Systematic and evolutionary studies of rissoellid microgastropods (Mollusca, Gastropoda, Heterobranchia)

（ガラスツボ科微小巻貝（軟体動物門・腹足綱・異鰓類）の体系学的および進化的研究）

A Ph.D. thesis

submitted

by

Luis Eduardo Chira Siadén

to

Graduate School of Science

Hokkaido University

Sapporo

Japan

in

March 2019

INDEX

ACKNOWLEDGEMENTS

Rallying past all the difficulties and hardships in this PhD journey would have not been possible without the support from different people, herein, I take the opportunity to thank all of them.

First, I would like to thank the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT) for the PhD scholarship grant that has enabled me to study for four years (second year of master course and PhD) here in Hokkaido University.

I would like to express my special appreciation and thanks to my supervisor Prof. Dr. Hiroshi Kajihara for the continuous support during my Ph.D. study, for his kindness, patience, motivation, and immense knowledge. His guidance helped me in all the time of this research and writing of this thesis. His advices on both research as well as on my career have been invaluable.

Besides my supervisor, I would like to express my sincerest gratitude to Dr. Kevin Wakeman for all his help with all the little and large problems occurring during my PhD, and of course for his sincere friendship. I also thank him for providing me the opportunity to join his research team, which gave me the chance to collect material in New Zealand. Without his precious support it would not be possible to conduct this research.

I would like to thank to the rest of my thesis committee: Dr. Takeo Horiguchi, and Dr. Kazuhiro Kogame, for their insightful comments and encouragement.

For helping me during the sampling trips, I thank to my girlfriend Yukina Katsurada, and my dear friends Yu Lu, Saori Takase, and Taichi Miura. We had so many enjoyable moments collecting samples.

I would also like to thank to the members of the Cawthron Institute, New Zealand for supporting me during my research trip in Nelson, New Zealand. Particularly, I would like to extend my sincerest gratitude to Dr. Steve Webb for his help in the processing of samples and his honest friendship, but also for all our candid conversations in Spanish.

Last but not the least, I would like to thank my family: my parents Silvia Siadén Ortega and Eduardo Chira Mino, for being always there, supporting me spiritually throughout my life, and keeping me happy despite our long distance.

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

In this study, I studied microgastropods belonging to the family Rissoellidae. The term "microgastropods" is here used to species that are less than 5 mm as adults. Rissoellidae comprises minute marine gastropods, measuring approximately 1 mm in shell length. Those microgastropods have a smooth, transparent/translucent or sometimes whitish shell. They also possess an operculum with unique morphology, which is one of the defining characteristic of the family. The operculum is yellowish, transparent, with 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. 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, similary to other basal heterobranchs. Rissoellids are hermaphroditic and undergo direct development, having a simple penis and a simultaneous hermaphrodite gonad. Rissoellids have a worldwide distribution and can be found in different habitats such as soft, sandy bottoms or hard substrata with algae, but they mostly associate with algae in shallow waters, where they rasp the algal surface and are believed to feed on microalgae and detritus matter. Rissoellidae remains one of the least studied taxon despite its worldwide distribution and common presence in the intertidal zone. Furthermore, although the presence of rissoellids in Japanese waters has been reported, most of them remain undescribed. Thus, the aims of this study are to unveil the species diversity of rissoellids based on morphological and molecular (barcode) studies from Hokkaido, Japan (Chapter 1) and to discuss evolutionary characters of one of

the most important organs, i.e. radula (Chapter 2) based on multigene phylogenetic data in rissoellids collected in Japan and New Zealand.

Chapter 1 deals with the species diversity of microgastropods of the family Rissoellidae in Japan, and the description of a new species is included. *Rissoella elatior* (Golikov, Gulbin & Sirenko, 1987), *Rissoella golikovi* (Gulbin, 1979), *Rissoella japonica* n. sp., and *Rissoella* sp. 1 were collected in different locations around Hokkaido, Japan. To species identification, I applied the most traditional morphological characters (body color pattern, shell, and radular morphology) in the taxonomy of rissoellids. This information was complemented with the amplification of a region of the mitochondrial cytochrome c oxidase subunit I (COI) gene. *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. has *i*) five teeth per row on the radula; *ii*) a central tooth that is higher than wide; *iii*) lateral and marginal teeth that are narrow, with an outer lateral projection at the base; and *iv*) every tooth of the radula 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 another new species, additional specimens are needed to complete its morphological description. This study represents the first insight into the genetic diversity of the family Rissoellidae. While four morphospecies were recognized, the addition of COI data raised the count to eight potential species, suggesting the existence of cryptic species among rissoellids. Admittedly, I was 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). This is the first time that molecular techniques have been applied in the taxonomy of this family.

Chapter 2 provides evolutionary hypotheses of the rissoellid radular morphology based on a multigene phylogenetic approach using mitochondrial (COI and 16S rDNA) as well as nuclear (18S rDNA and 28S rDNA) sequences. The radula is one of the most important morphological characters in the taxonomy of rissoellids because it shows a great interspecific variation. For that reason, it is important to explain the evolutionary transformation of this (the radula) taxonomic character which might help to understand species diversification, specialization. I analyzed specimens assigned to nine different *Rissoella* species collected in Hokkaido, Japan, and the South Island of New Zealand, using molecular markers, combined with radular morphology. A vast interspecific variation was found in the rissoellid radular morphology; three major groups, however, are recognized based on the number of teeth per row (three, five, and seven teeth). The phylogenetic tree showed that species with seven teeth per row formed a clade, while those with five teeth did not; only one species with three teeth was included in the analysis. The molecular analyses also suggest that the major event that has characterized the rissoellid radular evolution would involve the development of the marginal teeth. The analyses also revealed that the plate-like outer marginal teeth did not represent vestigial teeth, but a derived state. My results suggest that a factor that could influence the radular morphology in the Rissoellidae is the diet rather than the substrate. The inclusion of other species in future studies is necessary to provide a better understanding about the evolutionary radular transformation in the Rissoellidae.

Disclaimer

Under Article 8.3 of the International Code of Zoological Nomenclature (International Commission on Zoological Nomenclature 1999), all of the names and nomenclatural acts in this dissertation are not available.

GENERAL INTRODUCTION

Gastropoda is the largest and most diverse class of the phylum Mollusca, exhibiting the highest diversity in morphology (Geiger 2006). However, there are still numerous unidentifiable or undescribed species, the majority of these being microgastropods (Bouchet *et al.* 2002; Sasaki 2008). The term "microgastropods" is here used to species that are less than 5 mm as adults.

One of the most diverse taxa in the Gastropoda is the Heterobranchia, which comprises large-sized species such as opisthobranchs and pulmonates, as well as some families that have exclusively evolved to be miniaturized (microgastropods) (Omalogyridae, Pyramidellidae, Rissoellidae, among others). Due to this great diversity, the Heterobranchia is still a subject of various studies. However, the relationship among heterobranchs and their taxonomic positions are still unresolved (Dinapoli & Klussmann-Kolb 2010). This may be due to the fact that some heterobranchs are poorly studied. One of these families, the Rissoellidae, remains one of the least studied taxon despite its worldwide distribution and common presence in the intertidal zone (Sasaki 2008).

Rissoellidae comprises minute marine gastropods, measuring approximately 1 mm in shell length. Rissoellids have smooth, transparent/translucent or sometimes whitish (opaque, sometimes with a band) shells. The contemporary understanding of this group contains only a few taxonomic characters that can be used to delimit closely related species. They also possess an operculum with unique morphology, which is one of the defining characteristic of the family as a whole, but does not present interspecific difference. The operculum is

yellowish, transparent, with concentric growth lines and a nucleus near the mid region on the inner edge. It 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 (Fig. 1). Ctenidium, esophageal glands, and crystalline styles are lacking, similary to 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). Rissoellids have a worldwide distribution and can be found in different habitats such as soft, sandy bottoms or hard substrata with algae (Caballer *et al.* 2014), but they mostly associate with algae in shallow waters (Caballer *et al.* 2011), where they rasp the algal surface and are believed to feed on microalgae and detritus matter (Olabarria 2002). Although the presence of rissoellids in Japanese waters has been reported, most of them remain undescribed (Hasegawa 2000, 2017).

The aims of this study are *i*) to increase our knowledge of the diversity of rissoellids, which is the basement in advancing biological studies (e.g. ecology and conservation); *ii*) to make rissoellid species identification easier and more reliable by combining morphological identifications with molecular techniques such as DNA barcoding; *iii*) to understand the interspecific phylogenetic relationships within the Rissoellidae; and *iv)* to infer the

evolutionary transformations of one of the most important morphological characters in the taxonomy of gastropods, the radula, using the Rissoellidae as a model.

This thesis is divided into two chapters. Chapter 1 deals with the rissoellid microgastropods in Hokkaido, Japan, in order to clarify the diversity of rissollids in the northern Japanese island. In the taxonomy of the Rissoellidae, species identification has been traditionally based on morphological characters such as shell, body color pattern, and radula. Until now, these morphological features (especially the radula) have been demonstrated to be very useful tools to distinguish species in this family. However, using only morphological characters could lead to either *i*) an overestimation of species number because of high phenotypic plasticity or *ii*) an underestimation because morphological characters do not always reveal the presence of cryptic species (Dasmahapatra *et al.* 2010; Maturana *et al.* 2011; Barco *et al.* 2016). Thus, this work intends to present a more accurate picture rissoellid species diversity by combining morphological and molecular approaches. This is the first time that molecular techniques have been applied in the taxonomy of this family.

Chapter 2 provides evolutionary hypotheses of the rissoellid radular morphology based on a multigene phylogenetic approach using mitochondrial (COI and 16S rDNA) as well as nuclear (18S rDNA and 28S rDNA) sequences. The radula is one of the most important morphological characters in the taxonomy of rissoellids because it shows a great interspecific variation. For that reason, it is important to explain the evolutionary transformation of this (the radula) taxonomic character which might help to understand species diversification, specialization.

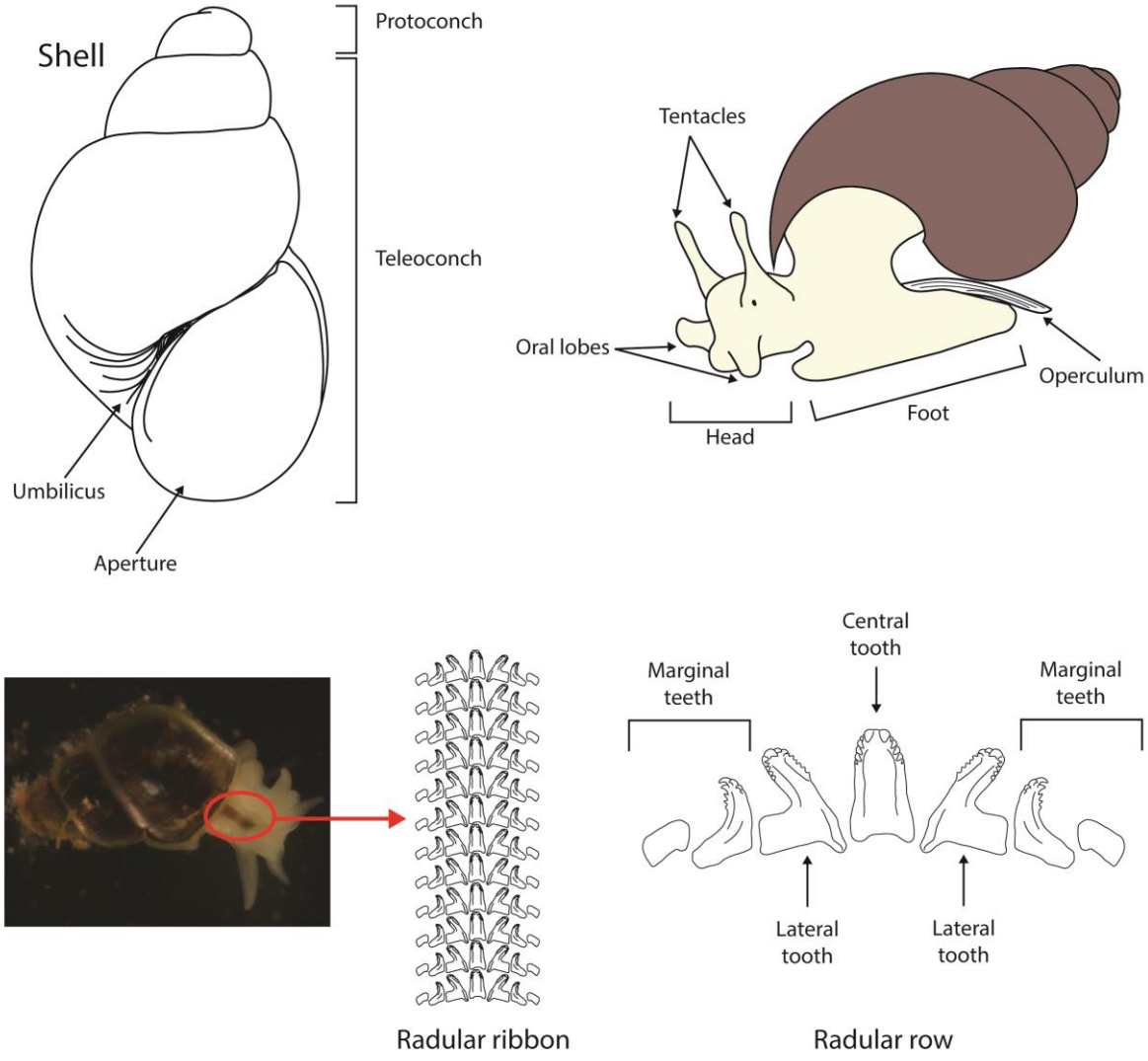

Figure 1. Morphological characters used in this study.

CHAPTER 1

Morphological and molecular diversity of rissoellids from Hokkaido, Japan

ABSTRACT

This chapter 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 Hokkaido, Japan. Light and scanning electron microscopy (SEM) was 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. has *i*) five teeth per row on the radula; *ii*) a central tooth that is higher than wide; *iii*) lateral and marginal teeth that are narrow, with an outer lateral projection at the base; and *iv*) every tooth of the radula 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 another new species, additional specimens are needed to complete its morphological description. This study represents the first insight into the genetic diversity of the family Rissoellidae. While four morphospecies were recognized, the addition of COI data raised the count to eight potential species, suggesting the existence of cryptic species among rissoellids.

INTRODUCTION

To date, approximately 60 species have been described in the Rissoellidae. Hasegawa (2000, 2017) mentioned that more than 10 species are distributed in Japanese waters, but most of them are still undescribed. To identify species in this family, contemporary works have used shell morphology complemented with other external and internal characters like radula morphology (Ponder & Yoo 1977), a combination of radula morphology and anatomy (Simone 1995; Wise 1998), diversity of 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 to complement morphological studies and to solve problems associated with species identification, especially among very similar (cryptic) 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, b; 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 on the Rissoellidae. A limited amount of genetic 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, I 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. My study represents the first insight into the phylogeny of rissoellids and presents the first use of molecular techniques as a complement of morphological information to distinguish species in the Rissoellidae, 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. 2). 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 Nikon SMZ1500 dissecting stereomicroscope (Nikon, Tokyo, Japan) and photographed with a Nikon D5200 digital camera (Nikon, Tokyo, Japan) attached to the stereomicroscope.

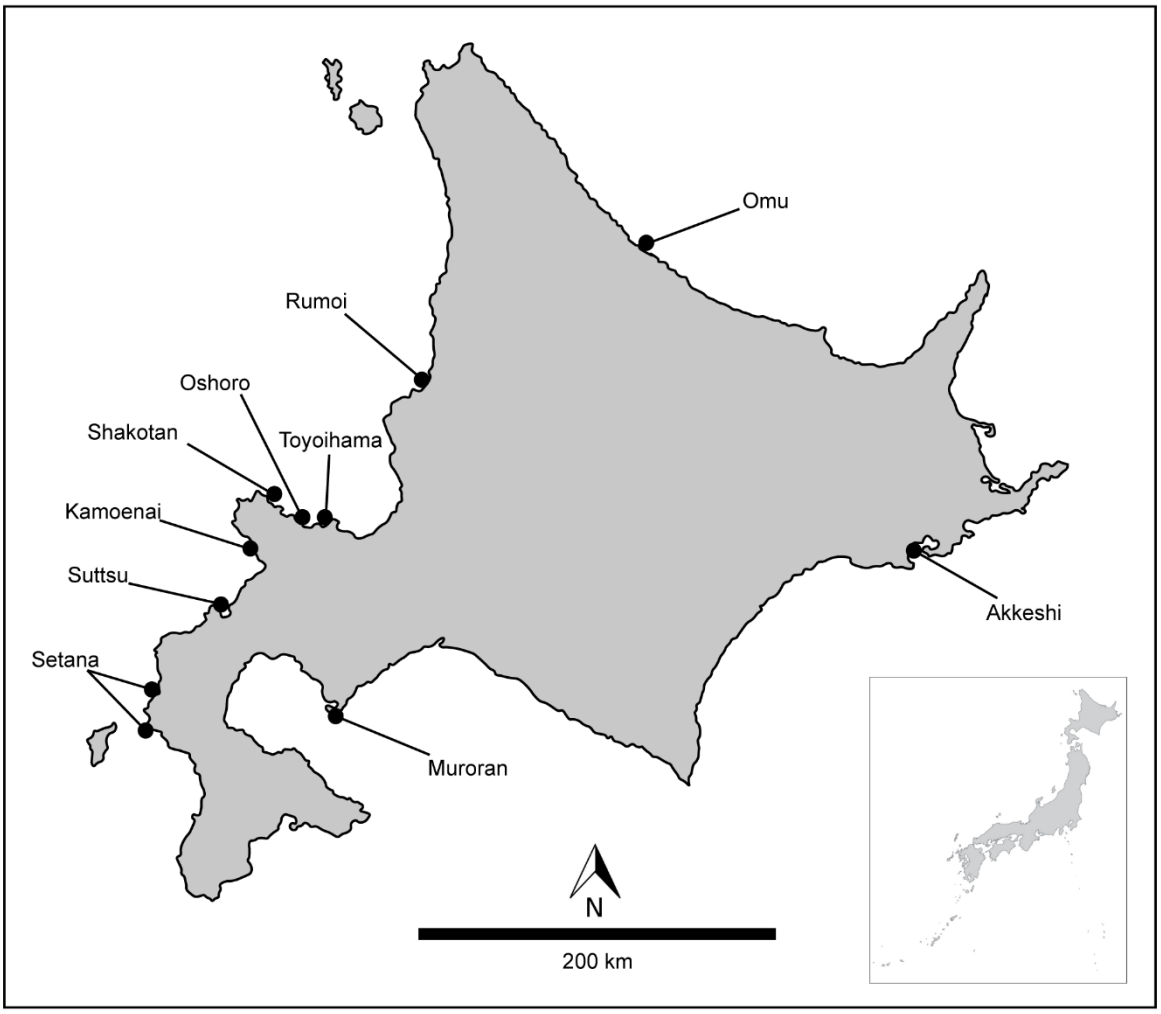

Figure 2. Sampling sites surveyed in this study.

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 scanning electron microscope (Hitachi, Tokyo, Japan). 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 accession numbers MK210173–MK210198.

Analyses of molecular data and species delimitation. Sequenced fragments were assembled using Geneious ver. 10.2.3 (Biomatters, Auckland, New Zealand; Kearse *et al.* 2012). 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/).

Table 1. Specimens analyzed in this study.

Morphospecies	Specimen Voucher	Locality (Japan)	Latitude / Longitude	Collection Date	GenBank Accession
Rissoella elatior	ICHUM 5780	Oshoro	43°12′33.7″N, 140°51′30.3″E	28/07/2017	MK210173
Rissoella elatior	ICHUM 5781	Oshoro	43°12′33.7″N, 140°51′30.3″E	28/07/2017	—
Rissoella elatior	ICHUM 5782	Oshoro	43°12′33.7″N, 140°51′30.3″E	28/07/2017	—
Rissoella elatior	ICHUM 5783	Shakotan	43°18′06.2″N, 140°35′55.6″E	04/10/2016	MK210174
Rissoella elatior	ICHUM 5784	Shakotan	43°18′06.2″N, 140°35′55.6″E	04/10/2016	MK210175
Rissoella elatior	ICHUM 5785	Kamoenai	43°08′10.5″N, 140°25′43.1″E	06/11/2016	—
Rissoella elatior	ICHUM 5786	Kamoenai	43°08′10.5″N, 140°25′43.1″E	06/11/2016	MK210176
Rissoella elatior	ICHUM 5787	Muroran	42°18′18.4″N, 140°59′02.7″E	05/11/2016	MK210177
Rissoella elatior	ICHUM 5788	Toyoihama	43°13′34.7″N, 141°00′58.3″E	14/04/2017	MK210178
Rissoella elatior	ICHUM 5789	Toyoihama	43°13′34.7″N, 141°00′58.3″E	14/04/2017	MK210179
Rissoella elatior	ICHUM 5790	Suttsu	42°47′15.1″N, 140°18′27.7″E	28/05/2017	MK210180
Rissoella elatior	ICHUM 5791	Setana	42°27′45.7″N, 139°51′04.6″E	08/10/2017	MK210181
Rissoella elatior	ICHUM 5792	Setana	42°27′45.7″N, 139°51′04.6″E	08/10/2017	—
Rissoella golikovi	ICHUM 5793	Oshoro	43°12′33.7″N, 140°51′30.3″E	28/07/2017	MK210186
Rissoella golikovi	ICHUM 5794	Oshoro	43°12′33.7″N, 140°51′30.3″E	28/07/2017	MK210187
Rissoella golikovi	ICHUM 5795	Oshoro	43°12′33.7″N, 140°51′30.3″E	28/07/2017	—
Rissoella golikovi	ICHUM 5796	Kamoenai	43°08′10.5″N, 140°25′43.1″E	28/05/2017	MK210188
Rissoella golikovi	ICHUM 5797	Muroran	42°18′18.4″N, 140°59′02.7″E	05/11/2016	MK210189
Rissoella golikovi	ICHUM 5798	Muroran	42°18′18.4″N, 140°59′02.7″E	05/11/2016	MK210190
Rissoella golikovi	ICHUM 5799	Muroran	42°18′18.4″N, 140°59′02.7″E	05/11/2016	MK210191
Rissoella golikovi	ICHUM 5800	Toyoihama	43°13′34.7″N, 141°00′58.3″E	14/04/2017	MK210192
Rissoella golikovi	ICHUM 5801	Toyoihama	43°13′34.7″N, 141°00′58.3″E	14/04/2017	—
Rissoella golikovi	ICHUM 5802	Toyoihama	43°13′34.7″N, 141°00′58.3″E	14/04/2017	—
Rissoella golikovi	ICHUM 5803	Omu	44°35′23.5″N, 142°57′40.0″E	11/06/2017	MK210193
Rissoella golikovi	ICHUM 5804	Akkeshi	43°01′08.7″N, 144°50′05.8″E	02/07/2017	MK210194
Rissoella golikovi	ICHUM 5805	Akkeshi	43°01′08.7″N, 144°50′05.8″E	02/07/2017	MK210195
Rissoella golikovi	ICHUM 5806	Akkeshi	43°01′08.7″N, 144°50′05.8″E	02/07/2017	MK210196
Rissoella japonica n. sp.	ICHUM 5807	Oshoro	43°12′33.7″N, 140°51′30.3″E	28/07/2017	MK210182
Rissoella japonica n. sp.	ICHUM 5808	Oshoro	43°12′33.7″N, 140°51′30.3″E	28/07/2017	MK210183
Rissoella japonica n. sp.	ICHUM 5809	Kamoenai	43°08′10.5″N, 140°25′43.1″E	06/11/2016	MK210184
Rissoella japonica n. sp.	ICHUM 5810	Kamoenai	43°08′10.5″N, 140°25′43.1″E	06/11/2016	MK210185
Rissoella japonica n. sp.	ICHUM 5811	Shakotan	43°18′06.2″N, 140°35′55.6″E	28/05/2017	—
Rissoella sp. 1	ICHUM 5812	Shakotan	43°18′06.2″N, 140°35′55.6″E	25/08/2016	MK210197
Rissoella sp. 1	ICHUM 5813	Shakotan	43°18′06.2″N, 140°35′55.6″E	25/08/2016	MK210198

RESULTS

Systematics

Family Rissoellidae Gray, 1850

Genus *Rissoella* Gray, 1847

Rissoella elatior (Golikov, Gulbin & Sirenko, 1987)

(Figs. 3A–F, 4A–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 5780, 5781, 5782, 5783, 5784, 5785, 5786, 5787, 5788, 5789, 5790, 5791, and 5792). For information on specimen 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 (Fig. 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 (Fig. 3A–F). 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 (Fig. 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 (Fig. 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 (Fig. 4E, H). Outer marginal teeth reduced, simple, plate-like (width almost 200% of length) (Fig. 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, my 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. 3D) the mantle coloration is brighter than others, the

former being pale brown with yellowish white asymmetrical patches and a brown patch centrally placed on the dorsal portion of the body whorl.

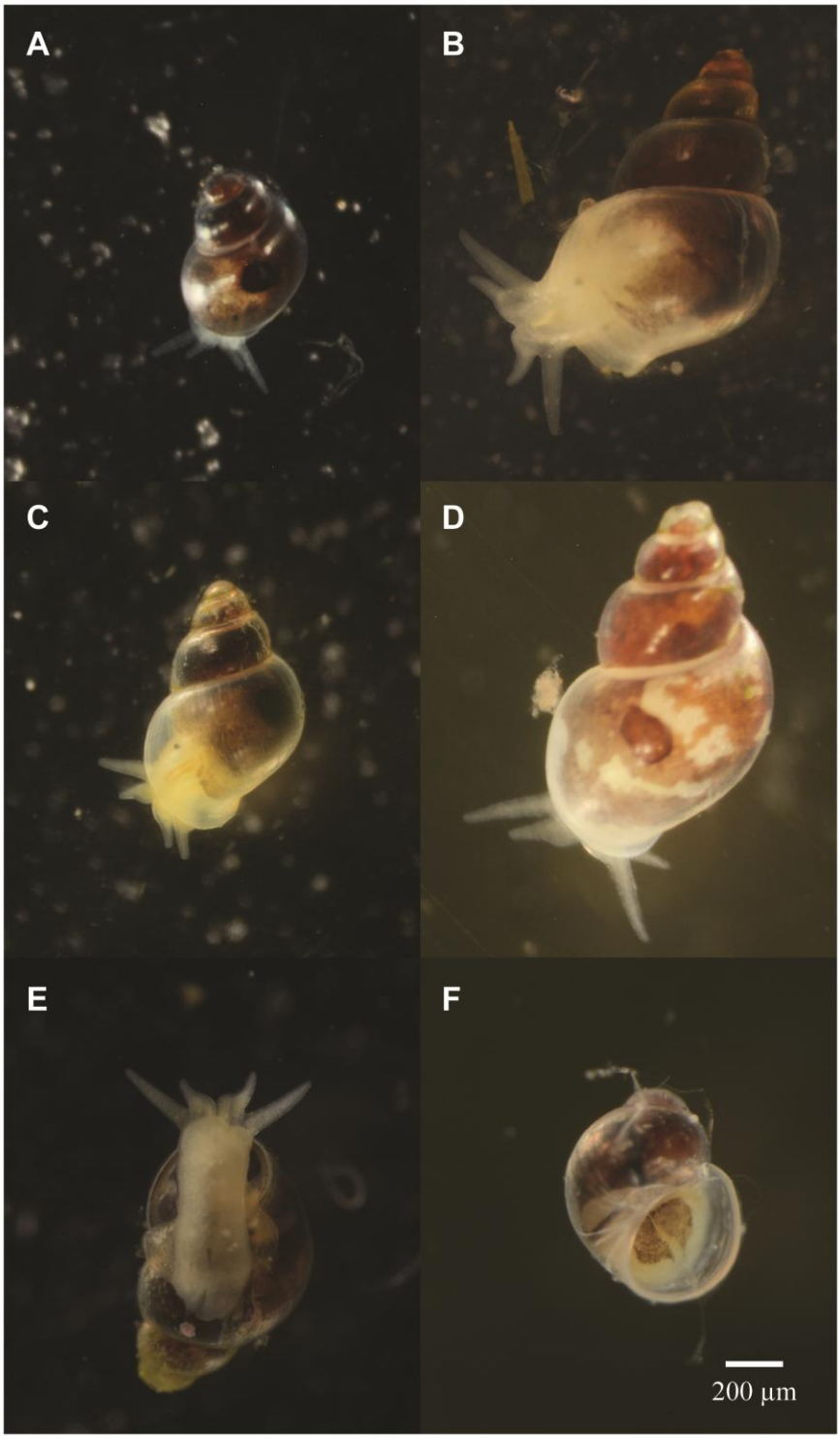

Figure 3. Body color pattern of living specimens of *Rissoella elatior*. (A–D) Abapertural view. (E–F) Apertural view.

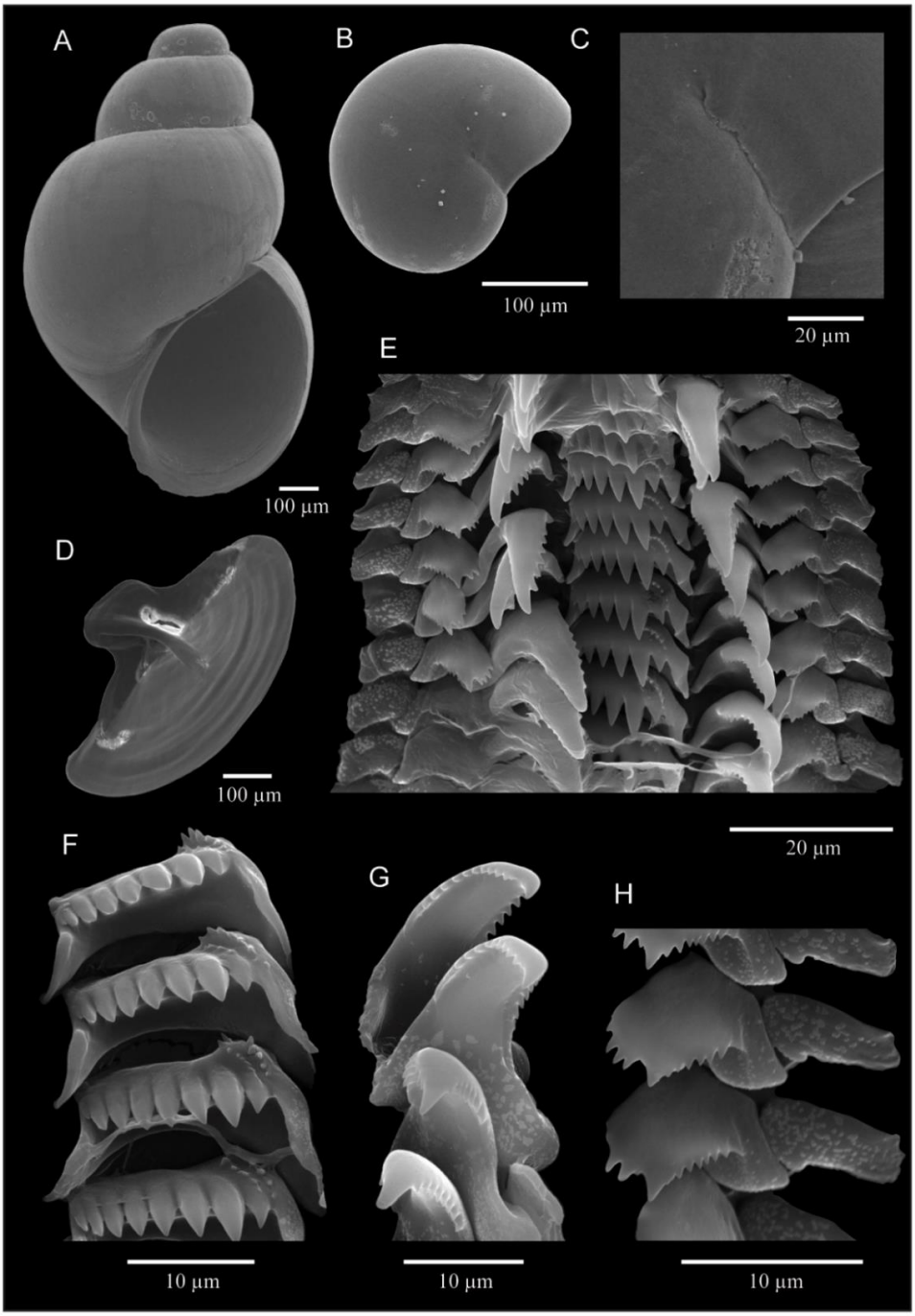

Figure 4. SEM images of *Rissoella elatior*. (A) Apertural view of the shell. (B) Apical view of the protoconch. (C) Apical view of the protoconch showing the protoconch suture. (D) Inner face view of the operculum. (E) Radula. (F) Details of the central tooth. (G) Details of the lateral teeth. (H) Details of the inner and outer marginal teeth.

***Rissoella golikovi* (Gulbin, 1979)**

(Figs. 5A, B, 6A–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 5793, 5794, 5795, 5796, 5797, 5798, 5799, 5800, 5801, 5802, 5803, 5804, 5805, and 5806). For information on specimen collection locality and GenBank accession numbers see Table 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. 6A). Protoconch smooth, of about one whorl (Fig. 6B, 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. 6D). 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 (Fig. 5A, B). Radular formula 12–13 × 1.R.1 (Fig. 6E). 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 (Fig. 6F, 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 (Fig. 6E, 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, my 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).

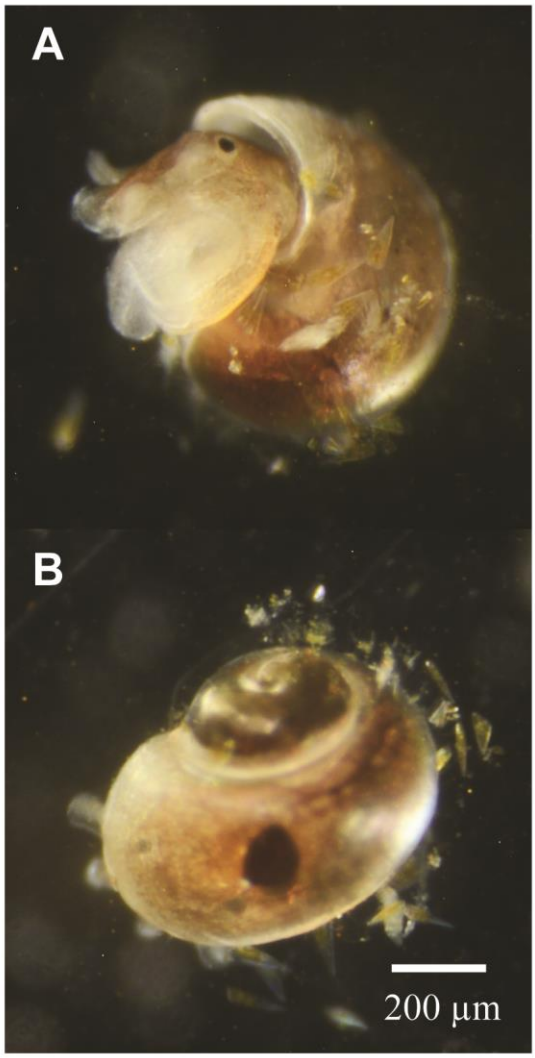

Figure 5. Body color pattern of living specimens of *Rissoella golikovi*. (A) Basal view. (B) Abapertural view.

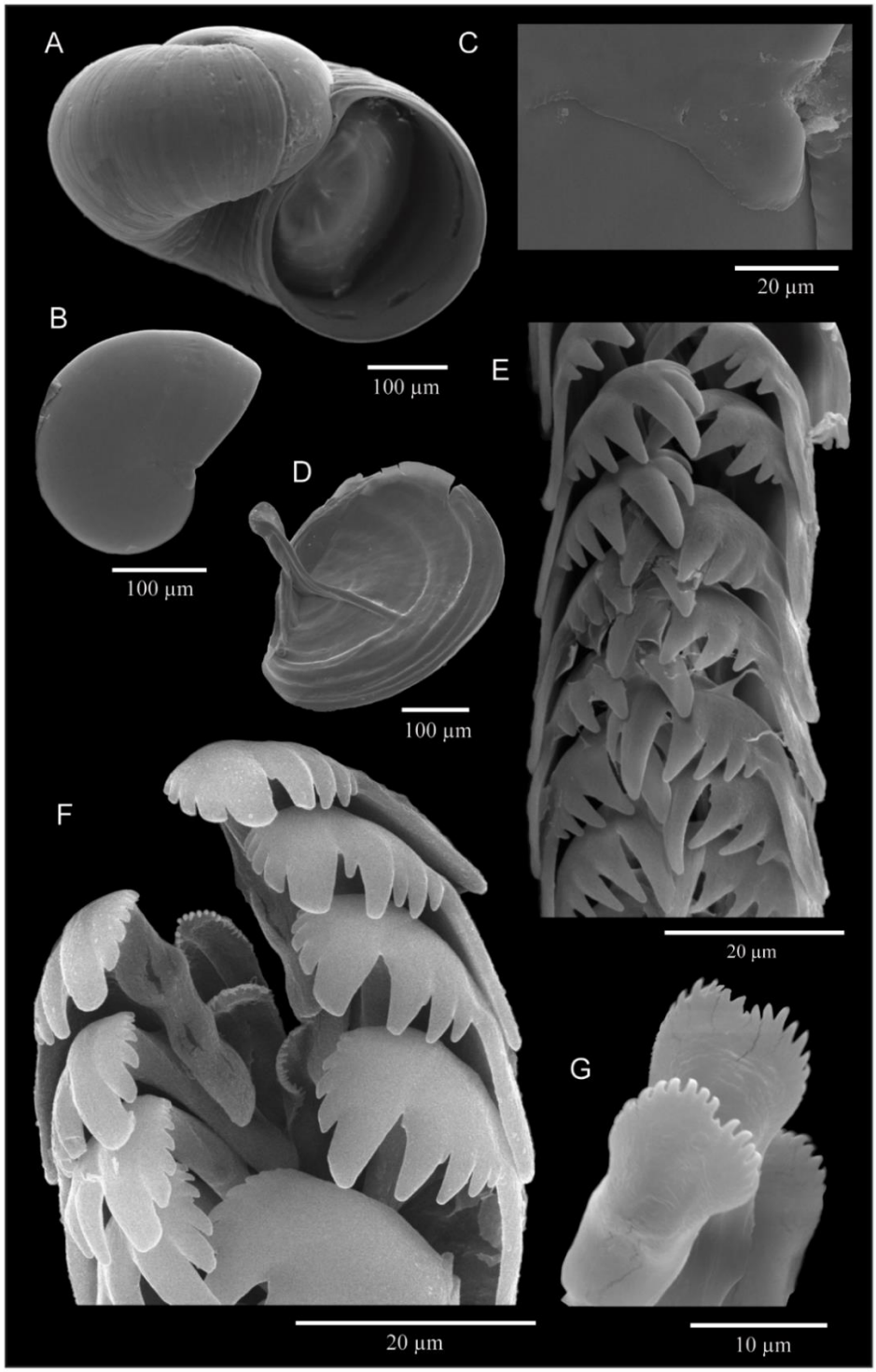

Figure 6. SEM images of *Rissoella golikovi*. (A) Apertural view of the shell. (B) Apical view of the protoconch. (C) Apical view of the protoconch showing the protoconch suture. (D) Inner face view of the operculum. (E–F) Radula. (G) Details of the central tooth.

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

(Figs. 7A–D, 8A–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 5809); Kamoenai, Hokkaido, Japan, 43°08′10.5″N, 140°25′43.1″E, 6 November 2016. *Paratypes:* 3 specimens (ICHUM 5807, 5808, 5810) from Oshoro Bay, Hokkaido, Japan; 1 specimen (ICHUM 5811); Shakotan, Hokkaido, Japan. For information on specimen 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. 8A). Protoconch smooth, of about 1 whorl, with rippled sculpture along suture

(Fig. 8B, 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. 8D). 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 (Fig. 7A–D). Radular formula 11–13 × 1.1.R.1.1 (Fig. 8E). Central tooth higher than wide (width about 48% of length), cutting edge with 10–13 sharp cusps of different sizes (Fig. 8E, 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 (Fig. 8E, 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 (Fig. 8E, 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.

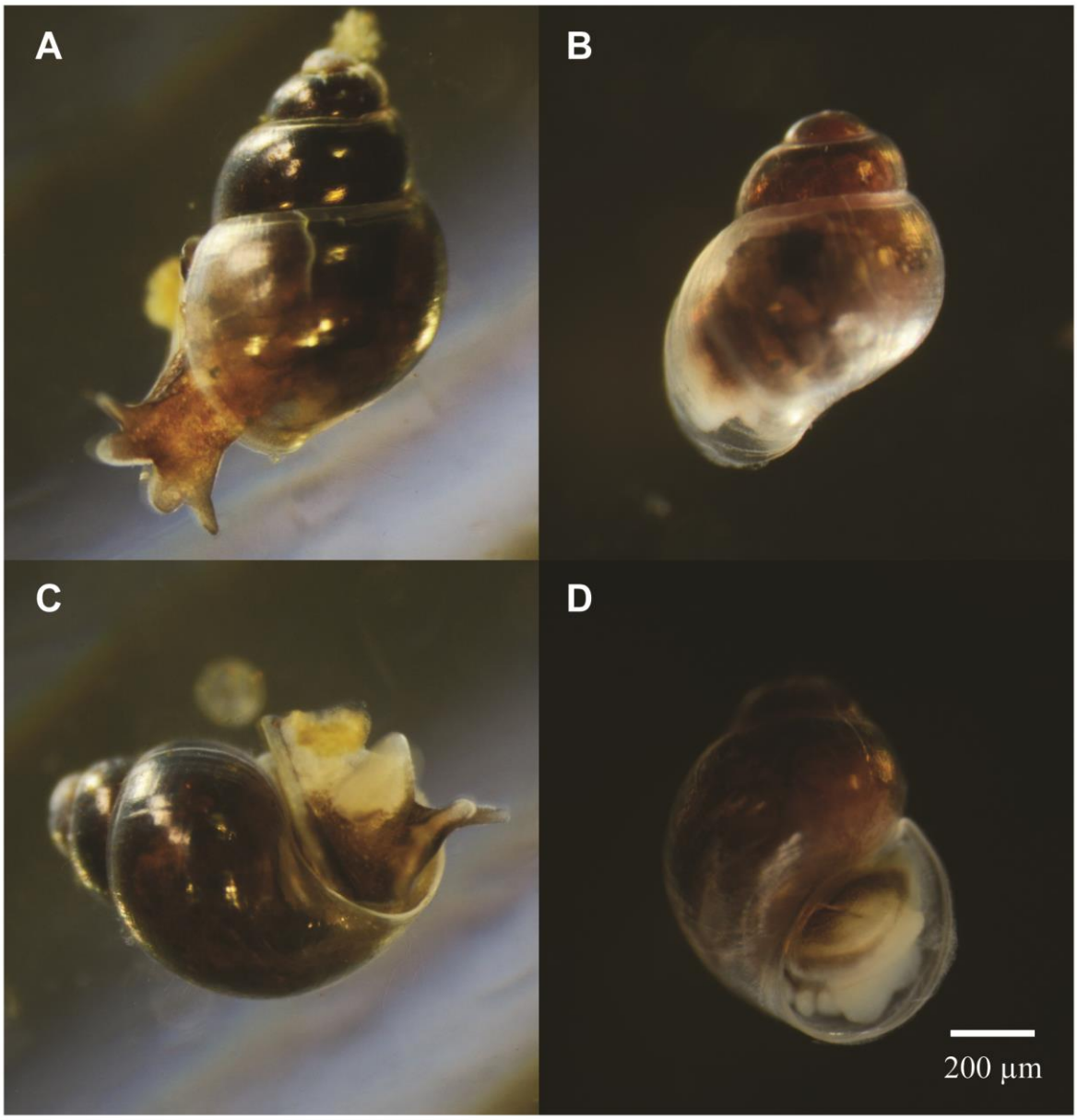

Figure 7. Body color pattern of living specimens of *Rissoella japonica* n. sp. (A–B) Abapertural view. (C) Lateral view. (D) Apertural view.

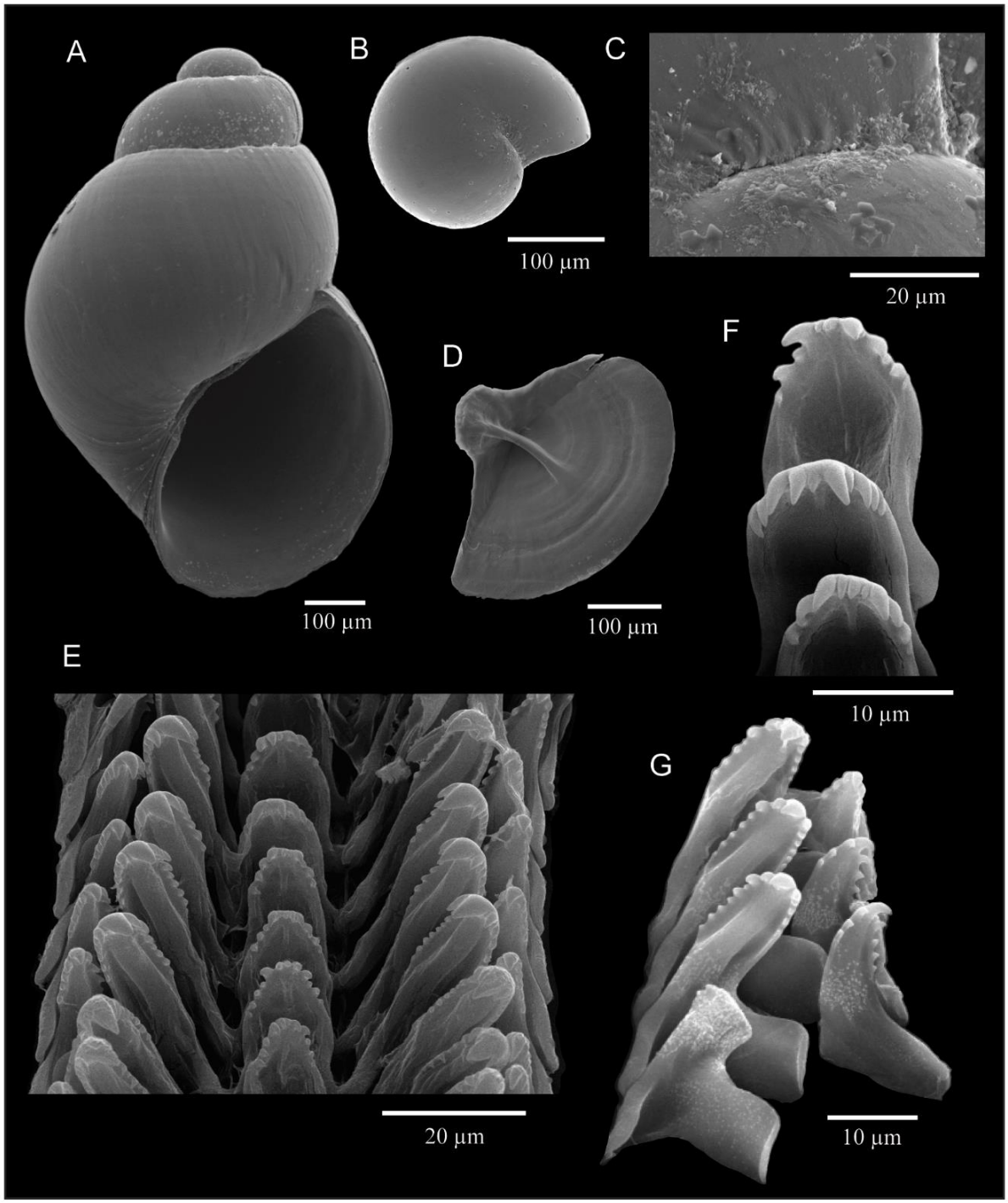

Figure 8. SEM images of *Rissoella japonica* n. sp. (A) Apertural view of the shell. (B) Apical view of the protoconch. (C) Apical view of the protoconch showing the protoconch suture. (D) Inner face view of the operculum. (E) Radula. (F) Details of the central tooth. (G) Details of the lateral and marginal teeth.

***Rissoella* sp. 1**

(Fig. 9A, B)

Material examined. Two mature specimens: ICHUM 5812, 5813; 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 specimen 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 (Fig. 9A, B).

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

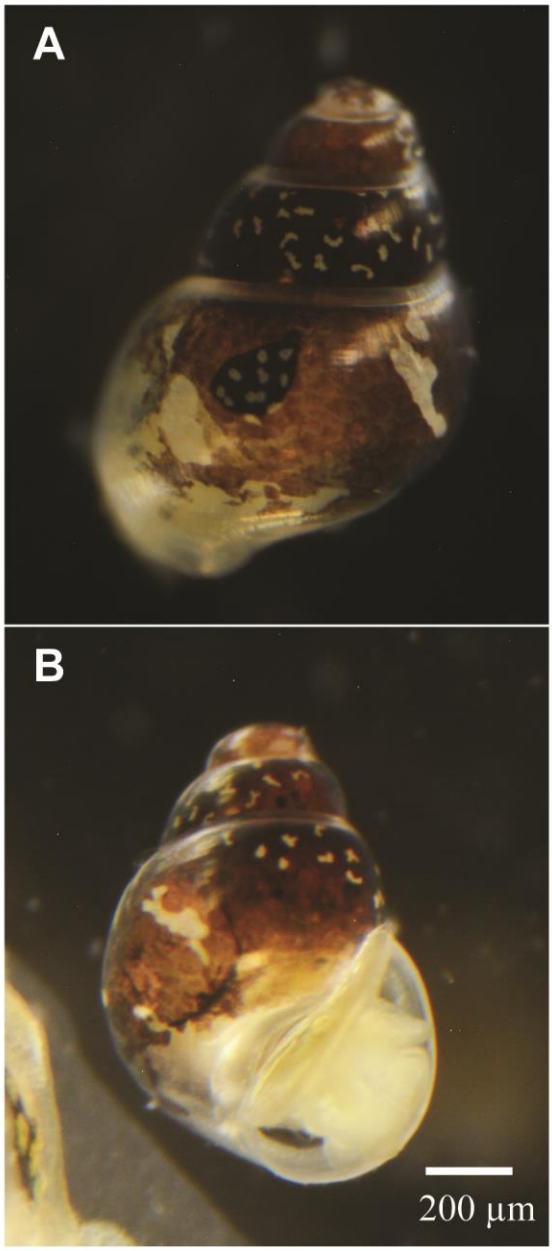

Figure 9. Body color pattern of living specimens of *Rissoella* sp. 1. (A) Abapertural view. (B) Apertural view.

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 described above as highly supported clades. 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. 10). 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 Table 2. 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% (Table 3). 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%) (Table 4).

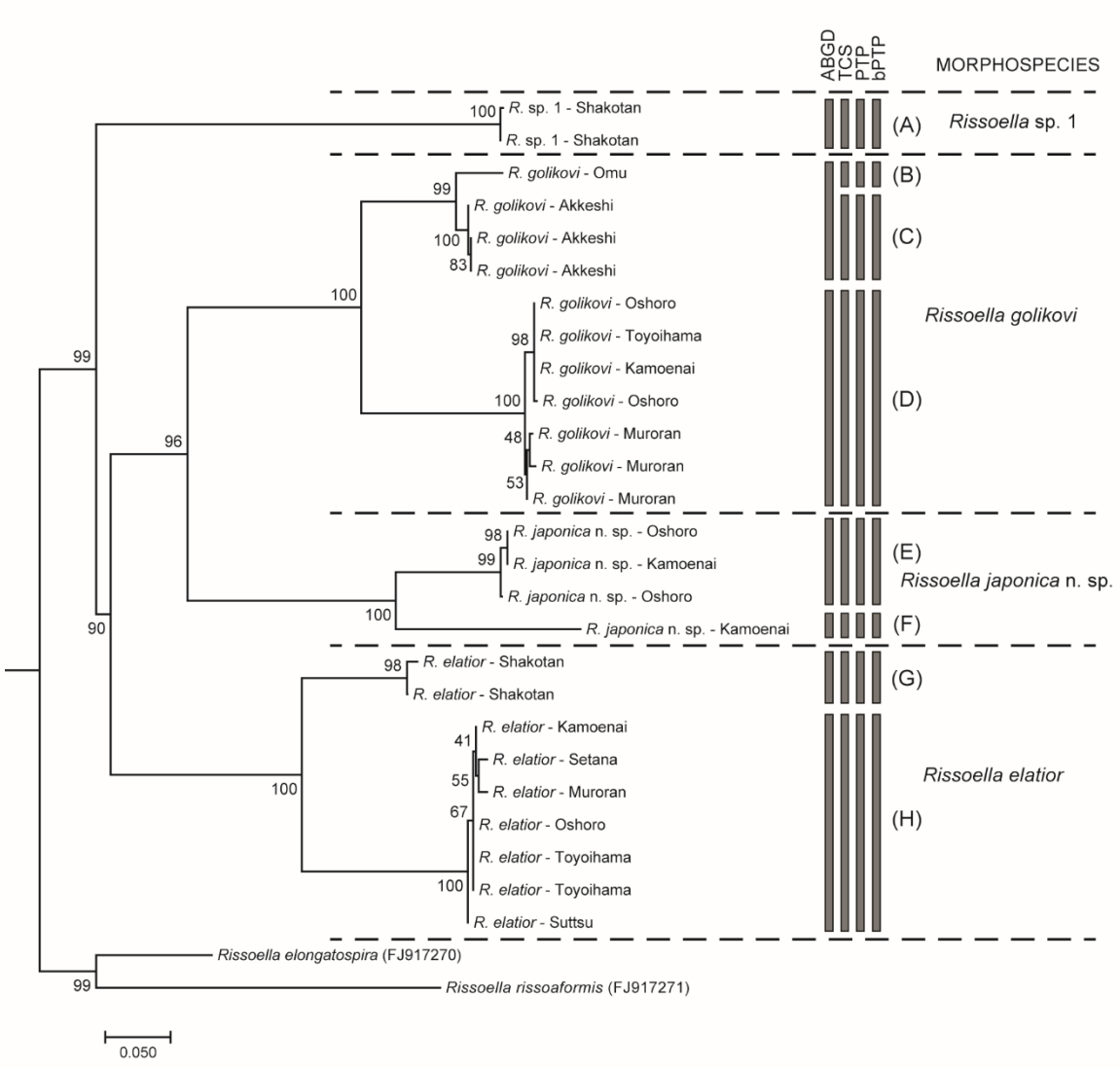

Figure 10. Maximum-likelihood tree analysis of the COI sequences for *Rissoella* morphospecies from Hokkaido, Japan. Scale bars represent raw percentage sequence divergence. MOTUs are indicated with letters in parenthesis.

Table 2. Pairwise sequence distances based on K2P (in percentage) between the analyzed specimens.

Specimens	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1. *Rissoella elatior* – Oshoro	-																										
2. *Rissoella elatior* – Shakotan	16.6	-																									
3. *Rissoella elatior* – Shakotan	16.6	0.6	-																								
4. *Rissoella elatior* – Kamoenai	0.2	16.9	16.9	-																							
5. *Rissoella elatior* – Muroran	1.0	16.9	16.9	0.8	-																						
6. *Rissoella elatior* – Toyoihama	0.0	16.6	16.6	0.2	1.0	-																					
7. *Rissoella elatior* – Toyoihama	0.0	16.6	16.6	0.2	1.0	0.0	-																				
8. *Rissoella elatior* – Suttsu	0.4	16.1	16.1	0.6	1.0	0.4	0.4	-																			
9. *Rissoella elatior* – Setana	1.0	16.6	16.6	0.8	1.2	1.0	1.0	1.0	-																		
10. *Rissoella japonica* n. sp. – Oshoro	27.4	27.4	27.1	27.1	27.4	27.4	27.4	26.8	26.6	-																	
11. *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	-																
12. *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	-															
13. *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	-														
14. *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	-													
15. *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	0.2	-												
16. *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	0.2	0.0	-											
17. *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	1.2	1.0	1.0	-										
18. *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	1.0	0.8	0.8	0.2	-									
19. *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	1.4	1.2	1.2	0.4	0.6	-								
20. *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	0.2	0.0	0.0	1.0	0.8	1.2	-							
21. *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	15.3	15.0	15.0	15.0	14.8	14.8	15.0	-						
22. *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	14.8	14.5	14.5	14.5	14.3	14.3	14.5	4.2	-					
23. *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	15.0	14.8	14.8	14.8	14.5	14.5	14.8	4.4	0.2	-				
24. *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	15.0	14.8	14.8	14.8	14.5	14.5	14.8	4.4	0.2	0.0	-			
25. *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	30.1	29.8	29.8	29.8	29.5	29.5	29.8	31.0	29.5	29.8	29.8	-		
26. *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	30.5	30.1	30.1	30.1	29.8	29.8	30.1	31.0	29.5	29.8	29.8	0.2	-	
27. *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	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	-
28. *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	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 3. Mean pairwise sequence distances based on K2P (in percentage) within and between morphospecies.

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

Table 4. Mean pairwise sequence distances based on K2P (in percentage) within and between MOTUs.

MOTUs	A	B	C	D	E	F	G	H
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

DISCUSSION

In the present study I explored 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, I 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 Australia, *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* is 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. My 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, I was 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). My 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). I follow this taxonomic scheme at the moment. A revision of the Rissoellidae in genus and subgenus levels is beyond the scope of this paper.

In this study I reported a new rissoellid species, and have generated the first molecular data of rissoellids from Japan. My COI sequence data provide 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.

CHAPTER 2

Molecular phylogeny of rissoellids and its correlation with radular morphology

ABSTRACT

I analyzed specimens assigned to nine different *Rissoella* species using molecular markers, combined with radular morphology. A vast interspecific variation was found in the rissoellid radular morphology; three major groups, however, are recognized based on the number of teeth per row (three, five, and seven teeth). The phylogenetic tree showed that species with seven teeth per row formed a clade, while those with five teeth did not; only one species with three teeth was included in the analysis. The molecular analyses also suggest that the major event that has characterized the rissoellid radular evolution would involve the development of the marginal teeth. The analyses also revealed that the plate-like outer marginal teeth did not represent vestigial teeth, but a derived state. My results suggest that a factor that could influence the radular morphology in the Rissoellidae is the diet rather than the substrate.

INTRODUCTION

The radula is a complex feeding organ common to most molluscs and consists of several repeated transverse rows of teeth attached to a basal membrane. Teeth are variable in shape and size, often with cusps on the cutting edge which are also distinct in shape and size (Guralnick & Lindberg 1999; Reid & Mak, 1999; Shaw *et al.* 2010). Radular morphology has been traditionally used in gastropod taxonomy (Reid 2000). It has been also used in phylogenetic reconstruction helping to understand the evolutionary paths in distinct gastropods and other molluscs (Hickman 1980, 1983; Shimek & Kohn 1981; Padilla 1985, 1989; Reid & Mak 1999; Guralnick & Lindberg 1999; Padilla 2003; Mutlu 2004; Herbert *et al*. 2007; Fedosov *et al*. 2011; Puillandre & Tenorio 2017). In gastropods, the evolutionary aspects of the radula are still little known (Padilla 2003). In fact, this sort of studies has been mainly carried out for the members in the superfamily Conoidea (Kantor & Puillandre 2012).

In the Rissoellidae, radular morphology has been well described in most of the species, showing a great interspecific variability (Ponder & Yoo 1977). Teeth are different in size and shape, with cusps (variable in number, shape, and size) on the cutting edge; except on the outer marginal teeth which are plate-like (Ponder & Yoo 1977). Despite the vast radular morphology observed among rissollids, three major groups can be recognized based on the number of teeth per row: *i*) one central tooth and a pair of lateral teeth, *ii*) one central tooth, a pair of lateral teeth, and a pair of marginal teeth, and *iii*) one central tooth, a pair of lateral teeth and two pairs of marginal teeth. Although there are observable differences in the interspecific radular patterns and variations among rissoellids, there are no studies that have addressed the evolutionary considerations of radular teeth form and number in this family.

Radular characters in combination with molecular approaches might help to better understanding of the evolution and phylogenetic affinity within Rissoellidae. However, most of the works in this family have been focused on species descriptions (Ponder & Yoo 1977; Simone 1995; Wise 1998; Ortea *et al.* 2004; Caballer *et al.* 2011, 2014), while only a few species have been included in molecular phylogenetic studies (Giribet & Wheeler 2002; McArthur & Harasewych 2003; Dinapoli *et al*. 2006; Dinapoli & Klussmann-Kolb 2010).

Thus, the aim of the present study is to better understand the diversity and evolution of radular types among rissoellids. Here, I present a more extensive genetic data of rissoellids using a combination of genetic markers (mitochondrial COI + 16S rDNA and nuclear 18S rDNA + 28S rDNA), specifically examining the group's radular morphology with the intention of understanding the morphological features of rissoellid radulae in an evolutionary context, and discussing the phylogenetic relationship of rissoellid species collected in Japan and New Zealand.

MATERIAL AND METHODS

Collection and processing of samples. Specimens were collected by snorkeling (washing different macro algae vigorously in a 30 µm planktonic mesh), and by SCUBA diving (using an airlift sampler pipe) in different localities around Hokkaido (Japan) and the South Island (New Zealand). Other details in collecting and processing the material are the same as in Chapter 1.

Radular morphology. Buccal bulbs were dissected, placed in commercial bleach diluted to 15% until the tissues were dissolved. Then the cleaned radulae were washed in 70% ethanol and observed with a Hitachi S-3000N scanning electron microscope (SEM) (Hitachi, Tokyo, Japan). Voucher material has been deposited at the Invertebrate Collection of the Hokkaido University Museum (ICHUM), Sapporo, Japan. Radular description of *Rissoella* sp. 1 and *R. rissoaformis* were taken from Hasegawa (1994) and Ponder & Yoo (1977), respectively.

DNA extraction, PCR amplification, and sequencing. Total DNA was extracted from foot-tissue or whole specimens using a DNeasy Blood & Tissue Kit (Qiagen, USA). Sequences of the partial COI, partial mitochondrial 16S rDNA, partial nuclear 18S rDNA, and partial nuclear 28S rDNA, were amplified. 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). Primer designs and PCR conditions are shown in the Table 5. 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 and all accession numbers are detailed in Table 6.

Table 5. Sequences of the primers used in the PCRs and lengths of amplified fragments.

Gene	Primers	Sequence 5′-3′	Direction	Reference
COI	LCOI	GGT CAA CAA ATC ATA AAG ATA TTG G	Forward	Folmer *et al.* (1994)
	HCOI	TAA ACT TCA GGG TGA CCA AAA AAT CA	Reverse	Folmer *et al.* (1994)
16S	16S-H	CGC CTG TTT ATC AAA AAC AT	Forward	Simon *et al.* (1994)
	16S-R	CCG GTC TGA ACT CAG ATC ACG T	Reverse	Simon *et al.* (1994)
18S	PF1	GCG CTA CCT GGT TGA TCC TGC C	Forward	—
	KWF2	GAT CCC GGA GAG GGA GCT TGA G	Forward	—
	KWR1	CGG TGT GTA CAA ACG GCA GGG AC	Reverse	—
	SSUR4	GATCCTTCTGCAGGTTCACCTAC	Reverse	—
28S	D1RF1	ACC CGC TGA ATT TAA GCA TA	Forward	Takano & Horiguchi (2005)
	305F-27	CGA TAG CAA ACA AGT ACC ATG AG	Forward	Yamada *et al*. (2013)
	D3A	GAC CCG TCT TGA AAC ACG GA	Forward	Takano & Horiguchi (2005)
	25R1	CCT TGG TCC GTG TTT CAA GA	Reverse	Takano & Horiguchi (2005)
	852R-70	CGA ACG ATT TGC ACG TCA AG	Reverse	Yamada *et al*. (2013)
	28-1483R	GCT ACT ACC ACC AAG ATC TGC	Reverse	Takano & Horiguchi (2005)

Table 6. Information for specimens analyzed in this study. Taxa collected for this study by the authors are marked with an +. Gene sequences amplified for this study are marked with an *.

Taxon	Locality	Radular morphology	GenBank accession number			
			COI	16S	28S	18S
RISSOELLIDAE						
Rissoella cystophora +	Tahuna, New Zealand	This study	—	(xxxxxx)*	—	—
Rissoella elongatospira +	Kaikoura, New Zealand	This study	(xxxxxx)*	(xxxxxx)*	—	(xxxxxx)*
Rissoella elatior +	Setana, Hokkaido, Japan	This study	(xxxxxx)*	(xxxxxx)*	(xxxxxx)*	(xxxxxx)*
Rissoella golikovi +	Oshoro, Hokkaido, Japan	This study	(xxxxxx)*	(xxxxxx)*	(xxxxxx)*	(xxxxxx)*
Rissoella japonica +	Oshoro, Hokkaido, Japan	This study	(xxxxxx)*	(xxxxxx)*	(xxxxxx)*	(xxxxxx)*
Rissoella rissoaformis	unknown	Ponder & Yoo (1977)	FJ917271	FJ917252	FJ917226	FJ917214
Rissoella sp. 1 +	Shakotan, Hokkaido, Japan	Hasegawa (1994)	(xxxxxx)*	(xxxxxx)*	(xxxxxx)*	(xxxxxx)*
Rissoella vitrea +	Tahuna, New Zealand	This study	(xxxxxx)*	—	—	—
Rissoella wilfredi +	Kaikoura, New Zealand	This study	(xxxxxx)*	(xxxxxx)*	(xxxxxx)*	(xxxxxx)*
OUTGROUP						
ACTEONIDAE						
Pupa solidula	unknown	—	DQ238006	EF489319	AY427481	AY427516
Rictaxis punctocaelatus	unknown	—	EF489393	EF489318	EF489370	EF489346
CAENOGASTROPODA						
Pomacea bridgesii	unknown	—	DQ916496	DQ093480	DQ279984	DQ093436
Aperostoma palmeri	unknown	—	DQ093523	DQ093479	DQ279983	DQ093435

Analysis of molecular data. Sequenced fragments were edited and assembled with MEGA7 (Kumar *et al.* 2016), and BLAST searched (Altschul *et al.* 1990) in GenBank database (www.ncbi.nlm.nih.gov/Genbank/index.html) to check if the correct genes had been amplified. Additional GenBank sequences of outgroup taxa were added to the dataset, which included a closely related species (*Pupa solidula*) and two other distant species (*Aperostoma palmeri* and *Pomacea bridgesii*). Individual COI, 16S rDNA, 18S rDNA, and 28S rDNA sequences were aligned using the online version of MAFFT (Katoh *et al.* 2002). The 16S, 18S and 28S sequences were characterized by several gap regions with ambiguous alignment, and such regions were excluded automatically by the software Gblocks v. 0.91b (Castresana 2000). And sequences were concatenated with MEGA7 (Kumar *et al.* 2016). JMODELTEST2 (Darriba *et al.* 2012) was used to determine the best substitution model on the individual and concatenated alignments. Maximum-likelihood (ML) trees (Felsenstein 1981) were estimated individually for each gene and also for a concatenate data set using RAxML v. 7.4.7 (Stamatakis 2014) on the CIPRES Science Gateway online service, to provide a graphical overview of genetic distances across the data set. Node support was inferred with bootstrap analysis (1,000 pseudoreplicates). (http://www.phylo.org/portal2/). Bayesian marginal posterior probabilities were calculated using MrBayes (Huelsenbeck *et al*. 2001).

RESULTS

Of the nine rissoellid species included in this study, seven were available for analysis of the radular (Table 7 and Figs. 11, 12). Due to a limited number of collected specimens, the

radular description of *Rissoella* sp. 1 was taken from Hasegawa (1997). Both morphological and genetic information of *R. rissoaformis* were taken from Ponder & Yoo (1977) and Dinapoli & Klussmann-Kolb (2010), respectively. All species were highly divergent in terms of tooth shape and number of cusps (Table 7 and Fig. 12). The radulae of *Rissoella elatior*, *R. rissoaformis*, and *R. wilfredi* were characterized by the presence of seven teeth per row which includes a plate-like outer marginal tooth on both sides. *Rissoella cystophora*, *R. elongatospira*, *R. japonica*, *R.* sp. 1, and *R. vitrea* were all found to have five teeth per row in the radula. *Rissoella golikovi* was unique among the species examined in this study in that the radula possessed only three teeth per row.

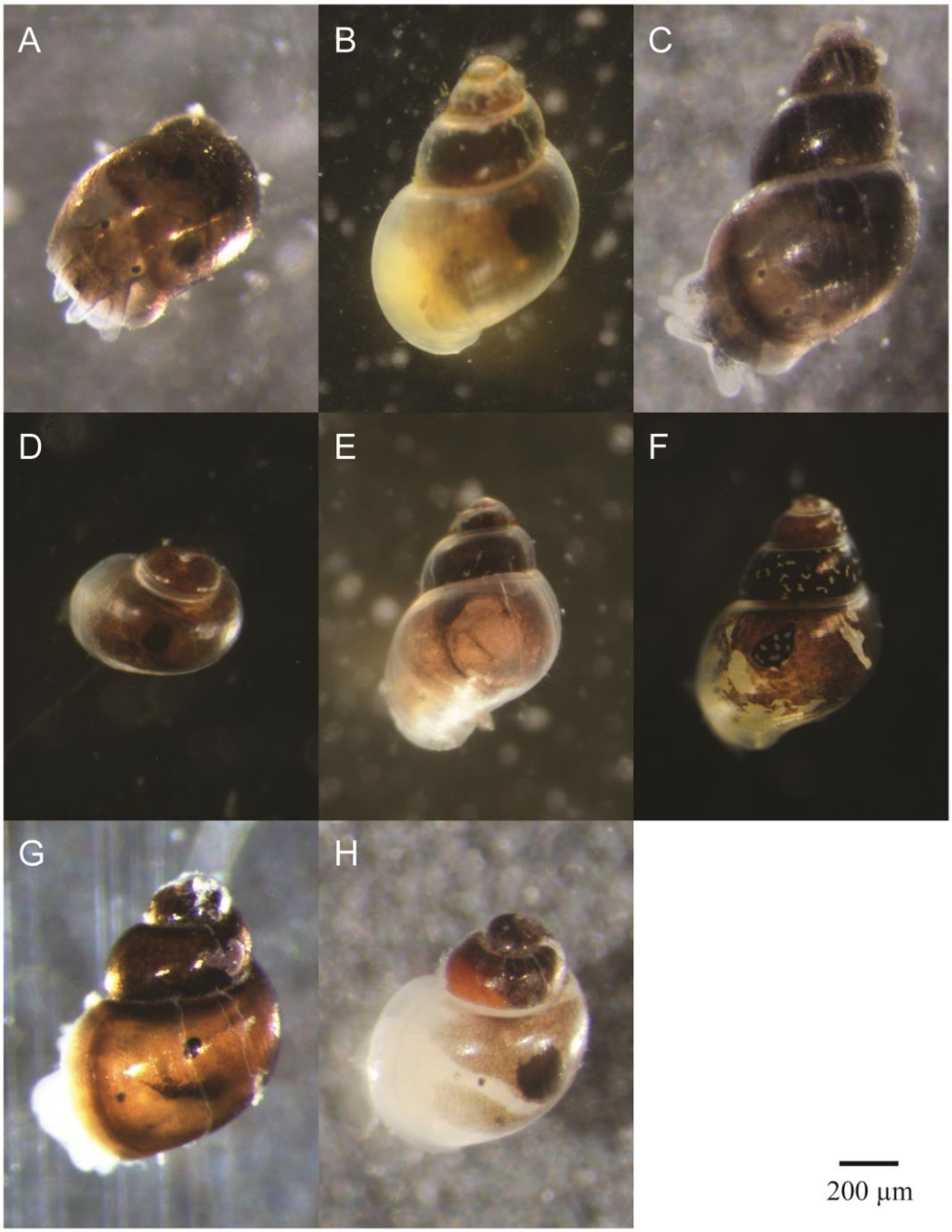

Figure 11. Rissoellid species included in this study: (A) *Rissoella cystophora*. (B) *Rissoella elatior*. (C) *Rissoella elongatospira*. (D) *Rissoella golikovi*. (E) *Rissoella japonica*. (F) *Rissoella* sp. 1. (G) *Rissoella vitrea*. (H) *Rissoella wilfredi*.

Table 7. Radular descriptions.

Species	Radula				Distribution	Reference
	Formula	Central tooth	Lateral teeth	Marginal teeth		
Rissoella cystophora	1.1.R.1.1	triangular with two cusps on the cutting edge and a central basal projection	wider than large with 8-10 sharp cusps gradually decreasing in size from the inner to the outer side	simple, plate-like	Australia and New Zealand	Ponder & Yoo (1977); this study
Rissoella elatior	2.1.R.1.2	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	elongate-triangular (width about 78% of length), each with large, sharp, smooth median cusp, and 8-12 smaller cusps along inner and outer margins	Inner 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. Outer teeth reduced, simple, plate-like (width almost 200% of length)	Russia and Japan	Golikov, Gulbin & Sirenko (1987); this study
Rissoella elongatospira	1.1.R.1.1	large, strongly convex cutting edge with 15-17 irregular, sharp cusps. A pair of basal projections	with a strong, rather long ridge on its inner side and a relatively short, strongly convex cutting edge, which has one prominent median cusp and 15-18 smaller cusps. The base is about twice the width of the cutting edge	rather large, with 11-14 sharp cusps, the apical one stronger and larger than the others	Australia and New Zealand	Ponder & Yoo (1977); this study
Rissoella golikovi	1.R.1	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	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		Russia and Japan	Gulbin (1979); this study
Rissoella japonica	1.1.R.1.1	higher than wide (width about 48% of length), cutting edge with 10-13 sharp cusps of different sizes	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	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	Japan	Chira Siadén *et al.* (*in press*); this study

Continue the Table 7.

Rissoella rissoaformis	2.1.R.1.2	with a larger median cusp, flanked by 4-5 smaller cusps gradually decreasing in size from the inner to the outer side. Two lateral basal projections	large, with a large cusp flanked by smaller irregular cusps. the base is wider	inner similar in shape to the lateral teeth, with a larger central cusp and flanked by smaller irregular cusps. Outer teeth simple, plate-like	New Zealand	Ponder & Yoo (1977)
Rissoella sp. 1	1.1.R.1.1	large and broad. Long and narrow basal processes. Biggest central and 5-10 flanking irregular cusps on each side	large main cusp and many cusps on both sides. Right tooth is much larger than left	inner: well-developed with many cusps	Japan	Hasegawa (1994)
Rissoella vitrea	1.1.R.1.1	large, strongly convex cutting edge with 10-13 irregular, sharp cusps. A pair of basal projections	with a strong, rather long ridge on its inner side and a relatively short, strongly convex cutting edge, which has one prominent median cusp and 13-18 smaller cusps. The base is about twice the width of the cutting edge	rather large, with 11-14 sharp cusps, the apical one stronger and larger than the others	Australia and New Zealand	Ponder & Yoo (1977); this study
Rissoella wilfredi	2.1.R.1.2	wider than large with 3 to 4 symmetrical cusps (1 to 2 on right of the largest cusp, and 1 on left). A row of very small denticles merging with the right basal process	broad bases, long outer portion, short inner portion. Large median cusp with many minor cusps on both sides	inner teeth similar in shape and cusp formation to lateral teeth, but with a prominent outer basal cusp. Outer teeth simple, plate-like	Australia and New Zealand	Ponder & Yoo (1977); this study

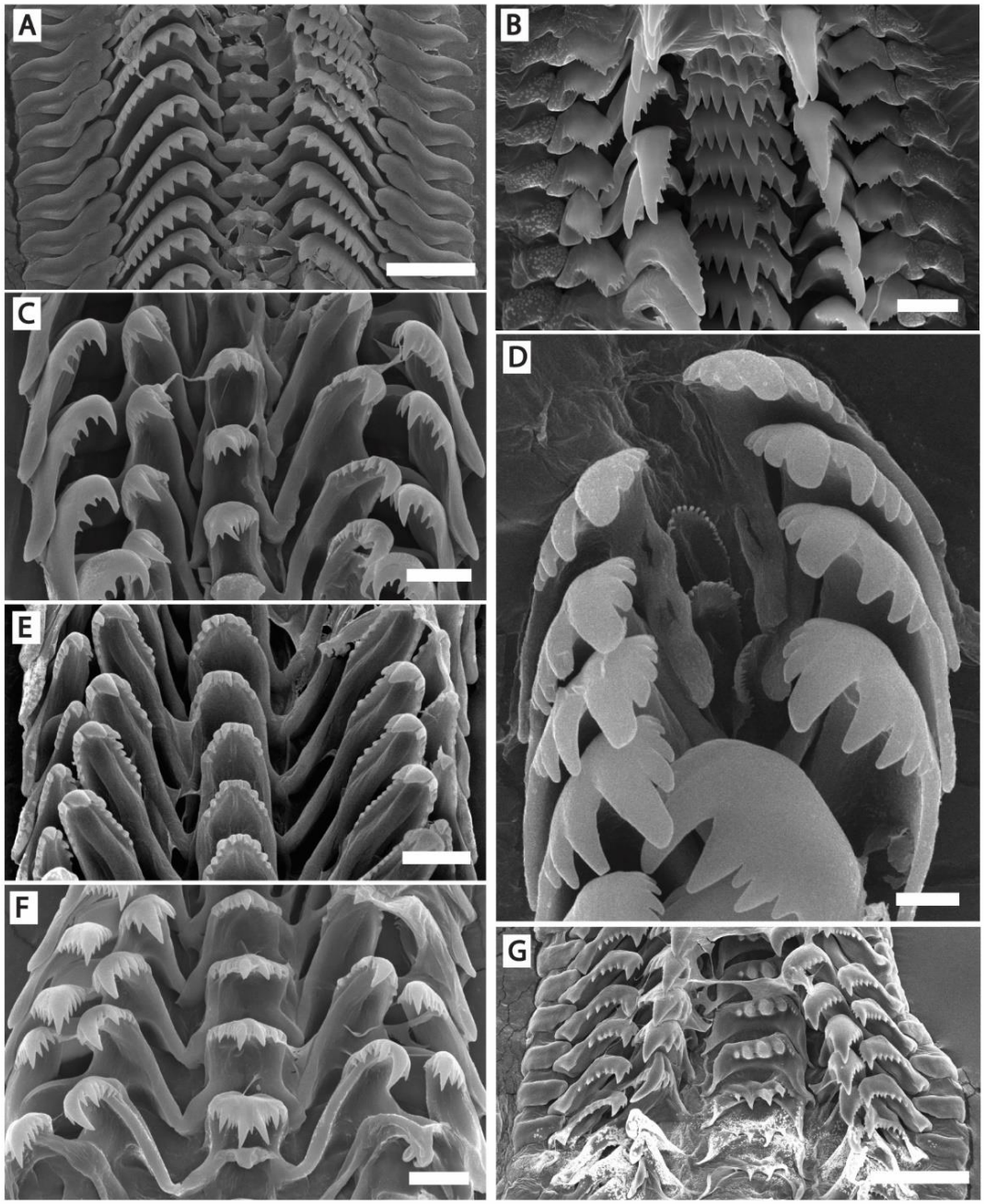

Figure 12. Radular morphology: (A) *Rissoella cystophora* (25 µm). (B) *Rissoella elatior* (10 µm). (C) *Rissoella elongatospira* (10 µm). (D) *Rissoella golikovi* (5 µm). (E) *Rissoella japonica* (10 µm). (F) *Rissoella vitrea* (10 µm). (G) *Rissoella wilfredi* (50 µm).

The trees reconstructed by the four individual gene markers, COI, 16S, 18S, and 28S were poorly resolved in my analyses. In *R. vitrea* and *R. cystophora*, only the COI and 16S regions could be amplified and sequenced, respectively. Due to the missing data in those two species, I generated two concatenated datasets. The first dataset included only seven rissoellid species (Fig. 13A), which recovered a highly supported monophyly of Rissoellidae (PP = 1.00, BS = 100). *Rissoella wilfredi*, *R. rissoaformis*, and *R. elatior* formed a clade (BS = 75). *Rissoella elongatospira* was sister to *R. japonica* (PP = 1.00, BS = 98). *Rissoella* sp.1 was sister to a clade containing *R. wilfredi*, *R. rissoaformis, R. elatior, R. elongatospira*, and *R. japonica* (PP = 1.00, BS = 99); to this clade, *R. golikovi* was a sister taxon (PP = 1.00, BS = 100). The second dataset included both *R. vitrea* and *R. cystophora* (Fig. 13B). Here, *Rissoella wilfredi*, *R. rissoaformis*, and *R. elatior* did not change their internal relation and position in relation to the first tree (BS = 76). *Rissoella vitrea*, for which only the COI region could be amplified, was sister to *R. elongatospira*; the clade comprised of these two was in turn sister to *R. japonica*, with the three species altogether forming another high supported clade (PP = 1.00, BS = 98). *Rissoella* sp. 1, *R. golikovi*, and *R. cystophora* branched at the base of rissoellids.

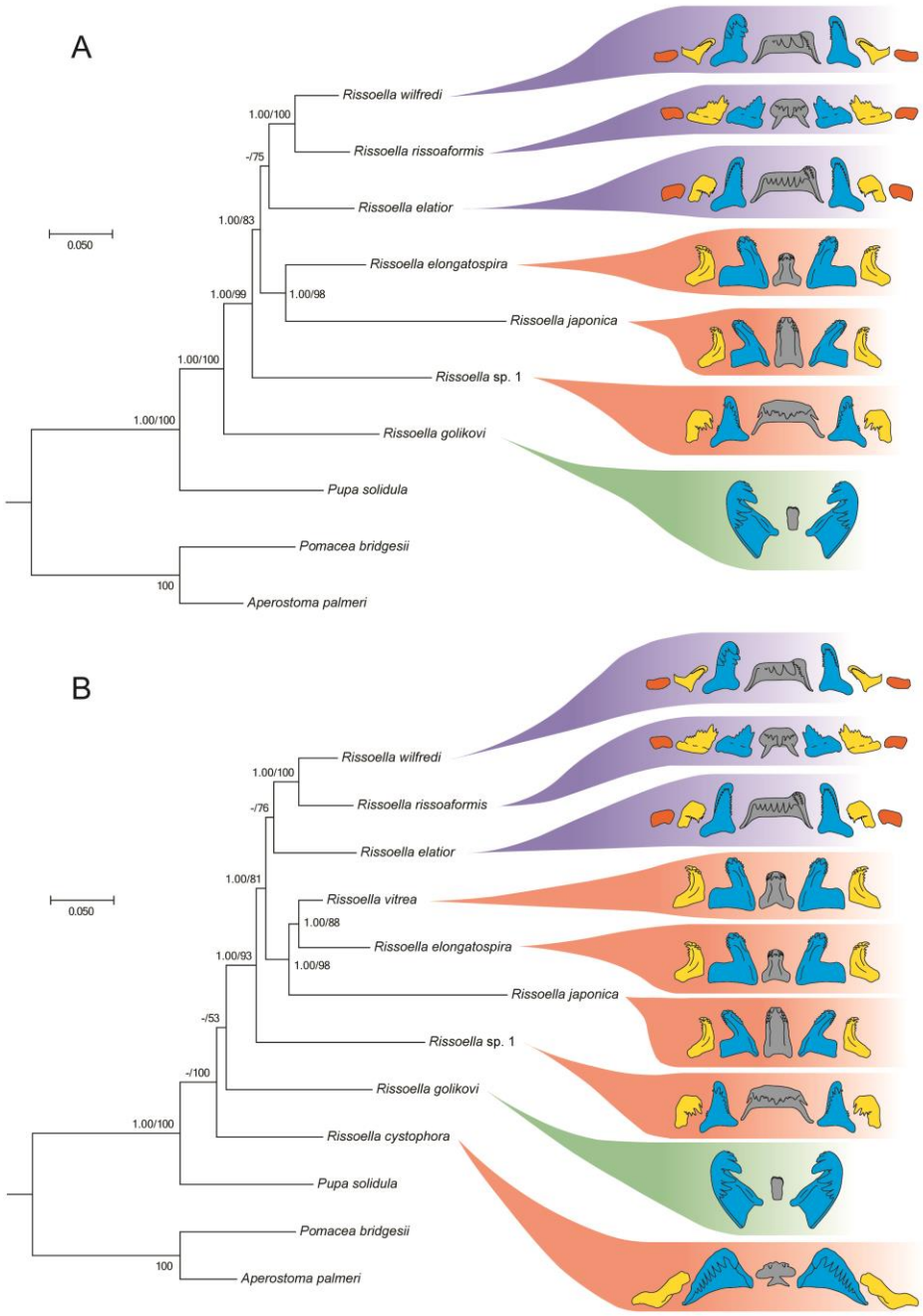

Figure 13. Bayesian inference phylogram based on a concatenated alignment of COI, 16S rDNA, 18S rDNA, 28S rDNA. (A) Tree based on dataset of only seven rissoellid species. (B) Tree including both *R. vitrea* and *R. cystophora* Support values are posterior probabilities (Bayesian analysis) and bootstrap values (maximum-likelihood analysis, as percentage). Only support values above 0.5 and 50, respectively, are given. Radular morphology: Central tooth (grey), lateral teeth (sky-blue), and marginal teeth (inner: yellow; outer: orange).

DISCUSSION

The combination of molecular and morphological data revealed that the plate-like outer marginal teeth do not represent vestigial teeth, but a derived state. To date, even though the radular morphology has been well described in most of the rissoellid species (Ponder & Yoo 1977), there has not been any study that has attempted to infer the interspecific evolutionary changes in radular form among rissoellids, or to explain the basis of these variations. Fortunately, the three major groups (three, five, and seven teeth per row) recognized in the radular morphology of rissoellids have been included in this study. Those three groups are determined based on the presence/absence and the number of the marginal teeth. Among the species included in this analysis, *R. wilfredi*, *R. rissoaformis*, and *R. elatior* are characterized by the presence of seven teeth per row, with the outer-most, plate-like teeth that are devoid of any cusps. In the resulting trees (Fig. 13A, B), these three species formed a highly supported clade. Species with this combination of characters were once classified in the subgenus *Jeffreysiella* (Ponder & Yoo 1977). However, placing these species in a separated subgenus, or even a different genus, would make the other species paraphyletic. Some authors have included more than one genus in the Rissoellidae (Gulbin, 1979; Golikov *et al.* 1987). However, my results are in favor of a different classification, in which only *Rissoella* is accepted in the family (e.g., WoRMS, http://www.marinespecies.org/aphia.php?p=taxdetails&id=138438).

My analysis failed to illustrate with certainty the character transformation that has led to *R. golikovi*. Among the species included in the analysis, *R. golikovi* shows a unique radular morphology in that it has *i*) three teeth per row, *ii*) a small central tooth, and *iii*) no marginal

teeth. *Rissoella globularis* shows a similar radular morphology, but has not been included in the present analysis. In the resulting tree based on the 7-species dataset, *R. golikovi* appeared to be the sister taxon to all the rest of the ingroup species (Fig. 13A). In this tree topology, two equally parsimonious interpretations are possible as to the ancestral state for all the rissoellids in terms of the number of teeth: *i*) three, followed by acquisition of inner marginal teeth in the lineage leading to the non-*R. golikovi* clade; and *ii*) five, followed by reduction of inner marginal teeth in the lineage leading to *R. golikovi.* In the 9-species tree (Fig. 13B), the branching order as to *R. golikovi* and *R. cystophora* near the root of *Rissoella* was uncertain due to low supporting values, the latter species having five teeth per row (Fig. 13A). Because *R. cystophora* was represented by only 16S, additional data of other gene markers are necessary to clarify the position of this species in the phylogenetic tree. Also, inclusion of other species in future studies, specifically *R. globularis*, is necessary to provide a better understanding about the evolutionary radular transformation in the Rissoellidae.

Rissoella golikovi would likely to have evolved to compensate the less number of teeth with bigger teeth to decrease stress during feeding. Padilla (2003) has shown that gastropods suffer stress during grazing, and reductions in the number of teeth per row can increase the stress at the tip of each of the remaining teeth. The small number of teeth per row (1.R.1) and the probable less efficient central tooth (very small) in *R. golikovi* might create stress during feeding. Although *R. golikovi* was the smallest species in this study, its radula possesses the largest lateral teeth among all the rissoellids presented here. It appears that the relation between the tooth size and the number of teeth per row is important in feeding.

I believe that the diet is the factor that has more influence on the radular morphology in this family. Studies on the radular variation in gastropods have suggested factors such as

diet and feeding strategies (Shimek & Kohn 1981; Kantor & Puillandre 2012), substrate/habitat type (Padilla 1998; Reid & Mak 1999), species ontogeny (Warén 1990), and shell size (Meirelles & Matthews-Cascon 2003) can influence radular morphology, specifically the tooth shape. More than one rissoellid species can co-exist on the same algal individual, and every rissoellid species can be found in several algal species without showing intraspecific radular variations. These observations allow me to infer that the substrate/habitat type does not affect the tooth shape in the Rissoellidae. This is because rissoellids do not "specifically feed on the macroalga" where they live, but mostly rasp the algal surface eating the epibiotic microalgae and other detritus material (Olabarria 2002; Caballer *et al.* 2011). Rissoellids might have a selective diet, creating an adaptive radular morphology. Based on the conclusions by Rosewater (1980), I assume that *Rissoella elatior*, *R.* sp. 1, and *R. wilfredi* feed on macroalgae because of their broad central tooth, while *R. elongatospira*, *R. japonica*, and *R. vitrea* probably graze microorganisms because of their narrow central tooth. These speculations should be tested in future studies because there is practically no information on the diet as well as the functioning mode of each tooth within the radula in rissoellids.

Describing the transformations of the radula is important for understanding the evolution of rissoellids in general. With nine species studied here, I am covering about the 15% of the rissoellid diversity, inferring the transformation of the rissoellid radulae in an evolutionary context. So, this study is a base for further studies to discover the factors that lead to a species diversification in the Rissoellidae or any other group of microgastropods.

REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman D.J. (1990) Basic local alignment search tool. *Journal of Molecular Biology*, 215(3): 403–410.

Barco, A., Raupach, M.J., Laakmann, S., Neumann, H. & Knebelsberger, T. (2016) Identification of North Sea molluscs with DNA barcoding. *Molecular ecology*, 16: 288–297.

Bouchet, P., Lozouet, P., Maestrati, P. & Héros, V. (2002) Assessing the magnitude of species richness in tropical marine environments: exceptionally high numbers of molluscs at a New Caledonia site. *Biological Journal of the Linnean Society*, 75: 421–436.

Caballer, M., Ortea, J. & Narciso, S. (2011) Description of two new species of *Rissoella* Gray, 1847 (Mollusca, Gastropoda, Heterobranchia) from Venezuela, with a key to the Caribbean species known for the genus. *ZooKeys*, 115: 1–18.

Caballer, M., Ortea, J. & Redfern, C. (2014) On the Genus *Rissoella* Gray, 1847 (Gastropoda: Heterobranchia: Rissoellidae) in the Bahamas. *American Malacological Bulletin*, 32 (1): 104–121.

Castresana, J. (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution*, 17: 540–552.

Chira Siadén, L.E., Wakeman, K.C., Webb, S.C., Hasegawa, K. & Kajihara, H. (*in press*) Morphological and molecular diversity of rissoellids (Mollusca, Gastropoda, Heterobranchia) from the Northwest Pacific island of Hokkaido, Japan. *Zootaxa*.

Clement, M., Posada, D. & Crandall, K.A. (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, 9: 1657–1659.

Darriba, D., Taboada, G.L., Doallo, R. & Posada, D. (2012) jModelTest 2: more models, new heuristics and parallel computing. Nature Methods, 9(8): 772.

Dasmahapatra, K.K., Elias, M., Hill, R.I., Hoffmans, J.I. & Mallet J. (2010) Mitochondrial DNA barcoding detects some species that are real, and some that are not. *Molecular Ecology*, 10: 264–273.

Dinapoli, A., Tamer, C., Franssen, S., Naduvilezhath, L. & Klussmann-Kolb, A. (2006) Utility of H3-Gene sequences for phylogenetic reconstruction—a case study of heterobranch Gastropoda. *Bonner zoologische Beiträge*, 55: 191–202.

Dinapoli, A. & Klussmann-Kolb, A. (2010) The long way to diversity – phylogeny and evolution of the Heterobranchia (Mollusca: Gastropoda). *Molecular Phylogenetics and Evolution*, 55 (1): 60–76.

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, 32 (5): 1792–1797.

Espinosa, J. & Ortea, J. (2009) Nueva especie de *Rissoella* J.E. Gray, 1847 (Gastropoda: Heterobranchia) de la Península de Guanahacabibes, Pinar del Río, Cuba. *Revista de la Academia Canaria de Ciencias*, XX (4): 15–17. [in Spanish]

Fedosov, A., Watkins, M., Heralde III, F.M., Showers Corneli, P., Concepcion, G.P. & Olivera, B.O. (2011) Phylogeny of the genus *Turris*: Correlating molecular data with

radular anatomy and shell morphology. *Molecular Phylogenetics and Evolution*, 59: 263–270.

Felsenstein, J. (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution*, 17 (6): 368–376.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology Biotechnology*, 3 (5): 294–299.

Fretter, V. (1948) The structure and life history of some minute prosobranchs of rock pools: *Skeneopsis planorbis* (Fabricius), *Omalogyra atomus* (Philippi), *Rissoella diaphana* (Alder) and *Rissoella opalina* (Jeffreys). *Journal of the Marine Biological Association of the United Kingdom*, 27 (3): 597–632.

Galindo, L.A., Puillandre, N., Strong, E.E. & Bouchet, P. (2014) Using microwaves to prepare gastropods for DNA barcoding. *Molecular Ecology Resources*, 14 (4): 700–705.

Geiger, D.L. (2006) Marine Gastropoda. In: The Mollusks: *A Guide to Their Study, Collection, and Preservation*, edited by Sturn C.F., T.A. Pearce, and A. Valdés. American Malacological Society, pp. 295–312.

Geiger, D.L. (2008) Micromolluscs in molecular systematics: Experiences and the best practices. *Zoosymposia*, 1: 39–45.

Giribet, G. & Wheeler, W. (2002) On bivalve phylogeny: a high-level analysis of the Bivalvia (Mollusca) based on combined morphology and DNA sequence data. Invertebrate Biology, 121(4): 271–324.

Golikov, A.N., Gulbin, V.V. & Sirenko, B.I. (1987) Prosobranch from Moneron Island shelf (Sea of Japan). I. Orders Patelliformes–Calyptraeiformes. In: *Fauna and Distribution of Molluscs, North Pacific and Polar Basin, edited by A. Kafanov. Far Eastern Science Center of the USSR Academy of Science*, pp. 20–40.

Gulbin, V.V. (1979) A new species of the littoral gastropod Rissoellidae from the Far-eastern seas. Academy of Sciences of the USSR, Vladivostok. Far East Science Center. *Marine Biology*, 3: 88–89. [in Russian]

Guralnick, R.P. & Lindberg, D.R. (1999) Integrating developmental evolutionary patterns and mechanisms: A case study using the gastropod radula. Evolution, 53: 447–459.

Hasegawa, K. (1997) Systematic study on the phytal gastropods in Japan. Doctoral thesis, Tokyo University, Tokyo, pp. 479.

Hasegawa, K. (2000) Rissoellidae. In: *Marine Mollusks in Japan*, edited by Okutani T. Tokai University Press, Tokyo, pp. 700–701.

Hasegawa, K. (2017) Rissoellidae. In: *Marine Mollusks in Japan*, the Second Edition, edited by Okutani T. Tokai University Press, Tokyo, pp. 398, 1063.

Hebert, P.D.N., Cywinska, A., Ball, S.L. & Dewaard, J.R. (2003a) Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences*, 270 (1512): 313–321.

Hebert, P.D.N., Ratnasingham, S. & Dewaard, J.R. (2003b) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society B: Biological Sciences*, 270 (Suppl 1): S96–S99.

Herbert, G.S, Merle, D. & Gallardo C.S. (2007) A developmental perspective on evolutionary innovation in the radula of the predatory neogastropod family Muricidae. American Malacological Bulletin, 23: 17–32.

Hickman, C.S. (1980) Gastropod radulae and the assessment of form in evolutionary paleontology. Paleobiology 6: 276–294.

HickmanC.S. (1983) Ecological and phylogenetic implications of the unusual radula of Laevinesta atlantica (Mollusca, Gastropoda). Veliger, 25: 323–325.

Huelsenbeck J.P., Ronquist, F. & Hall, B. (2001) MrBayes: Bayesian inference of phylogeny. Bioinformatics, 17: 754–755.

Kano, Y., Chikyu, E. & Warén, A. (2009) Morphological, ecological and molecular characterization of the enigmatic planispiral snail Genus *Adeuomphalus* (Vetigastropoda: Seguenzioidea). *Journal of Molluscan Studies*, 75 (4): 397–418.

Kantor, Y.I. & Puillandre, N. (2012) Evolution of the radular apparatus in Conoidea (Gastropoda: Neogastropoda) as inferred from a molecular phylogeny. Malacologia, 55(1): 55–90.

Kantor, Y.I. & Sysoev, A.V. (2006) *Marine and Brackish Water Gastropoda of Russia and Adjacent Countries: An Illustrated Catalogue*. KMK Scientific Pres Ltd., Moscow, pp. 371, 140 pls.

Katoh K, Misawa K, Kuma K, Miyata T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research*, 30: 3059–3066.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., *et al*. (2012) Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, 28 (12): 1647–1649.

Kimura, M. (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, 16 (2): 111–120.

Kristof, A., De Oliveira, A.L., Kolbin, K.G. & Wanninger, A. (2016) A putative species complex in the Sea of Japan revealed by DNA sequence data: a study on *Lottia* cf. *kogamogai* (Gastropoda: Patellogastropoda). *Journal of Zoological Systematics and Evolutionary Research*, 54 (3): 177–181.

Kumar, S., Stecher, G. & Tamura, K. (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molecular Biology and Evolution, 33(7): 1870–1874.

Maturana, C.S., Moreno, R.A., Labra, F.A., González-Wevar, C.A., Rozbaczylo, N., Carrasco, F.D. & Poulin, E. (2011) DNA barcoding of marine polychaetes species of southern Patagonian fjords. *Revista de Biologia Marina Y Oceanografia*, 46 (1): 35–42.

McArthur, A.G. and Harasewych, M.G. (2003) Molecular systematics of the major lineages of the Gastropoda. In: *Molecular Systematics and Phylogeography of Mollusks*, edited by Lydeard, Charles and Lindberg, D. R., pp. 140–160.

Miller, M.A., Pfeiffer, W., and Schwartz, T. (2010) "Creating the CIPRES Science Gateway for inference of large phylogenetic trees" in Proceedings of the Gateway Computing Environments Workshop (GCE), 14 Nov. 2010, New Orleans, LA pp 1 - 8.

Mutlu, E. (2004) Sexual dimorphisms in radula of Conomurex persicus (Gastropoda: Strombidae) in the Mediterranean Sea. Marine Biology 145: 693–698.

Olabarria, C. (2002) Role of colonization in spatio-temporal patchiness of microgastropods in coralline turf habitat. *Journal of Experimental Marine Biology and Ecology*, 274 (2): 121–140.

Ortea, J. & Espinosa, J. (2004) Una combinación de ciencia, arte y naturaleza: Especies nuevas del género *Rissoella* J. E. Gray, 1847 (Gastropoda, Heterobranchia) descritas en homenaje a las artistas de la plástica cubana. *Avicennia*, 17: 77–94. [in Spanish]

Ortea, J., Espinosa, J. & Magaña, J. (2004) Descripción de una nueva especie del género *Rissoella* J. E. Gray, 1847 (Mollusca, Gastropoda, Heterobranchia) del Pacifico de Costa Rica. *Avicennia*, 17: 95–100. [in Spanish]

Padilla, D.K. (1985) Structural resistance of algae to herbivores. A biomechanical approach. *Marine Biology* 90: 103–109.

Padilla, D.K. (1989) Structural defenses of algae: The importance of form and calcification in resistance to tropical limpets. *Ecology*, 70: 835–842.

Padilla, D.K. (2003) Form and function of radular teeth of herbivorous molluscs: Focus on the future. *American Malacological Bulletin*, 18(1): 163–168.

Ponder, W.F. & Yoo, E.K. (1977) A revision of the Australian species of Rissoellidae (Mollusca: Gastropoda). *Records of The Australian Museum*, 31 (4): 133–185.

Puillandre, N., Lambert, A., Brouillet, S. & Achaz, G. (2012) ABGD, automatic barcode gap discovery for primary species delimitation. *Molecular Ecology*, 21 (8): 1864–1877.

Puillandre, N. & Tenorio, M.J. (2017) A question of Rank: DNA sequences and radula characters reveal a new genus of cone snails (Gastropoda Conidae). *Journal of Molluscan Studies*, 83(2): 200–210.

Reid D.G. (2000) The use of the radula in the taxonomy and phylogeny of gastropods: cautionary cases of convergence, intraspecific variation and plasticity. *Phuket Marine Biological Center Special Publication* 21: 329–345.

Reid, D.G. & Mak Y.M. (1999) Indirect evidence for ecophenotypic plasticity in radular dentition of *Littoraria* species (Gastropoda: Littorinidae). Journal of Molluscan Studies, 65: 355–370.

Rolán, E. & Hernández, J.M. (2004) Dos nuevas especies de *Rissoella* (Mollusca: Rissoellidae) de las islas Canarias y Senegal. *Revista de la Academia Canaria de Ciencias*, XV (3–4): 229–235. [in Spanish]

Rosewater J. (1980) A close look at *Littorina* radulae. *Bulletin of the American Malacological Union*, 1959: 5-8.

Sars, G.O. (1878) Mollusca regionis Arcticae Norvegiae. In: *Bidrag til Kundskaben om Norges Arktiske Fauna*. Chritiania: Brøgger, 466 pp.

Sasaki, T. (2008) Micromolluscs in Japan: Taxonomic composition, habitats, and future topics. *Zoosymposia*, 1: 147–232.

Shaw, J.A., Macey, D.J., Brooker, L.R. & Clode P.L. (2010) Tooth use and wear in three iron-biomineralizing mollusc species. Biological Bulletin, 218(2): 132–144.

Shimek, R.L. & Kohn, A.J. (1981) Functional morphology and evolution of the toxoglossan radula. *Malacologia*, 20: 423–438.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H. & Flook, P. (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annual Review of Ecology, Evolution, and Systematics*, 87: 651–701.

Simone, L. (1995) *Rissoella ornata*, a new species of Rissoellidae (Mollusca: Gastropoda: Rissoelloidea) from the southeastern coast of Brazil. *Proceedings of the Biological Society of Washington*, 108 (4): 560–567.

Stamatakis, A. (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics*, 22 (21): 2688–2690.

Sun Y., Li Q., Kong, L. & Zheng, X. (2012) DNA barcoding of Caenogastropoda along coast of China based on the COI gene. *Molecular Ecology Resources*, 12 (2): 209–218.

Syromyatnikov, M.Y., Golub, V.B., Kokina, A.V., Soboleva, V.A. & Popov, V.N. (2017) DNA barcoding and morphological analysis for rapid identification of most economically important crop-infesting Sunn pests belonging to *Eurygaster* Laporte, 1833 (Hemiptera, Scutelleridae). *Zookeys*: 706, 51–71.

Takano, Y. & Horiguchi, T. (2005) Acquiring scanning electron microscopical, light microscopical and multiple gene sequence data from a single dinoflagellate cell. Journal of Phycology, 42: 251–256.

Thiele, J. (1929–1935) *Handbuch der systematischen Weichtierkunde*. Gustav Fischer, Jena. 1154 pp.

Weigand, A.M., Jochum, A., Pfenninger, M., Steinke, D. & Klussmann-Kolb, A. (2011) A new approach to an old conundrum—DNA barcoding sheds new light on phenotypic plasticity and morphological stasis in microsnails (Gastropoda, Pulmonata, Carychiidae). *Molecular Ecology Resources*, 11 (2): 255–265.

Weigand, A.M., Jochum, A., Slapnik, R., Schnitzler, J., Zarza, E. & Klussmann-Kolb, A. (2013) Evolution of microgastropods (Ellobioidea, Carychiidae): integrating taxonomic, phylogenetic and evolutionary hypotheses. *BMC Evolutionary Biology*, 13 (1): 1–24.

Wise, J.B. (1998) Morphology and systematic position of *Rissoella caribaea* Rehder, 1943 (Gastropoda: Heterobranchia: Rissoellidae). *The Nautilus*, 111 (1): 13–21.

Yamada, N., Terada, R., Tanaka, A. & Horiguchi, T. (2013) Bispinodinium angelaceum gen. Et sp. Nov. (Dinophyceae), a new sand-dwelling dinoflagellate from the seafloor off Mageshima Island, Japan. Journal of Phycology, 49: 555–569.

Zhang, J., Kapli, P., Pavlidis, P. & Stamatakis, A. (2013) A general species delimitation method with applications to phylogenetic placements. *Bioinformatics*, 29 (22): 2869–2876.