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New species of *Sargassum*-boring *Limnoria* Leach, 1814 (Crustacea, Isopoda, Limnoriidae) from Japan

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Abstract

A new marine *Sargassum*-boring species of *Limnoria* (Limnoriidae) is described on the basis of specimens obtained at Kamogawa-shi, Chiba Prefecture, Japan. *Limnoria aspera* **sp. nov.** shares a reduced mandibular palp to a seta, algal-feeding, and the clavate shaped epipod of the maxilliped with the other species of non-mandibular-palp group. *L. aspera* **sp. nov.** differs morphologically from the congeneric species by secondary unguis of pereopods and unique carinae of pleonite 5 and pleotelson. We describe the sequences of the mitochondrial COI gene and the nuclear 28S rDNA gene. *L. aspera* **sp. nov.** differs by 14.2–18.0% in *p*-distance based on COI sequences from other Japanese species, *L. furca* and *L. nagatai*.

Key words: *Limnoria aspera* **sp. nov.**, Pacific Ocean, COI, 28S rDNA, taxonomy

Introduction

The isopod family Limnoriidae is represented by 3 genera: *Limnoria* Leach, 1814, *Lynseia* Poore, 1987, *Paralimnoria* Menzies, 1957 (Cookson 1991; Cookson & Poore 1994). *Limnoria* includes wood, algae, and seagrass feeders (Cookson 1991; Yoshino *et al.* 2017). Until recently, 3 species of algal-feeding *Limnoria* are recorded in Japan; *Limnoria furca* Yoshino & Ohsawa, 2019, which bore into the holdfasts of the brown kelp *Eisenia bicyclis* along the Sea of Japan coasts: *L. nagatai* Nunomura, 2012 which bores into the holdfasts of *E. bicyclis* and *E. nipponica* along the Pacific coasts; and *L. segnoides* Menzies, 1957, which was collected from the red alga *Corallina* at Misaki, Kanagawa Prefecture (Menzies 1957; Nunomura 2012; Yoshino *et al.* 2018; Yoshino & Ohsawa 2019).

We collected *L. aspera* **sp. nov.** from the holdfasts of the brown kelp, *Sargassum*, in the subtidal zone along the Pacific coast of Japan. The holdfasts of some kelps of *S. ringgoldianum* and *S. siliquastrum* were hollowed out by their feeding activity. This study presents the description of this new species based on morphological characters and the sequences of the mitochondrial COI genes and the nuclear 28S rDNA genes. Specimens we collected are deposited with the Chiba Museum, Zoology, Crustacea (CBM-ZC), Chiba Prefecture, Japan.

Materials and methods

Sargassum ringgoldianum and *S. siliquastrum* were collected using a knife and scissors, from the subtidal zone (0–1 m in depth) off the Marine Biosystems Research Center, Kamogawa-shi, Chiba Prefecture, Japan. The algal samples were taken immediately to the laboratory.

The individuals of *Limnoria* were picked out from the holdfasts with tweezers in the laboratory and fixed in 99.5% ethanol. After DNA extraction, we stored the specimens in 70% ethanol. Morphological observations and drawings were prepared with dissection microscope (SZX10; Olympus, Tokyo, Japan). Some samples were

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observed under SEM (JSM-6010LA; JEOL, Tokyo, Japan) and Keyence digital microscope VHX-7000 with SEM head system VHX-D500/VHX-D510 (Keyence, Osaka, Japan).

To preserve the body of the collected *Limnoria* for morphological observations, total DNA was extracted using a nondestructive chloroform extraction method as described by Yoshino *et al.* (2018). DNA extraction solution with 100 µL SNET buffer (SDS 0.3%, NaCl 400 mM, EDTA 5 mM, Tris-HCl pH 8.0 20 mM) + 2 µL proteinase K (200 µg/mL proteinase K) was prepared per individual and incubated overnight. The Cytochrome c oxidase I (COI) region of mitochondrial genes was amplified using the primers LCO1718 (5′- TW GGD GCN CCD GAY ATG GCH TTY CCD CG -3′) and HCO2386 (5′- AA AAT TTT AAT TCC AGT AGG AAC TGC AAT AAT TAT -3′) (Yoshino *et al.* 2018) and PCR was carried out in a thermocycler using the following profile: initial denaturation phase of 2 min at 95 °C; 41 cycles of 50 s at 95 °C, 1 min 30 s at 45 °C, 1 min 30 s at 72 °C; final extension step of 10 min at 72 °C. The 28S rRNA was amplified using the primers 28SniphF1 (5′- CAAGTACCGTGAGGGAAAGTT -3′) and 28SniphR1 (5′- GTTCACCATCTTTCGGGTC -3′) (Lefébure *et al.* 2006) and PCR was carried out using the following profile: initial denaturation phase of 10 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 57 °C, 2 min at 72 °C; final extension step of 2 min at 72 °C (Haye *et al.* 2012). The PCR products were purified by the enzymatic method with ExoSAP-IT (USB Corporation, Cleveland, OH, USA) modified from Dugan *et al.* 2002. Namely, 10 µL of PCR product mixed with 0.2 µL of ExoSAP-IT and 1.8 µL of Milli-Q water were incubated with 1 U of each enzyme at 37 °C for 30 min. The enzymes were inactivated at 80 °C for 15 min, and the PCR products were stored at –20 °C. Purified DNA was quantified with a concentration marker and sequenced with a BigDye Terminator v3.1 Sequencing Standard Kit (Applied Biosystems, Foster City, CA, USA). The obtained electropherograms were verified and nucleotide sequences were aligned manually by using MEGA5 (Tamura *et al.* 2011). The genetic distances between sequences were calculated by the *p*-distance method using MEGA5. The sequences from *L. chilensis* (Acc. No. FJ541244–FJ541250), *L. furca* (Acc. No. LC146617–LC146632), *L. furca* misidentified as *L. segnoides* in Song & Min (2017) (Acc. No. KX171203, KX171204), *L. nagatai* (Acc. No. LC146527–LC146616), *L. quadripunctata* (Acc. No. FJ541253–FJ541265) and *L.* sp. (Acc. No. FJ541251, FJ541252) were used for the genetic analyses of COI, and *L. chilensis* (Acc. No. FJ611318, FJ611320), *L. quadripunctata* (Acc. No. FJ611321) and *L.* sp. (Acc. No. FJ611319, FJ611322, FJ611323) sequences for 28S rRNA. The sequence alignment was carried out with MEGA5, and Kakusan4 (Tanabe 2011) was used for the substitution model selection. RaxML (Stamatakis 2014) was used to create the maximum likelihood (ML) tree, where the bootstrap values were calculated with a trial frequency of 1,000. The sequence of *Armadillidium vulgare* (Acc. No. EF643519) was used as outgroup.

Taxonomy

Limnoriidea Poore, 2002

Limnoriidae White, 1850

Genus *Limnoria* Leach, 1814

Limnoria aspera Yoshino & Ohsawa, sp. nov.

Figs 1–8

Material examined. *Holotype*: male 2.6 mm, Kamogawa-shi, Chiba prefecture, Japan, 35°07’N, 140°10’E, subtidal zone (0–1 m in depth), *Sargassum ringgoldianum* holdfasts, Takeshi A. Ohsawa and Hiroki Yoshino, 9 September 2020 (CBM-ZC 16564).

***Paratypes*:** male 2.8 mm, Kamogawa-shi, Chiba prefecture, Japan, 35°07’N, 140°10’E, subtidal zone (0–1 m in depth), *Sargassum ringgoldianum* holdfasts, Yuriko Kambara, 14 July 2014 (CBM-ZC 16565). male 2.7 mm, Kamogawa-shi, Chiba prefecture, Japan, 35°07’N, 140°10’E, subtidal zone (0–1 m in depth), *Sargassum siliquastrum* holdfasts, Takeshi A. Ohsawa and Hiroki Yoshino, 9 September 2020 (CBM-ZC 16566). male 3.2 mm, Kamogawa-shi, Chiba prefecture, Japan, 35°07’N, 140°10’E, subtidal zone (0–1 m in depth), *Sargassum siliquastrum* holdfasts, Takeshi A. Ohsawa and Hiroki Yoshino, 9 September 2020 (CBM-ZC 16567).

Type locality. Kamogawa-shi, Chiba Prefecture, Japan.

Description. *Body* oblong, dark yellow to light brown in 70% ethanol (Figs. 1, 2A). *Head* almost globular.

Eyes black in color, each with 8 ommatidia. *Pleonite* composed of 5 distinct segments. Pereonal segment 1 longest, approximately 1.5–1.7 times longer than segment 2 (Fig. 1), with 1 thinly V-shaped groove. Segments 2–4 subequal length. Posterior pereonal segments 4–7 progressively shorten. Coxal plates of pereonal segments 2–4 rectangular in shape and those of posterior segments prolonged acutely at posterior angle. *Pereonites 6, 7* and pleonites 1 to 4 each with transverse row of many small setae.

Pleonite 5 approximately 0.6 times as long as pleotelson (Figs 2, 3A). Pleonite 5 dorsomedially with Y- or I-shaped indistinct longitudinal carina on which few scale spikes form line (Figs 2B, 3A). Pleonite 5 covered with fused scales, which make tile mosaics (Fig. 2C).

FIGURE 1. *Limnoria aspera* **sp. nov.**, paratype (male, 3.2 mm, CBM-ZC 16567). Body; dorsal view. Stereo microscope image (SZX10; Olympus). Scale bar: 1 mm.

Pleotelson 0.6–0.8 times as long as wide, medially with 1 tubercle on which few scale spikes follow its line (Figs 2, 3A). Pleotelson covered with fused scales, which present tiled pattern on surface (Fig. 2C). Lateral crests and posterior margin of pleotelson margined with sets of about 2–5 directed upward scale spikes. Posterior edge of pleotelson with fringe of long sheathed setae and many short setae (Fig. 3B).

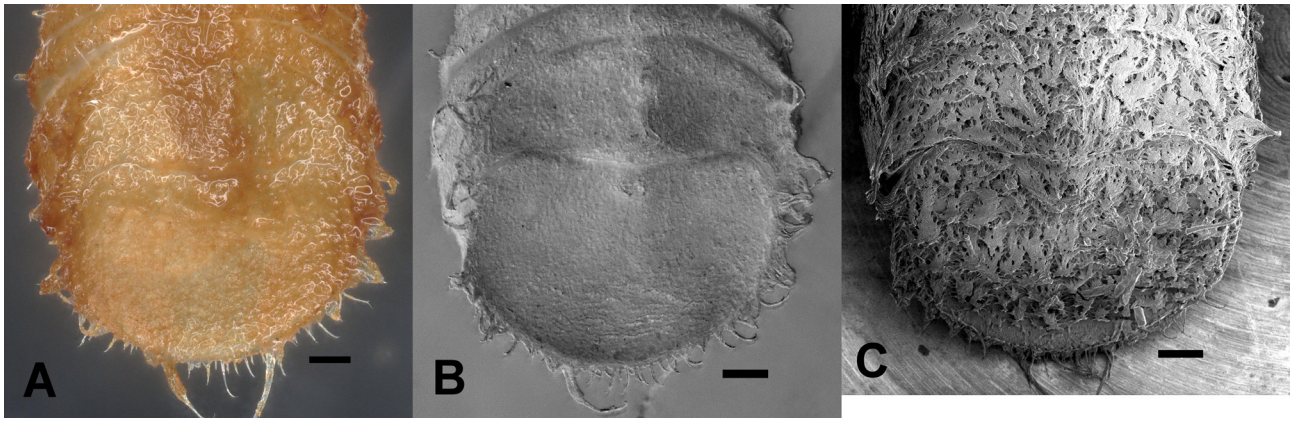

FIGURE 2. *Limnoria aspera* **sp. nov.**, A–B, holotype (male, 2.6 mm, CBM-ZC 16564); C, paratype (male 2.8 mm, CBM-ZC 16565). A, pleotelson, dorsal view, Optical microscope image (VHX-7000; Keyence); B, pleotelson, dorsal view, 3D digital microscope image (VHX-7000; Keyence); C, pleotelson, dorsal view, SEM image (JSM-6010LA; JEOL). Scale bars: A–C = 0.1 mm.

Antenna 1 with 4 flagellar articles; second article with 7 aesthetascs (Fig. 3D). Flagellum of antenna 2 with 4 articles (Fig. 3E).

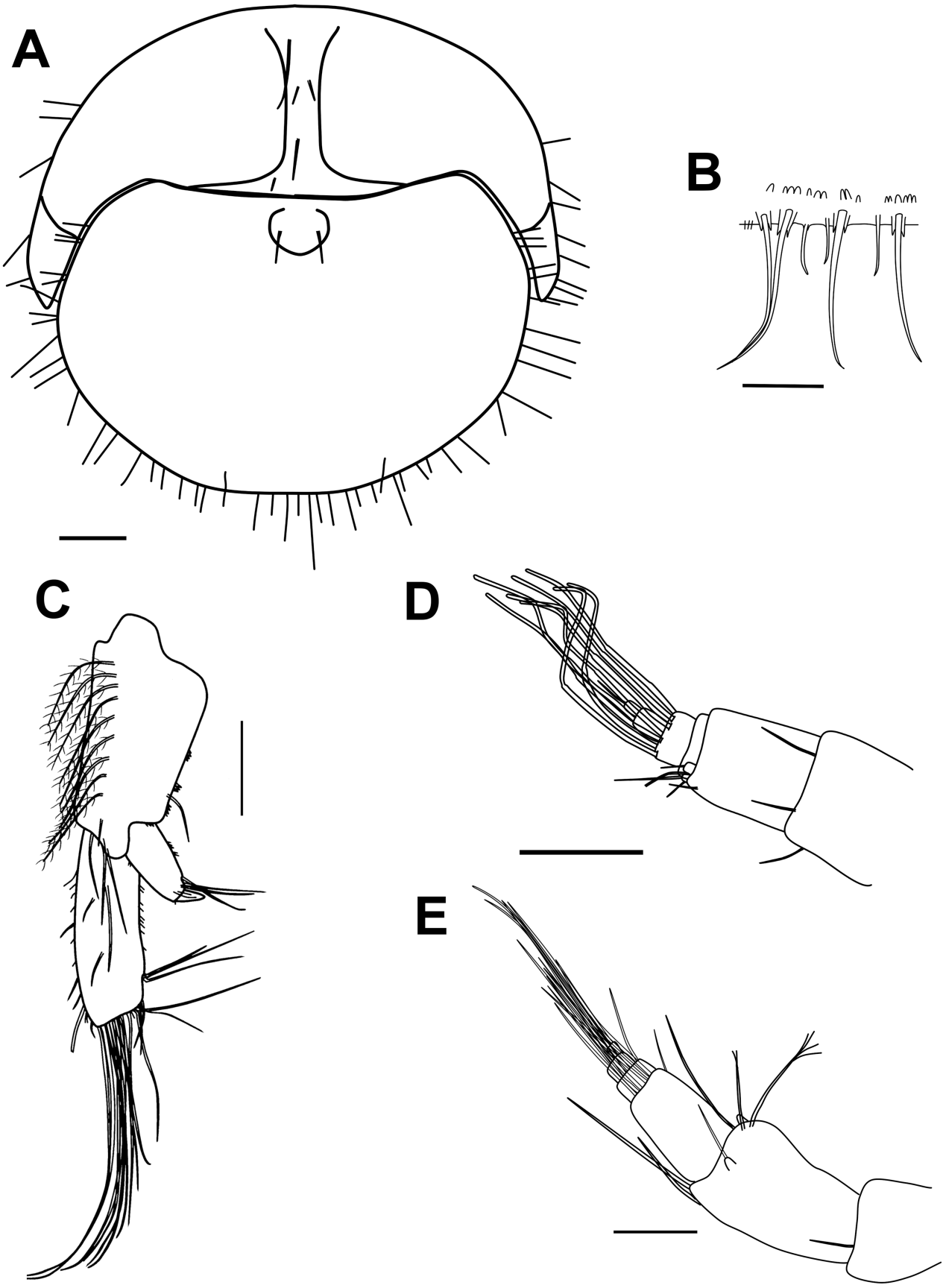

FIGURE 3. *Limnoria aspera* **sp. nov.**, holotype (male, 2.6 mm, CBM-ZC 16564). A, pleonite 5 and pleotelson; B, posterior margin of pleotelson; C, uropod; D, antenna 1; E, antenna 2. Scale bars: A, C–E = 0.1 mm, B = 0.05 mm.

Mandibular palp lacking, replaced by single long stout seta (Figs 4A, B). Mandibular incisors lack rasp and file (Figs 4A–B, 8A). Lacinia mobilis of right mandible branched at intermediate point, branches gradually curving 90-degrees and serrated on anterior side (Fig. 4B). Posterior branch almost same length as anterior branch. Lacinia mobilis of left mandible with 1 serrated seta.

Epipod of *maxilliped*, clavate, approximately 3 times as long as wide, reaching articulation of palp, with simple true setae (Fig. 4E).

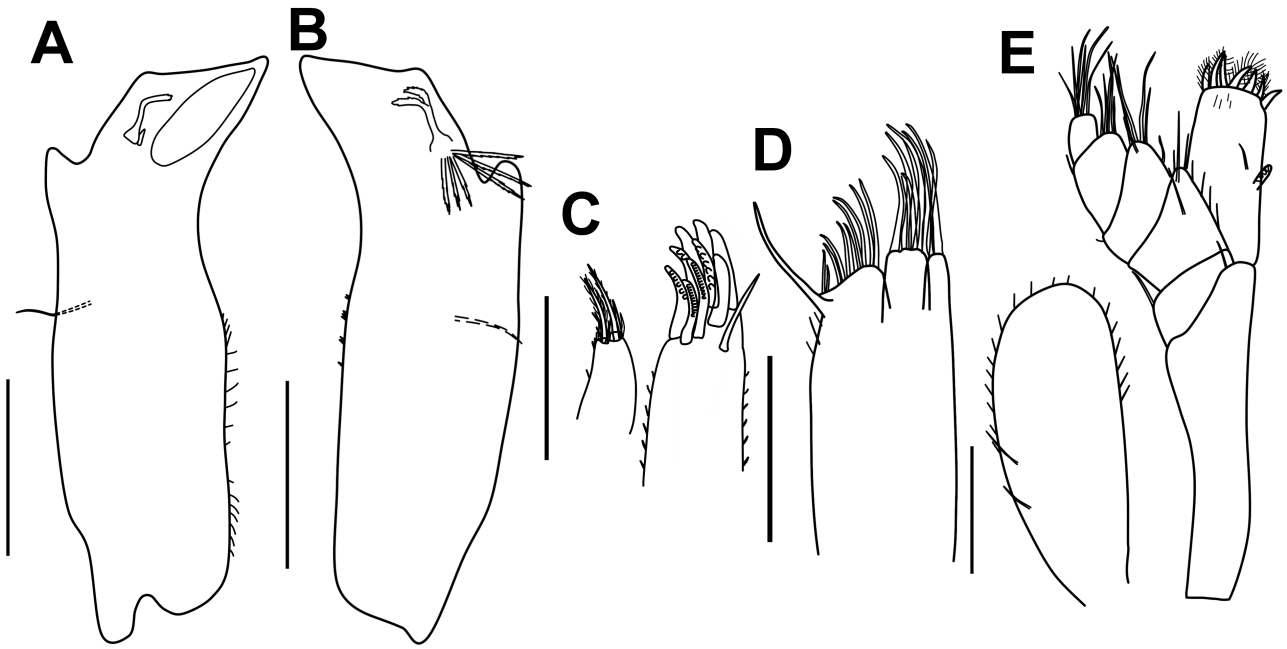

FIGURE 4. *Limnoria aspera* **sp. nov.**, holotype (male, 2.6 mm, CBM-ZC 16564). A, left mandible; B, right mandible; C, maxilla 1; D, maxilla 2; E, maxilliped. Scale bars: A–E = 0.1 mm.

Secondary unguis on *pereopod* 1 undivided, without spinule or with 1–7 spinules (Fig. 5A). Secondary unguis on pereopods 2–6 undivided (Figs 5B, D, 6A–B) or slightly bifid (Fig. 5C). Secondary unguis on pereopod 7 undivided with 4–7 spinules (Fig. 6C). Ventral comb seta absent on merus and present on carpus of pereopods 6 and 7. Propodus of pereopods 3–5 with prominent barbed projection opposing secondary unguis, projection reduced on pereopod 2 (Figs 5, 6, 8B).

Pleopod 2 with plumose setae up to 0.9 times length of exopod (Fig. 7B). Appendix masculina long, reaching beyond endopod tip, articulating near midlength of endopod. Endopod of pleopod 5, oval (Fig. 7C). Peduncle of pleopod 5 with simple seta laterally. Peduncles with coupling hook sequence 32220.

Uropod exopod with laterally recurved apical claw (Figs 3C, 8C). Row of simple long setae on endopod placed apically. Uropod peduncle about 1.1 times as long as endopod, with many short simple setae and row of plumose setae. Exopod about 0.4–0.5 times as long as peduncle.

Molecular data. We deposited the nucleotide sequences of COI region of mitochondrial genes (Acc. no. LC610781, LC610782) and 28S rRNA of nuclear genes (Acc. no. LC612562, LC612563) obtained from the holotype and paratype in GenBank.

Substrate. *Sargassum ringgoldianum*, *S. siliquastrum* holdfasts.

Distribution. Known only from the type locality.

Etymology. From the Latin *aspera* (= rough; Gender feminine), referring to the rough exoskeleton on pleonite 5 and pleotelson.

Remarks. Mandibular palp of *Limnoria aspera* **sp. nov.** is reduced to a seta. This feature is also found in 7 species: *L. bacescui*, *L. bituberculata*, *L. furca*, *L. nagatai*, *L. segnoides*, *L. uncapedis* and *L. zinovae* (Cookson 1991; Pillai 1957; Kussakin 1963; Menzies 1957; Nunomura 2012; Ortiz & Lalana 1988; Yoshino & Ohsawa 2019). Cookson 1991 stated that "*L. uncapedis*, *L. segnoides* and *L. bituberculata* all share the following features: broad maxillipedal epipod, loss of mandibular palp, similar shape of the lacinia mobilis of the right mandible, and modification of the secondary unguis on pereopod 1", and Yoshino & Ohsawa (2019) said that the species of non-

mandibular-palp group are algal-feeding and shared the broad epipod of maxilliped, except seagrass-feeding *L. zinovae*, the shape of epipod of maxilliped of which is unknown. *L. aspera* **sp. nov.** fits into this group, as an algal-feeding species that also has the broad epipod of maxilliped (Table 1).

L. bacescui, *L. segnoides*, and *L. zinovae* differ from *L. aspera* **sp. nov.** by the number of flagellar articles of antenna 2, the secondary unguis on the pereopods, lacinia mobilis of right mandible, and the sculpturing of pleonite 5 and pleotelson.

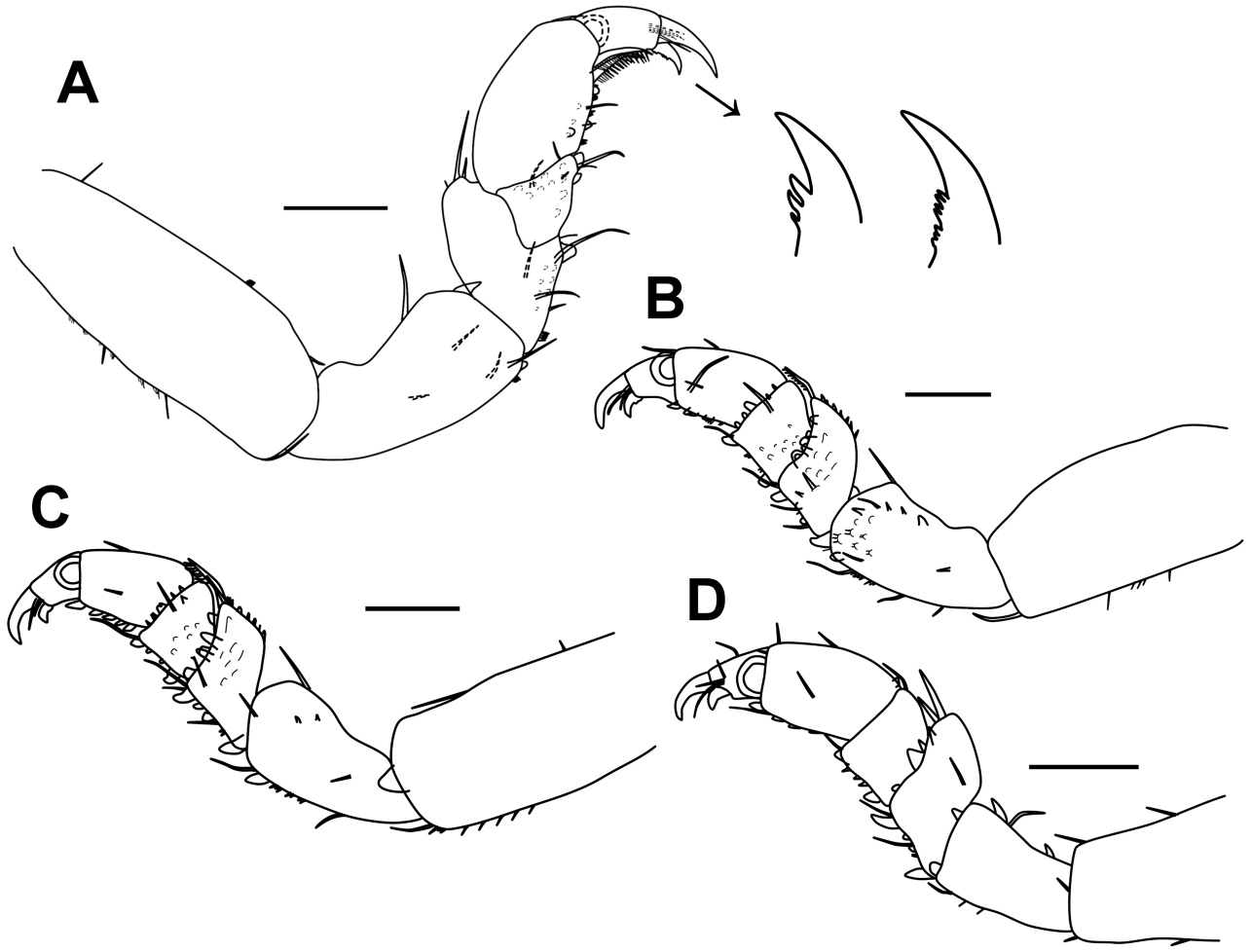

FIGURE 5. *Limnoria aspera* **sp. nov.**, holotype (male, 2.6 mm, CBM-ZC 16564). A, pereopod 1 and secondary unguis of pereopod 1; B–D, pereopods 2–4. Scale bars: A–D = 0.1 mm.

L. aspera **sp. nov.** is similar to *L. uncapedis* in the number of flagellar articles of antennae 1 and 2, secondary unguis of pereopod 1, lacinia mobilis of right and left mandible, and pereopods 2–5 with barbed projection on the propodus. However, *L. uncapedis* is pale yellow and has no carinae on pleonite 5 and pleotelson, while *L. aspera* **sp. nov.** is dark yellow and has Y- or I-shaped carina on pleonite 5 and 1 tubercle on pleotelson.

Similarly to *L. aspera* **sp. nov.**, *L. bituberculata* has 4 flagellar articles of antenna 1, spinules on the secondary unguis of pereopod 1, and a dorsomedial longitudinal carina between 2 longitudinal grooves on pleonite 5. However, *L. bituberculata* is pale yellow and has 2 prominent tubercles on the pleotelson, while *L. aspera* **sp. nov.** is dark yellow and has 1 medial tubercle on the pleotelson.

L. aspera **sp. nov.** seems to be most similar to *L. furca*, and *L. nagatai*. *L. aspera* **sp. nov.** is distinguished from *L. furca*, and *L. nagatai* by dark yellow body color (*L. furca*, and *L. nagatai* pale yellow), undivided or slightly bifid secondary unguis of pereopods (*L. furca* bifid or trifid, *L. nagatai* bifid), and the medial tubercle on the pleotelson lacks the attachment of two inverted V-shaped carinae (*L. furca*, and *L. nagatai* with medial tubercle followed by a pair of subparallel carinae).

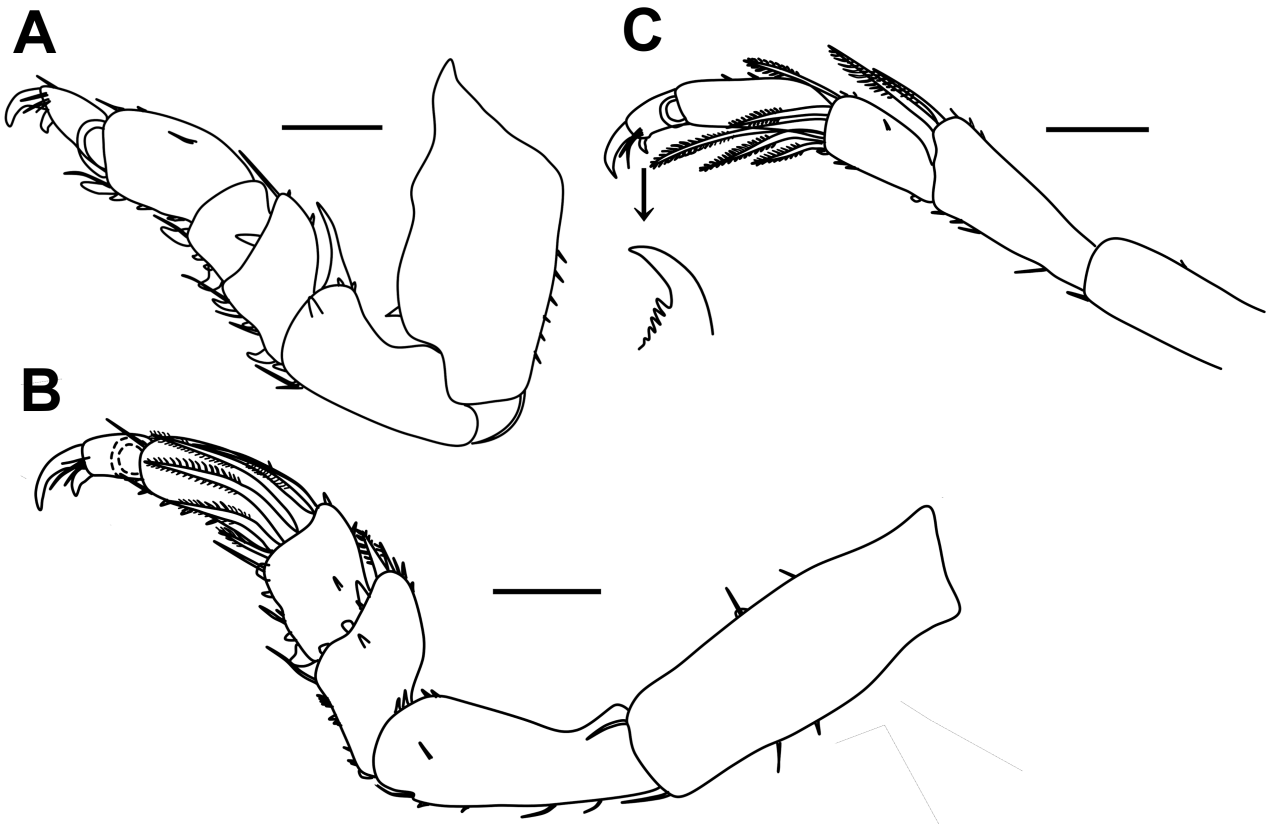

FIGURE 6. *Limnoria aspera* **sp. nov.**, holotype (male, 2.6 mm, CBM-ZC 16564). A–C, pereopods 5–7. Scale bars: A–C = 0.1 mm.

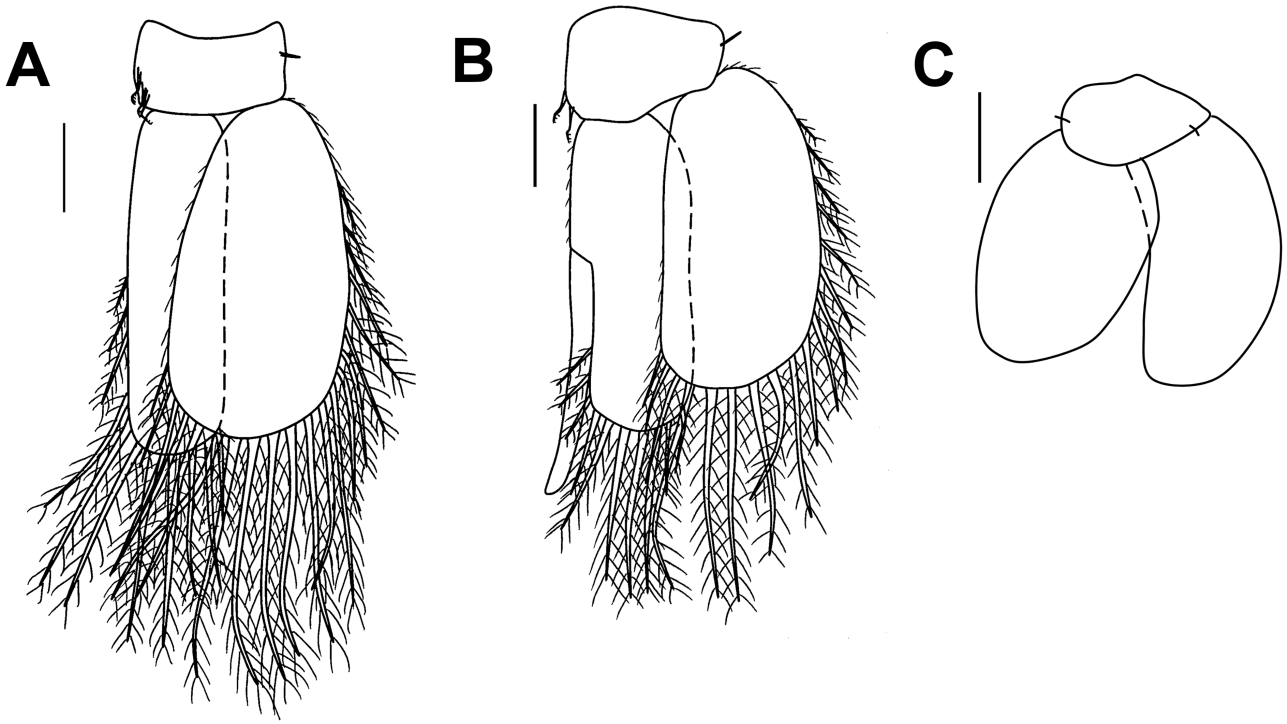

FIGURE 7. *Limnoria aspera* **sp. nov.**, holotype (male, 2.6 mm, CBM-ZC 16564). A, pleopod 1; B, pleopod 2; C, pleopod 5. Scale bars: A–C = 0.1 mm.

TABLE 1. Comparative table of morphological characters of non-mandibular-palp group of *Limnoria* (Isopoda, Limnoriidae).

	L. aspera **sp. nov.**	*L. bacescui*	*L. bituberculata*	*L. furca*
Distribution	Japan	Cuba	India	Japan, South Korea
Depths	Sublittoral zone (0–1 m)	2.5 m	Littoral zone	sublittoral zone, 10–15 m
Substrate	Holdfasts of brown algae *Sargassum ringgoldianum*, and *S. siliquastrum*	Brown alga *Stypopodium zonale*	Holdfasts of brown alga *Sargassum*	Holdfasts of brown alga *Eisenia bicyclis*, rinsing rhizome of red alga *Corallina* sp.
Color	Dark yellow	-	Pale yellow or cream yellow	Pale yellow
Number of flagellar articles of antenna 1	4	-	4	4
Number of flagellar articles of antenna 2	4	3	4	3
Epipod of maxilliped	Clavate	Clavate	Clavate	Clavate
Rasp and file on mandibular incisors	Absent	Absent?	Absent	Absent
Secondary unguis of pereopod 1	Undivided without spinule or with 1–4 spinules	Absent	Undivided with 3–4 spinules	Bifid or trifid
Lacinia mobilis of right mandible	2 serrated branches	1 branch	-	2 serrated branches
Lacinia mobilis of left mandible	1 serrated seta	-	-	1 serrated seta
Pereopod with barbed projection on propodus	2–5	At least 1?	At least 2	2–5
Carina on pleonite 5	Dorsomedial Y- or I-shaped indistinct longitudinal carina	Absent?	Dorsomedial convex with 1 pair of submedian longitudinal grooves	Ill-defined Y-shaped carinae
Tubercle and carina on pleotelson	1 medial tubercle	Absent?	Proximal median convex and 2 prominent tubercles	1 medial tubercle followed by faint pair of subparallel, inverted V-shaped carinae

.....continued on the next page

TABLE 1. (Continued)

	L. nagatai	*L. segnoides*	*L. uncapedis*	*L. zinovae*
Distribution	Japan	Japan, Madagascar	Australia	Japan, Russia
Depths	Sublittoral zone	Low tide zone	1–21 m	0–20 m
Substrate	Holdfasts of brown algae *Eisenia bicyclis*, and *E. arborea*	Washed from red alga *Corallina*	Red and brown algae, possibly bryozoans and sponges	Rhizomes of seagrass *Phyllospadix iwatensis*
Color	Pale yellow or pale red, head red	-	Pale yellow	-
Number of flagellar articles of antenna 1	4	-	4	4
Number of flagellar articles of antenna 2	3	-	4	4
Epipod of maxilliped	Clavate	Clavate	Clavate	-
Rasp and file on mandibular incisors	Absent	Absent	Absent	Absent
Secondary unguis of pereopod 1	Bifid	Undivided without spinule	Undivided with several spinules	Bifid
Lacinia mobilis of right mandible	2 serrated branches	2 serrated branches	2 serrated branches	1 marginally serrated branch
Lacinia mobilis of left mandible	1 serrated seta	-	1 thick serrated seta	-
Pereopod with barbed projection on propodus	2–5	-	2–5	-
Carina on pleonite 5	Dorsomedial Y-shaped carina	Dorsomedial V-shaped carina	Absent	Dorsomedial Y-shaped carina
Tubercle and carina on pleotelson	1 medial tubercle followed by 1 pair of subparallel, inverted V-shaped carinae	1 medially elevated anteriorly located region followed by 2 subparallel carinae	Absent	Dorsomedial tubercle and 2 short indistinct carinae

-: unknown

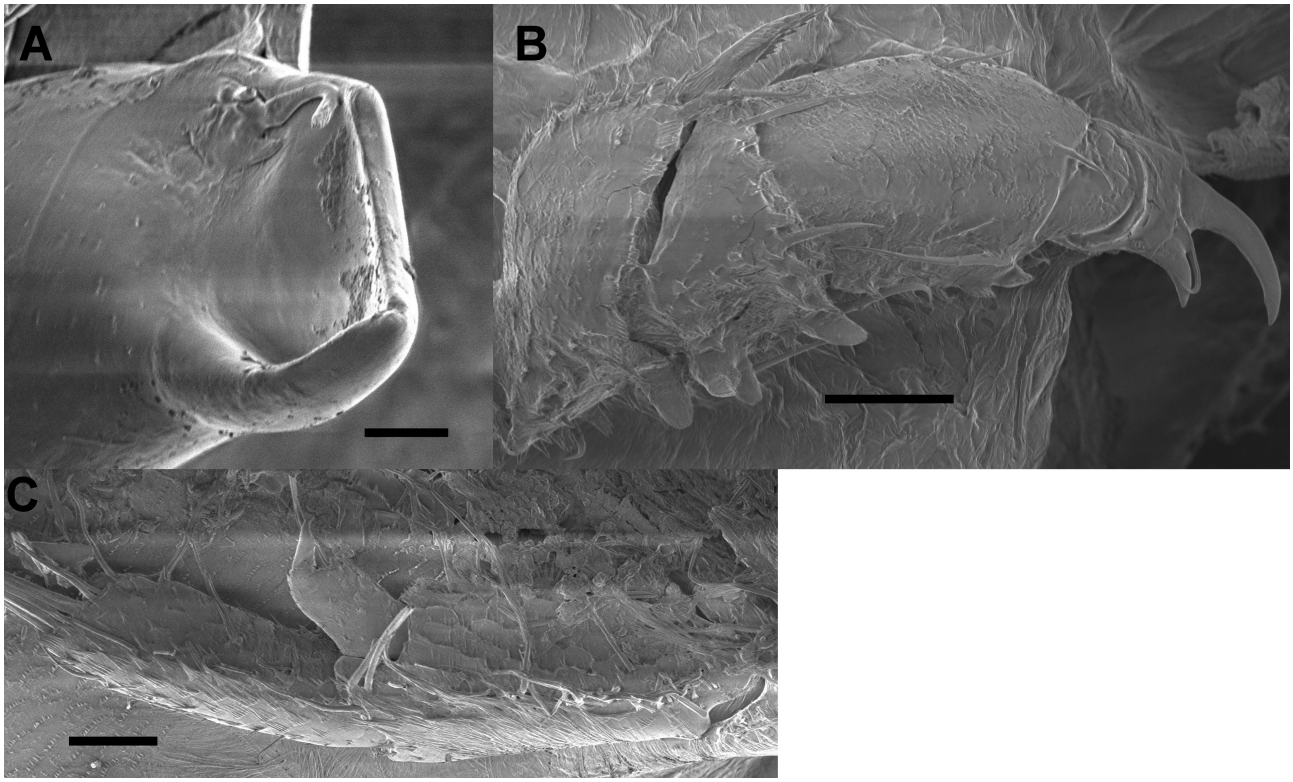

FIGURE 8. *Limnoria aspera* **sp. nov.,** paratype (male 2.8 mm, CBM-ZC 16565). A, left mandible, apical view, SEM image (JSM-6010LA; JEOL); B, pereopod 2, lateral view, SEM image (JSM-6010LA; JEOL); uropod, ventral view, SEM image (JSM-6010LA; JEOL). Scale bar: A = 0.02 mm, B, C = 0.05 mm.

Discussion

Limnoria aspera **sp. nov.** is eighth species to be described as non-mandibular-palp *Limnoria*, where the mandibular palp is replaced with a seta. Most limnoriids have mandibular palp with 1–3 articles. The dorsal surface of the pleotelson of some species in the non-mandibular-palp group, such as *L. nagatai*, is covered with small pores, but that of *L. aspera* **sp. nov.** is covered with fused scales resembling mosaic tiles. Cookson (1991) indicated that *L. uncapedis* lived on fragile substrates, which may have led to the development of prominent pereopodal barbs in order to grip on substrates. The prominent barbs of pereopods 2–5 are also found in *L. aspera* **sp. nov.**, *L. furca* and *L. nagatai* (Yoshino unpublished), that are collected in the holdfasts of brown algae (Yoshino & Ohsawa 2019).

The percentage of difference of COI sequences in *p*-distance differs by 15.3–18.0% between *L. aspera* **sp. nov.** and *L. nagatai*, and 14.2–15.1% between *L. aspera* **sp. nov.** and *L. furca*. Similarly, the percentage of difference of 28S rRNA sequences in *p*-distance differs by 14.5–20.5% between *L. aspera* **sp. nov.** and these other species. The differences are of the same order of magnitude as found among several isopods species (Wetzer 2001). The phylogenetic tree of COI shows that the sequences of *L. aspera* **sp. nov.** are included a clade formed with the non-mandibular-palp group, *L. furca* and *L. nagatai* with bootstrap value 70% (Fig. 9). *L. furca* is much closer to *L. nagatai* than *L. aspera* **sp. nov.**, which is congruent with morphological similarities as well. *L. bituberculata* was also collected from the holdfasts of *Sargassum*, in the littoral zone in India (Pillai 1957). However, *L. aspera* **sp. nov.** is morphologically more similar to *L. furca* and *L. nagatai* from *Eisenia*, than to *L. bituberculata*. Further experiments are required to understand the change of host preferences within the non-mandibular-palp group.

Sargassum with *L. aspera* **sp. nov.** seemed more easily detached from the seabed than that without *Limnoria*. The holdfasts bored by *Limnoria* were hollowed out, which appears to weaken their adhesion to the seafloor substrate. This kind of damage to holdfasts causes some seaweeds to come adrift (Jones 1971).

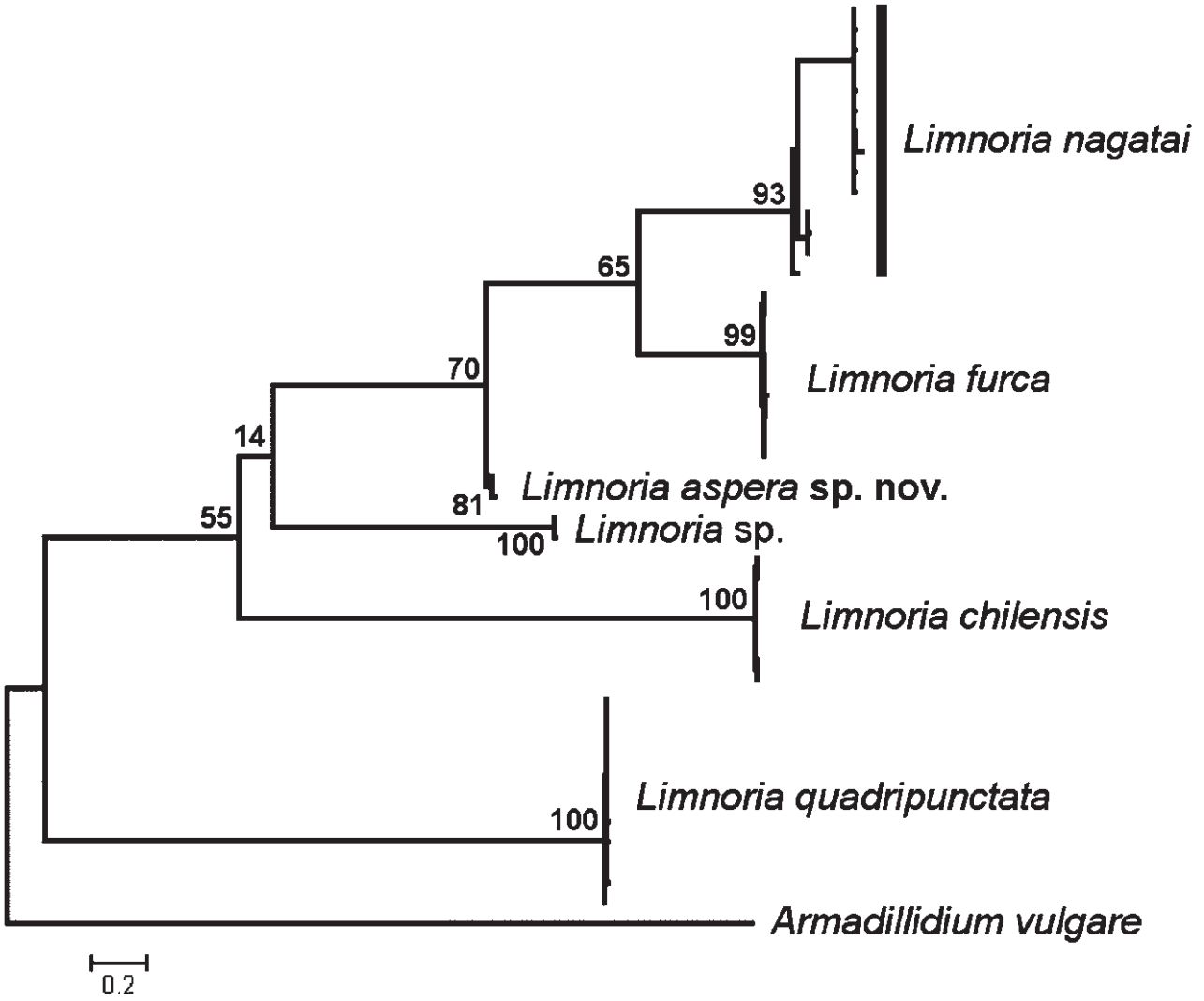

FIGURE 9. ML tree based on the COI sequences of *Limnoria aspera* **sp. nov.** and other *Limnoria* obtained from GenBank as well as of *Armadillidium vulgare* as outgroup. Numbers along branches correspond to bootstrap values (1000 replicates). The scale bar represents the number of substitutions per site.

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