Zootaxa 4551 (4): 415–431
https://www.mapress.com/j/zt/

Article

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

https://doi.org/10.11646/zootaxa.4551.4.2
http://zoobank.org/urn:lsid:zoobank.org:pub:03FFCA27-5928-48D2-9E30-672A878C59A3

Morphological and molecular diversity of rissoellids (Mollusca, Gastropoda, Heterobranchia) from the Northwest Pacific island of Hokkaido, Japan

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Abstract

This study deals with four species of marine microgastropods of the family Rissoellidae. *Rissoella elatior* (Golikov, Gulbin & Sirenko, 1987), *R. golikovi* (Gulbin, 1979), *R. japonica* **n. sp.**, and *Rissoella* sp. 1 were collected in different locations around the island of Hokkaido, Japan. Light and scanning electron microscopy (SEM) were used to study the general morphology of the shell and radula, and a region of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene was amplified for 26 specimens. *Rissoella elatior* is morphologically characterized by a highly asymmetrical radula with a deep notch encircled by 10–13 minute secondary cusps on the left dorsal margin of the central tooth. *Rissoella golikovi* is characterized by a skeneiform shell and possession of three teeth per row on the radula. ***Rissoella japonica*** **n. sp.** shows five teeth per row on the radula; central tooth higher than wide; lateral and marginal teeth narrow with an outer lateral projection at the base; all teeth presenting numerous small cusps on the cutting edge. *Rissoella* sp. 1 is distinguished from *R. japonica* **n. sp.** in having *i*) very short oral lobes, *ii*) a mantle with a large, black patch and whitish blotches inside, and *iii*) different color patterns associated with the visceral mass. Although *Rissoella* sp. 1 probably represents an undescribed species, additional specimens are needed to complete its description. This study represents a first molecular approach to the family Rissoellidae. Studies of traditional morphological characters indicated four species, the addition of COI data raised the count to eight potential species, suggesting the occurrence of cryptic species among rissoellids.

Key words: COI gene, Rissoelidae, *Rissoella*, morphospecies, integrative taxonomy, ABGD

Introduction

The family Rissoellidae comprises minute marine gastropods, measuring approximately 1 mm in shell length. Rissoellids have smooth, transparent to translucent or sometimes whitish (opaque, sometimes with a band) shells with only a few taxonomic characters that can be used to delimit closely related species. They also possess an operculum with unique morphology, which is a defining characteristic of the family as a whole, but does not present interspecific difference. It is yellowish, transparent, with concentric growth lines and a nucleus near the mid region on the inner edge. The operculum also has a sharp ridge on the internal side along the columellar ridge, from which a vertical, blunt peg arises, and a short, rounded ridge passes across a portion (less than half) of the operculum (Simone 1995). The head bears a pair of oral lobes arising from the snout, and a pair of longer cephalic tentacles. The soft body is colorful, especially around the hypobranchial gland. Other characteristics of rissoellids include a variable radula, with a central tooth, a lateral tooth, and 0-2 marginal teeth per row. Ctenidium, esophageal glands, and crystalline styles are lacking, like in other basal heterobranchs. It is thought that the function of the ctenidium has been replaced with a ciliated tract that runs forward from the anus (Fretter 1948; Simone 1995). Rissoellids are hermaphroditic and undergo direct development, having a simple penis and a simultaneous hermaphrodite gonad (Fretter 1948; Simone 1995; Wise 1998).

Accepted by V. Padula: 10 Dec. 2018; published: 4 Feb. 2019

Rissoellids can be found in different habitats such as soft, sandy bottoms or hard substrata with algae (Caballer *et al.* 2014), but they are mostly associate with algae in shallow waters (Caballer *et al.* 2011), where they rasp the algal surface and probably feed on microalgae, diatoms, and detritus (Olabarria 2002). The family has a worldwide distribution, and although the presence of rissoellids in Japanese waters has been reported (58 species), most of them remain undescribed (Hasegawa 2000, 2017). To identify species in this family, contemporary works have used the shell morphology complemented with other external and internal characters like radula morphology (Ponder & Yoo 1977), radula morphology and anatomy (Simone 1995; Wise 1998), body color patterns (Ortea *et al.* 2004; Ortea & Espinosa 2004; Rolán & Hernández 2004; Espinosa & Ortea 2009), operculum and jaw structure, as well as modeling the digestive and reproductive systems (Caballer *et al.* 2011, 2014).

At present, molecular approaches are widely used in order to complement morphological studies and to solve problems associated with species identification, especially among very similar species (Maturana *et al.* 2011; Weigand *et al.* 2013; Kristof *et al.* 2016; Syromyatnikov *et al.* 2017). A gene fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI) is one of the most commonly used gene markers and its suitability for species identification has been demonstrated in a wide range of animal taxa (Hebert *et al.* 2003a, 2003b; Sun *et al.* 2012). However, although molecular techniques have been applied in some taxonomic works covering microgastropods (Kano *et al.* 2009; Weigand *et al.* 2011), there is not any similar study focused in the Rissoellidae. A limited amount of COI data is available for members of the Rissoellidae, where only two species, *Rissoella elongatospira* Ponder, 1966 and *Rissoella rissoaformis* (Powell, 1939) have been sequenced (Dinapoli & Klussmann-Kolb 2010).

In this study we describe a previously unrecognized diversity of rissoellids around Hokkaido, the Northwest Pacific island of Japan. Species are described morphologically and supported by a DNA-based species delimitation analysis. Our study represents the first insight in the phylogeny of rissoellids and presents the first use of molecular techniques as a complement of morphological information to distinguish species in the family, including the description of a new species.

Material and methods

Collection and processing of samples. Specimens were collected through snorkeling (washing different macro algae vigorously in a 30 µm planktonic mesh), and through SCUBA diving (using an airlift sampler pipe) in different localities around Hokkaido, Japan (Fig. 1). The airlift consisted of a polyvinyl chloride tube of a minimum length of 110 cm and of 5.5 cm diameter, with a SCUBA cylinder supplying air. The end of the tube was affixed to a 0.5 mm mesh nylon bag that can be removed, closed and replaced underwater. Airlift suction sampling was used on the rhizome layer of several algae and adjacent rocky substrata. Living specimens were sorted under a dissecting stereomicroscope Nikon SMZ1500 (Nikon, Tokyo, Japan) and photographed with a Nikon D5200 (Nikon, Tokyo, Japan) digital camera attached to the stereomicroscope.

To facilitate fixation and separation of the soft part from the shell, specimens were placed in small plastic containers with enough seawater to encourage the animals to extend from the aperture and then placed in a microwave oven (MWO) (700 W for 3–5 s) (Galindo *et al.* 2014), and preserved in 99% ethanol. After separating the soft part from the shell, a piece of the foot-head was cut for molecular analysis and the rest was placed in 30% bleach to dissolve the tissue and extract the operculum and radula. In the case of *Rissoella* sp. 1, the whole material was homogenized for DNA extraction, because only two specimens were collected. Shell, operculum, and radula were cleaned with commercial bleach diluted to 15%, washed in 70% ethanol, and observed with a Hitachi S-3000N (Hitachi, Tokyo, Japan) scanning electron microscope. Voucher material has been deposited at the Invertebrate Collection of the Hokkaido University Museum (ICHUM), Sapporo, Japan.

DNA extraction, PCR amplification and sequencing. Total DNA was extracted from foot tissue or whole specimens (Table 1) using a DNeasy Blood & Tissue Kit (Qiagen, USA). A partial region of the COI gene was amplified using a universal primer pair, LCO1490 and HCO2198 (Folmer *et al.* 1994). Each PCR reaction mixture contained 2 µl DNA and 8 µl PCR-mix (5.75 µl sterile dH_2O, 1 µl of 10 × buffer, 0.83 µl of 2.5 mM dNTP, 0.33 µl of 10 µM forward primer, 0.33 µl of 10 µM reverse primer, and 0.05 µl of *Taq*-polymerase). Thermocycler conditions included initial denaturation at 94 °C for 3 min; 35 cycles of denaturation at 94 °C for 40 s, annealing at 42 °C for 1 min, elongation at 72 °C for 1 min; and final elongation at 72 °C for 5 min. Amplified products were confirmed by electrophoresis in 1% agarose gel. Sequencing reactions were performed with BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, USA) using 0.8 pmol/µl of the primers used for amplification. Sequencing was done using ABI Prism 3730 Genetic Analyzer (Applied Biosystems, USA). Novel sequences generated in this study have been deposited in GenBank under the following accession numbers: MK210173–MK210198.

Analysis of molecular data and species delimitation analyses. Sequenced fragments were assembled using Geneious ver. 10.2.3 (Biomatters, Auckland, New Zealand; Kearse *et al.* 2012), and BLAST searches were performed to check for amplification of contaminants. *Rissoella rissoaformis* and *Rissoella elongatospira* were used as outgroups. Sequences were aligned using MUSCLE (Edgar 2004). Sequence divergence was calculated by the Kimura 2-parameter (K2P) substitution model (Kimura 1980), the standard model used in DNA barcoding studies. A maximum-likelihood (ML) tree (Felsenstein 1981) was created using RAxML (Stamatakis 2006) to provide a graphical overview of genetic distances across the data set. Node support was inferred with bootstrap analysis (1000 pseudoreplicates).

Four different methods were used in the species delimitation analyses: automatic barcode gap discovery (ABGD) (Puillandre *et al.* 2012); TCS (Clement *et al.* 2000); Poisson tree processes (PTP) (Zhang *et al.* 2013); and Bayesian PTP (bPTP) (Zhang *et al.* 2013). The ABGD analysis was carried out with the aligned sequence dataset and performed in a web-based interface (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html). The TCS analysis was conducted using the aligned sequence dataset, with the parsimony connection limit set at 95%. The PTP/bPTP analyses were performed with the ML tree in a web-based interface (http://species.h-its.org/ptp/).

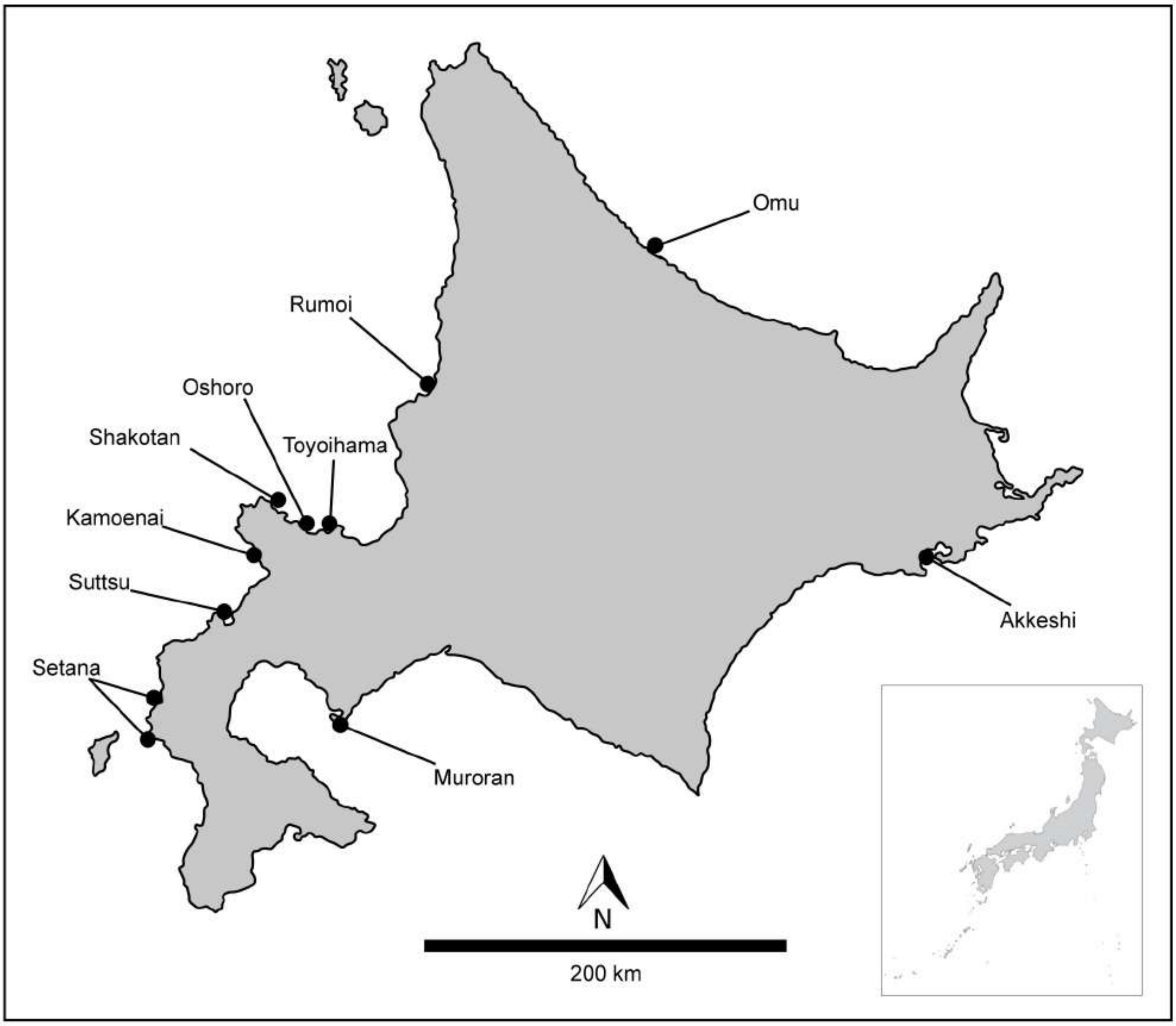

FIGURE 1. Sampling sites in Hokkaido island, Japan.

Results

Molecular diversity, phylogenetic analysis, and species delimitation. The four morphospecies initially identified in this study were examined by applying a combination of four molecular delimitation approaches based on the mitochondrial COI sequence data. In total, 26 specimens were successfully sequenced (Table 1). The aligned final dataset was 503 bp in length. The phylogenetic tree recognized four morphospecies previously described as clades highly supported. Those clades were split into eight Molecular Taxonomic Units (MOTUs) based on the TCS, PTP, and bPTP species delimitation analyses, and this was congruent with the eight MOTUs recovered by the ML analysis. The ABGD species delimitation analysis detected seven MOTUs (Fig. 2). Three of the four morphospecies turned out to comprise more than one clade: *Rissoella golikovi* comprised three distinct MOTUs (B, C, and D); *Rissoella japonica* **n. sp.** comprised two MOTUs (E and F); *Rissoella elatior* comprised two MOTUs (G and H); while *Rissoella* sp. 1 comprised a single MOTU (A).

Pairwise distance comparisons based on K2P between the analyzed specimens are shown in the Supplementary Material (Table S1). Mean K2P distances within morphospecies ranged from 0.2% to 8.2%, and the interspecific comparisons were much higher and ranged from 26.0% to 30.0% (Supplementary Material, Table S2). The K2P distances within MOTUs ranged from 0.1% to 0.7%; while the distance between MOTUs ranged between 25.0% to 31.0%, with exception to the comparison of the MOTUs B-C (4.4%), B-D (15.0%), C-D (14.7%), E-F (16.0%), and G-H (16.6%) (Supplementary Material, Table S3).

Systematics

Family Rissoellidae Gray, 1850

Genus *Rissoella* Gray, 1847

Rissoella elatior (Golikov, Gulbin & Sirenko, 1987)

(Figs 2A–D, 3A–H)

Jeffreysina elatior Golikov, Gulbin & Sirenko, 1987: 35, pl. 3, fig. 6; Kantor & Sysoev, 2006: 248, pl. 123, fig. D (holotype 36525/1; Moneron Island, Russia). Type material not available for analyses.
Jeffreysiella elatior—Hasegawa, 2017: 398, 1063, pl. 355, fig. 6.

Material examined. Thirteen mature specimens (ICHUM RT1001, RT1002, RO1001, RO1002, RO1003, RSH1001, RSH1002, RK1001, RK1002, RM1001, RSU1001, RSE1001, and RSE1002). For information on specimens collection locality and GenBank accession numbers see Table 1.

Description. Shell minute (800–1270 µm) but relatively larger if compared to other species described here, thin, fragile, translucent or whitish opaque, elongate (width about 63% of length), with narrow umbilicus, spire of about 25% of total length (Fig. 4A). Protoconch smooth, slightly pointed, of approximately one whorl, without sculpture at suture (Figs. 4B, C). Teleoconch smooth except for faint markings of growth lines; with deep suture; up to 3 ½ convex whorls; aperture simple, entire, semicircular, slightly shorter or almost 50% of total length. Operculum typical of family (Fig. 4D). Head–foot opaque white, with slender oral lobes and longer cephalic tentacles. Mantle brown or black pigmented, with black patch centrally placed on dorsal portion of body whorl. Black patch hardly recognized in specimens with black mantle (Figs. 3B–D). Radular formula 15–16 × 2.1.R.1.2 (Fig. 4E). Central tooth wide (width about 61% of length), with 7–8 sharp cusps, latter gradually increasing in size from left to right until 6th (or in some cases 7th); right-most cusp slightly smaller than left ones. Group of 10–13 minute secondary cusps encircling upper margin of last right cusp (Figs. 4E, F). Lateral teeth elongate-triangular (width about 78% of length), each with large, sharp, smooth median cusp, and 8–12 smaller cusps along inner and outer margins (Figs. 4E, G). Inner marginal teeth represented by small, curved plates (width about 93% of length), each with large, sharp, smooth median cusp, flanked by 4–5 (along inner margin) or 5–7 (along outer margin) smaller cusps (Figs. 4E, H). Outer marginal teeth reduced, simple, plate-like (width almost 200% of length) (Figs. 4E, H).

Distribution and microhabitat. Originally reported from the northern part of the Sea of Japan (Moneron Island) (Golikov *et al.* 1987), Russia, and subsequently reported to be widely distributed along the Japanese

Archipelago from Hokkaido to Miyako Island, Okinawa (Hasegawa 2017). Material in this study was collected from Rumoi to Setana on the Sea of Japan; and on the Pacific coast near Muroran, Japan. It was found on various algae including the coralline algae *Corallina* spp.

Remarks. Although the type specimens of *Rissoella elatior* have not been examined in this work, our newly sampled material agrees with the original description of this species (Golikov *et al.* 1987), as well as the photograph of the holotype (Kantor & Sysoev 2006: pl. 123, fig. D). In some specimens (Fig. 3A) the mantle coloration is brighter than others (Figs. 3B–D), the former being pale brown with yellowish white asymmetrical patches and a brown patch centrally placed on the dorsal portion of the body whorl.

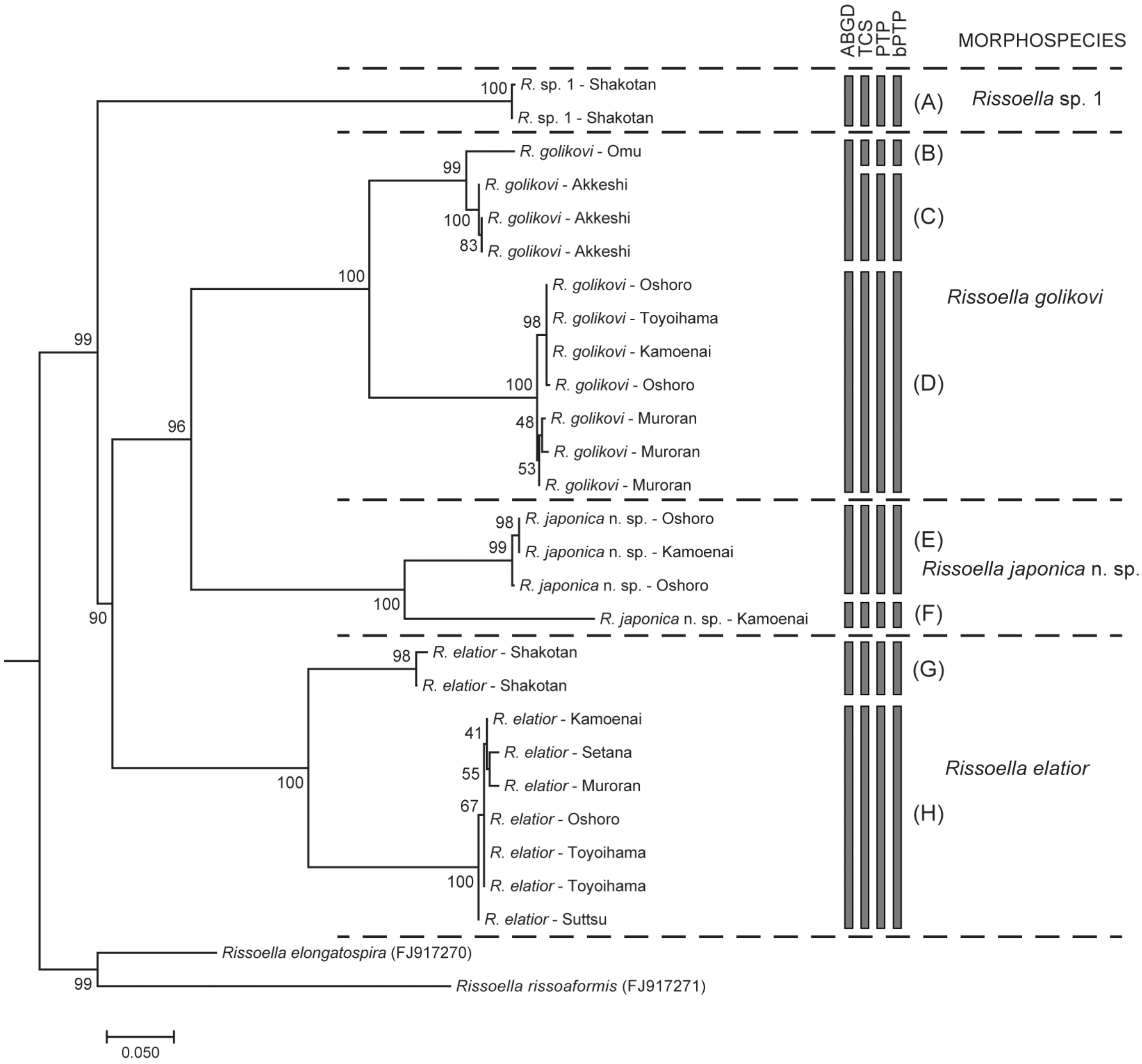

FIGURE 2. Maximum-likelihood tree analysis of the COI sequences of *Rissoella* from Hokkaido, Japan. Scale bars represent raw percentage sequence divergence. MOTUs are indicated with letters in parenthesis.

Rissoella golikovi (Gulbin, 1979)

(Figs 3E–F, 5A–G)

Jeffreysina golikovi Gulbin, 1979: 89, figs. 1–2 (holotype ZIN 41388/1; Vanino Bay, Sea of Japan, Russia); Kantor & Sysoev, 2006: 248, pl. 123, fig. E (paratype); Hasegawa, 2017: 398, 1063, pl. 355, fig. 7. Type material not available for analyses.

Material examined. Fourteen mature specimens (ICHUM RT3001, RT3002, RT3003, RO3001, RO3002, RO3003, RK3001, RM3001, RM3002, RM3003, ROM3001, RA3001, RA3002, RA3003). For information on specimens collection locality and GenBank accession numbers see Table 1.

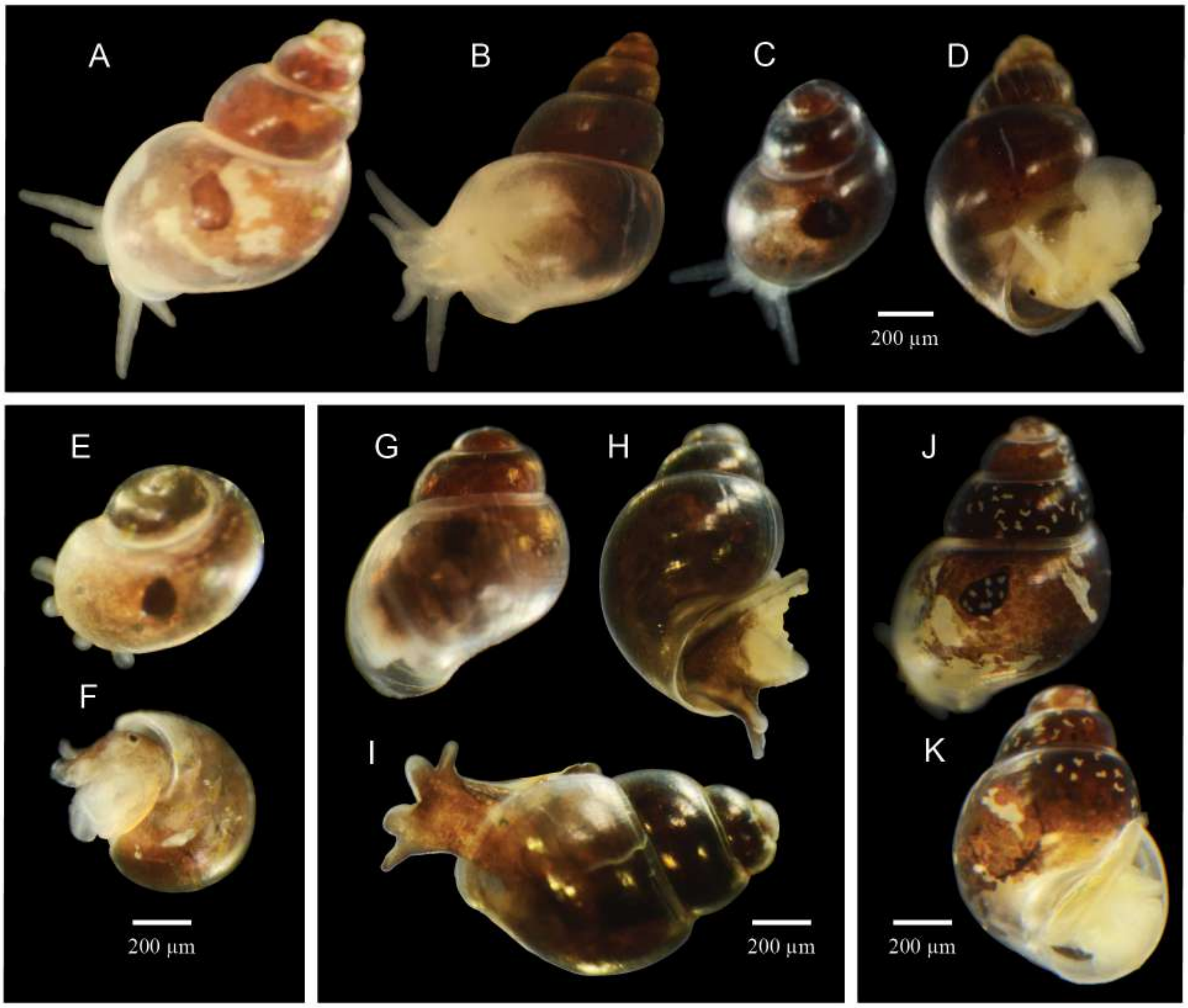

FIGURE 3. Living specimens. (A–D) *Rissoella elatior*. (E–F) *Rissoella golikovi*. (G–I) *Rissoella japonica* **n. sp.** (J–K) *Rissoella* sp. 1.

Description. Shell minute, smaller (296–450 µm) in comparison to other rissoellids, thin, extremely fragile, translucent or whitish opaque, skeneiform (width about 150% of length), with deep, widely perforate umbilicus (Fig. 5A). Protoconch smooth, of about one whorl (Figs. 5B, C). Teleoconch smooth with distinct growth lines, slightly deep suture, of about three convex whorls; aperture simple, entire, nearly circular but with margin adjacent to previous whorl flattened. Operculum typical of family (Fig. 5D). Head–foot brown or dark grey with colorless sole; oral lobes short; cephalic tentacles slightly longer than oral lobes; oral lobes and cephalic tentacles proximally having similar coloration to head, gradually becoming transparent in distal portion. Mantle dark brown or black pigmented, with black or darker brown patch on center of dorsal portion of body whorl; another smaller dark patch placed on left of neck (Figs. 3E, F). Radular formula 12–13 × 1.R.1 (Fig. 5E). Central tooth higher than wide (width about 52% of length), with medial narrow ridge, cutting edge with one small central sharp cusp flanked by several larger cusps (Figs. 5F, G). Lateral teeth triangular (width about 41% of length), each with median ridge; cutting edge with larger median cusp, flanked by 4–5 sharp cusps, consecutively decreasing in size (Figs. 5E, F).

Distribution and microhabitat. Known from Vanino Bay, as well as middle Kurile Islands, Russia. Material in this study was collected from Hokkaido, Japan: Otaru and Kamoenai (Sea of Japan), near Omu (Sea of Okhotsk), and Akkeshi and Muroran (Pacific). It was found on various algae including the coralline algae *Corallina* spp.

Remarks. The type material of *Rissoella golikovi* was not examined, due to restrictions on shipping biological material. However, our newly sampled material agrees with its description. The present morphospecies is nearly identical in radula morphology to *Rissoella globularis* (Forbes & Hanley), which has been reported from France to northern Norway, as illustrated by Sars (1878). Nevertheless, these two species can clearly be distinguished by shell morphology. In *R. globularis*, the shell is depressed conical, while it is skeneiform in *R. golikovi*. The skeneiform shell of this species makes it easily distinguishable from other species in the family where shells are either ovate or elongate. *Rissoella golikovi* was first described by Gulbin (1979) from Vanino Bay, Russia, and was subsequently recorded from the eastern part of Hokkaido (Hasegawa 2017).

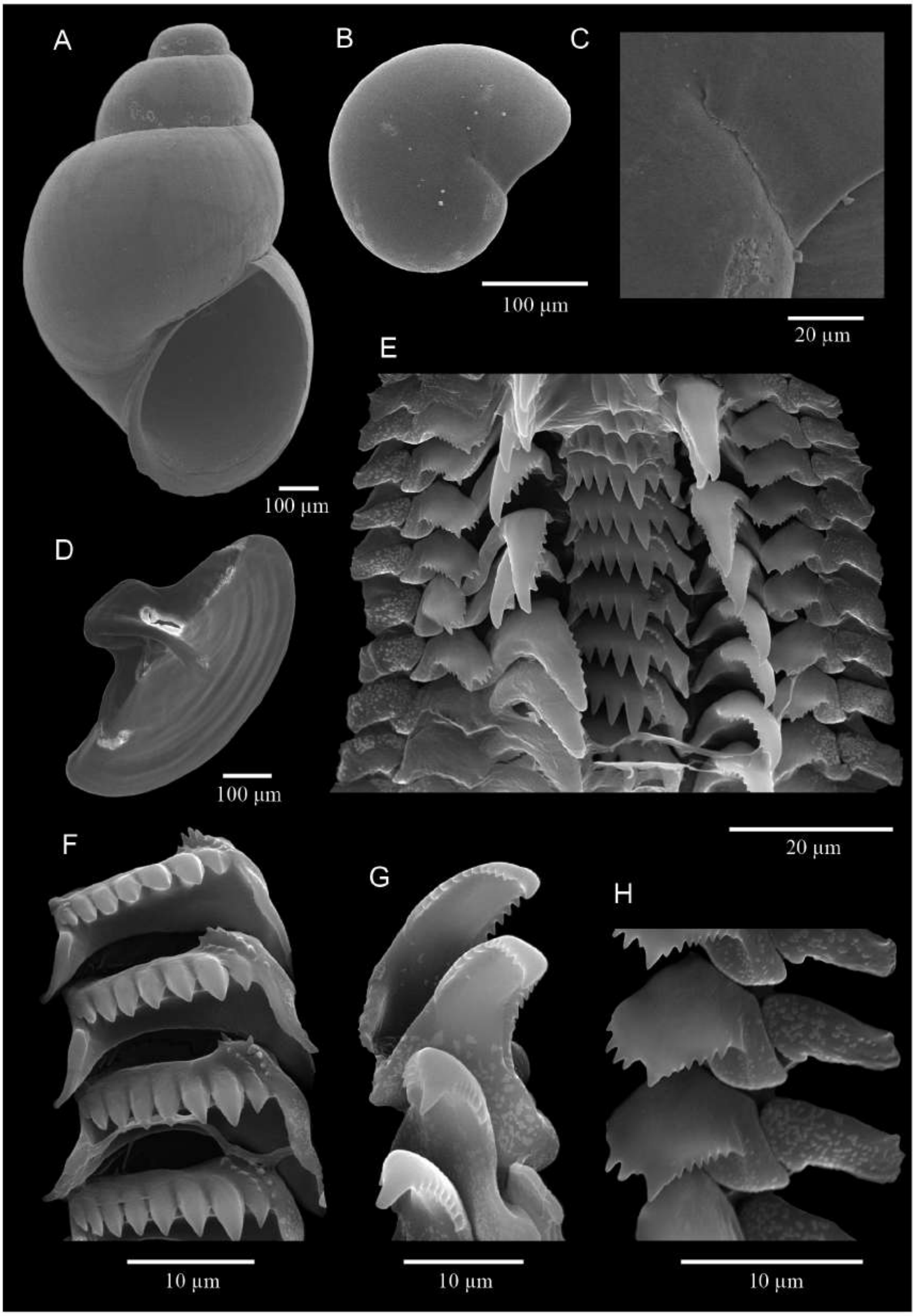

FIGURE 4. SEM micrographs of *Rissoella elatior*. (A) Apertural view of the shell. (B) Apical view of the protoconch. (C) Detail of the protoconch showing the suture. (D) Inner face view of the operculum. (E) Radula. (F) Central teeth. (G) Lateral teeth. (H) Inner and outer marginal teeth.

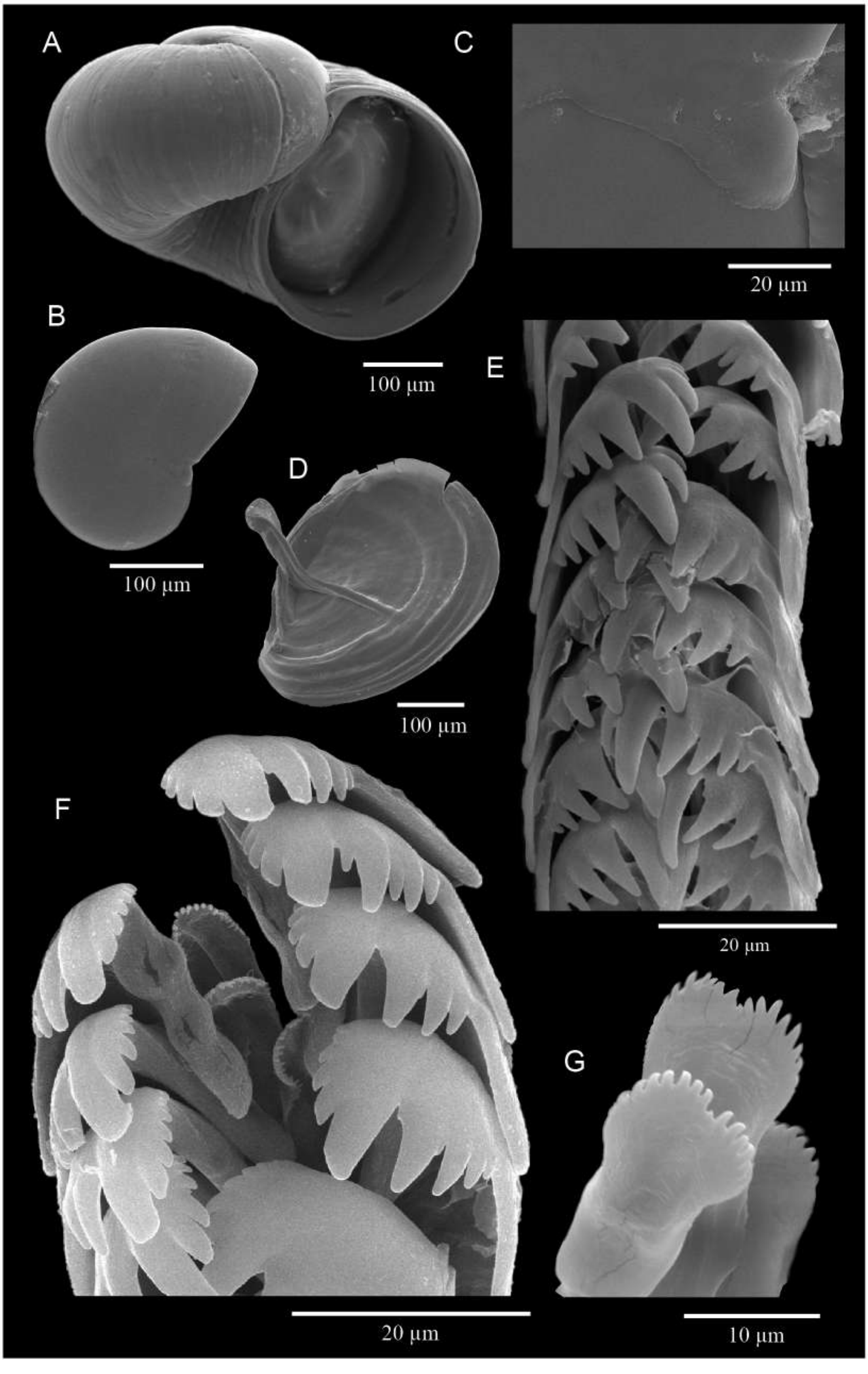

FIGURE 5. SEM micrographs of *Rissoella golikovi*. (A) Apertural view of the shell. (B) Apical view of the protoconch. (C) Detail of the protoconch showing suture. (D) Inner face view of the operculum. (E–F) Radula. (G) Central teeth.

Rissoella japonica **Chira & Hasegawa, n. sp.**

(Figs 3G–I, 6A–G)

Rissoella sp.—Hasegawa, 2000: 700-701, plate 349, fig. Rissoellidae-1; Hasegawa, 2017: 398, 1063, pl. 355, fig. 5.

Type material. Holotype: adult, 0.9 mm (ICHUM RK2001); Kamoenai, Hokkaido, Japan, 43°08′10.5″N 140°25′43.1″E, 6 November 2016. **Paratypes:** 3 specimens (ICHUM RK2002, RO2001, RO2002) from Oshoro Bay, Hokkaido, Japan; 1 specimen (RSH2001); Shakotan, Hokkaido, Japan. For information on specimens collection locality and GenBank accession numbers see Table 1.

ZooBank registration: urn:lsid:zoobank.org:act:E3102674-B307-40F6-A700-7696AE32FCA8

Etymology. The species name, *Rissoella japonica*, refers to the geographical distribution from where the species was found.

Diagnosis. Protoconch with rippled sculpture at suture. Radula, central tooth with 10-13 sharp cusps on cutting edge. Lateral teeth narrow, with median ridge becoming basal process, and outer lateral projection on base; cutting edge with major median cusp, flanked by 5-6 (along outer margin) or 7-9 (along inner margin) sharp cusps. Marginal teeth similar in shape to lateral one but smaller, cutting edge with median cusp, flanked by 3-5 smaller sharp cusps on each side.

Description. Shell minute (764–1091 µm), thin, fragile, translucent or whitish opaque, elongate (width about 67% of length), with narrow umbilicus, spire of about 30% of total length (Fig. 6A). Protoconch smooth, of about 1 whorl, with rippled sculpture along suture (Figs. 6B, C). Teleoconch smooth, with distinct growth lines, deep suture, about 2 ½ convex whorls; aperture simple, entire, semicircular, slightly longer than 50% of total length. Operculum typical of family (Fig. 6D). Head–foot dark brown with colorless sole; oral lobes and tentacles dark brown. Mantle dark brown or black pigmented, with black patch placed slightly to left on dorsal portion of body whorl (Figs. 3G-I). Radular formula 11-13 × 1.1.R.1.1 (Fig. 6E). Central tooth higher than wide (width about 48% of length), cutting edge with 10–13 sharp cusps of different sizes (Figs. 6E, F). Lateral teeth narrow (width about 23% of length), with median ridge becoming basal process, outer lateral projection on base; cutting edge with larger median cusp, flanked by 5–6 (along outer margin) or 7–9 (along inner margin) sharp cusps (Figs. 6E, G). Marginal teeth with similar shape to lateral one but smaller (width about 33% of length); cutting edge with median cusp, flanked by 3–5 smaller sharp cusps on each side (Figs. 6E, G).

Distribution and microhabitat. In the Sea of Japan from Otaru to Setana, Japan. It was found in the intertidal zone on various algae including the coralline algae *Corallina* spp.

Remarks. Both *R. japonica* **n. sp.** and *R. elatior* occur sympatrically in some localities, and they might be confused. However, they can be distinguished by the head-foot coloration (being dark brown in *R. japonica* **n. sp.** and white in *R. elatior*) and the radula morphology, as well as by conchological characters such as spire/total length and aperture/total length ratios. Based on radula morphology, *R. japonica* **n. sp.** belongs to a group containing the type species of *Rissoella s.s.*, *R. diaphana* illustrated by Thiele (1929–1935; as *R. glabra*), in having a symmetrical configuration with five teeth per row. *Rissoella japonica* **n. sp.** can be distinguished from *R. diaphana* by the relatively narrower and smaller central tooth.

Rissoella **sp. 1**

(Figs 3J, 3K)

Material examined. Two mature specimens: ICHUM RSH4001, RSH4002; Shakotan, Hokkaido, Japan, 43°18′06.2″N 140°35′55.6″E, 25 August 2017. Both specimens were used for DNA extraction. For further information on specimens collection locality and GenBank accession numbers see Table 1.

Remarks. Shell minute, thin, fragile, translucent or whitish, elongate. Head–foot translucent white; with very short round oral lobes, long cephalic tentacles with tapering tip, translucent as well. Mantle pigmented in light brown with three or four big yellowish asymmetrical patches. Big black patch, with few small whitish blotches inside, centrally placed on the dorsal portion of body whorl. Visceral mass dark brown to black, with several elongate whitish blotches (Figs. 3J, K).

Distribution and microhabitat. Found in Shakotan, Hokkaido, Japan; in the subtidal zone on red algae *Gelidium* spp.

TABLE 1. Specimens analyzed in this study.

Species	Specimen Voucher	Locality (Japan)	Latitude / Longitude	Collection Date	GenBank Accession
Rissoella elatior	RO1001	Oshoro	43°12′33.7″N, 140°51′30.3″E	28/07/2017	MK210173
Rissoella elatior	RO1002	Oshoro	43°12′33.7″N, 140°51′30.3″E	28/07/2017	-
Rissoella elatior	RO1003	Oshoro	43°12′33.7″N, 140°51′30.3″E	28/07/2017	-
Rissoella elatior	RSH1001	Shakotan	43°18′06.2″N, 140°35′55.6″E	04/10/2016	MK210174
Rissoella elatior	RSH1002	Shakotan	43°18′06.2″N, 140°35′55.6″E	04/10/2016	MK210175
Rissoella elatior	RK1001	Kamoenai	43°08′10.5″N, 140°25′43.1″E	06/11/2016	-
Rissoella elatior	RK1002	Kamoenai	43°08′10.5″N, 140°25′43.1″E	06/11/2016	MK210176
Rissoella elatior	RM1001	Muroran	42°18′18.4″N, 140°59′02.7″E	05/11/2016	MK210177
Rissoella elatior	RT1001	Toyoihama	43°13′34.7″N, 141°00′58.3″E	14/04/2017	MK210178
Rissoella elatior	RT1002	Toyoihama	43°13′34.7″N, 141°00′58.3″E	14/04/2017	MK210179
Rissoella elatior	RSU1001	Suttsu	42°47′15.1″N, 140°18′27.7″E	28/05/2017	MK210180
Rissoella elatior	RSE1001	Setana	42°27′45.7″N, 139°51′04.6″E	08/10/2017	MK210181
Rissoella elatior	RSE1002	Setana	42°27′45.7″N, 139°51′04.6″E	08/10/2017	-
Rissoella golikovi	RO3001	Oshoro	43°12′33.7″N, 140°51′30.3″E	28/07/2017	MK210186
Rissoella golikovi	RO3002	Oshoro	43°12′33.7″N, 140°51′30.3″E	28/07/2017	MK210187
Rissoella golikovi	RO3003	Oshoro	43°12′33.7″N, 140°51′30.3″E	28/07/2017	-
Rissoella golikovi	RK3001	Kamoenai	43°08′10.5″N, 140°25′43.1″E	28/05/2017	MK210188
Rissoella golikovi	RM3001	Muroran	42°18′18.4″N, 140°59′02.7″E	05/11/2016	MK210189
Rissoella golikovi	RM3002	Muroran	42°18′18.4″N, 140°59′02.7″E	05/11/2016	MK210190
Rissoella golikovi	RM3003	Muroran	42°18′18.4″N, 140°59′02.7″E	05/11/2016	MK210191
Rissoella golikovi	RT3001	Toyoihama	43°13′34.7″N, 141°00′58.3″E	14/04/2017	MK210192
Rissoella golikovi	RT3002	Toyoihama	43°13′34.7″N, 141°00′58.3″E	14/04/2017	-
Rissoella golikovi	RT3003	Toyoihama	43°13′34.7″N, 141°00′58.3″E	14/04/2017	-
Rissoella golikovi	ROM3001	Omu	44°35′23.5″N, 142°57′40.0″E	11/06/2017	MK210193
Rissoella golikovi	RA3001	Akkeshi	43°01′08.7″N, 144°50′05.8″E	02/07/2017	MK210194
Rissoella golikovi	RA3002	Akkeshi	43°01′08.7″N, 144°50′05.8″E	02/07/2017	MK210195
Rissoella golikovi	RA3003	Akkeshi	43°01′08.7″N, 144°50′05.8″E	02/07/2017	MK210196
Rissoella japonica **n. sp.**	RO2001	Oshoro	43°12′33.7″N, 140°51′30.3″E	28/07/2017	MK210182
Rissoella japonica **n. sp.**	RO2002	Oshoro	43°12′33.7″N, 140°51′30.3″E	28/07/2017	MK210183
Rissoella japonica **n. sp.**	RK2001	Kamoenai	43°08′10.5″N, 140°25′43.1″E	06/11/2016	MK210184
Rissoella japonica **n. sp.**	RK2002	Kamoenai	43°08′10.5″N, 140°25′43.1″E	06/11/2016	MK210185
Rissoella japonica **n. sp.**	RSH2001	Shakotan	43°18′06.2″N, 140°35′55.6″E	28/05/2017	-
Rissoella sp. 1	RSH4001	Shakotan	43°18′06.2″N, 140°35′55.6″E	25/08/2016	MK210197
Rissoella sp. 1	RSH4002	Shakotan	43°18′06.2″N, 140°35′55.6″E	25/08/2016	MK210198

Discussion

In the present study we explore the consistency of traditional morphological features (shell and radula morphology, and body color pattern) to identify species within the Rissoellidae, and compared this to a molecular framework based on the phylogenetic analysis of COI sequence data. Using a contemporary set of morphological characters, we could distinguish four morphospecies: *Rissoella elatior*, *R. golikovi*, *R. japonica* **n. sp.**, and the unidentified *Rissoella* sp. 1. The first morphospecies, *R. elatior*, has a very characteristic radula which is also similar to a species described from Australian, *R. colleenae* Ponder & Yoo, 1977; however, they can be distinguished by the number of minute secondary cusps encircling the upper margin of the last right cusp, and the inner marginal teeth morphology. In *R. colleenae*, there are eight secondary cusps and the cutting edges of the inner marginal teeth are bluntly rounded, while in *R. elatior* there are 10–13 secondary cusps and the cutting edges of the marginal teeth have a distinct number of cusps. The second morphospecies, *Rissoella golikovi*, has a skeneiform shell, making it easily distinguishable from other species in the family where shells are either ovate or elongate. It shows resemblance to *R. globularis*, distributed in northeastern Atlantic, in radula morphology. Further information of *R. golikovi* and *R. globularis* are needed—specifically in terms of the radula using SEM and molecular analysis—to confirm the relationship between these two species. The third, *R. japonica*, is described here as a new species. The novelty of *R. japonica* is clearly defined, compared to other species, by radula morphology. The last morphospecies, *Rissoella* sp. 1, shows a distinct color pattern that differs from any other known species in the family. It may represent another undescribed species; however, the only two specimens available were used for DNA extraction. New specimens and SEM observations are needed to describe in detail the shell and radula, and to confirm the novelty of *Rissoella* sp. 1.

In the molecular phylogenetic analysis, the 26 COI sequences taken from the specimens in this study clustered with their respective morphological identities (sequences generated from specimens identified as *R. golikovi*, were more closely related to each other, than to other specimens). Each of the clades, with the exception of *Rissoella* sp. 1, was composed of more than one MOTUs. Adding more specimens from *Rissoella* sp. 1 into future molecular analyses may result in the discovery of additional cryptic species. Our species delimitation analyses supported that these MOTUs potentially represent (at most) eight possible cryptic species, because the intra-MOTU genetic distances were generally high: *Rissoella golikovi* (14.7%), *R. japonica* **n. sp.** (16.0%), and *R. elatior* (16.6%). Comparison between mitochondrial and nuclear gene trees might help our understanding of the nature of these MOTUs. In this study, morphospecies were identified using a contemporary set of morphological characteristics including body color pattern, shell morphology, and radula morphology. Admittedly, we were not able to distinguish hidden lineages using those morphological traits alone. To distinguish those “possible” cryptic species might require, perhaps, detailed study of the internal anatomy (e.g. genitalia).

To date, there is little agreement among specialists as to the generic or subgeneric placement of some rissoellid species, and some species have been regarded as members of *Jeffreysia* Alder in Forbes & Hanley, 1850; *Jeffreysiella* Thiele, 1912; *Jeffreysiopsis* Thiele, 1912; *Jeffreysina* Thiele, 1925; *Jeffreysilla* Thiele, 1925; and *Zelaxitas* Finlay, 1927; besides *Rissoella*. Those genera or subgenera were originally distinguished based on significant differences in radular characters. In later studies, Ponder & Yoo (1977), in a comprehensive study of Australian species, recognized four subgenera, *Rissoella s.s.*, *Jeffreysilla*, *Jeffreysiella*, and *Zelaxitas*, in the single genus *Rissoella*. Some researchers generally separate *Jeffreysina* as a distinct genus (e.g., Kantor & Sysoev 2006). Our studied material of *R. golikovi* is nearly identical to *R. globularis*, the type species of *Jeffreysina*, based on radula morphology as illustrated by Sars (1878). They differ from other genera/subgenera in the family in possessing only three teeth per row, in comparison to 5–7 in others. Nevertheless, most of the recent researchers generally accept only one genus, *Rissoella*, in the family (e.g., WoRMS, http://www.marinespecies.org/aphia.php?p=taxdetails&id=138438). We follow this taxonomic scheme at the moment. A revision of Rissoellidae in genus and subgenus levels is beyond the scope of this paper.

In this study we reported a new rissoellid species, and have generated the first molecular data of rissoellids from Japan. The COI sequence data generated provides some of the first insights into potential cryptic speciation within the family Rissoellidae, providing support for the use of additional morphological features which may be useful in reconciling cryptic diversity and identification, even among closely related species within this group of microgastropods.

Acknowledgements

We are grateful to Dr. Vladimir V. Gulbin (A.V. Zhirmunskii Institute of Marine Biology, Vladivostock, Russia) for providing information related to the species descriptions. We are also indebted to three anonymous reviewers who improved this manuscript with their comments and suggestions. This study was funded by a MEXT PhD Scholarship (no number) to LECHS, as well as a joint JSPS/MBIE-RSNZ (PG7M170001) to KCW and SCW, and JSPS KAKENHI (17K07520) to HK.

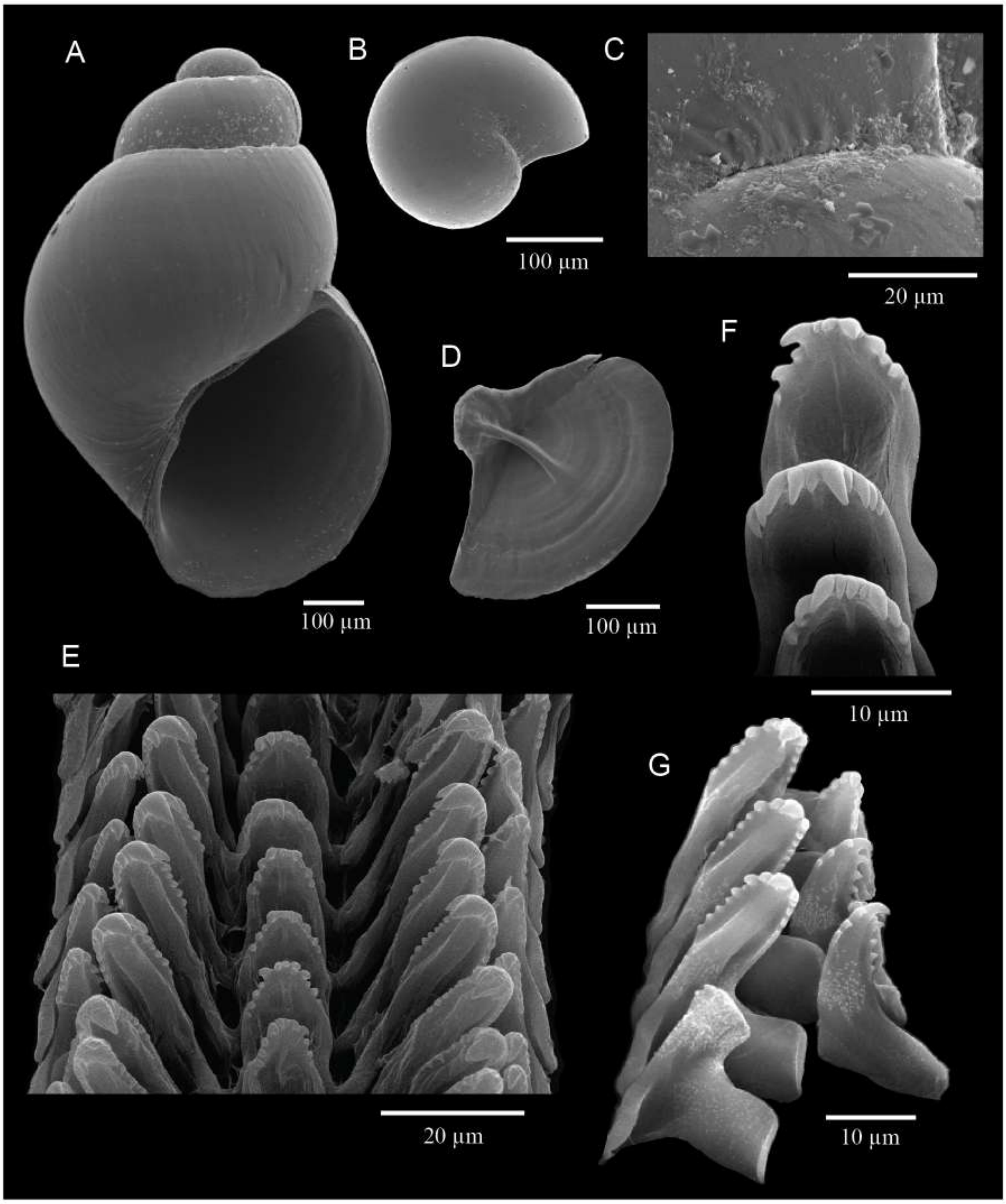

FIGURE 6. SEM micrographs of *Rissoella japonica* **n. sp.** (A) Apertural view of the shell. (B) Apical view of the protoconch. (C) Detail of the protoconch showing the suture. (D) Inner face view of the operculum. (E) Radula. (F) Central teeth. (G) Lateral and marginal teeth.

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TABLE S1. K2P pairwise sequence distances (in percentage) between the analyzed specimens.

Specimens	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Rissoella elatior – Oshoro	-												
Rissoella elatior – Shakotan	16.6	-											
Rissoella elatior – Shakotan	16.6	0.6	-										
Rissoella elatior – Kamoenai	0.2	16.9	16.9	-									
Rissoella elatior – Muroran	1.0	16.9	16.9	0.8	-								
Rissoella elatior – Toyoihama	0.0	16.6	16.6	0.2	1.0	-							
Rissoella elatior – Toyoihama	0.0	16.6	16.6	0.2	1.0	0.0	-						
Rissoella elatior – Suttsu	0.4	16.1	16.1	0.6	1.0	0.4	0.4	-					
Rissoella elatior – Setana	1.0	16.6	16.6	0.8	1.2	1.0	1.0	1.0	-				
Rissoella japonica n. sp. – Oshoro	27.4	27.4	27.1	27.1	27.4	27.4	27.4	26.8	26.6	-			
Rissoella japonica n. sp. – Oshoro	27.4	27.7	28.0	27.1	27.4	27.4	27.4	26.8	26.6	0.6	-		
Rissoella japonica n. sp. – Kamoenai	27.4	27.4	27.1	27.1	27.4	27.4	27.4	26.8	26.6	0.0	0.6	-	
Rissoella japonica n. sp. – Kamoenai	31.4	28.9	29.2	31.7	31.7	31.4	31.4	30.8	31.4	15.9	16.2	15.9	-
Rissoella golikovi – Oshoro	25.4	27.1	27.1	25.1	25.4	25.4	25.4	24.8	24.8	26.6	26.8	26.6	29.0
Rissoella golikovi – Oshoro	25.1	26.8	26.8	24.8	25.1	25.1	25.1	24.6	24.6	26.3	26.6	26.3	28.7
Rissoella golikovi – Kamoneai	25.1	26.8	26.8	24.8	25.1	25.1	25.1	24.6	24.6	26.3	26.6	26.3	28.7
Rissoella golikovi – Muroran	25.4	27.4	27.4	25.1	25.4	25.4	25.4	24.8	25.4	27.1	27.4	27.1	29.3
Rissoella golikovi – Muroran	25.1	27.1	27.1	24.8	25.1	25.1	25.1	24.6	25.1	26.8	27.1	26.8	29.0
Rissoella golikovi – Muroran	25.1	27.1	27.1	24.8	25.1	25.1	25.1	24.5	25.1	26.8	27.1	26.8	28.7
Rissoella golikovi – Toyoihama	25.1	26.8	26.8	24.8	25.1	25.1	25.1	24.6	24.6	26.3	26.6	26.3	28.7
Rissoella golikovi – Omu	28.0	26.0	26.0	27.7	28.0	28.0	28.0	27.4	28.0	26.6	27.4	26.6	27.5
Rissoella golikovi – Akkeshi	26.6	25.7	26.3	26.3	26.6	26.6	26.6	26.0	26.6	25.7	25.4	25.7	28.1
Rissoella golikovi – Akkeshi	26.9	26.0	26.6	26.6	26.9	26.9	26.9	26.3	26.9	26.0	25.7	26.0	28.4
Rissoella golikovi – Akkeshi	26.9	26.0	26.6	26.6	26.9	26.9	26.9	26.3	26.9	26.0	25.7	26.0	28.4
Rissoella sp. 1 – Shakotan	30.1	29.2	29.5	29.8	30.1	30.1	30.1	29.5	30.4	28.9	29.2	28.9	30.1
Rissoella sp. 1 – Shakotan	30.1	29.2	29.5	29.8	30.1	30.1	30.1	29.5	30.4	28.6	28.9	28.6	30.1
Rissoella rissoaformis	27.3	27.5	27.5	27.0	26.4	27.3	27.3	26.7	26.4	28.3	28.3	28.3	32.4
Rissoella elongatospira	26.6	21.3	21.6	26.3	26.9	26.6	26.6	26.0	26.3	24.0	23.7	24.0	26.0

……*continued on the next page*

TABLE S1. (Continued)

Specimens	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**
Rissoella elatior – Oshoro														
Rissoella elatior – Shakotan														
Rissoella elatior – Shakotan														
Rissoella elatior – Kamoenai														
Rissoella elatior – Muroran														
Rissoella elatior – Toyoihama														
Rissoella elatior – Toyoihama														
Rissoella elatior – Suttsu														
Rissoella elatior – Setana														
Rissoella japonica n. sp. – Oshoro														
Rissoella japonica n. sp. – Oshoro														
Rissoella japonica n. sp. – Kamoenai														
Rissoella japonica n. sp. – Kamoenai														
Rissoella golikovi – Oshoro	-													
Rissoella golikovi – Oshoro	0.2	-												
Rissoella golikovi – Kamoneai	0.2	0.0	-											
Rissoella golikovi – Muroran	1.2	1.0	1.0	-										
Rissoella golikovi – Muroran	1.0	0.8	0.8	0.2	-									
Rissoella golikovi – Muroran	1.4	1.2	1.2	0.4	0.6	-								
Rissoella golikovi – Toyoihama	0.2	0.0	0.0	1.0	0.8	1.2	-							
Rissoella golikovi – Omu	15.3	15.0	15.0	15.0	14.8	14.8	15.0	-						
Rissoella golikovi – Akkeshi	14.8	14.5	14.5	14.5	14.3	14.3	14.5	4.2	-					
Rissoella golikovi – Akkeshi	15.0	14.8	14.8	14.8	14.5	14.5	14.8	4.4	0.2	-				
Rissoella golikovi – Akkeshi	15.0	14.8	14.8	14.8	14.5	14.5	14.8	4.4	0.2	0.0	-			
Rissoella sp. 1 – Shakotan	30.1	29.8	29.8	29.8	29.5	29.5	29.8	31.0	29.5	29.8	29.8	-		
Rissoella sp. 1 – Shakotan	30.5	30.1	30.1	30.1	29.8	29.8	30.1	31.0	29.5	29.8	29.8	0.2	-	
Rissoella rissoaformis	27.5	27.2	27.2	27.8	27.5	27.5	27.2	29.1	30.6	30.9	30.9	31.1	31.4	-
Rissoella elongatospira	27.1	26.9	26.9	26.9	26.9	26.6	26.9	25.5	25.7	26.0	26.0	24.0	24.0	22.5

TABLE S2. Mean K2P pairwise sequence distances (in percentage) within and between morphospecies.

Morphospecies	1	2	3	4
Rissoella elatior	6.9			
Rissoella japonica **n. sp.**	28.2	8.2		
Rissoella golikovi	26.0	27.0	8.0	
Rissoella sp. 1	29.9	29.2	30.0	0.2

TABLE S3. Mean pairwise sequence distances based on K2P (in percentage) within and between MOTUs.

MOTUs	1	2	3	4	5	6	7	8
A	0.2							
B	31.0	-						
C	29.7	4.4	0.1					
D	29.9	15.0	14.7	0.7				
E	28.9	26.9	25.8	26.7	0.4			
F	30.1	27.5	28.3	28.8	16.0	-		
G	29.4	26.0	26.2	27.0	27.5	29.1	0.6	
H	30.0	27.9	26.6	25.0	27.2	31.4	16.6	0.6