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A New Genus and Species of the Spring-endemic Ostracoda (Cypricercinae, Cyprididae) and its Genetic Population Structure among Rheocrenic Springs in Japan

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We describe the ostracod *Lissostrandesia fonticola* gen. et sp. nov. in the subfamily Cypricercinae McKenzie, 1971, collected from six rheocrenic springs in Japan. The populations sampled were separated by up to 1000 km, and some of them by one or two marine straits, which comprise significant barriers for freshwater animals. *Lissostrandesia* differs from the other 13 cypricercine genera in (1) lacking a groove and inner list on the anterior inner margin of the left valve; (2) having b and d setae on the fifth limb; (3) having d1 and d2 setae on the sixth limb; (4) having a stout attachment of the caudal ramus; (5) having a Triebel's loop on the dorsal branch of the attachment; and (6) having a long free ventral branch, its length more than twice its width, and (7) having a free dorsal branch contributing to a tip on Triebel's loop. These differences were enough to warrant establishment of a new tribe, Lissostrandesiini, to accommodate the new genus. We present a key to the genera in Cypricercinae. The maximum *p*-distance for the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene among six local populations was 0.662%, a low value indicative of conspecificity. In addition, four populations with sample sizes of 13–21 individuals shared two main *COI* haplotypes, indicating high apparent connectivity. A trend of decreasing genetic diversity from south to north suggests *L. fonticola* has had a longer history on Honshu Island and dispersed northward from there. Using the 16S rRNA gene as a marker, we detected the endosymbiotic bacterium *Cardinium*, a group of "reproduction-manipulating" bacteria, in five populations, suggesting that *L. fonticola* is parthenogenetic. Passive dispersal is the most likely explanation for the broad distribution of this species across strong geographic barriers.

Key words: Cox1, Cypridoidea, Genetic distance, Taxonomy, 18S

BACKGROUND

Cypricercinae McKenzie, 1971, one of 23 subfamilies in the ostracod family Cyprididae Baird, 1845, is characterized morphologically by having a Triebel's loop on the distal part of the caudal ramus attachment (hereafter "CR attachment"). This subfamily contains more than 170 species in 13 genera (Ferreira et al. 2019; Meisch et al. 2019; Martens et al. 2023). Savatenalinton and Martens (2009b) revised this subfamily and proposed three tribes based mainly on characters on the inside of the valve margins and the

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CR attachment, including Triebel's loop on the distal part of the attachment. Martens et al. (2023) proposed a fourth tribe. The four tribes are Bradleystrandesiini Savatenalinton and Martens, 2009b, with three genera; Cypricercini McKenzie, 1971, with five genera; Lithocypridini Martens, de Almeida and Higuti, 2023 in Martens et al. (2023), with one genus; and Nealecypridini Savatenalinton and Martens, 2009b, with four genera. Species in Cypricercinae inhabit freshwater environments, and representatives have been found in all zoogeographical regions except Antarctica.

Five cypricercine species have been reported from Japan to date: *Bradleycypris vittata* (Sars, 1903); *Bradleytriebella lineata* (Victor and Fernando, 1981); *Bradleytriebella tuberculata* (Hartmann, 1964); *Pseudostrandesia tenebrarum* Smith and Ozawa, 2021 in Smith et al. 2021; and *Tanycypris alfonsi* Nagler, Geist, and Matzke-Karasz, 2014 (Okubo 1972 1990 2004; Okubo and Ida 1989; Ishii et al. 2017; Smith et al. 2011 2021). All except *P. tenebrarum*, which is known only from aquaria in pet shops, have been reported from lentic habitats such as rice fields and ponds.

Springs are isolated, disjunct freshwater ecosystems having a characteristic, well-differentiated biological community (Cantonati et al. 2012). High endemism is expected for spring-dwelling organisms with limited dispersal ability (*e.g.*, benthic animals lacking planktonic stages), because each spring is geographically isolated, physically and chemically distinct, and often minimally affected by human disturbance. The ostracod species diversity in springs has been well studied in Europe (*e.g.*, Roca and Baltanás 1993; Mezquita et al. 1999; Rosati et al. 2014) but not in Japan (*e.g.*, Broodbakker 1988; Smith and Kamiya 2006; Smith 2011).

Within the past decade, the population-genetic structures of ostracods have been investigated with genetic markers, mostly the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene but also several nuclear genes, including elongation factor I alpha (EF-1α), internal transcribed spacer (ITS), and 28S rRNA (*e.g.*, Koenders et al. 2012 2017; Martens et al. 2013 2015; Shearn et al. 2017; Schön et al. 2018a). One molecular study of the cypridid *Callistocypris* sp. from neotropical forests in Mexico revealed a single species inhabiting phytotelmata separated by more than 200 km, suggesting that this species can disperse between isolated bodies of water (Mercado-Salas et al. 2021). Another study, on cypridids in the genus *Bennelongia*, which is endemic to Western Australia, detected five species with restricted distributions (collected from only one site or several sites near one another), and two more broadly distributed species, detected at sites over 800 km apart (Martens et al. 2015).

During field surveys of ostracods in freshwater springs in Japan, we collected morphologically identical cypricercine individuals from six disjunct rheocrenic springs, in three regions separated by straits, a significant geographical barrier for freshwater animals: one locality on Rishiri Island, three on Hokkaido Island, and two on Honshu Island. We could not assign this morphospecies to any known cypricercine genus, so herein we erect a new species, genus, and tribe for it; describe the species; and present a sequence for its 18S rRNA gene.

Because benthic ostracods are typically lacking planktonic stages, and the groups of springs where we collected specimens are separated by marine barriers, we expected to detect several lineages within the morphospecies. We thus also investigated its population structure based on *COI* sequences from individuals from four disjunct sampling areas. Additionally, for information relevant to the reproductive mode of the species, we used a molecular marker to check for the presence of the endosymbiont bacterium *Cardinium*, a group of "reproduction-manipulating" bacteria (cf. Ma and Schwander 2017; Schön et al. 2018b; Schön and Martens 2020). Finally, we constructed a key to the genera of Cypricercinae.

MATERIALS AND METHODS

Sampling

We obtained ostracods from six rheocrenic springs around volcanoes (Fig. 1; Table 1): Kanro-sensui (KS) on Rishiri Island; springs near the Mikuni-bashi Bridge (MB), Daisetsu Asahidake Spring (AS), and Genshi-no-izumi (GI) on Hokkaido; and Gudari-numa (GN) and Metori Spring (MS) on Honshu. Bottom sediment was stirred in ambient water in a bucket, and all but the sediment was filtered through a 63-μm-mesh plankton net. This process was repeated several times at each locality. Ostracods were picked from the residue under an SZX9 stereomicroscope (Olympus, Japan). Geographical coordinates and elevations were obtained from GSI Maps (Geospatial Information Authority of Japan 2023). Water temperature was measured by using an O-274 thermometer (DRETEC, Japan).

Morphological observations

Ostracods were fixed and preserved in 80% or 100% ethanol. The methods used for dissection, preparation of slides, light microscopy, scanning electron microscopy (SEM), and drawing were as described by Munakata et al. (2021). The material

studied has been deposited in the Invertebrate Collection of the Hokkaido University Museum (ICHUM), Sapporo, or in the Rishiri Town Museum, under catalog numbers ICHUM-8603 to 8615, 8625, and 8626, and RTMCRU216 (for details, see Table S1).

The following abbreviations are used in the text: Ca, carapace; LV, left valve; RV, right valve; H, height; L, length; W, width; An1, antennula; An2, antenna; Md, mandible; Mx, maxillula; L5–7, fifth to seventh limbs; CR, caudal ramus; db, dorsal branch; vb, ventral

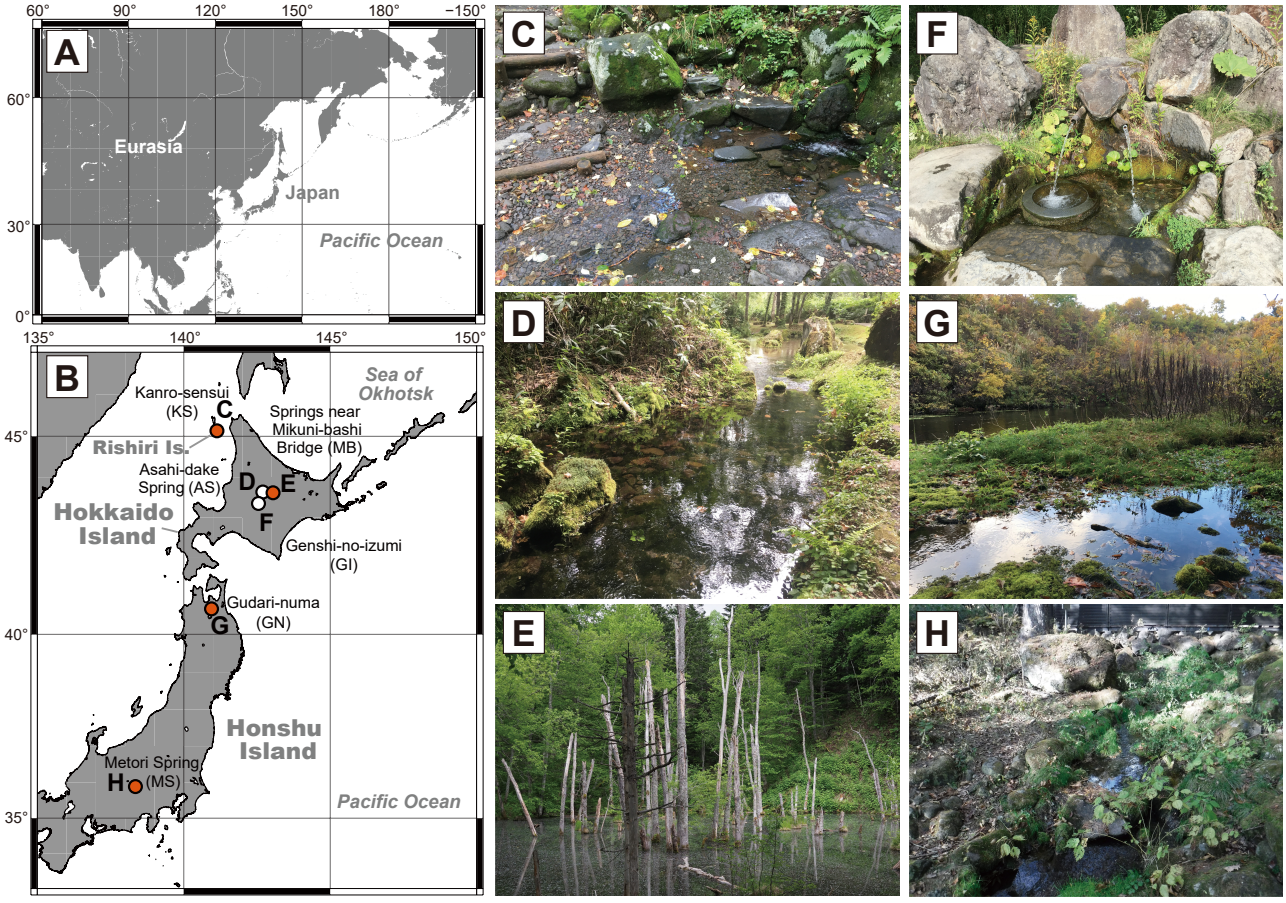

Fig. 1. Sampling localities for *Lissostrandesia fonticola* gen. et sp. nov. in Japan. (A) Map of eastern Asia and the western Pacific. (B) Map showing northern to central Japan, with letters C–H indicating sampling localities (circles); red circles indicate populations included in population-genetic analyses. (C–H) Photographs of sampling sites. (C) Kanro-sensui (KS). (D) Springs near Mikuni-bashi Bridge (MB). (E) Daisetsu Asahidake Spring (AS). (F) Genshi-no-izumi (GI). (G) Gudari-numa (GN). (H) Metori Spring (MS). Maps were generated with GMT6 (Wessel et al. 2019).

Table 1. Sampling localities for *Lissostrandesia fonticola* gen. et sp. nov.

Locality name (abbriviation)	Environment	Latitude	Longitude	Altitude (m)	Water temperature (°C)	Sampling date
Kanro-sensui (KS)	Springhead and springbrook	45°13.11'N	141°13.03'E	267	6.7	05.x.2020, 29.v.2023
Springs near to Mikuni-bashi Bridge (MB)	Springbrook	43°37.19'N	143°03.26'E	905	No data	14.vi.2022
Daisetsu Asahidake Spring (AS)	Springbrook	43°37.59'N	142°41.31'E	445	7.0	26.viii.2020
Genshi-no-izumi (GI)	Springhead	43°21.22'N	142°32.31'E	419	7.2	27.viii.2020
Gudari-numa (GN)	Springhead	40°40.11'N	140°57.08'E	586	8.2	24.x.2021, 13.x.2022
Metori Spring (MS)	Springhead and springbrook	35°53.32'N	138°20.39'E	1175	8.0	27.iii.2019, 02.xi.2021

branch; TL, Triebel's loop. The appendage chaetotaxy follows Broodbakker and Danielopol (1982) for An1, Md, and Mx; Martens (1987) for An2; Meisch (2000) for L5–7; and Meisch (2007) for CR. In addition to vb, db, and main branch used in Savatenalinton and Martens (2009b), we proposed the following terms: shared vb, the region of the vb shared with TL; free vb, the remaining portion of the vb; shared dv, the region of the db shared with TL; free db, the remaining portion of the db; and bridge, the connection between vb and db that makes the loop in TL (for details, see Fig. 7B). The following measurements were made from digital images by using ImageJ (Schneider et al. 2012): L and H of the LV and RV (LV-L, LV-H, RV-L, and RV-H) and W of the carapace (Ca-W). Measurements in the text are in millimeters, followed by the mean value and sample size in parentheses.

Molecular analysis

Total DNA was extracted from the soft parts of 24, 32, 22, and 27 specimens from the GN, KS, MB, and MS populations, respectively, and one specimen from each of the local populations AS and GI, by using a NucleoSpin Tissue XS Kit (Macherey-Nagel, Germany) following the manufacturer's protocol. The primers used for PCR amplification and sequencing for *COI*, 18S, and *Cardinium* 16S are listed in Munakata et al. (2021). We designed the specific primer Cypricer_LCO_inner (AGCCATGCTAGGAACAGCTT) and used it instead of primer LCO1490 in cases where PCR amplification with the LCO1490/HCO2198 primer pair failed. The amplicon from primer LCO1490 was 658 bp long, whereas that from Cypricer_LCO_inner was 608 bp long. PCR amplification conditions for *COI* and *Cardinium* 16S with TaKaRa Ex Taq DNA polymerase (TaKaRa Bio, Japan) and for 18S with KOD FX Neo (Toyobo Life Science, Japan) were as described by Munakata et al. (2021). All nucleotide sequences were determined by direct sequencing in the forward and reverse directions with a BigDye Terminator Kit ver. 3.1 and a 3730 DNA Analyzer (Life Technologies, USA). Fragments were concatenated by using MEGA 11 (Tamura et al. 2021). Ambiguous positions due to double peaks in sequencing chromatograms were assigned a letter in the IUPAC nucleotide ambiguity code (Johnson 2010). BLAST (Altschul et al. 1990) was used to search the International Nucleotide Sequence Database (INSD; International Nucleotide Sequence Database Collaboration 2023) for sequences most similar to ours. The 18S dataset for phylogenetic analysis included one sequence we determined from a specimen collected from MS, the type locality (ICHUM-8610; accession number LC789200), and 32 sequences from 31 cypridoidean species and one outgroup taxon (*Pontocypris mytiloides*, Pontocypridoidea) taken from the INSD (Table S2). Detailed methods for 18S sequence alignment and the phylogenetic analysis, and the results from the analysis are provided as files S1–3.

Population genetics

COI sequences were determined for 13 individuals from the GN, 21 from KS, 20 from MB, and 19 from MS population. After alignment by means of Clustal W (Thompson et al. 1994), these sequences were trimmed to the shortest length among them (608 bp), and three sites containing ambiguous bases (as indicated by IUPAC codes; see above) were removed with MEGA 11. An integer neighbor-joining (IntNJ) network was constructed with PopART v.1.7 (Leigh and Bryant 2015) at 0.50 reticulation tolerance. Haplotype diversity (h), nucleotide diversity (π), and Tajima's D (Tajima 1989) were calculated with DnaSP v.6.12.03 (Rozas et al. 2017). Fu's F_S (Fu 1997) was calculated with Arlequin v. 3.5.2.2 (Excoffier and Lischer 2010).

RESULTS

TAXONOMY

Superfamily Cypridoidea Baird, 1845
Family Cyprididae Baird, 1845
Subfamily Cypricercinae McKenzie, 1971

Tribe Lissostrandesiini trib. nov.

urn:lsid:zoobank.org:act:77659c20-0d1a-4b8e-832c-8f35d462023a

Type genus: *Lissostrandesia* gen. nov.

Diagnosis: Inner lamella on LV without groove or inner list (Type A, see Savatenalinton and Martens 2009b); L5 with b, d setae; L6 with d1, d2 setae; CR attachment stout, with TL on db; vb with long, free part (free vb; length more than twice width); dorsal branch of TL with free part contributing to tip of TL (free db).

Genus *Lissostrandesia* gen. nov.

urn:lsid:zoobank.org:act:3ae75bca-c259-40c5-a325-4b717128d779

Type species: *Lissostrandesia fonticola* gen. et. sp. nov.

Etymology: Derived from the ancient-Greek adjective *lissos* (smooth), referring to the smooth inner margin of the valves, lacking any grooves or lists,

combined with the generic name *Strandesia*, a common group in this subfamily. Gender feminine.

New Japanese name: *Hira-maruwa-kaimijinko* combines the Japanese word *hira* (smooth) with *maruwa-kaimijinko*, the name for the subfamily Cypricercinae (Okubo 2004).

Diagnosis: Same as the diagnosis for the tribe.

Lissostrandesia fonticola **gen. et sp. nov.**

(Figs. 2–4)

urn:lsid:zoobank.org:act: 5ab1df55-6ebf-46a9-9428-dff0ba4391af

Cypricercinae sp.: Munakata et al. (2023), 9–10, figs. 2A, 3A, B, 4.

Type locality: Springhead of Metori Spring, Nagasaka Town, Hokuto City, Yamanashi Prefecture, Japan (35°53.32'N, 138°20.39'E); in sediment.

Material examined: Holotype: female, ICHUM-8615, two slides. Paratypes (14 females): ICHUM-8603–8610, two slides for each; ICHUM-8611, 8612, one SEM stub and one slide for each; ICHUM-8613, 8614, one SEM stub for each; ICHUM-8625, 8626, undissected, one vial for each. All individuals except ICHUM-8626 were collected by MM at the type locality; ICHUM-8626 was collected by Fuga Matsui from a spring near Mikuni-bashi Bridge (Table S1).

Etymology: The specific name is from the Latin *fontis*, genitive singular of *fons* (fountain or spring), and *-cola*, nominative singular of the adjective-forming suffix "*cola*" (inhabit).

New Japanese name: *Shimizu-hira-maruwa-kaimijinko*. Because this species was collected only in springs, the Japanese word *shimizu* (cold, clear freshwater) was prefixed to the generic name.

Diagnosis: Same as the generic diagnosis.

Description of Lissostrandesia fonticola: Measurements (in millimeters, except for ratios) of carapace and valves: LV-L 0.99–1.05 (1.02, n = 3), LV-H 0.51–0.56 (0.53, n = 3), LV-H/LV-L 0.52–0.53 (0.53, n = 3); RV-L 1.00–1.03 (1.02, n = 3), RV-H 0.50–0.53 (0.52, n = 3), RV-H/RV-L 0.50–0.51 (0.51, n = 3); Ca-W 0.41–0.42 (0.42, n = 3), Ca-W/LV-L 0.40–0.42 (0.41, n = 3).

Carapace (Figs. 2, 3) translucent, with violet-colored area dorsally; eyes black (Fig. 2A). Carapace outer surface smooth, with sparse tiny setae; widest point at about mid-length (Fig. 3A, B); anterior and posterior ends rounded in dorsal view (Fig. 3A), outer list present on ventral and posterior margins of both valves (Fig. 3B). RV slightly overlapping LV along entire margin, with slight ventral expansion (Fig. 3A, B); greatest height at mid-length of RV; anterodorsal and posterodorsal margins smooth; posterior margin slightly more sharply rounded than anterior; ventral margin slightly concave; apex of anterior margin below mid-height of RV and higher than apex of posterior margin (Fig. 3C, E); in inner view, inner list and groove absent along entire margin; calcified inner lamella well developed on anterior, posterior, and ventral margins (Fig. 3C). LV similar to RV in shape; ventral margin nearly straight (Fig. 3D, F). Two oblong mandibular muscle scars and five oblong adductor muscle scars on LV and RV (Fig. 3C, D). Hinge adont.

An1 (Fig. 4A) with seven podomeres. Podomere length ratio from second to seventh podomeres 5: 7: 3: 3: 3: 3. First podomere with one dorsal and two ventrodistal plumed setae; without Wouters organ. Second podomere with dorsodistal plumed seta not reaching middle of third podomere; without Rome organ. Third podomere with dorsodistal plumed seta reaching beyond end of seventh podomere and ventrodistal plumed seta reaching end of fourth podomere. Fourth podomere with two dorsodistal setae reaching tips of long setae on sixth podomere and two ventrodistal plumed setae reaching end of fifth podomere. Fifth podomere with two long dorsodistral setae reaching tips of long setae on seventh podomere and two shorter ventrodistal plumed setae extending

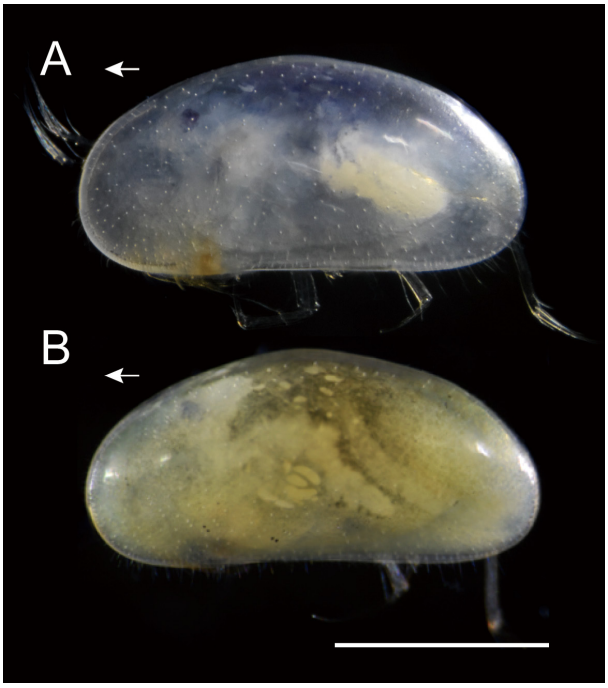

Fig. 2. Left views of fixed specimens of *Lissostrandesia fonticola* gen. et sp. nov. (A) Form with translucent carapace and dorsal violet area; female from Metori Spring (paratype ICHUM-8625). (B) Pale yellowish form; female from springs near Mikuni-bashi Bridge (paratype ICHUM-8626). Arrows indicate anterior. Scale bar = 0.5 mm.

to end of seventh podomere. Sixth podomere with four outer distal long setae (as long as podomeres 1–7) and shorter inner distal seta. Seventh podomere with three distal setae (two long, reaching tips of long setae on sixth podomere; one short, ca. one-fifth length of long setae) and aesthetasc ya (two-fifths length of long setae).

An2 (Fig. 4B, C) with five podomeres. First podomere (coxa; not illustrated) with three ventral plumed setae. Second podomere (basis) with ventro-subdistal seta reaching middle of third podomere. Exopodite with one plumed long and two unequal short setae. Third (first endopodal) podomere with six inner subdistal natatory setae (one long seta not extending beyond half of fourth podomere and five short setae just reaching end of third podomere), ventrodistal plumed seta reaching tip of fourth podomere, and mid-ventral aesthetasc Y not reaching beyond end of third podomere. Fourth podomere undivided, with two mid-dorsal setae, dorso-subdistal setae z1–3 of unequal length, mid-ventral plumed setae t1–4 reaching middle of claws G1 and G3, mid-ventral short aesthetasc y1, ventrodistal short aesthetasc y2, and distal claws G1–3; claw G2 ca. 90% length of claws G1, G3. Fifth podomere (Fig. 4C) with plumed seta g reaching to ca. three-fourths length of claw GM and bifurcate aesthetasc y3 (longer than half length of claw GM); Gm ca. three-fifths length of GM; GM reaching tips of claws G1, G3.

Md (Fig. 5A) with coxa, palp comprising four podomeres (one basal, three endopodal), and vibratory plate. Coxa with distal teeth and two subdistal plumed setae. First podomere (basis) with one ventrodistal seta, ventrodistal setae S1 and S2, and ventrodistal short seta α (ca. one-third length of seta S2); seta S1, S2 unequal in length, bearing row of long setules. Vibratory plate (exopodite; not illustrated) with four rays. Second (first endopodal) podomere with four dorsodistal setae of unequal length (longer two setae reaching end of fourth podomere); one mid-ventral, one long plumed, and three smooth setae not extending beyond tips of claws on fourth podomere; and mid-ventral plumed short seta ß (ca. one-fourth length of mid-ventral smooth setae). Third podomere with four dorso-subdistal and two ventro-subdistal setae; inner region with distal plumed

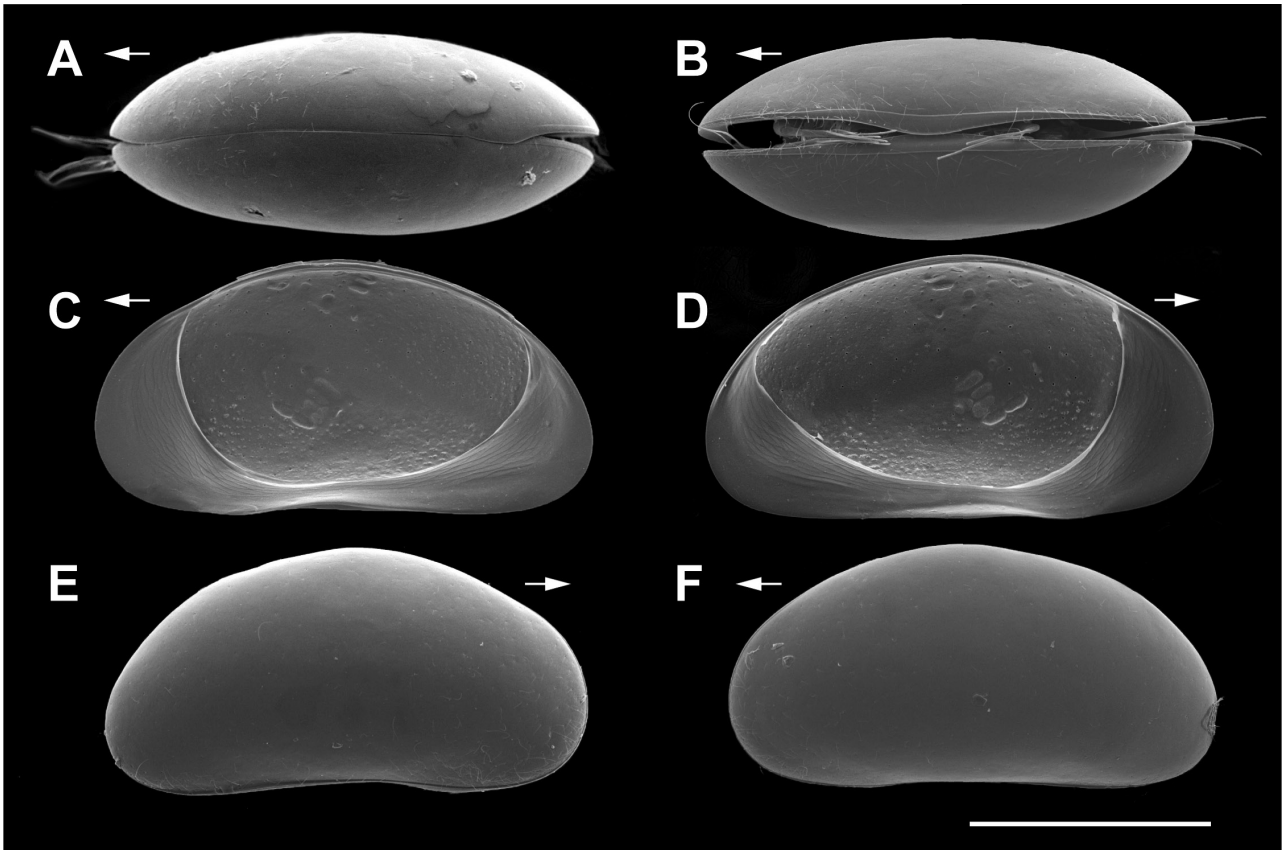

Fig. 3. SEM images of carapaces and valves of female *Lissostrandesia fonticola* gen. et sp. nov. (A) Paratype ICHUM-8613; (B) Paratype ICHUM-8614; (C, D) Paratype ICHUM-8611; (E, F) Holotype, ICHUM-8612. (A, B) Dorsal and ventral views of whole carapace. (C, D) Inner views of left and right valves. (E, F) Outer views of left and right valves. Arrows indicate anterior. Scale bar = 0.5 mm.

seta γ and three distal plumed setae. Fourth podomere with distal seta and four distal claws.

Mx (Fig. 5B) with palp comprising two podomeres, three endites, and vibratory plate. First palpal podomere with one subdorsodistal and six dorsodistal plumed setae reaching tips of claws on second palpal podomere. Second palpal podomere rectangular, L/W ca. 2, with three distal setae and three distal claws. First endite with two ventroproximal plumed setae, and eight smooth and two plumed distal setae. Second endite with eight distal setae. Third endite with two distal serrated spines and seven distal setae (two of them plumed). Vibratory plate (not illustrated) with more than 10 rays.

L5 (Fig. 6A) with protopod, palp, and vibratory plate. Protopod with two setae a, plumed seta b, plumed seta d, and 12 distal plumed setae of unequal lengths. Palp with distal plumed setae h1–3. Vibratory plate (not illustrated) with several rays.

L6 (Fig. 6B) with six podomeres (border between first and second podomeres indistinct). Length ratio for third to sixth podomeres and terminal claw (h_2) 14: 7: 10: 3: 24. First and second podomeres (protopod) with setae d_1, d_2. Third (first endopodal) podomere with ventrodistal plumed seta e not reaching middle of fifth podomere. Fourth podomere not fused to fifth podomere, with ventrodistal plumed seta f reaching end of sixth podomere. Fifth podomere with short ventrodistal plumed seta g. Sixth podomere with dorsodistal plumed seta h3, ventrodistal plumed seta h1, and distal curved claw h_2.

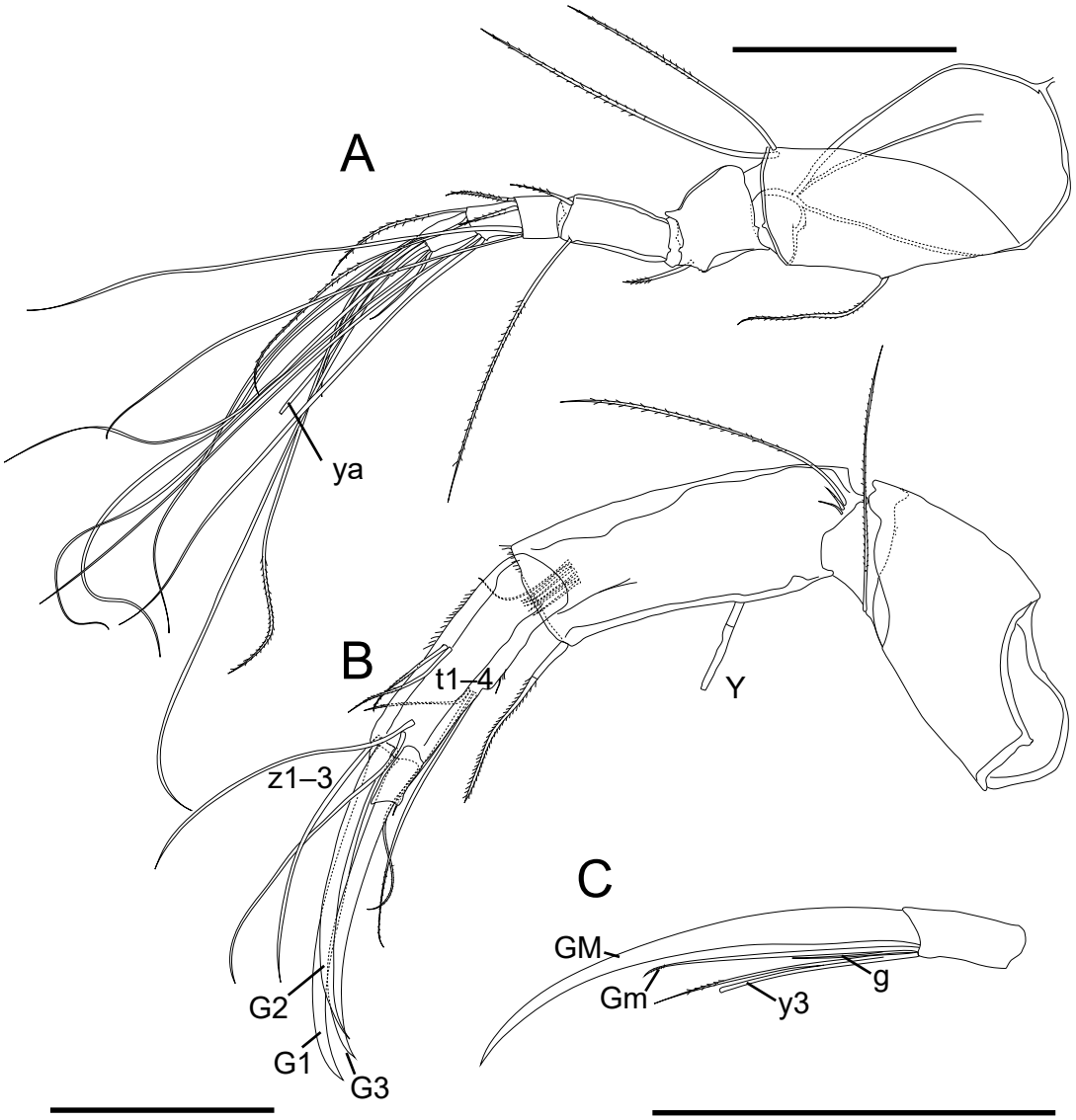

Fig. 4. *Lissostrandesia fonticola* gen. et sp. nov., holotype female (ICHUM-8612). (A) Antennula. (B) Antenna, outer view; coxa, setae, and claws on fifth podomere omitted. (C) An2, fifth podomere. Scale bars = 0.1 mm.

L7 (Fig. 6C) with four podomeres, bearing pincer organ formed by third and fourth podomeres. First podomere (protopod) with plumed setae d_1, d_2, d_p. Second (first endopodal) podomere with ventrodistal plumed seta e not extending beyond end of fused third and fourth podomeres. Third podomere with mid-ventral plumed seta f reaching tip of seta h1. Fourth podomere with long plumed seta h3, hook-like seta h2, and tiny seta h1.

CR (Fig. 7A) with CR-L/CR-W ratio ca. 17. L ratio of ramus, plumed seta Sa, claw Ga, claw Gp, and plumed seta Sp 17: 3: 9: 7: 2.

CR attachment (Fig. 7B) stout, with TL on db, and with free db contributing to acute tip; vb with well-developed free part, with L of free vb about 10 times W; vb with swollen end.

Male unknown.

Variation: We observed two types of carapace coloration, depending on the population. In individuals from MS, KS, AS, and GI, the carapace was translucent, with a violet area on the dorsal side (Fig. 2A); in individuals from GN and MB, the carapace was pale yellowish, with a dark green area on the dorsal side (Fig. 2B). These colors were retained in ethanol.

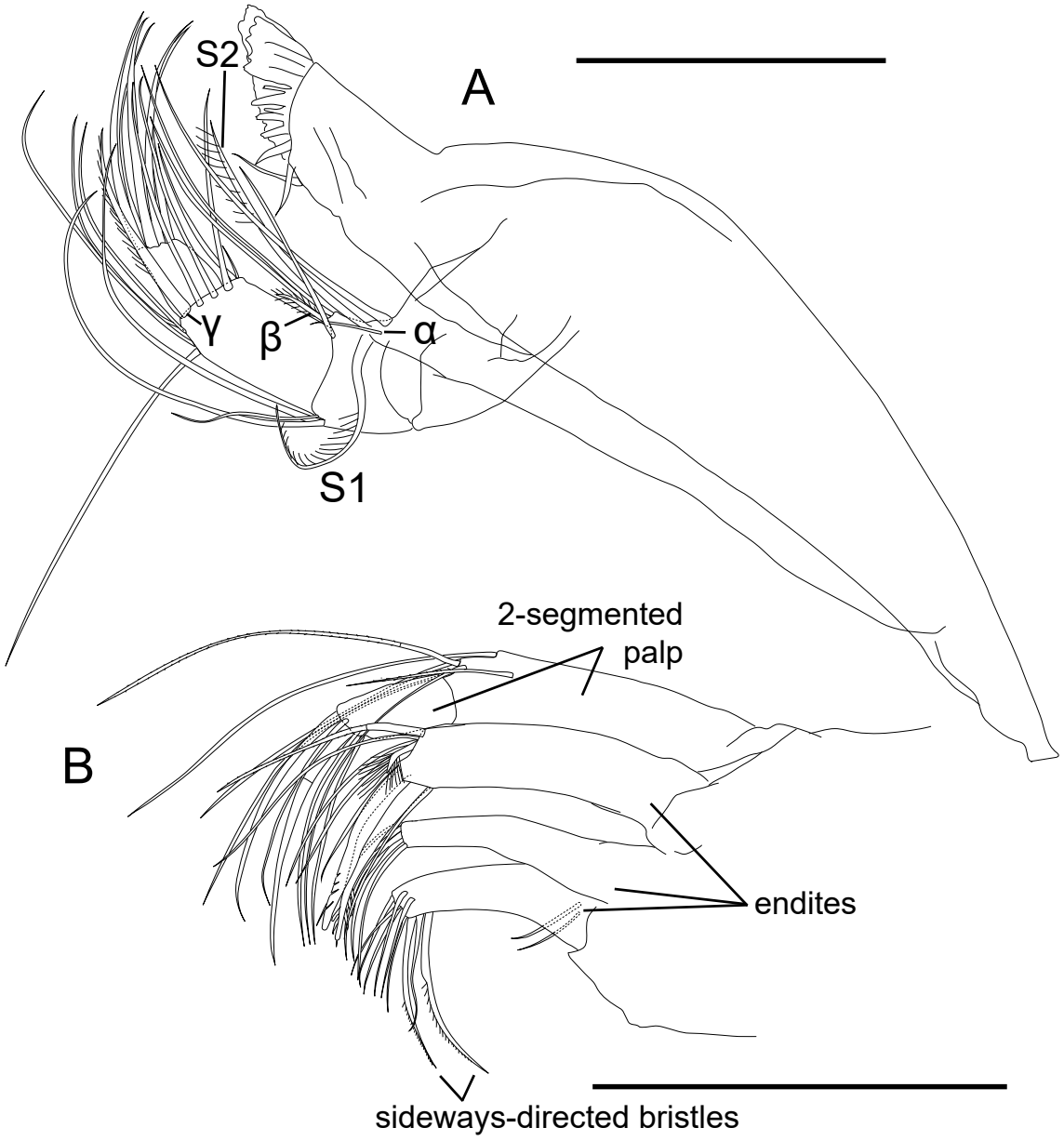

Fig. 5. *Lissostrandesia fonticola* gen. et sp. nov., holotype female (ICHUM-8612). (A) Mandible, inner view, vibratory plate omitted. (B) Maxillula. Scale bars = 0.1 mm.

Molecular information

We determined *COI* sequences for 19 individuals from the MS population (608 or 658 bp, encoding 202 or 218 amino acids; INSD accession numbers LC789259–LC789277); these differed by 0–4 nucleotide substitutions in 608 bp. In BLAST searches, the most similar sequence to ours was from "Ostracoda sp." (MF751183.1; identity score 84.33%, query cover 98%; deWaard et al. 2019).

We also determined *COI* sequences (608 or 658 bp; INSD accession numbers LC789204–LC789258, LC726439) from ostracods from the other five populations: 21 from KS, 20 from MB, one from AS, one from GI, and 13 from GN. Mean *p*-distances (608 bp) within populations for which more than one individual was sequenced were 0.016–0.193% and those between them were 0.00–0.229%. Maximum *p*-distances within and between populations were 0.500% and 0.662%, respectively (Table 2).

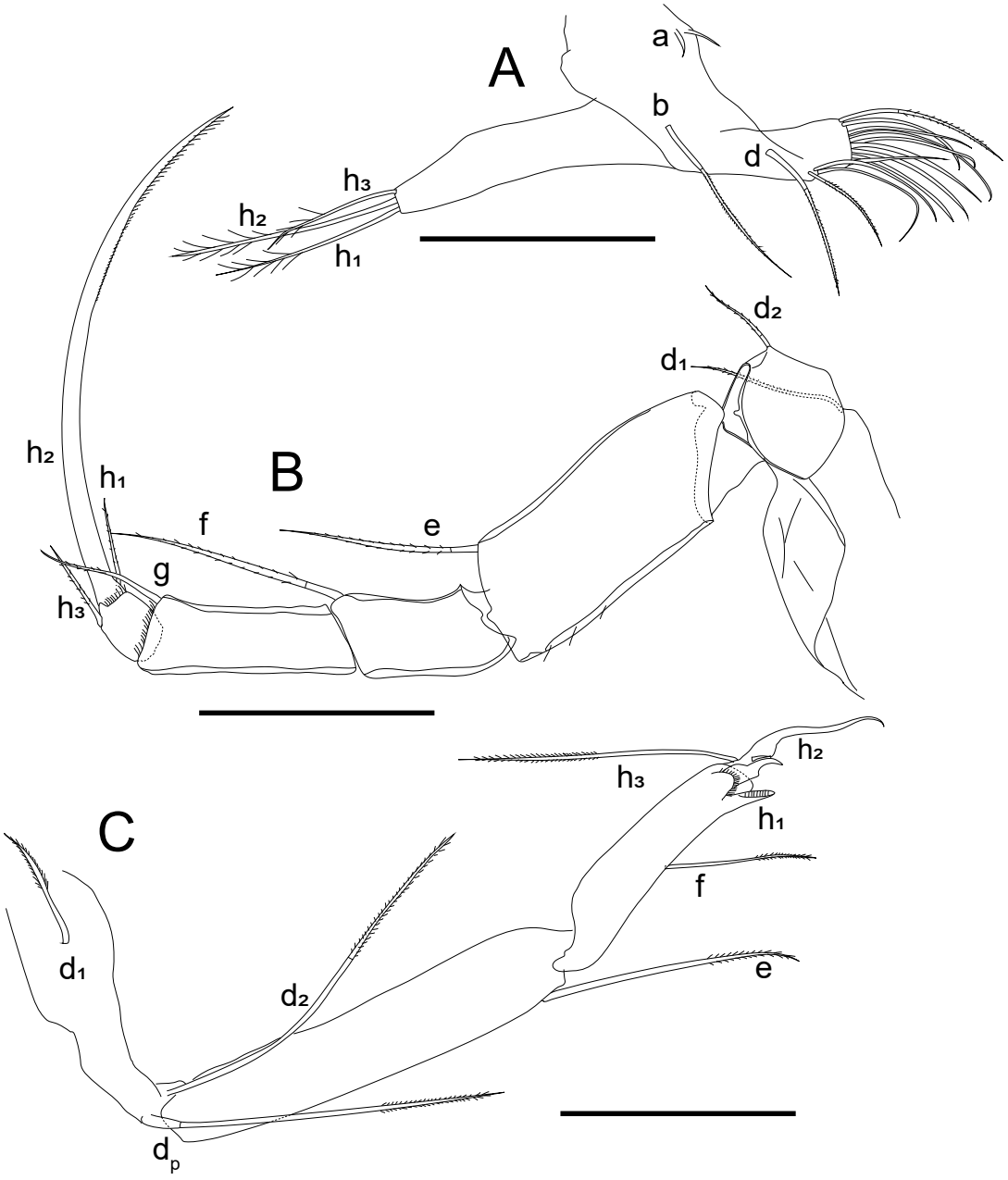

Fig. 6. *Lissostrandesia fonticola* gen. et sp. nov., holotype female (ICHUM-8612). (A–C) Fifth to seventh limbs; setules of distal setae on protopod, palp of fifth limb, and vibratory plate on fifth limb omitted. Scale bars = 0.1 mm.

We determined a nearly complete 18S sequence (1735 bp; LC789200) and a partial 16S sequence for *Cardinium* (907 bp; LC789280) from one individual from the MS population. BLAST searches found the most similar sequences to ours to be the 18S sequence from *Cypretta seurati* (AB674999.1; identity score 96.95%, query cover 100%; Hiruta et al. 2016) and the 16S sequence from *Cardinium* from *Cavernocypris hokkaiensis* (LC666825.1; identity score 98.68%; query cover 100%; Munakata et al. 2022). The 18S and *Cardinium* 16S sequences were also determined from one individual each from the KS, MB, GI, and GN populations (Table S1). The *p*-distances among the five 18S sequences ranged from 0.00 to 0.23% (mean 0.092%). The *Cardinium* 16S sequences from the five populations were identical. In our 18S-based ML tree (File S1), *L. fonticola* forms a weakly supported clade (59% ultrafast bootstrap support, or uBS) as the sister taxon to a well-supported clade (96% uBS) containing the three taxa in Cypricericinae included in the analysis.

Genetic diversity and neutrality tests

Nine haplotypes were detected (Fig. 8) among the 73 *COI* sequences from the four *L. fonticola* populations (KS, MB, GN, and MS) represented by more than one individual. Two haplotypes were detected at KS, two at MB, four at GN, and eight at MS. The most

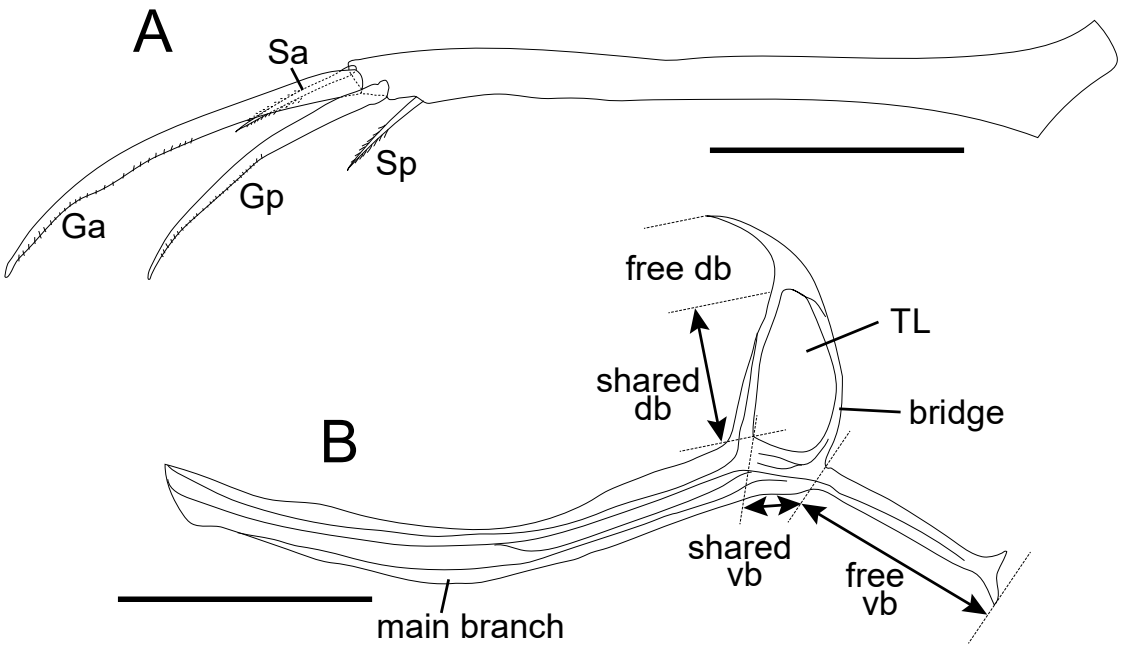

Fig. 7. *Lissostrandesia fonticola* gen. et sp. nov., holotype female (ICHUM-8612). (A) Caudal ramus. (B) Caudal ramus attachment. Abbreviations: db, dorsal branch; vb, ventral branch; Sp, posterior seta; Sa, anterior seta; Gp, posterior claw; Ga, anterior claw; TL, Triebel's loop. Scale bars: 0.1 mm.

Table 2. Inter- and intra-population genetic distances (*p*-distance, as percentages) for *COI* (608 bp) within and among six populations of *Lissostrandesia fonticola* gen. et sp. nov. in Japan. Numbers shaded in gray are within-population distances; numbers in parentheses in the table are mean values

Population	AS	GI	GN	KS	MB	MS	Overall
AS	-						
GI	0–0 (0)	-					
GN	0–0.331 (0.064)	0–0.331 (0.064)	0–0.331 (0.110)				
KS	0–0.165 (0.157)	0–0.165 (0.157)	0–0.331 (0.173)	0–0.165 (0.016)			
MB	0–0.165 (0.124)	0–0.165 (0.124)	0–0.331 (0.149)	0–0.165 (0.045)	0–0.166 (0.065)		
MS	0–0.331(0.104)	0–0.331 (0.104)	0–0.661 (0.155)	0–0.500 (0.229)	0–0.500 (0.202)	0–0.500 (0.193)	
Overall	-	-	-	-	-	-	0–0.661 (0.139)

common haplotypes, H-1 and H-4 (detected in 25 and 37 individuals, respectively), were found in all four populations. The single individuals from each of AS and GI were haplotype H-1. Five haplotypes were unique to MS, and one was unique to GN. Haplotype H-2 was shared only by populations from Honshu (GN and MS). Haplotype and nucleotide diversities were highest at MS (h = 0.772, π = 0.00193) and lowest at KS (h = 0.095, π = 0.00016) (Table 3).

The Tajima's *D* values were not significant for any population or overall (Table 3). Fu's F_S values were significant negative values (Table 3) for the MS population and overall ($P < 0.02$; Holsinger 2022), but were not significant for other populations.

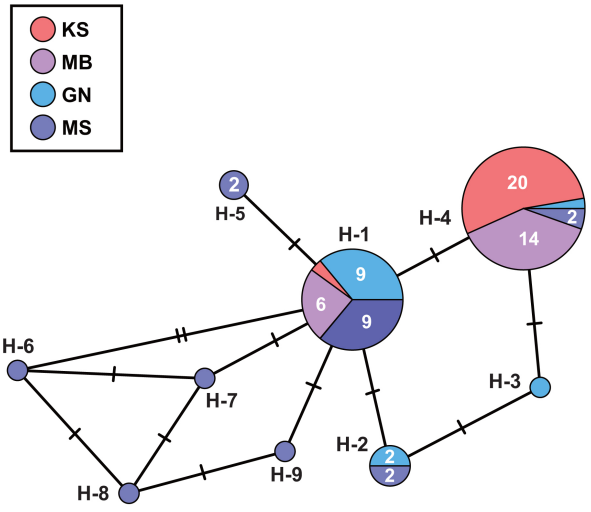

Fig. 8. IntNJ network for COI haplotypes H-1 to H-9 (605 bp) from four populations of *Lissostrandesia fonticola* gen. et sp. nov.: GN, Gudari-numa (N = 13 individuals); KS, Kanro-sensui (N = 21), MB, Mikuni-bashi Bridge (N = 20); MS, Metori Spring (N = 19). Circles indicate haplotypes, with the size of each circle proportional to the frequency of the haplotype. Numbers of individuals greater than one are labelled inside the circles. Each hatch mark on lines between circles represents one nucleotide substitution.

DISCUSSION

Taxonomy

Lissostrandesia gen. nov. can be distinguished from the other 13 genera in Cypricercinae by the following combination of characters: (1) the inner margin in the anterior part of LV lacks a groove and inner list; (2) b and d setae are present on L5; (3) d_1 and d_2 setae are present on L6; (4) the CR attachment is stout; (5) TL is located on the db of the CR attachment; (6) the vb has a long free portion (free vb; L more than twice W); and (7) the db has a free portion that contributes to the tip of TL (free db). The conditions of these seven traits and four additional traits in all cypricercine genera are summarized in table 4.

Cypricercinae currently contains four tribes (Bradleystrandesiini, Cypricercini, Lithocypridini, and Nealecypridini) that are defined mainly by the morphology of the anterior part of LV in inner view, the position of TL on the CR attachment, and the degree of development of the vb and db of the CR attachment (Savatenalinton and Martens 2009b; Martens et al. 2023). Given the condition of the three characters in *Lissostrandesia* (Table 4), we concluded it does not fit well in any of the four existing tribes and requires a new tribe.

At present, the establishment of our new tribe and genus cannot be tested by molecular data sufficiently. It is because most genera of 13 known cypricercine genera lack any molecular information; 18S and *COI* sequences have been determined from members of three (*Bradleycypris*, *Strandesia*, and *Tanycypris*) and two (*Bradleystrandesia* and *Strandesia*) genera only. But at least, the conclusion from our morphological analysis was congruent with our 18S based phylogeny (File S1) and our BLAST result for *COI* in which "Ostracoda sp.", not species of *Bradleystrandesia* or *Strandesia*, was the top hit sequence.

Table 3. Genetic diversity and the results of neutrality tests based on *COI* sequences (605 bp) for four *Lissostrandesia fonticola* local populations in Japan

Population	N	Nh	Np	h	π	Tajima's *D*	Fu's F_S
GN	13	4	2	0.526	0.00110	0.580 ($P = 0.764$)	-1.389 ($P = 0.055$)
KS	21	2	1	0.095	0.00016	-1.164 ($P = 0.125$)	-0.919 ($P = 0.079$)
MB	20	2	1	0.442	0.00073	1.025 ($P = 0.829$)	1.169 ($P = 0.631$)
MS	19	8	5	0.772	0.00193	1.031 ($P = 0.174$)	-4.473* ($P = 0.001$)
Overall	73	9	5	0.630	0.00141	-0.738 ($P = 0.294$)	-4.255* ($P = 0.016$)

Abbreviations: N, number of individuals; Nh, number of haplotypes; Np, number of polymorphic sites; h, haplotype diversity; π, nucleotide diversity; *, significant at $P < 0.02$.

Habitat

Among six water bodies on Rishiri Island, Munakata et al. (2023) collected *L. fonticola* (as "Cypricercinae sp.") only from Kanro-sensui Spring, at 6.7°C. In reexamining ostracods collected from seven sites in Daisetsuzan National Park (Munakata et al. 2022), we found this ostracod species only in the sample from Daisetsu Asahidake Spring, at 7.0°C. So far, *L. fonticola* has been collected only from sediments in springheads and spring brooks having low water temperature (6.7–8.2°C) on Rishiri Island and on Hokkaido and Honshu Islands, but not from warmer water bodies or lentic environments such as rice field, ponds, and wetlands (unpublished data). *Lissostrandesia fonticola* can thus be described as a cryophilic, benthic species inhabiting rheocrenic springs.

Conspecificity and population structure among six populations of *L. fonticola*

Given that *L. fonticola* presumably has low dispersal capability and is restricted to springs, we expected disjunct populations to be genetically isolated, comprising local lineages. However, our study revealed low genetic distances among the populations, equivalent to those expected within populations. The maximum *p*-value distances observed within and between populations (0.500% and 0.662%, respectively) were far smaller than interspecific distances previously reported for other cypridoidean ostracod genera: a 6.1% *p*-distance in *Bennelongia* De Deckker and McKenzie, 1981 (Martens et al. 2013) and 4.0–6.1% K2P distances for *Physocypria* Vávra, 1897 (Karanovic 2015). We therefore concluded that our ostracods from the six populations are conspecific, and that this species has a broad distribution (spanning more than 1000 km) across significant geographical barriers (straits).

It is interesting that a single, cryophilic, benthic ostracod species inhabiting rheocrenic springs shows such a wide distribution across straits. As ostracods show direct development, their innate dispersal capability is restricted, so passive dispersal must be involved in achieving a broad distribution. We collected only female individuals of *L. fonticola* and detected the *Cardinium* 16S sequence from an individual in

Table 4. Comparative character matrix for the 13 genera in subfamily Cypricercinae (Savatenalinton and Martens 2009a b; Ferreire et al. 2019; Martens et al. 2023). Character states present in *Lissostrandesia* are shaded gray

	anterior part of internal LV		CR attachment				An2	Mx			L5		L6
	groove	inner list	main branch[1]	Triebel's loop	free vb[2]	free db	aesthetasc Y[3]	sideways-directed bristles	setae on 1st palpal podomere	setae on 2nd palpal podomere	d-seta	b-seta	d-setae
Bradleystrandesiini													
Bradleystrandesia	0 or 1	1	slender	db	long	present	short	2	6+1	3 claws+3 setae	present	present	d1 = d2
Bradleytriebella	1	1	slender	db	long	present	normal	2	6+1	3 claws+3 setae	absent	present	?
Spirocypris	1	1	slender	db	long	present	short	2	6+1	3 claws+3 setae	present	present	?
Cypricercini													
Bradleycypris	1	0	stout	middle	long	present	normal	2	6+1	3 claws+3 setae	present	present/absent	d1 > d2
Cypricercus	1	1 or 2	stout	middle	long	present	short	2	6+1	3 claws+3 setae	present	present	d1 > d2
Neostrandesia	1	0	stout	middle	long	present	normal	2	4+1	1 claw+3 setae	present	present	d1 > d2
Pseudostrandesia	1	1	stout	middle	long	present	normal	2	6+1	3 claws+3 setae	absent	present	d1 > d2
Strandesia	1	0 or 1	stout	middle	long	present	short	2	6+1	3 claws+3 setae	present	present	d1 > d2/d1 = d2
Lithocyprdini													
Lithocypris	0	0	slender	db	long	absent	normal	2	4+1	5 claws or setae	present	present	d1 > d2
Nealecypridini													
Astenocypris	0	0	stout	middle	short	present	short	0	6+1	2 claws+3 setae	absent	absent	d1 > d2
Diaphanocypris	0	0	stout	middle	absent	present	normal	2	5+1	2 claws+3 setae	absent	absent	no d1, 2
Nealecypris	0	0	stout	middle	absent	present	normal	2	6+1	3 claws+3 setae	absent	present	d1 > d2
Tanycypris	1	0	stout	middle	short	present	normal	2	6+1	3 claws+3 setae	present	present	d1 > d2
Lissostrandesiini trib. nov.													
Lissostrandesia gen. nov.	0	0	stout	db	long	present	normal	2	6+1	3 claws+3 setae	present	present	d1 > d2

Abbreviations: LV, left valve; An2, antenna; CR, caudal ramus; Mx, maxillula; L5, L6, fifth and sixth limbs; vb, ventral branch. [1]slender, Types D, E; stout, Types A–C (see fig. 2 in Savatenalinton and Martens [2009b]). [2]long, the free ventral branch is more than twice as long as wide; short, the free ventral branch is less than twice as long as wide. [3]short, distance between tip of Y and tip of podomere is about length of Y; normal, distance between tip of Y and tip of podomere is less than length of Y; long, tip of Y reaching beyond tip of podomere.

each of five populations, suggesting that *L. fonticola* is parthenogenetic (cf. Ma and Schwander 2017; Schön et al. 2018b; Schön and Martens 2020). Because one parthenogenetic individual is theoretically enough to found a new population, this trait along with passive transport by biotic or abiotic factors may contribute to the wide distribution of *L. fonticola*. Another ostracod similarly distributed across marine barriers is the cypridid *Cypridopsis vidua* (O. F. Müller, 1776) (cf. Munakata et al. 2023), which can reproduce parthenogenetically (Havel and Hebert 1989).

Values of h and π greater than 0.5 and 0.005, respectively, are considered large (Grant and Bowen 1998; otherwise, small). Our h values were large for the GN, MS, and overall populations but small for KS and MB, and π values were small for all populations. These results indicate that the KS and MB populations (with both values small) recently underwent a bottleneck or founder event, and that GN, MS, and the overall population (with large h and small π) underwent a bottleneck followed by rapid population growth and the accumulation of mutations (Grant and Bowen 1998). The same history for MS and the overall population was supported by Fu's F_S test, in which significant negative values indicate a recent population expansion. Our data show a trend of decreasing nucleotide and haplotype diversity with increasing latitude (Lat. °N, Table 3) from Honshu to Rishiri Island. This trend may reflect the past dispersal of *L. fonticola* from Honshu northward.

Ambiguity in *COI* sequences

During *COI* sequencing, double peaks were observed at three sites in the sequencing chromatograms in both directions for 16 individuals. These ambiguous sites all involved the third position of codons, and differences in the base at these sites were synonymous, *i.e.*, did not change the amino acid. It is possible that this species has heteroplasmic mtDNA, multiple functional copies of *COI* in the mitochondrial genome, or nuclear mitochondrial DNA (NUMTs). As pointed out by Parakatselaki and Ladoukakis (2021), these conditions could affect the validity of mtDNA as a genetic marker. False heteroplasmy due to NUMTs, or biased amplification and sequencing, can lead to comparisons between nuclear and real mtDNA sequences or solely between NUMT sequences, which obviously will lead to incorrect results.

The population-genetic analyses in this study may underestimate the true genetic diversity because we excluded the three ambiguous positions. If the ambiguity were resolved, the genetic differences among the six population would remain far smaller than the interspecific differences reported in related groups, and our conclusion that the six populations are conspecific would not change.

Key to the genera in Cypricercinae

This key is modified from Martens et al. (2023); for valve Types A–E indicated in the key, see Savatenalinton and Martens (2009b). The apparent absence of Wouters organ on An1 in step 4 means either that this organ is not present or that it is so small and cannot be detected under a light microscope.

1. TL on db of CR attachment 2
- TL in middle of distal part of CR attachment 6
2. Anterior part of internal LV without groove or inner list (Type A) 3
- Anterior part of internal LV with or without groove and with 1 inner list (Type D or F) 4
3. db lacking free db ..... *Lithocypris* Martens, de Almeida and Higuti, 2023 in Martens et al. (2023)
- db with free db ..... *Lissostrandesia* gen. nov.
4. L5 lacking d seta, carapace subtriangular in lateral view, An1 with Wouters organ ..... *Bradleytriebella* Savatenalinton and Martens, 2009b
- L5 with d seta, carapace not subtriangular in lateral view, An1 lacking Wouters organ 5
5. Carapace tumid in dorsal view, valves strongly ornamented with tubercules ..... *Spirocypris* Sharpe, 1903
- Carapace elliptical in dorsal view, subovate (not tumid), valves weakly ornamented with or lacking tubercules ..... *Bradleystrandesia* Broodbakker, 1983
6. L5 lacking b seta 7
- L5 with b seta 8
7. L6 lacking d1, d2 setae, CR attachment lacking vb ..... *Diaphanocypris* Würdig and Pinto, 1990
- L6 with d1, d2 setae, CR attachment with vb ..... *Astenocypris* G.W. Müller, 1912
8. L5 lacking d seta 9
- L5 with d seta (very large in *Neostrandesia*) 10
9. CR attachment lacking vb, carapace narrow in dorsal view ..... *Nealecypris* Savatenalinton and Martens, 2009b
- CR attachment with vb, carapace elliptical in dorsal view, subovate in lateral view ..... *Pseudostrandesia* Savatenalinton and Martens, 2009b
10. CR attachment with short vb 11
- CR attachment with long, well-developed vb 12
11. CR attachment with vb short and stout, distally rounded; Triebel's loop sometimes with 2 loci ..... *Tanycypris* Triebel, 1959
- CR attachment with vb short and narrow, distally acute; Triebel's loop always single ..... *Neostrandesia* Ferreira et al., 2019
12. Anterior part of internal LV without inner list (Type B), hemipenis with triangular lateral shield (Type D) ..... *Bradleycypris* McKenzie, 1982
- Anterior part of internal LV with 0–2 inner lists (Type C, D, or E), hemipenis with wing-like or subquadrate lateral shield (Type B or C) 13
13. Carapace in lateral view elliptical, subovate, not elongate (length < twice width), hemipenis with large, winglike lateral shield (Type B), Zenker organ with chitinous sheet on both ends ..... *Strandesia* Stuhlmann, 1888
- Carapace in lateral view elongate (length > twice width), hemipenis with small, subquadrate lateral shield (Type C),

Zenker organ with crown of petal-like structures on distal end plate ..... *Cypricercus* G.O. Sars, 1895

CONCLUSIONS

We have described the ostracod *Lissostrandesia fonticola* gen. et sp. nov. in the subfamily Cypricercinae McKenzie, 1971 from six rheocrenic springs in Japan. *Lissostrandesia* can be distinguished from the other 13 cypricercine genera by a particular combination of morphological characters. We have proposed Lissostrandesiini trib. nov. for *Lissostrandesia*. The maximum *COI p*-distance among six local populations was 0.662%; a low level indicative of conspecificity. In addition, four populations for which *COI* was sequenced for 13–21 individuals all shared two main haplotypes. The six populations we examined were disjunct from one another by up to 1000 km, with those on separate islands (Rishiri, Hokkaido, and Honshu) separated by marine straits, a significant geographical barrier for freshwater animals. The highest genetic diversity was observed in the population in Yamanashi Prefecture, central Japan, and a trend was evident for decreasing diversity with increasing latitude. Our analyses suggested the past dispersal of *L. fonticola* from Honshu northward.

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Authors' contributions: MM collected specimens, conducted the taxonomic and molecular analyses, and wrote the manuscript and the figures. HT and KK contributed to the manuscript.

Competing interests: MM, HT, and KK declare that they have no conflict of interest.

Availability of data and materials: All DNA sequence data from this study have been deposited in GenBank. A list of specimens and GenBank accession numbers, and the aligned DNA sequence dataset, are available as supplementary data in Excel and fasta formats, respectively.

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Ethics approval consent to participate: Not applicable.

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Supplementary materials

Table S1. List of *Lissostrandesia fonticola* specimens used in morphological or genetic analyses, with their museum catalog and GenBank accession numbers. Abbreviations: AS, Daisetsu Asahidake Spring; GI, Genshi-no-izumi; GN, Gudari-numa; KS, Kanro-sensui; MB, springs near Mikuni-bashi Bridge; MS, Metori Spring. (download)

Table S2. List of species included in the molecular phylogenetic analysis, with their GenBank accession numbers and source references. (download)

File S1. Detailed methods for the phylogenetic analysis, and the results of the analysis. (download)

File S2. Aligned 18S sequences used in the maximum-likelihood analysis, trimmed in MEGA 11 to the shortest length among them. (download)

File S3. Aligned 18S sequences used in the maximum-likelihood analysis, reduced to 1583 positions after the removal of alignment-ambiguous sites with Gblocks ver. 0.91b in NGPhylogeny.fr under "relaxed" parameters. (download)