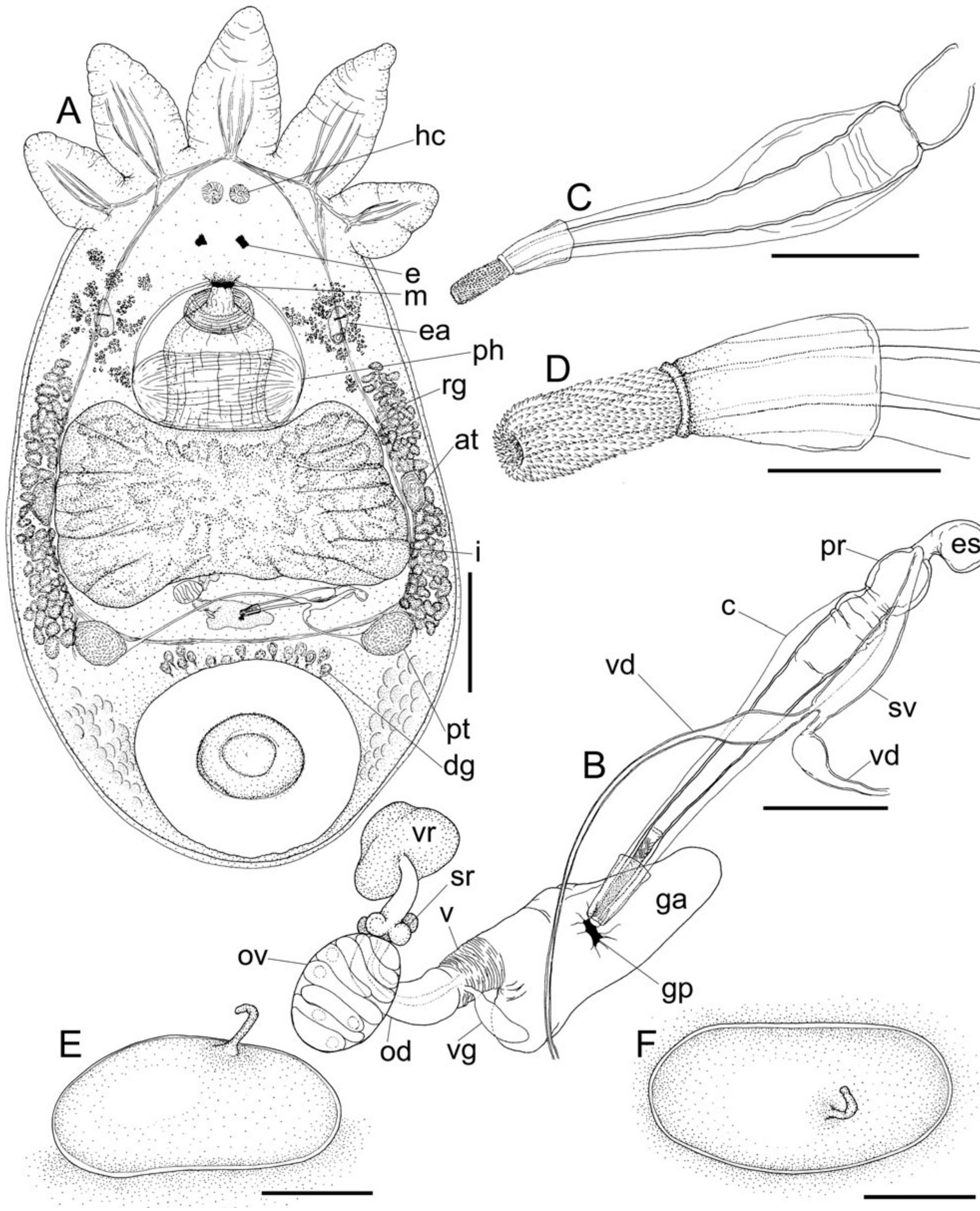

Fig. 2. *Temnosewellia* aff. *vietnamensis*, (a, b) specimen (NSMT-Pl 6494), (c, d) specimen (NSMT-Pl 6494), (e, f) egg (NSMT-Pl 6495). (a) habitus, ventral, hc = Haswell's cell, e = eye, m = mouth, ph = pharynx, i = intestine, ea = excretory ampulla, rg = rhabdite glands, dg = disc glands, at = anterior testis, pt = posterior testis; (b) male and female reproductive systems, ventral, vr = vesicula resorben, sr = seminal receptacle, ov = ovary, od = oviduct, vg = vaginal gland, v = vagina, ga = genital atrium, gp = gonopore, c = cirrus, pr = prostate, es = ejaculatory sac, sv = seminal vesicle, vd = vas deferens; (c) cirrus, ventral; (d) distal portion of cirrus; (e) egg, lateral view; and (f) same, apical view. Scale bars: 400 μm (a); 100 μm (b, c); 40 μm (d); and 300 μm (e, f).

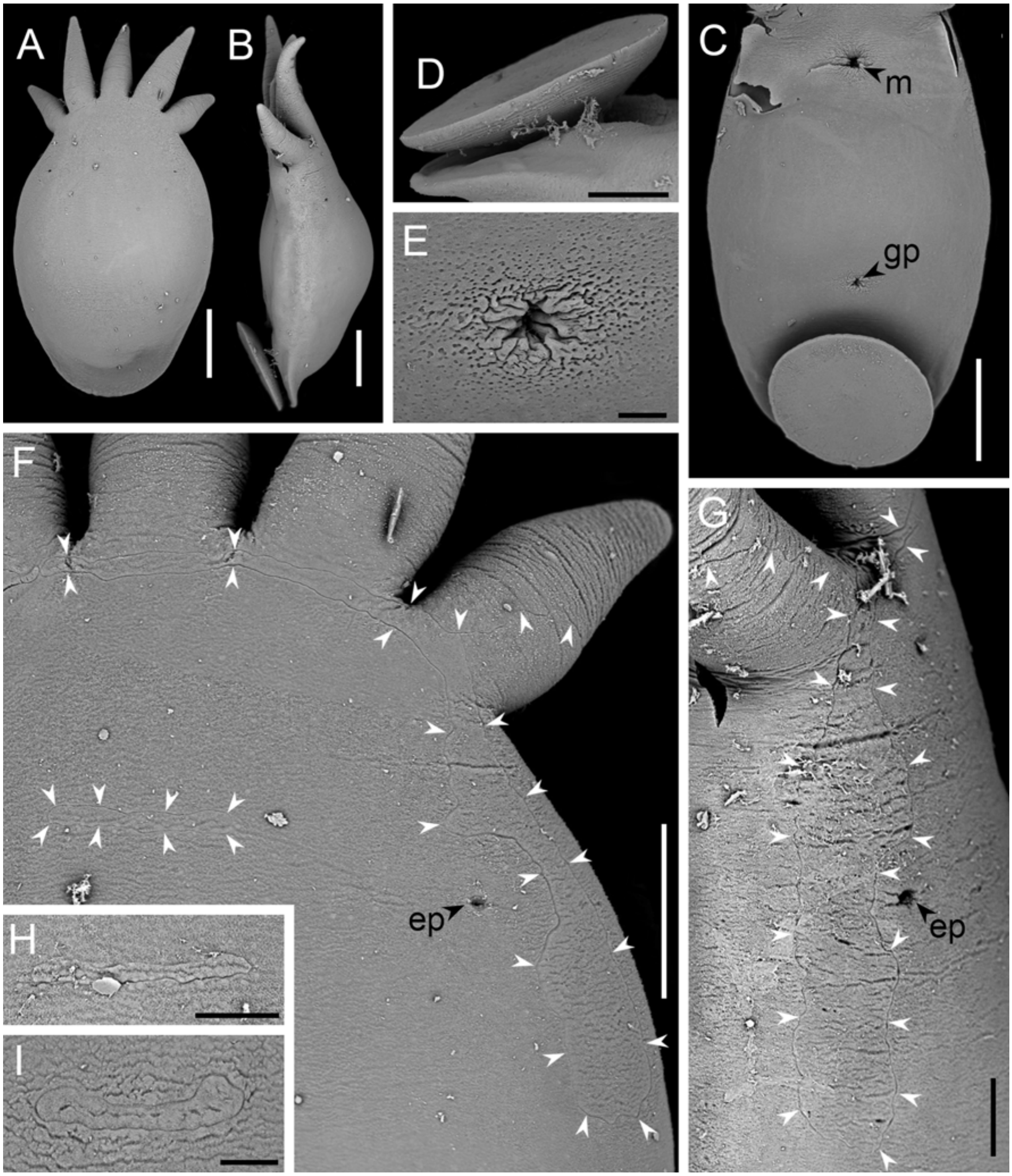

Fig. 3. *Temnosewellia* aff. *vietnamensis*, scanning electron microscope micrographs of specimens, m = mouth, gp = gonopore, ep = excretory pore, white arrowheads indicating edges of syncytial plates, (a–h) specimens attached on *Eriocheir japonica* (De Haan, 1835) from Oda River, (i) specimen attached on *Geothelphusa exigua* Suzuki & Tsuda, 1994 from a tributary of Kimotsuki River. (a) habitus, dorsal view; (b) same, lateral view; (c) habitus without tentacles, ventral view; (d) posterior part of habitus, lateral view; (e) gonopore; (f) partial habitus, right side of anterior part, dorsal view; (g) same, left side of anterior part, lateral view; and (h, i) horizontal bar-shaped syncytium situated posterior to post-tentacular syncytium. Scale bars: 400 µm (a–c); 200 µm (d, f); 40 µm (e); 100 µm (g, h); and 50 µm (i).

covered with fine spinules on the eversible region and the distal opening not or slightly oblique with. *Temnosewellia athertonensis* (Cannon, 1993), *Temnosewellia cita* (Hickman, 1967), *Temnosewellia maculata* Sewell *et al.*, 2006, *Temnosewellia rouxi*, *Temnosewellia semperi* and *T. vietnamensis*. The species differ from *T. athertonensis* and *T. maculata* by lacking the body pigmentation and the length of introvert of cirrus (42–85 µm vs. 15 µm and 96–98 µm, respectively) (see Cannon, 1993; Sewell *et al.*, 2006). The species is differentiated from *T. cita* by having the longer introvert (7–27 µm, see Hickman, 1967). The general shape and measurements of the cirrus of *T.* aff. *vietnamensis* are very similar to that of *T. vietnamensis* (i.e. cirrus length: 137–426 µm vs. 309.4 µm; cirrus width 13–68 µm vs. 73.78 µm; introvert length 42–77 µm vs. 59.26 µm, see Damborenea & Brusa, 2009; this paper). While the spinules on the introvert of *T.* aff. *vietnamensis* are sharp, those of *T. vietnamensis* are relatively small and fine (see Damborenea & Brusa, 2009, fig. 9; this paper, fig. 6). In addition to these, *T.* aff. *vietnamensis* has several minor differences on the female reproductive system from *T. vietnamensis*: the four seminal receptacles present (vs. two); bearing a long, curved oviduct with

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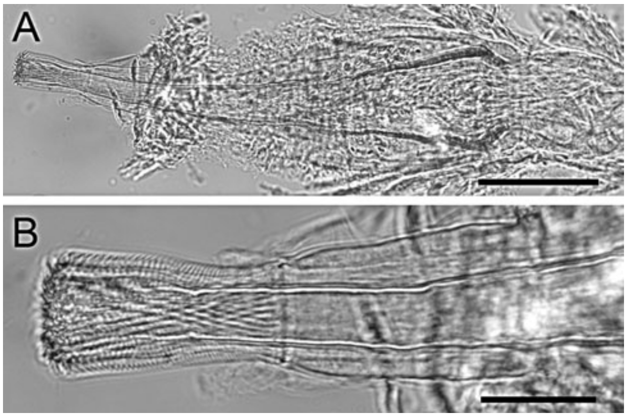

Fig. 4. *Temnosewellia* aff. *vietnamensis*, specimen (NSMT-Pl 6495), cirrus, ventral. (a) General view. (b) Distal portion. Scale bars: 100 μm (a), 30 μm (b).

vaginal gland (vs. a short oviduct without visible vaginal gland) (see Damborenea & Brusa, 2009, fig. 1b), but it is considered to be insufficient to separate those species clearly. The two congeners, *T. rouxi* and *T. semperi*, were regarded as similar species with *T. vietnamensis* by Damborenea & Brusa (2009) but the length of cirrus and introvert of those were not shown in the previous descriptions (see Semper, 1872; Weber, 1889; Merton, 1913, 1914; Cannon, 1991). *Temnosewellia* aff. *vietnamensis* is also similar to *T. semperi* but is distinguished by having the cylindrical introvert (vs. slightly dilated, i.e. scoop-shaped, see Semper, 1872; Weber, 1889). It is difficult to compare the morphology of cirrus to that of *T. rouxi* because the previous descriptions are elementary. In the previous descriptions of *T. rouxi*, a post-tentacular arc composed of two paired large gland cells are present (Merton, 1914; Cannon, 1991) while only one pair of the Haswell's cells are observed on *T.* aff. *vietnamensis* as well as *T. vietnamensis*. From Kagoshima, southern Japan, *T. minor* was recorded on the Australian crayfish *C. tenuimanus* from the culture ponds but its cirrus with a distinctly inflated introvert is clearly differentiated from that of *T.* aff. *vietnamensis* (see Oki *et al.*, 1995; Cannon & Sewell, 2001; this paper).

Molecular phylogeny

Monophyletic clades were formed by the phylogenetic analysis based on partial sequences of both 28S and COI of *T.* aff. *vietnamensis* (fig. 6). Small differences in nucleotide sequences were shown between specimens from Oda River (LC651446, LC651447 and LC651452–LC651454) and a tributary of Kimotsuki River (LC651441, LC651443–LC651445 and LC651448–LC651451). These localities are on adjacent but unconnected rivers, and the distance between them is approximately 7 km. The geographical variations among them were one base substituted within the 741 bases of 28S, and 24 bases substituted within the 569 bases of COI (amino acid substitution was observed in one residue between sites. One more residue was substituted in the single individual from Oda River). They constituted species-specific clades from other congeners.

Discussion

Phylogenetic position of T. *aff. vietnamensis with other temnocephalids*

Hoyal Cuthill *et al.* (2016) conducted the phylogenetic analyses using 28S and COI with 23 species of *Temnosewellia* associated

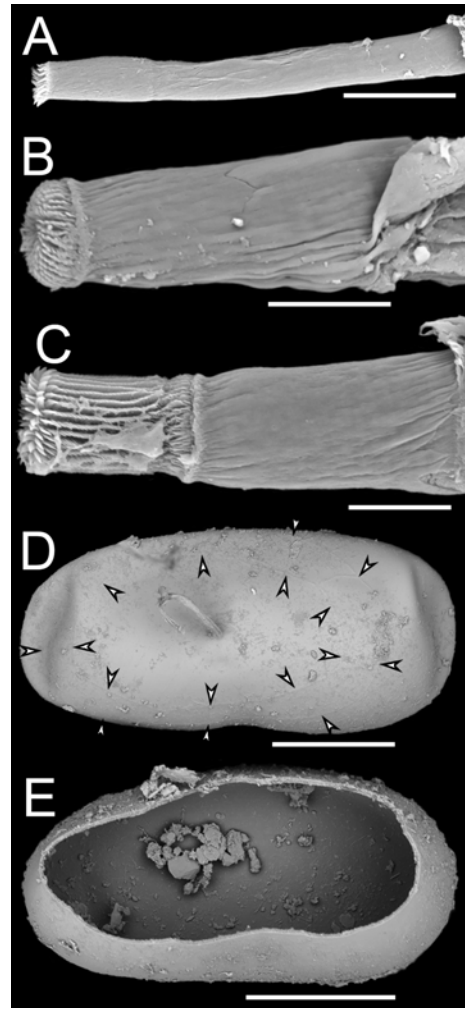

Fig. 5. *Temnosewellia* aff. *vietnamensis*, scanning electron microscope micrographs of cirri and eggs. (a) distal portion of cirrus with spined region of introvert not exposed; (b) distal portion of cirrus with spined region of introvert slightly exposed; (c) distal portion of cirrus, spined region of introvert exposed; (d) egg attached on host, arrowheads indicating both outer and inner borders of opercular plates, apical view; and (e) egg shell hatched along outer margin of opercular plates, apical view. Scale bars: 40 μm (a); 20 μm (b, c); and 500 μm (d, e).

with the crayfishes of *Euastacus* Clark, 1936 from eastern Australia and *T. minor* and *T. dendyi* as outgroups. In our analyses using *Diceratocephala boschmai* Baer, 1953 as an outgroup, the clade of *T.* aff. *vietnamensis* was located between those of the 23 species and *T. minor* and *T. dendyi* in 28S (fig. 6). Two sequences registered as *T. fasciata* (KC869888) and *T. minor* (AY157164) in GenBank are doubted to possibly be *T. minor* and *T. dendyi*, respectively (see Vecchioni *et al.*, 2021). In COI, the clade of *T.* aff. *vietnamensis* formed another clade with *T. minor*. While the monophyletic clades were formed by *T.* aff. *vietnamensis* both in the trees of 28S and COI (fig. 6), the almost sequences used in the phylogenetic analysis are of congeners from eastern Australia obtained by Hoyal Cuthill *et al.* (2016). Although *T.* aff. *vietnamensis* is morphologically similar to Asian species, that is, *T. semperi* and *T. vietnamensis*, there is no genetic data published for these species. Further phylogenetic analysis is needed to reveal the phylogenetic relationship among Asian species. Along with this, as Vecchioni *et al.* (2021) pointed out, sequences available in the public

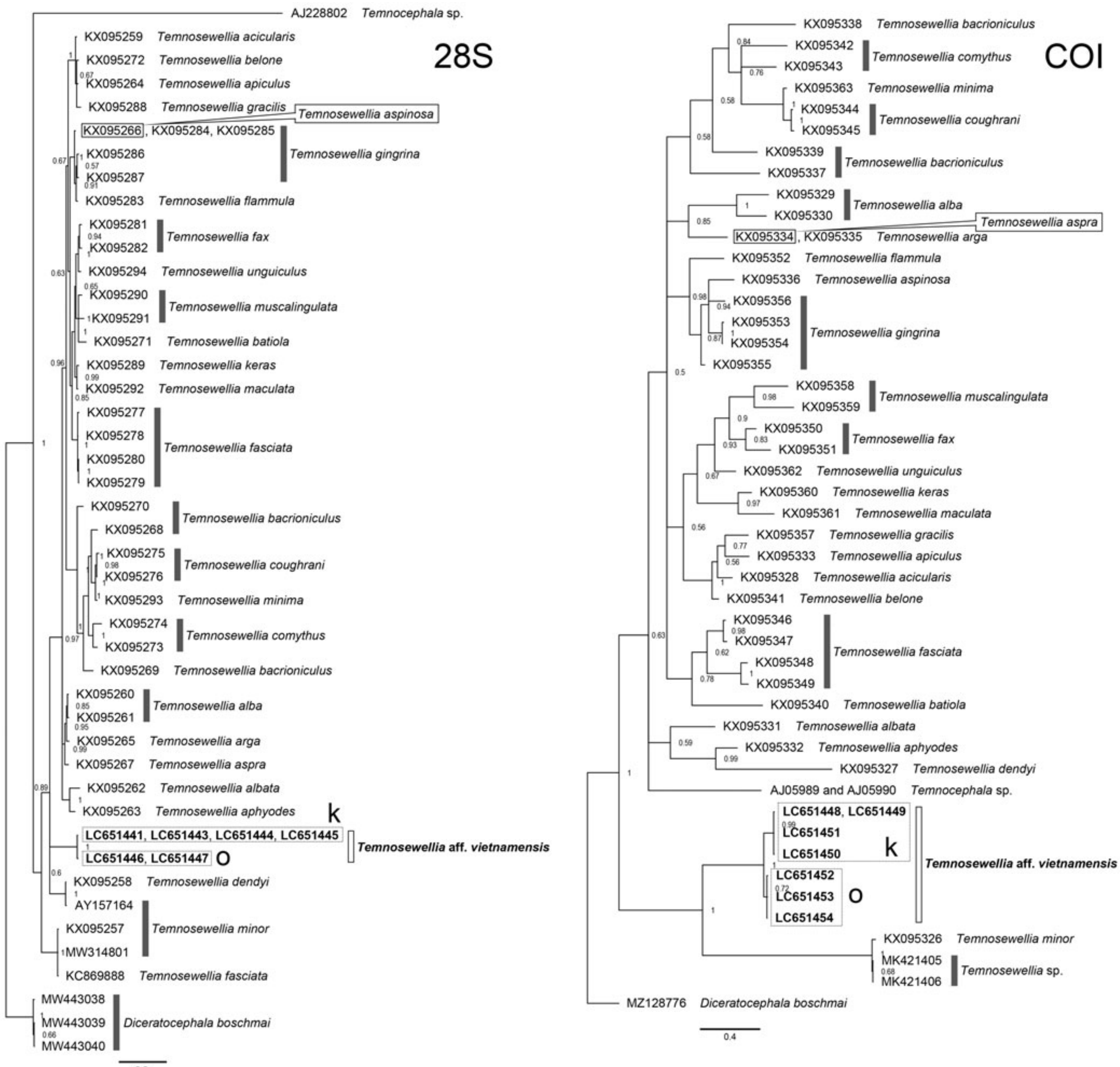

Fig. 6. Bayesian inference trees for the genus *Temnosewellia* derived from the analysis of fragments of nuclear 28S rDNA (556–596 base pairs (bp)) and cytochrome c oxidase subunit I (COI) (526 bp) sequences. *Diceratocephala boschmai* (accession numbers MW443038–443040 and MZ128776) was used as an outgroup for rooting the trees. The posterior probability values are indicated on the branches. The scale bars correspond to the substitutions per nucleotide site. *k* = sequences of *Temnosewellia* aff. *vietnamensis* from a tributary of Kimotsuki River. o = sequences of *T.* aff. *vietnamensis* from Oda River. COI sequences, AJ05989 and AJ05990, were combined to be used for the analysis.

databases must correspond to the comprehensive and systematic revision of temnocephalans.

In this study, the specimens of *T.* aff. *vietnamensis* collected from two adjacent rivers which belong to different water systems show the geographical variations for both 28S and COI. These might indicate that the individuals do not migrate between rivers with hosts via land or sea while *Eriocheir japonica* is catadromous and migrate downstream into the sea to mate and lay eggs (e.g. Kobayashi, 1998). In the future study, it will be necessary to be confirmed with sequences of a larger number of individuals.

Records of Temnosewellia *in Japan*

The previous records of the species which seems to be the members of the genus *Temnosewellia* from East to South Asia are plotted in fig. 7. In southern Japan, since the record of a species of *Temnocephala* from Okinawa Island by Okada (1938), its unidentified congeners have been reported several times in various localities. From Iriomote and Tanegashima Islands, the Ryukyu Islands, species of *Temnocephala* were recorded from *Geothelphusa marginata fulva* Naruse, Shokita & Shy, 2004 (=*G. miyazakii*) (Potamidae)

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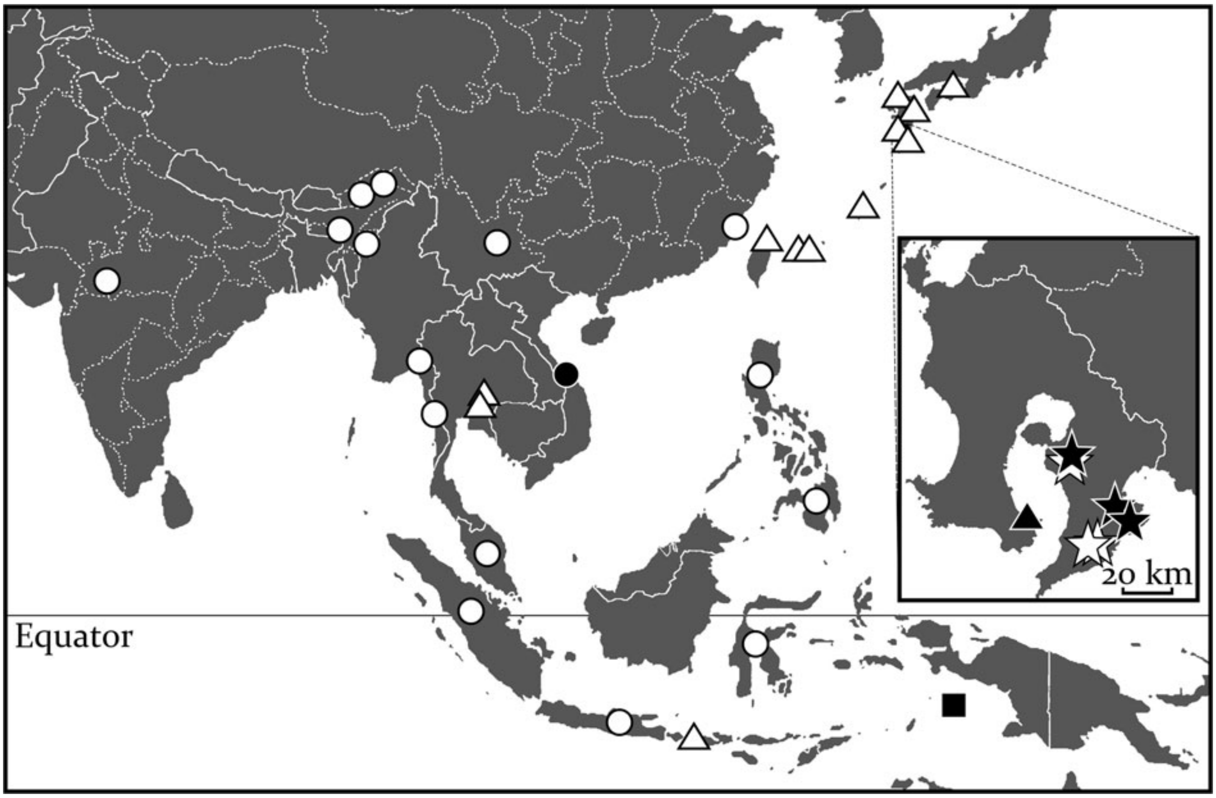

Fig. 7. Map of East to South Asia showing the distributional records of the genus *Temnosewellia* Damborenea et Cannon, 2001. Black filled star = *Temnosewellia* aff. *vietnamensis* newly collected from the Osumi Peninsula, Kagoshima, Japan in this study; open star = same from the Osumi Peninsula, Kagoshima, Japan by Okawachi *et al.* (2013); black filled triangle = *Temnosewellia minor* from Ibusuki, Kagoshima, Japan (Tamura *et al.*, 1985; Oki *et al.*, 1995); open circle = *Temnosewellia semperi* from Sumatra, Java, and various sites of Sulawesi, Indonesia (Weber, 1889), Luzon and Mindanao, Philippines (Semper, 1872), Malaysia (Rohde, 1966; Cannon, 1991), Dawna Hills and Dawei, Myanmar (Gravely, 1913), Narmada River in Madhya Pradesh, Jaintia Hills, and Manipur Hills, India (Gravely, 1913; Chauhan & Ramakrishna, 1953), Yembung and Siyom Rivers in Abor Country (Wood-Mason, 1875; Gravely, 1913; Chauhan & Ramakrishna, 1953), and Yunnan and Foochow, southern China (Gravely, 1913; Lee, 1936); black filled square = *Temnosewellia rouxi* from Aru Island, Indonesia (Merton, 1914); black filled circle = *Temnosewellia vietnamensis* from Quangnam, Vietnam (Damborenea & Brusa, 2009); and open triangle = unidentified species of *Temnosewellia* from Tokushima, Saga, Miyazaki, Kagoshima, Tanegashima Island, Okinawa Island, Ishigaki Island, and Iriomote Island, Japan (Okada, 1938; Suzuki *et al.*, 1983; Shimazu, 2003; Iwata *et al.*, 2004; Kawakatsu *et al.*, 2007a), Taipei, Taiwan (Okada, 1938), Lombok, Indonesia (Ng & Anker, 2016), and eastern Thailand (Ngamniyom *et al.*, 2019).

and *E. japonica*, respectively (Suzuki *et al.*, 1983). Although Kawakatsu *et al.* (2004) listed the species as *Temnosewellia semperi* (=*Temnocephala semperi*), the authors also noted that it was not identified based on the specimens. Shimazu (2003) reported a finding of a species of temnocephalid that might be *T. semperi* associated with *E. japonica* from Tokushima Prefecture [listed as *Temnosewellia semperi* (Weber, 1889)? of Shikoku by Kawakatsu *et al.* (2007a)]. Iwata *et al.* (2004) recorded a species of *Temnocephala* associated with *E. japonica* from the northern part of Miyazaki Prefecture, and the authors implied the possibility of immigration of the species to Saga Prefecture. Further, Kawakatsu *et al.* (2007a) noted the findings of the congeners associated with *G. minei* Shy & Ng, 1998 and *E. japonica* from Ishigaki Island in the Ryukyu Islands and unspecified localities in Kagoshima Prefecture, respectively. Damborenea & Cannon (2001) reviewed the species of *Temnocephala* from Central to South America, and transferred all species previously assigned to *Temnocephala* in Australia and Southeast Asia to *Temnosewellia*. Since then, the distribution of all valid species of *Temnocephala* is restricted from Central to South America, and thus it is reasonable that the species previously reported from Japan are considered to be members of *Temnosewellia*. For the species level identification, re-examinations of all the previous records from the Ryukyu Islands, Kyushu and Shikoku based on the specimens are required. Specimens found from *E. japonica* and *G. exigua* in Kagoshima were provisionally identified as *Temnosewellia* sp. (Okawachi *et al.*, 2013), but they are considered to be *T.* aff. *vietnamensis*. Additionally, an alien species *T. minor* has been recorded from the culture ponds of the crayfish *C. tenuimanus* in Ibusuki, Kagoshima (Tamura *et al.*, 1985; Oki *et al.*, 1995; Kawakatsu *et al.*, 2007a, b).

Species diversity of Temnosewellia *from East to South Asia*

Previous records of the species of *Temnosewellia* in East to South Asia are shown in fig. 7. *Temnosewellia* aff. *vietnamensis* is similar to *T. semperi* as well as *T. vietnamensis*, which are known from freshwater crabs in tropical Asian countries. *Temnosewellia semperi* was originally described from Indonesia (i.e. Sumatra, Java and various sites of Sulawesi) based on the specimens associated with freshwater crabs [reported as species of *Telphusa* by Weber (1889)]. The species is now known from various freshwater crabs in Luzon and Mindanao, Philippines (Semper, 1872) and Malaysia including *Stoliczia rafflesi* (Roux, 1936) (=*Potamon rafflesi*) (Rohde, 1966; Cannon, 1991). *Temnosewellia semperi* has been found from *Barytelphusa lugubris* (Wood-Mason, 1871) (=*Paratelphusa* (*Barytelphusa*) *lugubris*), *Tiwaripotamon adiatretum* (Alcock, 1909) (=*Po. adiatretum*), *Indochinamon andersonianum* (Wood-Mason, 1871) (=*Po. andersonianum*), *Po.* (*Potamon*)

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manii (Rathbun, 1904) (=*Po. manii*), and *Alcomon superciliosum* (Kemp, 1913) (=*Po. superciliosum*) which were caught from Myanmar, India, southern China, and their border, Abor Country (Gravely, 1913; Lee, 1936; Chauhan & Ramakrishna, 1953). Furthermore, Gravely (1913) noted that *T. semperi* is often found from the members of *Geothelphusa* Stimpson, 1858 (=*Geotelphusa*) and *Potamon* Savigny, 1816 in large numbers. He also mentioned that a specimen which was accidentally attached to fishes from Daphla Hill, Abor Country and was identified as *Temnocephala chilensis* (Moquin-Tandon, 1846) by Wood-Mason (1875) was *T. semperi*. Recently, Ng & Anker (2016) reported a species that was possibly a member of *Temnosewellia* when the authors described the freshwater crab *Sundathelphusa tuerkayi* Ng & Anker, 2016 (Gecarcinucidae) from Lombok, Indonesia. Considering the locality and host, this species might be *T. semperi* or a closely related species. If all of those records are of true *T. semperi*, the facts mean that the species is widely distributed from isolated islands to Eurasian continent in tropical to temperate Asia. However, *T. vietnamensis* and *T.* aff. *vietnamensis*, which are similar to *T. semperi*, were found from Vietnam and Japan, respectively (Damborenea & Brusa, 2009; this paper). Furthermore, no species of the host that has such a wide distribution range is known. Based on these facts, it is doubtful that all of the records so far were conspecific. Re-examinations of previous records of temnocephalids identified as *T. semperi* are needed based on the specimens (see Kawakatsu *et al.*, 2007a), and redescription of the species is also strongly required. There are other records of the congener. From Taiwan, Okada (1938) found a congener from Yanmingshan, Taipei, and he mentioned that the species is conspecific to the unidentified congener from Okinawa Island, Japan. In eastern Thailand, an unidentified species of *Temnosewellia* was found from the red claw crayfish *Cherax quadricarinatus* von Martens, 1868 introduced from Australia (Ngamniyom *et al.*, 2019). Together with the host, the species of *Temnosewellia* might be invaded. Just as Ngamniyom *et al.* (2019) claimed that the species genetically similar to *T. minor* and *T. fasciata*, the species was formed a clade with *T. minor* by the partial sequences of COI in this study (fig. 6). Damborenea & Brusa (2009) suggested that great numbers of potential hosts of undescribed temnocephalids are present in Southeast Asia. Nevertheless, the freshwater crabs in tropical to temperate regions of East to South Asia are actually highly diverse, only four native species including *T.* aff. *vietnamensis* so far being recognized. Discoveries of many undescribed species in the regions from future studies using morphology and molecular characterization are expected.

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Conflict of interest. None.

Ethical standards. The authors assert that all procedures contributing to this study comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals. This study is not related to human experimentation.

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