



# Comparative morphology of glochidial shells of *Inversiunio* (Bivalvia: Unionidae) from Japan

ELENA M. SAYENKO<sup>1</sup> & YOSHIHIRO B. AKIYAMA<sup>2</sup>

<sup>1</sup> Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch of Russian Academy of Sciences, Vladivostok 690022, Russia (sayenko@ibss.dvo.ru).  <https://orcid.org/0000-0002-5459-2692> <sup>2</sup> Coastal, Marine and Disaster Prevention Department, National Institute for Land and Infrastructure Management, Ministry of Land, Infrastructure, Transport and Tourism, Kanagawa 239-0826, Japan.  <https://orcid.org/0000-0002-6116-5920> • Corresponding author: E.M. Sayenko.

**Abstract.** Glochidial characters may help to clarify evolutionary relationships among unionid taxa with unclear taxonomic positions. Mature glochidia of three species of *Inversiunio* Habe, 1991 that occur in Japan—*I. reinianus* Kobelt, 1879, *I. jokohamensis* (von Ihering, 1893), and *I. yanagawensis* Kondo, 1982—were examined with the aim of assessing the taxonomic utility of the morphological and morphometric characters of their glochidial shells. All morphological indices of glochidia showed no statistically significant differences and were regarded as not useful for identification among investigated *Inversiunio* species. Four different types of external glochidial valve fine sculpture were revealed that support proposed phylogenetic relationships based on genetic data.

**Key words.** Bivalvia, freshwater mollusk, glochidia.

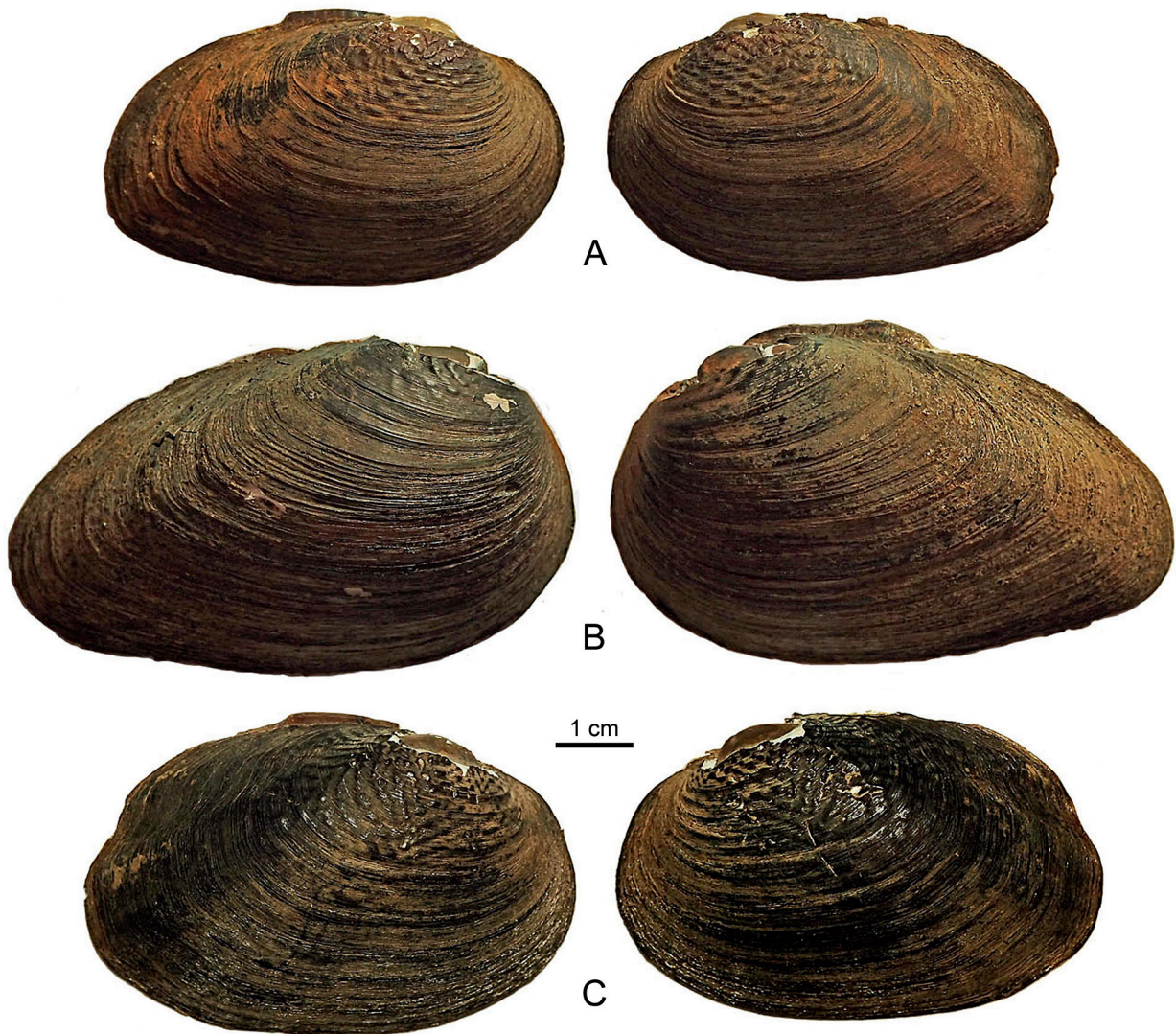
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## Introduction

Many questions of bivalve classification have remained unresolved to the present day. Among freshwater bivalves, the taxonomy of Unionidae Rafinesque, 1820 has the greatest number of questions. *Inversiunio* Habe, 1991 is one of the Asian unionid taxa with unclear taxonomic divisions. HABE (1991a) separated two species with hooked glochidia from *Inversidens* Haas, 1911 and combined these in the genus *Inversiunio*. Initially, *Inversidens* included species with two different glochidial (larval shell) morphologies, i.e., with semi-circular or semi-oval, unhooked glochidia or with subtriangular, hooked glochidia (INABA 1941, 1964, KONDO 1982). When first described, *Inversiunio* included two species: *I. reinianus* Kobelt, 1879, endemic to Lake Biwa, and *I. yanagawensis* Kondo, 1982, distributed in western Japan (HABE 1991b). Subsequently, mussels from central Japan, formerly identified as *Inversidens japonensis* (I. Lea, 1859) (KIMURA 1994, MASUDA 1997), were re-described as *Inversiunio jokohamensis* (von Ihering, 1893) (KONDO 1998). Today, *Inversiunio* includes four species, three of which occur in Japan (Fig. 1)—*I. jokohamensis* from Hokkaido and eastern Honshu, *I. reinianus*, endemic to the Lake Biwa basin, and *I. yanagawensis*

from western Honshu, Shikoku, and Kyushu. A fourth species, *I. verrucosus* Kondo, Hyun & Seung-Ho, 2007, occurs on the Korean peninsula (KONDO et al. 2007, KONDO 2008). All the Japanese *Inversiunio* species are included in the *Red List of Shellfishes* (MINISTRY OF THE ENVIRONMENT OF JAPAN 2019); *I. yanagawensis* and *I. reinianus* are listed as Vulnerable, and *I. jokohamensis* is listed as Near Threatened.

The validity of the genus *Inversiunio* was recently confirmed, with high statistical support, by an analysis of mitochondrial 16S ribosomal DNA sequences (SANO et al. 2017) and a COI+28S dataset (LOPES-LIMA et al. 2020). A phylogenetic analysis also showed the relationship of the genera *Inversiunio* and *Nodularia* Conrad, 1857 (SANO et al. 2017), and *Inversiunio* was placed in the tribe Nodulariini together with the genera *Cuneopsis* Simpson, 1900, *Nodularia*, and *Schistodesmus* Simpson, 1900 (LOPES-LIMA et al. 2020). However, the taxonomic composition of *Inversiunio* is not completely clear. Among the three Japanese species, *I. jokohamensis* was marginally supported, while *I. yanagawensis* was shown to be paraphyletic, forming a clade together with *I. reinianus*. In addition, the genetic distances between *I. yanagawensis* and *I. reinianus* were as low as the intra-specific genetic distances within *I. jokohamensis* (SANO et al. 2017). Using COI molecular data, LOPES-LIMA et



**Figure 1.** Shells of *Inversiunio* from Japan. **A.** *I. jokohamensis* (Inawashiro Lake, Fukushima Pref.). **B.** *I. reinianus* (Lake Biwa, Shiga Pref.). **C.** *I. yanagawensis* (channel of Yodo River, Kyoto Pref.).

al. (2020) distinguished two main clades: one containing only *I. jokohamensis*, and another with three species, *I. reinianus*, *I. yanagawensis*, and *I. verrucosus*, based on morphological differences (KONDO et al. 2007, KONDO 2008), instead of considering them as populations of *I. reinianus*.

The larvae (glochidia) of freshwater bivalves in the family Unionidae are obligate parasites of fishes and amphibians prior to their metamorphosis into juveniles. Morphology of mature glochidia has been used to classify unionids. The first descriptions of the glochidia of *Inversiunio* were published by INABA (1941). INABA's descriptions and all subsequent investigations used light microscopy (LM) only (HIGASHI & HAYASHI 1964, INABA 1964, KONDO 1982, 1998, NAITO 1986, MASUDA 1997, KONDO et al. 2007). According to some studies (BONETTO 1961, SAYENKO 2006, SAYENKO & SHEDKO 2005), the morphological characteristics of glochidia are not useful for distinguishing between species. However, comparative morphology of the glochidia, especially examination of the fine structure of glochidial shells

obtained by scanning electron microscopy (SEM), may have potential as a taxonomic tool, at both the species level (PANHA & EONGPRAKORNKEAW 1995, MANSUR & SILVA 1999, PIMPÃO et al. 2012, O'BRIEN et al. 2019) and among higher taxa (INABA 1964, HOGGARTH 1999, SAYENKO et al. 2005, 2017, SAYENKO 2016).

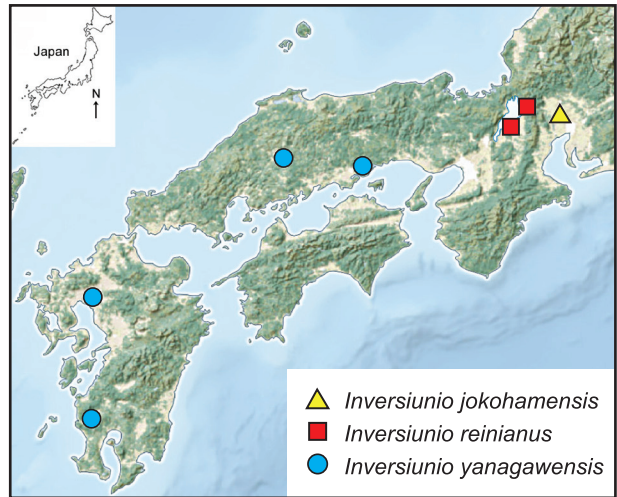
The glochidial shell of the discussed *Inversiunio* mussels has two dorsally articulated, subtriangular valves, with a slightly protruding basal edge forming a straight dorsal line. The shells of glochidia consist of two layers. The inner layer is thick, punctuated by pores, though their outer ends are covered by the thin outer layer that forms a special external microsculpture (KINZELBACH & NAGEL 1986). With high magnification, ridges and depressions, which appear to be organized in a regular manner, may be observed on the external shell surface. Previously it was shown that such exterior valve sculpture differs among unionid genera and can be used for identification of species and taxonomic revisions (PANHA & EONGPRAKORNKEAW 1995, HOGGARTH 1999, SAYENKO 2014, 2015a, 2016).

The present study describes the larvae of the Asian mussel genus *Inversiunio* using a morphological and a morphometric comparison of the glochidia of all three Japanese species and contributes to the taxonomy of these freshwater bivalves.

## Material and Methods

Glochidia were obtained by facilitating their evacuation from mussels by aeration (ALDRIDGE & MCIVOR 2003). The species identification of the glochidia was determined from the adult individuals from which they were extracted. After removing the soft part, the shells of adult mussels were washed, dried, labeled, and sorted in the laboratory; identification was made using regional literature studies (KONDO 2008). After identification, selected dry adult shells were photographed using a digital camera (Fig. 1); they are deposited at Osaka Museum of Natural History (OMNH, hereafter) with the following collection numbers: OMNH-KON-14032.1 (*I. jokohamensis*), OMNH-KON-15018.1 (*I. reinianus*), and OMNH-KON-14026.3 (*I. yanagawensis*). Samples of the three *Inversiunio* species were collected from seven localities in Japan. These were studied by using LM and SEM (Fig. 2; Table 1). All glochidia samples are deposited at Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch of the Russian Academy of Sciences, Vladivostok (FSCEATB FEB RAS, hereafter).

Qualitative comparisons of fine structure morphology among species were made using SEM. In this case, the quality of the investigated sculpturing depends on the methods of preservation (ethanol or formalin) and cleaning (maceration or chemicals) of the glochidial shells. For our investigation we used samples with glochidia initially preserved in 75% alcohol as well as 10% formalin. To prevent deformation or destruction of the hooks, only chemical cleaning was used. To prepare for LM and SEM work, the glochidia were washed several times in distilled water and cleaned of the soft tissues in a 5% KOH solution. To prevent destruction of the thin outer layer of the shells during this procedure, the glochidia were examined by LM every half an hour. The cleaned shells were washed several times in distilled water and



**Figure 2.** A map showing the collecting sites of *Inversiunio* in Honshu and Kyushu islands, Japan.

an alcohol series (80, 90, and 96%), after which they were mounted on permanent slides for LM as well as on stubs for SEM and photography.

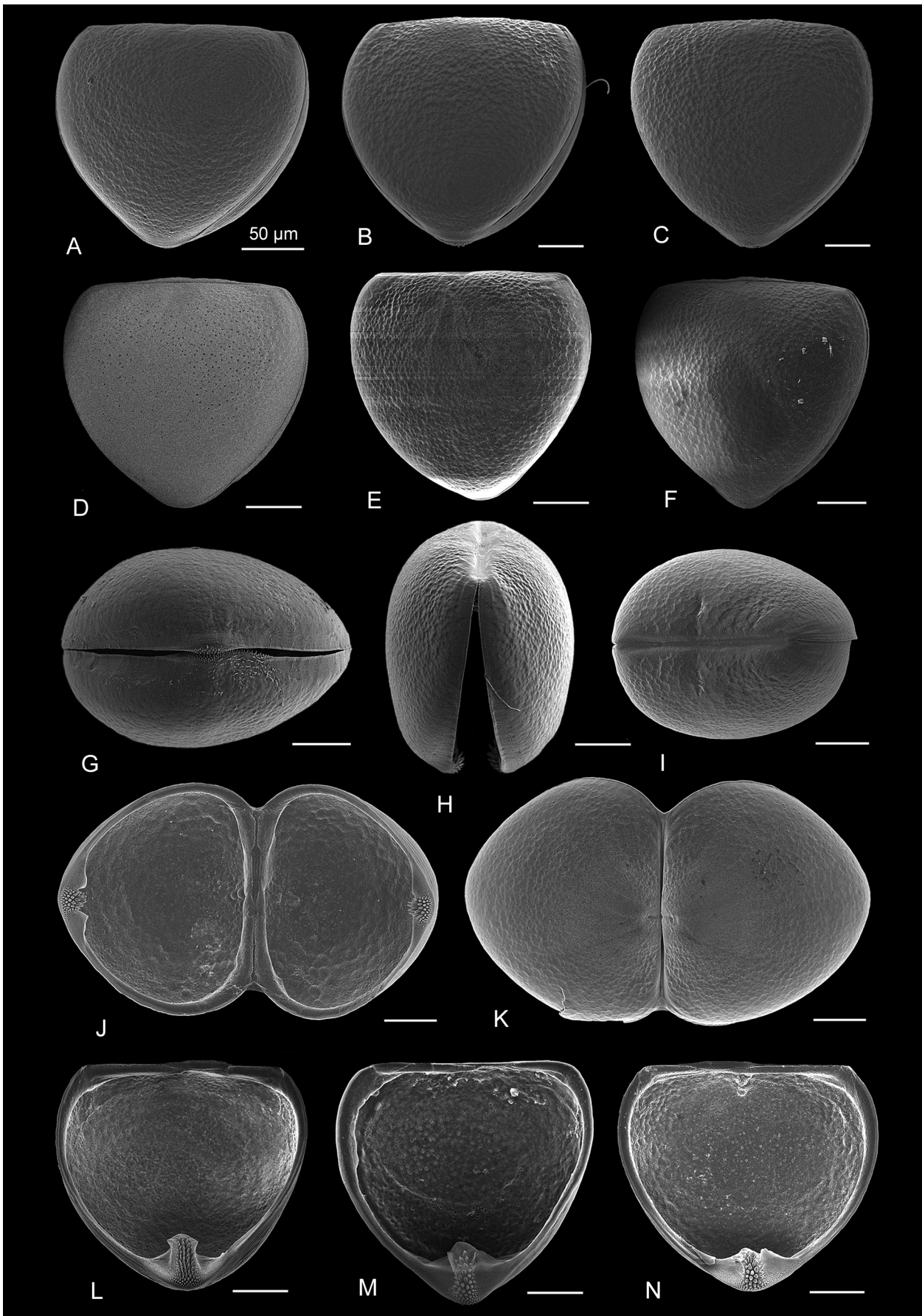
The exterior surface of glochidia from formalin samples (*I. yanagawensis* from Gion Creek, Okayama Pref., Honshu) had the worst quality, although the microsculpture could definitely be observed in these specimens too.

To avoid crushing the glochidia under the coverslip during the mounting process for LM permanent slides, each group of larvae was surrounded by a separate hair part. To exclude the possibility of deformation of the hooks and outer shell layer, chromium was sputtered immediately after drying the samples on a stub for SEM.

The definition of the anterior and posterior regions of the glochidial valves follows that of HOGGARTH (1987). The main conchological characters of the glochidial shells were measured under light microscope using an ocular micrometer and directly from SEM photographs. The description of the main conchological features of glochidial shells follows HOGGARTH (1999) and SAYENKO (2016). Measurements were made on at least 15 glochidia per adult female by LM (Nikon light microscope with ocular reticle) and on at least 10 glochidia per adult female by SEM. Not all measurements could be made on every glochidium. Both temporary water-

**Table 1.** Investigated *Inversiunio* species and sampling sites, date collected, and collector; *n* = number of investigated specimens.

Species	Sampling sites (listed from N to S)	Date collected	Collector	<i>n</i>
<i>I. jokohamensis</i>	Honshu, Gifu Pref., agricultural irrigation ditch ( <b>Nagara River basin</b> ) at Seki City	01.vi.2018	Kengo Ito	2
<i>I. reinianus</i>	Honshu, Shiga Pref., <b>northern Lake Biwa</b> at Nagahama City	03.xi.2016	Toshiaki Nishimura	1
<i>I. reinianus</i>	Honshu, Shiga Pref., <b>Lake Biwa</b> at Okishima, Oumihachiman City	03.xi.2016	Mio Kondo and Takeru Uechi	3
<i>I. yanagawensis</i>	Honshu, Okayama Pref., <b>Gion Creek</b> at Okayama City	13.xi.1993	Takaki Kondo	1
<i>I. yanagawensis</i>	Honshu, Hiroshima Pref., canal ( <b>Gouno river basin</b> ) at Shobara City	27.x.2017	Junichi Naito	2
<i>I. yanagawensis</i>	Kyushu, Fukuoka Pref., canal ( <b>Yabe river basin</b> ) at Yanagawa City	04.xi.2018	Takeru Uechi	1
<i>I. yanagawensis</i>	Kyushu, Kagoshima Pref., <b>Sendai River</b> at Satsuma City	03.xi.2018	Takeru Uechi	2



**Figure 3.** Closed (A–F, frontal view; G, ventral valve view; H, lateral view; I, view of ligament) and open (J, interior view; K, exterior view) glochidial shells and interior valves (L–N) of *Inversiunio*. A, I. *I. jokohamensis* (Nagara river basin). B, C, H. *I. yanagawensis* (Gouno river basin). D, E, K. *I. yanagawensis* (Sendai River). F, G. *I. reinianus* (northern Lake Biwa). J, L. *I. reinianus* (Lake Biwa at Okishima). M. *I. yanagawensis* (Gion Creek). N. *I. yanagawensis* (Yabe river basin). All scale bars = 50  $\mu$ m.

glycerin and permanent glycerin-gelatin preparations were used for this purpose. Glochidial valve length (L) was measured parallel to the hinge as the greatest distance from anterior to posterior margins. Glochidial valve height (H) was measured perpendicular to length as the greatest distance from dorsal to ventral margins. Hinge length, or hinge ligament length (lig)—as in glochidium the hinge ligament extends the entire length of the hinge—was measured in a straight line from the anterior to posterior points of intersects. Seen from the front of the glochidium, hook length (h) was measured from the tip to the distal end of the hook. To standardize the measurements, three ratios were calculated: H/L, lig/L, and h/H.

We examined the validity of the three standardized morphological indices (H/L, lig/L, h/H) of glochidial larvae for distinguishing between two *Inversiunio* species. To assess the validities of the morphological indices with consideration of differences in sampling localities reflecting the intraspecific variation, we employed generalized linear mixed models with binomial error distribution assumption and a logit-link function. First, a pair of mussel species was made in all combinations. As a dummy variable for response variable, each mussel species in a pair was given 0 or 1. Morphological indices were used as explanatory variable and the codes of sampling localities were used as random effect. Next, we fitted models using Markov chain Monte Carlo (MCMC) techniques from the MCMCglmm package (HADFIELD 2010). The chains were run for 11,000,000 iterations, discarding 1,000,000 iterations burn-in period from each chain, and thinning the remaining iterations so that we retained 1,000 samples from the posterior distribution of

the parameters (thinning interval: 10,000). Convergence of the models was assessed by visual inspection of traces. For the case of complete separation of morphological index data between two species (ALLISON 2008), following GELMAN et al. (2008), Gelman prior with a scale of  $\sigma + \pi^2/3$  was used for the fixed effect. For random factors, a parameter-expanded prior with a Cauchy distribution ( $V = 1$ ,  $\nu = 1$ ,  $\alpha \cdot \mu = 0$ , and  $\alpha \cdot V = 1000$ ) was used. The effects of the explanatory variables on the response variable were assessed by checking whether the 95% credible interval contains zero. If the interval did not contain zero, we determined that the explanatory variable was valid to distinguish between two mussel species. Statistical analysis was conducted using R version 3.6.1 (R CORE TEAM 2019).

The microsculpture of each larval shell was examined on at least at three points: closer to the ventral end (i.e., to the hook), at the center of the valve (the adductor region), and at the valve rim closer to the ligament. The description of the exterior valve microsculpture follows PANHA & EONGPRAKORNKEAW (1995), HOGGARTH (1999), and SAYENKO (2016).

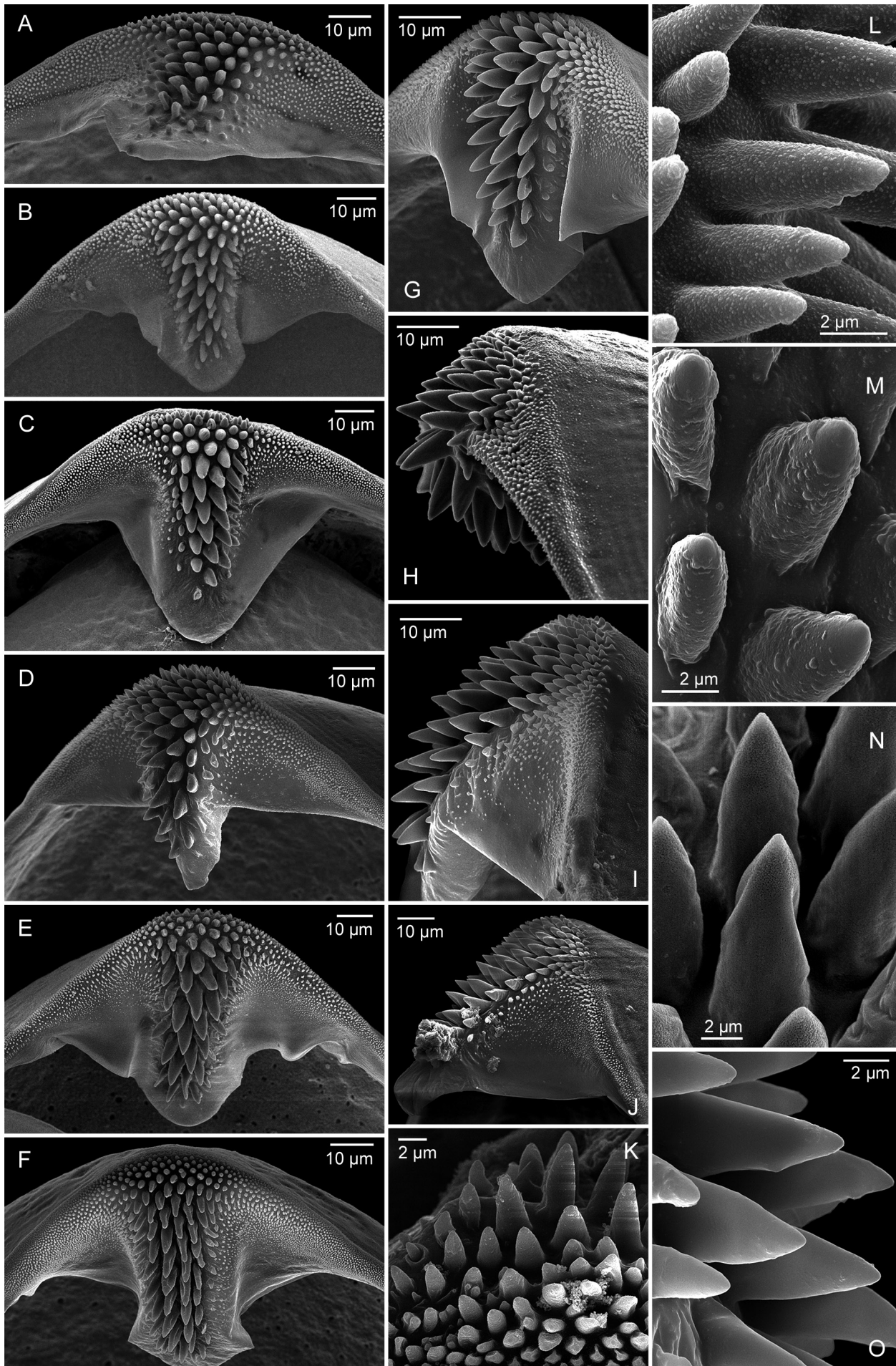
The photographs of glochidia were obtained on a Zeiss Merlin scanning electron microscope at the Biology and Genetic Engineering Center for Collective Use of the FSCEATB FEB RAS.

## Results

Mature glochidia from the investigated mussels had sub-triangular, moderately asymmetric shell shapes (Fig. 3), straight hinge lines and a large single styliform hook at the

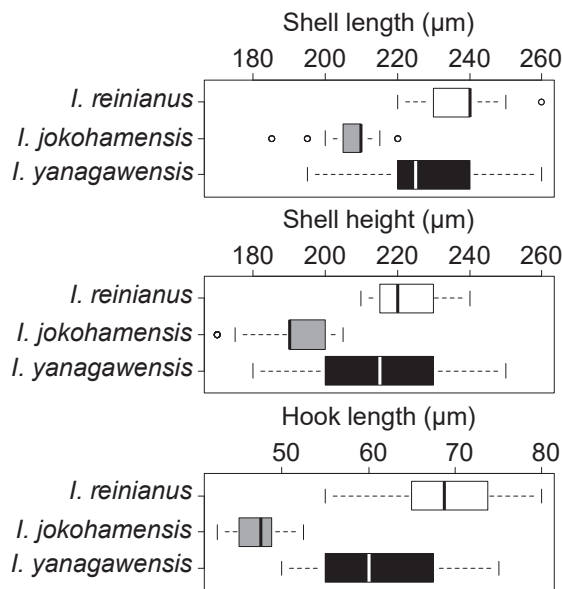
**Table 2.** Measurements of glochidia of *Inversiunio*: literature data. L = length of glochidial valve; H = height of glochidial valve; lig = hinge ligament length.

Species	Locality	H (µm)	L (µm)	lig (µm)	H/L	lig/L	Reference
<i>I. reinianus</i>	Lake Biwa, Shiga Pref., Honshu	229	239	181	0.96	0.76	INABA 1941, 1964
		240	245	185	0.98	0.76	
<i>I. reinianus</i>	Lake Biwa, Shiga Pref., Honshu	191	195	151	0.98	0.77	HIGASHI & HAYASHI 1964
		201	206	181	0.98	0.88	
		241	245	188	0.98	0.77	
<i>I. reinianus</i>	Lake Biwa, Shiga Pref., Honshu	231	235	–	0.98	–	KONDO 1998
<i>I. jokohamensis</i>	River at Karasue, Gifu Pref., Honshu	185	222	166	0.83	0.75	INABA 1941, 1964
<i>I. jokohamensis</i>	Tai River, Hyogo Pref., Honshu	180	200	–	0.90	–	MASUDA 1997
<i>I. jokohamensis</i>	Creek at Hokusei town, Mie Pref., Honshu	196	219	–	0.90	–	KONDO 1998
<i>I. jokohamensis</i>	Morodo River, Chiba Pref., Honshu	176	191	–	0.92	–	KONDO 1998
<i>I. jokohamensis</i>	Creek at Ichikai town, Tochigi Pref., Honshu	174	197	–	0.88	–	KONDO 1998
<i>I. yanagawensis</i>	Futatsukawa Creek, Fukuoka Pref., Kyushu	214	229	182	0.93	0.79	KONDO 1982
<i>I. yanagawensis</i>	Gion Creek, Okayama Pref., Honshu	232	242	189	0.96	0.78	KONDO 1982
<i>I. yanagawensis</i>	Eno River, Hiroshima Pref., Honshu	209	235	–	0.89	–	NAITO 1986
<i>I. yanagawensis</i>	Creek at Yagi town, Kyoto Pref., Honshu	205	232	–	0.88	–	KONDO 1998
<i>I. yanagawensis</i>	Creek at Kinomoto town, Shiga Pref., Honshu	236	246	–	0.96	–	KONDO 1998
<i>I. verrucosus</i>	Seomjin River, Kwanchon-myeon, Imsil-gun, Jeollabuk-do, Korea	236	257	199	0.92	0.77	KONDO et al. 2007



**Table 3.** Measurements of glochidia of *Inversiunio* species (our data); minimum–maximum values and mean arithmetical value (in parentheses) are given. L = length of glochidial valve; H = height of glochidial valve; lig = hinge ligament length.

Species	Locality	H (µm)	L (µm)	lig (µm)	H (µm)	H/L	lig/L	h/H
<i>I. reinianus</i>	Northern Lake Biwa	215–240 (229)	220–260 (241)	180–190 (184)	65–80 (72)	0.92–0.98 (0.95)	0.69–0.78 (0.76)	0.28–0.34 (0.32)
<i>I. reinianus</i>	Lake Biwa at Okishima	210–220 (219)	220–240 (235)	175–195 (185)	55–72.5 (66)	0.88–0.98 (0.93)	0.75–0.83 (0.79)	0.29–0.32 (0.30)
<i>I. jokohamensis</i>	Nagara river basin	170–200 (191)	185–215 (208)	150–180 (168)	42.5–52.5 (47)	0.85–0.92 (0.90)	0.76–0.86 (0.81)	0.22–0.26 (0.24)
<i>I. yanagawensis</i>	Gion Creek	210–240 (225)	225–245 (236)	180–195 (184)	55–75 (68)	0.93–0.98 (0.95)	0.77–0.79 (0.78)	0.26–0.33 (0.31)
<i>I. yanagawensis</i>	Gouno river basin	190–240 (229)	230–250 (244)	170–200 (189)	55–75 (67)	0.79–0.98 (0.92)	0.74–0.80 (0.77)	0.26–0.31 (0.29)
<i>I. yanagawensis</i>	Sendai River	190–215 (203)	195–230 (220)	160–190 (178)	50–60 (54)	0.82–0.96 (0.92)	0.78–0.86 (0.81)	0.25–0.30 (0.27)
<i>I. yanagawensis</i>	Yabe river basin	185–210 (195)	195–220 (207)	150–180 (159)	50–55 (53)	0.91–0.96 (0.94)	0.75–0.82 (0.77)	0.25–0.30 (0.27)



**Figure 5.** Boxplots of glochidia shell measurements among *Inversiunio* species. Thick vertical line is the median; box shows the interquartile range; whiskers extend to 1.5× interquartile range; circles are outliers.

ventral tip of each valve. The hooks are covered by lanceolate microstylets (Fig. 4). The larval thread is present.

The glochidial valves of all investigated *Inversiunio* were longitudinally extended; that is, their height was always less than their length (Tables 2, 3; Fig. 5). Mussels of *I. jokohamensis* from the Nagara river basin had the smallest glochidia, with glochidia height always less than 200 µm. Specimens of *I. yanagawensis* from Gion Creek had the largest glochidia, with glochidia height and length always more than 200 µm (Table 3).

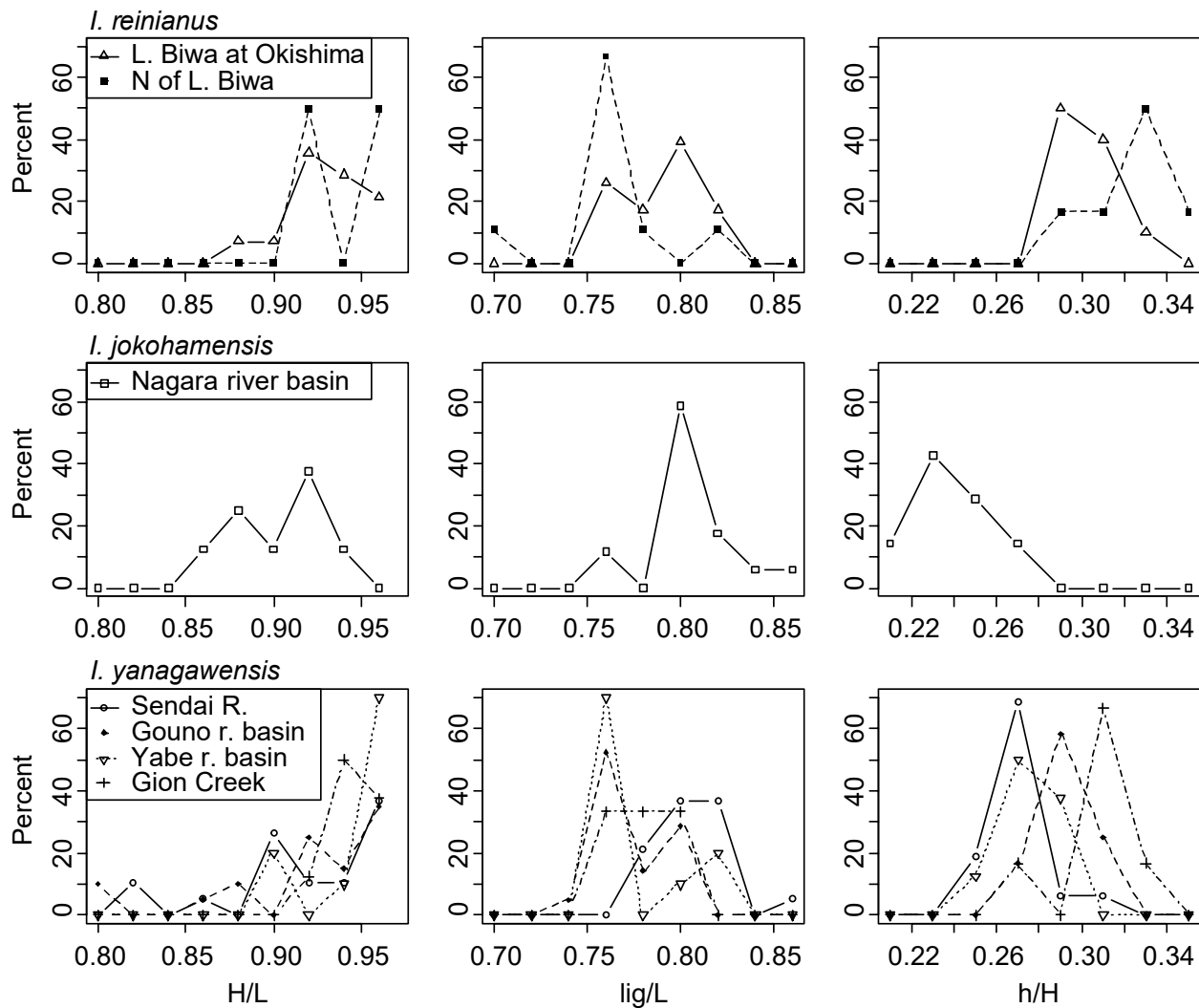
The absolute sizes of glochidial hooks differed greatly,

ranging from 42 to 80 µm in the studied species (Table 3; Fig. 5), although the relative sizes were approximately the same, not exceeding a third (22–34%) of the valve height (Table 3; Fig. 4).

Distributions of each morphological index wholly or partly overlapped among species (Fig. 6). Ranges of each index value between two species exhibited 40.7–55.2% overlap for H/L, 42.5–86.3% overlap for lig/L, and 0.0–48.0% overlap for h/H. The numbers of data in the overlap ranges were accounted for between 39.7% and 91.6% for H/L, 85.7% and 97.8% for lig/L, and 0.0% and 53.4% for h/H. In the comparison of h/H between *I. reinianus* and *I. jokohamensis*, the ranges of h/H values did not overlap at all, revealing complete separation of the data (Fig. 6). As the results of comparisons of morphological indices between two species, all morphological indices showed no statistically significant differences and were regarded as ineffective parameters for species identification (Table 4).

Microsculpture of *Inversiunio* glochidia is slightly different at various locations on the exterior valve surface. Among the investigated species the following types of microsculpture were distinguished: 1) from tight vermiculate and vermiculate at the ligament and central part of the valve to tight looped at ventral valve tip for *I. yanagawensis* (three localities: Gion Creek in Okayama Pref., Sendai River in Kagoshima Pref., and Gouno river basin in Hiroshima Pref.), 2) from tight vermiculate over most of the valve to loose vermiculate at the valve rim for *I. reinianus* (two localities in Lake Biwa), 3) from sparse globular at the ligament to loose vermiculate in the central parts of the valve for *I. yanagawensis* (Yabe River in Fukuoka Pref.), and 4) beaded over the entire valve

◀ **Figure 4.** Styliform hooks (A–J) and macrospines (K–O) of glochidia. A, L. *I. jokohamensis* (Nagara river basin). B, G. *I. yanagawensis* (Sendai River). C, H. *I. yanagawensis* (Gouno river basin). D, F, I, M, O. *I. reinianus* (Lake Biwa at Okishima). E, J, N. *I. yanagawensis* (Gion Creek). K. *I. yanagawensis* (Yabe river basin).



**Figure 6.** Distributions of the three morphological indices, H/L, lig/L, and h/H, for the glochidia of *Inversiunio* species for respective localities.

**Table 4.** Results of generalized linear mixed models for comparing glochidial morphological indices between *Inversiunio* species.

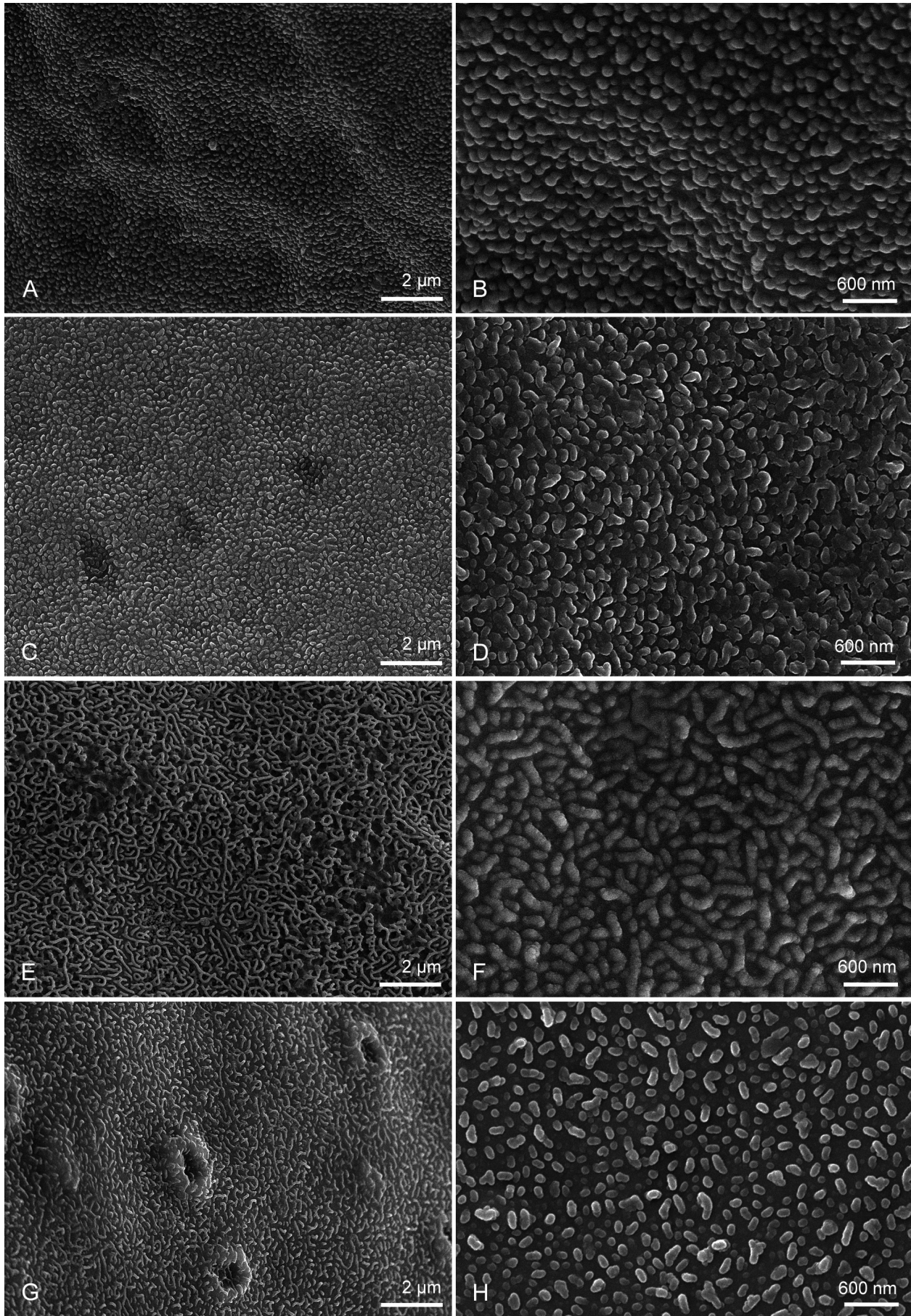
Response variable (species combination)	Fixed effect	Posterior mean	Lower 95% confidence limit	Upper 95% confidence limit	Effective sample size	MCMC $p$ -value
<i>I. reinianus</i> & <i>I. jokohamensis</i>	H/L	-575.6	-7560.9	5794.0	365	0.88
<i>I. reinianus</i> & <i>I. yanagawensis</i>		-164.6	-2345.7	1968.7	220	0.87
<i>I. jokohamensis</i> & <i>I. yanagawensis</i>		234.5	-1755.6	2811.5	234	0.86
<i>I. reinianus</i> & <i>I. jokohamensis</i>	lig/L	-91.3	-5475.4	5004.5	303	0.99
<i>I. reinianus</i> & <i>I. yanagawensis</i>		336.4	-2604.6	3724.7	144	0.90
<i>I. jokohamensis</i> & <i>I. yanagawensis</i>		-114.0	-3428.0	3122.0	176	0.95
<i>I. reinianus</i> & <i>I. jokohamensis</i>	h/H	-2.3	-58.0	52.7	1000	0.92
<i>I. reinianus</i> & <i>I. yanagawensis</i>		-921.4	-6201.8	3104.0	518	0.68
<i>I. jokohamensis</i> & <i>I. yanagawensis</i>		1871.3	-3293.8	7178.5	549	0.48

surface for *I. jokohamensis* (Nagara river basin in Gifu Pref.) (Fig. 7; Table 5).

Developing immature glochidia (at the stage with a shell already formed, but smaller than mature larvae and not yet exhibiting hooks) did not show marked microsculpture on the exterior valve surface.

## Discussion

In East Asia, two other genera of freshwater mussels have distributions that partly overlap with *Inversiunio*: *Nodularia* Conrad, 1853 and *Middendorffinaia* Moskvicheva & Starobogatov, 1973. The shell shapes and the positions



**Figure 7.** Exterior valve microsculpture. **A, B.** Beaded (*I. yokohamensis*, Nagara river basin). **C, D.** Tight vermiculate (*I. yanagawensis*, Sendai River). **E.** Tight looped (*I. yanagawensis*, Gion Creek). **F.** Vermiculate (*I. yanagawensis*, Gouno river basin). **G.** Loose vermiculate (*I. reinianus*, Lake Biwa at Okishima). **H.** Sparse globular (*I. yanagawensis*, Yabe River).

**Table 5.** Species-specific microsculpture on the external valve surfaces of *Inversiunio* glochidia.

Location on valve	<i>I. reinianus</i>	<i>I. jokohamensis</i>	<i>I. yanagawensis</i>	
	Lake Biwa	Nagara River	Gion Creek, Sendai River, Gouno river basin	Yabe River
Central part of ligament	Tight vermiculate	Beaded	Tight vermiculate, vermiculate	Sparse globular
Valve rim near ligament	Loose vermiculate	Beaded	Tight vermiculate, vermiculate	Tight vermiculate
Central part of valve	Tight vermiculate	Beaded	Tight vermiculate, vermiculate	Loose vermiculate
Ventral tip of valve	Tight vermiculate	Beaded	Tight looped	Vermiculate

of the posterior shell margin in these genera are quite variable (KONDO 2008, BOGATOV 2012, KLISHKO et al. 2018, 2019). Thus, it is very difficult to distinguish these three mussel genera from each other using only morphological characters, and, in some cases, it is impossible, especially if the geographical location of the collection is unknown. The validity of these three mussel genera and the relationship between *Nodularia* and *Inversiunio* were recently confirmed using molecular methods and techniques (SANO et al. 2017, KLISHKO et al. 2019, LOPES-LIMA et al. 2017, 2020). LOPES-LIMA et al. (2020) placed *Inversiunio*, together with the genera *Nodularia*, *Cuneopsis*, and *Schistodesmus*, in the tribe Nodulariini, while the genus *Middendorffinaia* was placed in a new tribe, Middendorffinaini Lopes-Lima, Bolotov & Bogan, 2020.

Glochidial shells, in addition to adult shells, exhibit remarkable size variation among habitats and often do not exhibit distinctive features necessary for distinguishing between species (SAYENKO & SHEDKO 2005, SAYENKO 2015a, 2016). Similarly, in the present study, all morphological indices among the glochidial characteristics were found to be not useful for identification of the investigated *Inversiunio* species.

In contrast, a multi-pronged approach that combines shell size variation with several glochidial morphological features can be diagnostic (Table 5). For example, the work of PIMPÃO et al. (2012) re-characterized some Amazonian Hyriidae based on comparisons of morphometric measurements and external morphology of larval shells. Similarly, glochidial characters including fine structure morphology have been shown to largely support proposed phylogenetic relationships based on genetic data (CHONG et al. 2008) and show the existence of two divergent clades among freshwater mussels of the genus *Anodonta* Lamarck, 1799 of the western United States and Canada (O'BRIEN et al. 2019).

Differences in the glochidial morphology separate the genus *Inversiunio* (with its subtriangular, hooked glochidia) from the genus *Inversidens* (with its semi-circular or semi-oval, unhooked glochidia) (KONDO 1982, 1998, 2008, HABA 1991a). Comparison of external glochidial valve sculpture can also be used as diagnostic feature at the generic level (PANHA & EONGPRAKORNKEAW 1995, HOGGARTH 1999, SAYENKO 2014, 2015a), as seen

in *Inversiunio*, *Middendorffinaia*, and *Nodularia*. Glochidia of *Middendorffinaia* show a beaded/globular microsculpture which is the most distinctive in comparison with the other two genera, and this fact confirms the separation of the genus into the tribe Middendorffinaini (LOPES-LIMA et al. 2020). In *Nodularia* the exterior valve microsculpture varies from loose- to tight-looped (SAYENKO & KHOLIN 2007, SAYENKO 2008, 2014, 2015b).

Genetic data showed that *I. yanagawensis* is paraphyletic, forming a clade together with *I. reinianus*, and that genetic distances between *I. yanagawensis* and *I. reinianus* are as low as the intraspecific genetic distances within *I. jokohamensis*. In other words, *I. jokohamensis* is marginally supported, while *I. yanagawensis* cannot be genetically well-distinguished from *I. reinianus* (SANO et al. 2017). The same results were received by LOPES-LIMA et al. (2020), with two main clades for *Inversiunio* species, where *I. jokohamensis* formed one clade and three species, with low genetic divergence, formed another clade.

Our data on external glochidial valve microsculpturing agree with the molecular data confirming the presence of three genera (*Inversiunio*, *Middendorffinaia*, and *Nodularia*) and emphasizing the taxonomic complexity of *Inversiunio*. Additional molecular analysis and data on glochidia morphology of all four species of *Inversiunio* from new localities are required to confirm the status of these taxa.

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