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Research Article

Taxonomic status of genera *Nodularia*, *Middendorffinaia* and *Inversiunio* (Bivalvia: Unionidae) from South-East Asia: morphometric, genetic and GenBank data

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Recent investigations of East Asian freshwater mussel genera *Nodularia*, *Middendorffinaia* and *Inversiunio* confirmed validity of the taxa, while their composition and taxonomic uncertainty concerning classification of these genera within a specific subfamily are still in question. The aim of the present study is to clarify the taxonomy and systematics of *Nodularia* and *Middendorffinaia* genera from the Russian Far East and *Inversiunio* from Japan, using a multiple dataset approach that combines morphology of glochidia, detailed conchological analyses with morphometry of adult and glochidial shells, and a molecular technique involving *COI* barcoding marker. As a result, three genetically distinct and highly divergent lineages were recognized confirming the existence of three distinct genera. The existence of five *Nodularia* species in Southeast Asia is shown. One of the sequences is shown to be incorrectly assigned to *N. jourdyi*, while in fact it also represents *N. douglasiae*, according to the BIN system. Existence of a single *Nodularia* species, namely *N. douglasiae*, and a single *Middendorffinaia* species, namely *M. mongolica*, in the Russian Far East is supported. We conclude that so-called comparatory species *Nodularia amurensis*, *N. schrencki*, and *N. vladivostokensis* are junior synonyms of the species *Nodularia douglasiae*; the comparatory species *Middendorffinaia ussuriensis* and *M. dulkeitiana* are junior synonyms of the species *Middendorffinaia mongolica*. Within *Inversiunio* specimens from Japan four species were recognized based on types of glochidial microsculpture of shells and genetic data. Gene sequences of *Inversiunio verrucosus*, endemic to the Seomjingang River basin in South Korea, and *I. yanagawensis* from Kyushu Island in Japan are very similar and they can be regarded as the same species, with *Inversiunio verrucosus* as a junior synonym of *Inversiunio yanagawensis*. Mussels from Honshu Island first referred to *I. yanagawensis* are shown to be a separate species.

Key words: comparatory species, gene *COI*, glochidia, *Inversiunio*, *Middendorffinaia*, *Nodularia*, shell morphometry

Introduction

Systematics of the Asian freshwater mussels abound with numerous questions and disputes. The system of unionids was originally based on conchological features, including shell shape and umbonal sculpture. Descriptions of new species from Russia and revisions of unionids were compared using the conchological characters such as the 'frontal contour' of a valve (the maximal convex section area of shells). This method, termed the Comparatory Method (CM), uses a single character to delimit bivalve species (Korniushin, 1998;

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Logvinenko & Starobogatov, 1971; Shikov & Zatravkin, 1991; Skarlato et al., 1990), and species described with the CM are called comparatory (Graf, 2007). As opposed to the CM, the Biological Method was recognized as more suitable by specialists with the argument that the comparatory taxa are simply more split than the lumped biological species (Graf, 2007; Korniushin, 1998). The history of the description, numerous redescriptions and revisions of Far Eastern mussels using CM clearly illustrates this situation.

Nodularia Conrad, 1853 was first described as a subgenus of *Unio* Philippson, 1788, with *Unio* (*Nodularia*) *douglasiae* Griffith & Pidgeon, 1833 as the type species, and raised to the genus level by Simpson (1900). In 1973 Moskvicheva and Starobogatov distinguished a new genus *Middendorffinaia* (type species *Unio mongolicus* Middendorff, 1851) from the Amur River basin and water bodies of Primorye in Russia. Description of this new genus was based on comparison of *Middendorffinaia* shells with the Japanese and Chinese bivalves only, while all differences with *Nodularia* from the Russian Far East were not discussed. Later Zatravkin and Bogatov (1987) focused on the position of the posterior end of the shells – below the shell mid-height or above the shell mid-height for *Nodularia* and *Middendorffinaia*, respectively, as well as differences of both the shell umbo sculpture and hinge teeth. Placement and composition of *Nodularia* and *Middendorffinaia* from the Far East of Russia varied considerably over the last centuries (Bogatov, 2000, 2012; Bogatov & Starobogatov, 1992; Moskvicheva, 1973; Moskvicheva & Starobogatov, 1973; Schrenck, 1867; Starobogatov et al., 2004; Westerlund, 1890, 1897; Zatravkin, 1983; Zatravkin & Bogatov, 1987; Zatravkin & Starobogatov, 1984; Zhadin, 1933, 1938, 1952). The situation of all taxonomic changes in the genus *Nodularia* was discussed in detail by Klishko et al. (2018). As a result, using the Comparatory Method, seven comparatory species of the genus *Nodularia* and eight comparatory species of the genus *Middendorffinaia* in eastern Russia were recognized (Bogatov, 2012; Starobogatov et al., 2004).

Graf (2007) considered the genus *Middendorffinaia* partly as *Unio crassus mongolicus* Middendorff, 1851 and partly as *Inversidens pantoensis* (Neumayr, 1899) although such separation was not discussed. The synonymy of *Middendorffinaia* species with the Chinese *Inversidens pantoensis* is not justified, because *Middendorffinaia* have hooked glochidia (Sayenko, 2015), while *Inversidens* Simpson, 1900 (at least the Japanese species of the genus) have unhooked glochidia (Kondo, 2008). Moreover, the shell of *Inversidens pantoensis* from China differs significantly from any species of *Middendorffinaia* (He & Zhuang, 2013). Later Graf and Cummings (2018) combined all of the comparatory species names within the subgenus *Middendorffinaia* (s.s.) as one species under genus *Nodularia*, i.e. *Nodularia mongolica*.

Lopes-Lima et al. (2017) based on two molecular markers (*COI* + 28*S*) consider *Nodularia* and *Inversiunio* Habe, 1991 as *incertae sedis* of the subfamily Unioninae, rejecting the proposal by Starobogatov and Zatravkin (published in: Zaytavkin & Bogatov, 1987) of the subfamily Nodulariinae with the two discussed Far Eastern genera, *Nodularia* and *Middendorffinaia*. The validity of the subfamily Nodulariinae and the validity of the genera *Nodularia* and *Middendorffinaia* were not recognized by many malacologists, who considered these two nominal genera to be junior synonyms of the genus *Unio*. However, based on conchological and anatomical analyses and molecular data (*COI*) it had been documented that there are no *Unio* species in the Russian Far East (Klishko et al., 2017, 2018), therefore it is incorrect to combine *Middendorffinaia* with *Unio crassus* and consider *Nodularia* and *Middendorffinaia* as a part of the genus *Unio.* Then, the existence of a single *Nodularia* species in the Russian Far East was shown, namely, *N. douglasiae* (Klishko et al., 2018). On the other hand, mussels assigned to *Middendorffinaia* and also collected from the Russian Far East are confirmed to be the only intraspecific forms of *M. mongolica* (Middendorff, 1851) (Klishko et al., 2019). In addition, genetic distances between *Middendorffinaia mongolica* and *Nodularia douglasiae* from the Russian Far East showed the presence of two independent species and genera (Klishko et al., 2019).

Sequence analyses of mitochondrial 16*S* ribosomal DNA showed that classification into two Japanese subspecies, *Nodularia douglasiae biwae* (Kobelt, 1879) and *N. d. nipponensis* (v. Martens, 1877), is not valid (Sano et al., 2017). Phylogenetic analysis of a fragment of the *COI* mitochondrial gene indicated that *N. douglasiae* inhabiting the middle and lower Yangtze River drainage are monophyletic with *N. douglasiae* from Japan, Russia, and South Korea (Liu et al., 2017).

Recently, based on morphological and molecular (*COI*, 16*S rRNA* and 28*S rRNA* gene fragments) data, it was demonstrated that the Asian genus *Oxynaia* Haas, 1911 is not monophyletic, and five species inhabiting Vietnam (including the type species *O. jourdyi* Morlet, 1886) belong to the genus *Nodularia* (Bolotov et al., 2018). A different view with the presence of two separate genera *Nodularia* and *Oxynaia* was presented but lacked any molecular data (Do et al., 2018).

Relatedness of the genera *Nodularia* and *Inversiunio* was recently shown in a phylogenetic analysis made by Sano et al. (2017). Difference in the glochidia morphology caused an isolation of *Inversiunio* (mussels with subtriangular hooked glochidia) from the genus *Inversidens* (semicircular or semi-oval unhooked glochidia) (Habe, 1991; Kondo, 1982, 1998, 2008). Later data on mitochondrial 16*S* ribosomal DNA sequences confirmed the validity of *Inversiunio* with high statistical support (Sano et al., 2017). As to the three *Inversiunio* species, separate status of *I. jokohamensis* (Ihering, 1893) was marginally supported, while *I. yanagawensis* (Kondo, 1982) could not be well-distinguished genetically from *I. reinianus* (Kobelt, 1879) (Sano et al., 2017).

Comparatory species *Nodularia lebedevi* Zatravkin & Starobogatov, 1984, known to inhabit the Lower Amur area, and all comparatory species of *Middendorffinaia* are listed in the Red Data Book of the Russian Federation (Russian Academy of Sciences (RAS), 2001) as endangered. Among 13 endangered Japanese unionoid species all species of the genus *Inversiunio* are also noted in the Red List of Shellfishes (Ministry of the Environment in Japan, 2019): *I. yanagawensis* and *I. reinianus* are marked as vulnerable species, and *I. jokohamensis* as a near-threatened species. While our paper was in review, another investigation has been published (Lopes-Lima et al., 2020) that deals with this same fauna and includes genetic data (*COI* and 28*S*) on the discussed three genera. However, for genera *Middendorffinaia* and *Nodularia* in Russia, this work refers to only published data. Effective conservation measures require an understanding of taxonomy and the recognition of different populations, so any investigation based on new molecular data from new localities and further biological characters, such as shell variability, glochidia morphology, are required.

Therefore, the aim of the present study is to investigate three Asian unionid mussel genera, notably *Nodularia* and *Middendorffinaia* from the Russian Far East and *Inversiunio* from Japan, using a multiple dataset approach. In detail, this study aims (i) to compare morphology of glochidia using both light and scanning electron microscopy, (ii) to prepare detailed conchological analyses with morphometry of adult and glochidial shells, (iii) to perform a genetic analysis using a molecular technique with *COI* barcoding marker, (iv) to establish the phylogenetic relationships of the discussed genera using a comprehensive approach based on all received data.

Materials and methods

Specimen sampling

In total, 606 dry shells from 108 localities (Table S1) and 31 preserved in ethanol specimens from 16 localities (Table S2) were used in the study. All glochidia samples are deposited at the Federal Scientific Centre of East Asia Terrestrial Biodiversity, the Far Eastern Branch of the Russian Academy of Sciences, Vladivostok (FSCEATB FEB RAS, hereafter). All samples of the soft parts used for DNA analysis are stored in the Institute of Biology, University of Szczecin, Poland.

Adult shells morphology and morphometry

To compare morphological characteristics among the three mussel genera, type material and the shell collections (Table S1) were gathered. Statistical analysis was carried out at different levels of the hierarchy, including river basins and species levels when molluscs of the same species from different river basins were compared, or when differences were observed between river basins reflecting the interspecific variation. In total, 128 shells of *Middendorffinaia* (with comparatory species *M. sujfunensis*–24 shells, *M. weliczkowskii*–67, *M. shadini*–10, *M. ussuriensis*–23, *M. mongolica*–4) from 15 localities, 128 shells of *Nodularia* (including comparatory species *N. amurensis*–71 shells, *N. middendorffi*–10, *N. schrencki*–44, *N. vladivostokensis*–3) from 13 localities, and 347 shells of *Inversiunio* (85 of *I. reinianus*, 202 of *I. jokohamensis*, 60 of *I. yanagawensis*) from 83 localities were used for the statistical analysis. Measurements of the type specimens of the comparatory species from literature (Bogatov, 2012; Moskvicheva, 1973; Moskvicheva & Starobogatov, 1973; Zatravkin & Bogatov, 1987) were also gathered. The main conchological characters of the shells such as length (L), height (H_{max} – maximal height, H_u – height at shell umbo), width (B, double value of a single valve was used if the other valve was destroyed) for each specimen were measured. In addition, the following character used in the key-books for the comparatory species identification of *Nodularia* and *Middendorffinaia* was analysed: length from the anterior shell edge to the umbo measured with a Vernier calliper parallel to the ventral valve edge (U_a) (Bogatov, 2012; Moskvicheva, 1973; Starobogatov et al., 2004). In order to use H_{max}, H_u, B and U_a as comparable morphological indices, these characters were normalized via dividing their values by the three reference lengths (L, H_{max} and H_u), thus forming six standardized morphological indices: H_{max}/L, H_u/L, B/L, U_a/L, B/H_{max}, and B/H_u.

Before making comparisons among genera, we examined the validity of the morphological indices for distinguishing between two mussel species with a sample size greater than two specimens. To assess the validity of the morphological indices with consideration of differences

in river basins that may reflect the intraspecific variation, generalized linear mixed models with binomial error distribution assumption and a logit-link function were employed. First, all possible pairs of mussel species were formed. As the response variable for designating a species, every pair of mussel species was categorized as either 0 or 1. Morphological indices were used as an explanatory variable and the codes of river basins were used as a random effect. When the river basins of sampling sites were the same in each of two species, random effect was not considered, and fixed effect generalized linear models were applied instead. Next, we fitted models using a Bayesian framework, running each of three Markov chains for 100,000 iterations, discarding 30,000 iterations burn-in period from each chain, and thinning the remaining iterations so that we retained 7,000 samples from the posterior distribution of the parameters. Convergence was assessed by ensuring that the scale reduction factor R-hat was < 1.1 (Gelman et al., 2014). 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 is valid to distinguish between two mussel species. A predictive model for mussel species identification based on a morphological index was made by a simple logistic regression model. Mussel species were identified using the model according to the following rule: when the value obtained from the predictive model is less than 0.5, the genus of the specimen is regarded as species 1, but when the value is equal to or more than 0.5, the genus of the specimen is regarded as species 2. Prediction accuracy for each predictive model was evaluated on the basis of the probability of correct identification. Statistical analysis was conducted using R version 3.5.0 (R Core Team, 2018).

Glochidia morphology

Glochidia were obtained from mussels by aeration or dissecting marsupia. In total, 18 samples of seven species collected from 11 localities were studied by scanning electron microscopy (SEM) (Table S2, see supplemental material online). As a preparation step prior to scanning, glochidia were cleaned from the soft tissues in a 5% KOH solution. To prevent destruction of the thin outer layer of the shells during this procedure, glochidia were examined every half an hour under a light microscope. The cleaned shells were washed several times in distilled water and a series of alcohols (80, 90, and 96%), after which they were mounted on a stub. Chromium was sputter coated immediately after drying the samples on a stub to exclude the possibility of deformation of the hooks and outer shell layer.

The microsculpture of each larval shell was examined at least at four points: closer to the ventral end (i.e., to the hook), at the centre of the valve (the adductor region), at the valve rim, and at the ligament. The description of the exterior valve microsculpture follows Panha and Eongprakornkeaw (1995) and Hoggarth (1999).

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

DNA extraction, amplification, and sequencing of gene fragments

For the genetic analyses, gill or foot tissue collected from specimens of the examined species and preserved in 96% ethanol were used. In total, six specimens belonging to two comparatory species of the genus *Middendorffinaia* (*M. ussuriensis* and *M. sujfunensis*), five specimens identified as comparatory species *Nodularia amurensis* and nine specimens of the genus *Inversiunio* (*I. jokohamensis*, *I. reinianus*, and *I. yanagawensis*) were analysed (Table S2, see supplemental material online).

Total genomic DNA was extracted with GeneMatrix Tissue DNA Purification Kit (EURx Ltd) according to manufacturer's procedure. Amplifications of 5′-end of the cytochrome oxidase subunit I mitochondrial gene (F-type *COI*) were performed, according to Promega (Madison, USA) procedure, in a reaction mixture composed of 0.25 units of Tag polymerase, 0.4 µM of each primer (Folmer et al., 1994), 200 µM of each dNTP, 2.0 mM $MgCl_2$ in a final volume of 10 µl. The PCR conditions were as follows: initial denaturation step of 2 min at 95 °C, followed by 5 cycles of 30 s at 95 °C (denaturation), 30 s at 45 °C (hybridization), 70 s at 72 °C (elongation), followed by 28 cycles of 30 s at 95 °C, 30 s at 55 °C, 60 s at 72 °C, and a final incubation of 5 min at 72 °C. After 1.5% agarose gel electrophoresis, the amplification products were viewed in UV light and purified using ExoSAP procedure (Werle et al., 1994) before direct sequencing was carried out by Macrogen, Seoul (South Korea, http://dna.macrogen.com/eng/).

Both DNA strands were sequenced and the obtained different haplotypes for analysed specimens and species were submitted to GenBank. Their accession numbers are shown in Table 1. Sequences with the same haplotypes were deposited only once.

The estimates of sequence divergence for pairwise distance (uncorrected *p*-distance) were assessed using MEGA 7.0 software (Kumar et al., 2016).

Table 1. List of all taxa and outgroups used and their GenBank accession numbers.

Taxon name	Locality	Voucher numbers/GenBank accession numbers (haplotypes)	Source
Ingroup Taxa			
Middendorffinaia mongolica (=*M. ussuriensis*)	Amur River basin, Russian Far East	430/MW139310 (H1), 428/MW139311 (H2)	This study
Middendorffinaia mongolica (=*M. sujfunensis*)	Gladkaya River, Russian Far East	431/MW139312 (H3)	This study
Middendorffinaia mongolica	Shilka River, Russian Far East	MH974547	Klishko et al., 2019
Middendorffinaia mongolica	Gladkaya River, Russian Far East	MH974548, MH974549, MH974550	Klishko et al., 2019
Middendorffinaia mongolica	Komarovka River, Russian Far East	MH974551	Klishko et al., 2019
Nodularia breviconcha (=*N. sinuolata*)	South Korea	GQ451864	Park et al., unpublished
Nodularia douglasiae (=*N. amurensis*)	Amur River basin, Russian Far East	432/MW139306 (h1), 433/MW139308 (h3), 435/MW139309 (h4)	This study
Nodularia douglasiae (=*N. amurensis*)	Khanka Lake basin, Russian Far East	450/MW139306 (h1), 449/MW139307 (h2)	This study
Nodularia douglasiae	Yangtze River basin, China	MG210495, MG210496, MG210498, MG210504, MG210509, MG210510, MG210516, MG210523, MG210524, MG210525, MG210526, MG210527, MG210528, MG210529, MG210530, MG210536, MG210546, MG210547, MG210558	Liu et al., 2017
Nodularia douglasiae	Asahi River, Japan	MF975688, MF975689	Klishko et al., 2018
Nodularia douglasiae	Amur River, Russian Far East	MF975692, MF975695, MF975696	Klishko et al., 2017
Nodularia douglasiae	Onon River, Russian Far East	MF975693, MF975694	Klishko et al., 2017
Nodularia douglasiae	Ussury River, Russian Far East	MF975697, MF975698	Klishko et al., 2017
Nodularia nipponensis	Abukuma River, Japan	MF975691	Klishko et al., 2018
Nodularia nuxpersicae	Song River, Vietnam	KX822654	Lopes-Lima et al., 2017
Nodularia jourdyi	Vietnam	MH248376	Bolotov et al., 2018
Inversiunio jokohamensis	Omono River basin, Japan	490/MW139316, 491/MW139317	This study
Inversiunio reinianus	Biwa Lake basin, Japan	478/MW139313, 485/MW139314, 487/MW139315	This study
Inversiunio cf. *yanagawensis* 1	Sendai River, Japan	483/MW139318	This study
Inversiunio cf. *yanagawensis* 1	Yabe River basin, Japan	484/MW139319	This study
Inversiunio cf. *yanagawensis* 2	Gouno River, Japan	479/MW139320, 480/MW139321	This study
Inversiunio yanagawensis	Japan	MT020654	Lopes-Lima et al., 2020
Inversiunio yanagawensis	Gion Creek, Japan	LC518986	Sano et al., 2020
Inversiunio verrucosus	South Korea	MT020660	Lopes-Lima et al., 2020
Outgroup Taxa			
Unio crassus	Poland	AF514296	Soroka, 2010
Unio pictorum	Poland	AF468684	Soroka, 2010

Before the phylogenetic analysis, the best-fit substitution model was fitted for every set of sequences independently, using the algorithm implemented in MEGA 7.0: Tamura–Nei with discrete Gamma distributed evolutionary rates among sites with five rate categories (+G) for *COI* alignment for *Nodularia*; Hasegawa–Kishino–Yano model (HKY, Hasegawa et al., 1985) for *COI* alignment for *Inversiunio*; and the Tamura–Nei model assuming that a certain fraction of sites are evolutionarily invariable (+I) for all received sequences of three genera plus GenBank data and *Unio crassus*, *U. pictorum* used as outgroup.

The Maximum likelihood trees were tested by bootstrap analysis with 1,000 replicates (Felsenstein, 1985) and the percentage of trees in which the associated taxa clustered together are shown next to the branches. Trees are drawn to scale, with branch lengths measured in the number of substitutions per site. All evolutionary analyses were conducted in MEGA 7.0 (Kumar et al., 2016).

Furthermore, all sequences of *Nodularia* and *Inversiunio* (own data and from GenBank) were tested to determine the recently proposed Barcode Index Number (BIN) system (Ratnasingham & Hebert, 2013). This system clusters sequences into Molecular Operational Taxonomic Units (MOTUs), regardless of their previous taxonomic assignment. BIN assignments are based on sequence divergence. Ultimately, the BIN system provides tried and tested means of checking the concordance between the morpho-taxonomic determination of species and *COI* sequence data. MOTUs clustering was performed with 97% threshold identity. Next, cluster representative sequences (MOTUs) were annotated according to BOLD Systems *COI* database and Species Level Barcode Records. Clustering and annotation results are presented in Figs S4–S5. Not all sequences of *Nodularia* from GenBank received a BIN assignment (Fig. S4, the group has been annotated to 'n/n', no number), although the BOLD database (http://v3.boldsystems.org/index.php/Public_BarcodeIndexNumber_Home) contains 11 MOTUs of 105 published records of *Nodularia douglasiae* specimens from four countries (China, Russia, South Korea and Japan). Because there was no match in the BOLD database for the *COI* sequences of *Inversiunio*, the MOTUs numbers were used for further visualizations (Fig. S5).

Results

Adult shells analysis

The shell shape of investigated mussels and the position of the posterior shell margin are quite variable. Beak and shell sculpture of adult *Inversiunio*, arranged in staggered rows and often covering most of the shell surfaces, resembles *Middendorffinaia* rather than *Nodularia*, as beak sculpture of the last genus is clearly visible only in young animals, but weakly marked or absent in adults (Figs 1, 2).

Distributions of each morphological index were wholly or partly overlapped in many pairs of intergeneric species (Figs S1–S2). On the other hand, the distribution ranges were entirely separated in 19 of 266 pairs of intergeneric species. More pairs exhibiting the entire separation were in the distributions of H_{max}/L (seven pairs, i.e., between *N. middendorffi* or *N. schrencki* and three *Inversiunio* species, and between *M. sujfunensis* and *I. reinianus*) (Fig. S1) and B/H_{max} (four pairs, i.e., between *N. middendorffi* or *N. schrencki* and *M. mongolica* or *I. yanagawensis*) (Fig. S2). The ranges of each index value between intergeneric species exhibited 0.0–92.6% overlap. The number of data in the overlap ranges accounted for between 0.0–99.1%.

As the result of comparisons of morphological indices between inter- and intrageneric species, for the 40 of 374 pairs of mussel species, the morphological index was found to be a valid parameter for species identification. Thirty-four of the 40 pairs exhibited morphological differences between intergeneric species. Particularly, H_{max}/L and B/H_{max} were found to represent valid indices for species identification in a comparatively large number of pairs of intergeneric species (14 pairs for H_{max}/L and 9 pairs for B/H_{max}).

Percentages of valid differences of morphological index values between inter- and intrageneric species are shown in Fig. S3. Higher percentages of morphological differences between intergeneric species were found in H_{max}/L and B/H_{max}. H_{max}/L values were significantly different in 53% of intergeneric species pairs between *Middendorffinaia* and *Inversiunio* and 44% of intergeneric species pairs between *Nodularia* and *Inversiunio*. B/H_{max} values were significantly different in 44% of interspecific species pairs between *Nodularia* and *Inversiunio*. Meanwhile, percentages of differences for all morphological indices between *Middendorffinaia* and *Nodularia* were equal to or less than 20%.

When results of all statistical analyses between species were integrated, percentages of valid differences of morphological indices between intergeneric species were the highest between *Nodularia* and *Inversiunio* (20%) and the lowest between *Middendorffinaia* and *Nodularia* (8%) (Fig. 3). These percentages were less than the highest percentage of the morphological difference between intrageneric species in *Inversiunio* (22%).

Comparatory species of *Nodularia* and *Middendorffinaia* (analysis of both the type material and collections was made) are poorly identified with a logistic regression model due to absence of clear interspecific heterogeneity (Fig. 3, Figs S1–S3).

Glochidia analysis

Glochidia from the investigated mussels are subtriangular, with moderately asymmetric shape and large styliform hooks covered by lanceolate microstylets. Size of glochidia of all three genera as well as the morphological indices are wholly or partly overlapped, and it is impossible to differentiate the taxa from each other by conchological features. Among the investigated genera, mussels of *Inversiunio jokohamensis* from Nagara River basin had the smallest glochidia (height

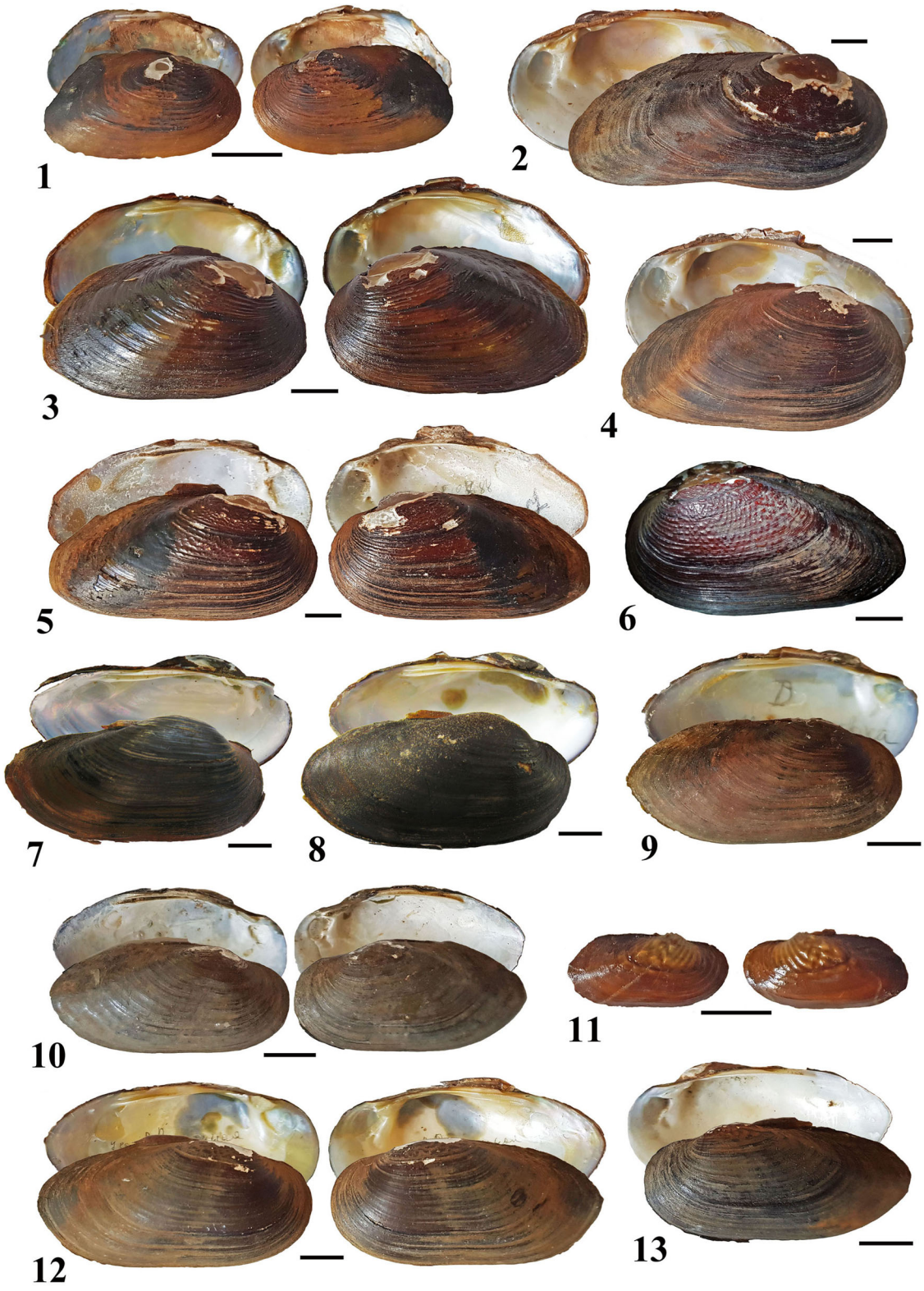

Fig. 1. Shell shape of the comparatory species of *Middendorffinaia* (**1.1–1.6**) and *Nodularia* (1.**8**–1.**13**) from eastern Russia: **1.1** – *M. mongolica* (Kukhtui River), **1.2** – *M. shadini* (Partizanskaya River), **1.3** – *M. sujfunensis* (Gladkaya River), **1.4** – *M. weliczkowskii* (Razdolnaya River), **1.5** – *M. sujfunensis* (Bolotnaya River), **1.6** – *M. dulkeitiana* (Solyonoe Lake), **1.7** – *N. amurensis* (Amur River), **1.8** – *N. schrencki* (Amur River), **1.9** – *N. schrencki* (Melgunovka River), **1.10** – *N. schrencki* (Astrakhanka River), **1.11** – juvenile *N. amurensis* (Khanka Lake basin), **1.12** – *N. vladivostokensis* (Razdolnaya River), **1.13** – *N. amurensis* (Spasovka River). Scale bar 1 cm.

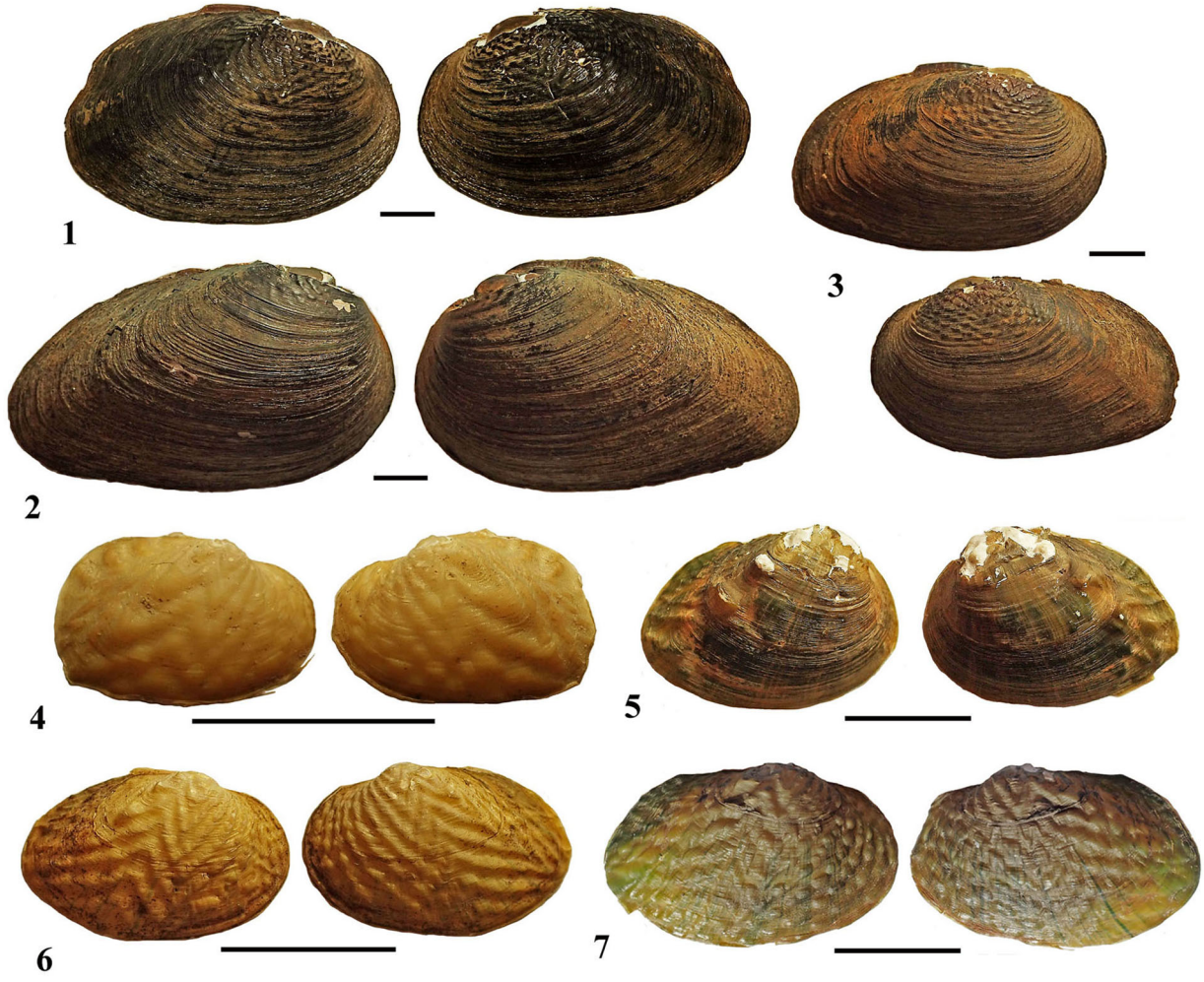

Fig. 2. Shell shape of adult (**2.1**–**2.3**) and juvenile (**2.4**–**2.7**) *Inversiunio* from Japan: **2.1** – *I. yanagawensis* (channel of Yodo River, Kyoto Prefecture), **2.2**, **2.5** – *I. reinianus* (Biwa Lake, Shiga Prefecture), **2.3** – *I. jokohamensis* (Inawashiro Lake, Fukushima Prefecture), **2.4** – *I. yanagawensis* (Yafusa River, Kagoshima Prefecture), **2.6** – *I. jokohamensis* (tributary of Koujiro River, Toyama Prefecture), **2.7** – *I. jokohamensis* (channel of Shadai River basin, Hokkaido Prefecture). Scale bar 1 cm.

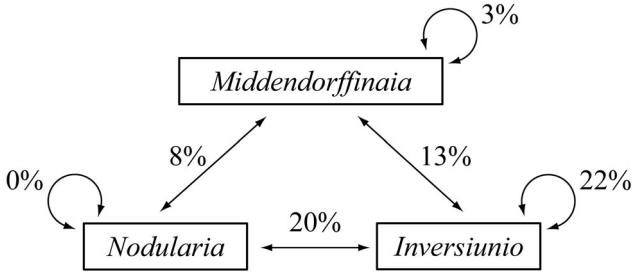

Fig. 3. Integrated percentages of valid differences of six morphological indices (H_{max}/L, H_u/L, B/L, U_a/L, B/H_{max} and B/H_u) between inter- and intrageneric species.

166.7–172.7 µm, length 183.3–206.3 µm) and bivalves of *I. yanagawensis* from Gion Creek had the largest glochidia (height 212.9–240 µm, length 225.9–247.5 µm). The valves are longitudinally extended, i.e. their height is always less than the length. Hooks 42–59 µm in length, or 18–35% of the valve height.

The distinct differences between the discussed groups of molluscs are supported by the data on the microsculpture of the outer surface of glochidial valves (Figs 4, 5). Glochidia of *Middendorffinaia* have the same beaded/globular microsculpture across the entire exterior surface of the valves (Fig. 4). Unlike glochidia of *Middendorffinaia*, the microsculpture of *Nodularia* and *Inversiunio* glochidia is slightly different at different points on the exterior valve surface. For *Nodularia*, microsculpture characteristics of exterior valve ranged from loose looped to tight looped (Fig. 4). The most complicated situation was found among investigated taxa of *Inversiunio*, where four types of microsculpture were distinguished: tight looped for *I. yanagawensis* (two localities – Gion Creek in Okayama Pref., and Gouno River

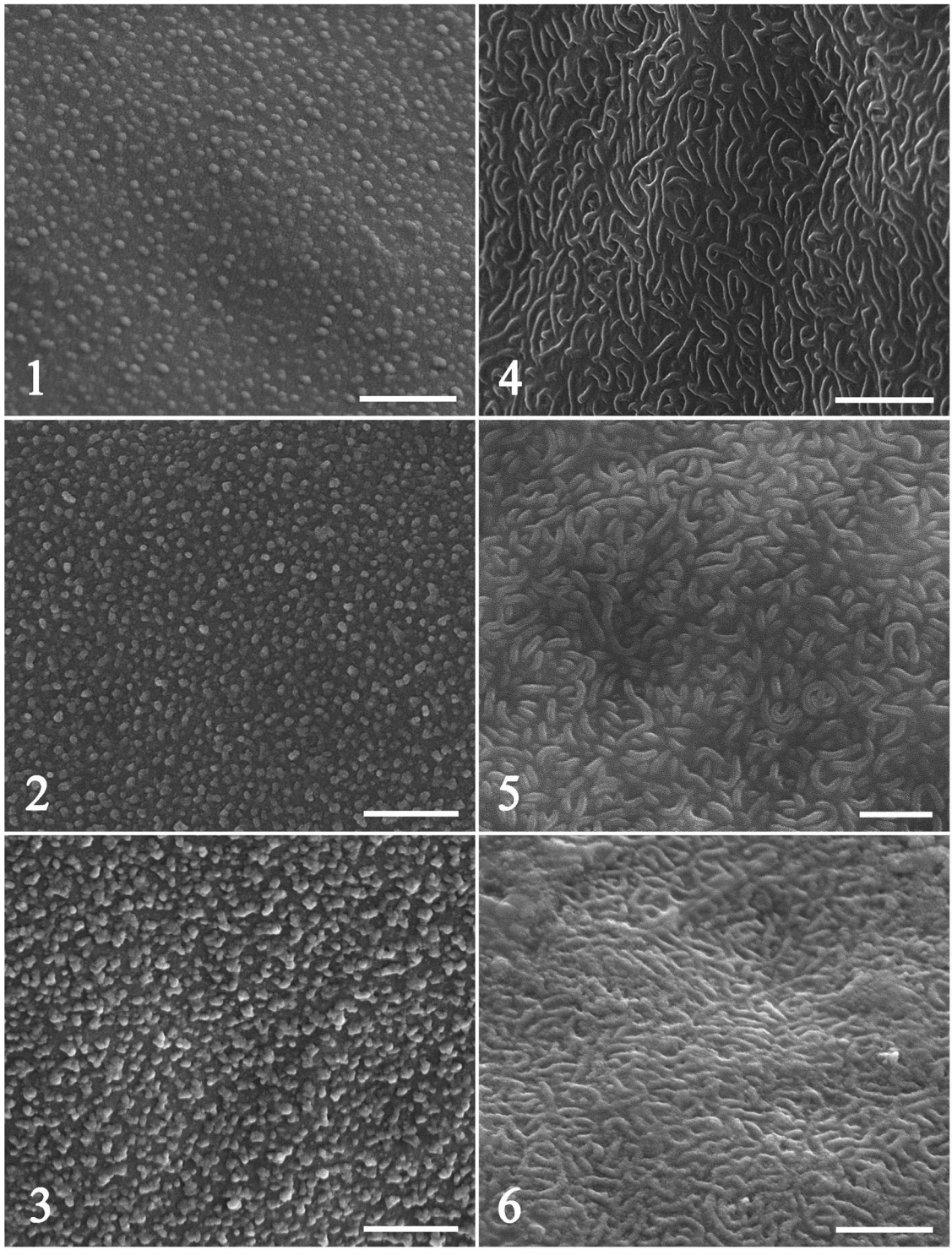

Fig. 4. Exterior valve microsculpture of *Middendorffinaia* and *Nodularia* glochidia (comparatory species names are used): **4.1** – *M. sujfunensis* from Artemovka River (beaded sculpture in central part of the valve); **4.2** – *M. dulkeitiana* from Solyonoe Lake (beaded sculpture in central part of the valve); **4.3** – *M. dulkeitiana* from Solyonoe Lake (globular sculpture near ligament); **4.4** – *N. amurensis* from Razdolnaya River (loose looped sculpture near ligament); **4.5** – *N. amurensis* from Razdolnaya River (tight looped sculpture in central part of the valve); **4.6** – *N. schrencki* from Ilistaya River basin (tight looped sculpture near ventral valve corner). Scale bar 1 μm.

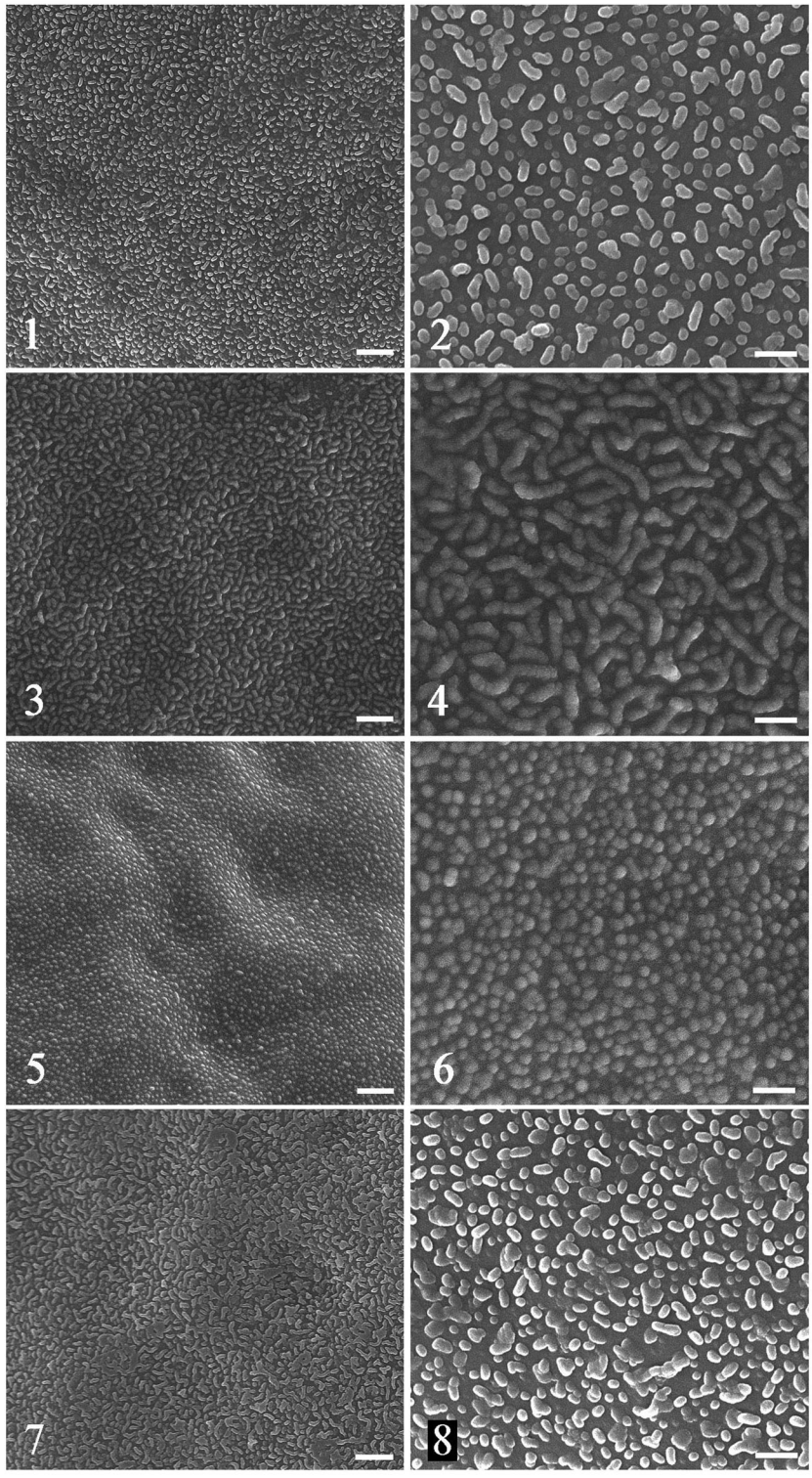

Fig. 5. Exterior valve microsculpture of *Inversiunio* glochidia: **5.1**, **5.2** – *I. yanagawensis* from Yabe River basin (sparse globular sculpture in central part of the valve); **5.3**, **5.4** – *I. yanagawensis* from Gouno River basin (tight looped sculpture in central part of the valve); **5.5**, **5.6** – *I. jokohamensis* from Nagara River basin (beaded sculpture in central part of the valve); **5.7**, **5.8** – *I. reinianus* from Biwa Lake at Okishima (loose vermiculate sculpture in central part of the valve and sparse globular sculpture near ligament, respectively). Scale bars 1 μm (**5.1**, **5.3**, **5.5**, **5.7**) and 1 nm (**5.2**, **5.4**, **5.6**, **5.8**).

Table 2. Average sequence diversity (*p*-distance) of *Nodularia jourdyi* (MH248376) with other *Nodularia douglasiae* species grouped as follows: Group 1 – our four haplotypes from South-East Asia; Group 2 – seven sequences from Russia (RUS) from Fig. S4; Group 3 – two sequences from Japan (JPN) from Fig. S4; Group 4 – three sequences with GenBank accession numbers MG210516, MG210527, MG210536 from Yangtze River, China (CHN), and *N. nipponensis* (MF975691). Average *p*-distances within groups are given in the diagonal of the table.

	N. jourdyi	**Group 1**	**Group 2**	**Group 3**	**Group 4**	***N. nipponensis***
N. jourdyi	–					
Group 1	0.017	**0.019**				
Group 2	0.013	0.018	**0.011**			
Group 3	0.010	0.013	0.010	**0.002**		
Group 4	0.074	0.076	0.081	0.080	**0.001**	
N. nipponensis	0.048	0.047	0.050	0.051	0.067	–

basin in Hiroshima Pref.), from sparse globular to tight vermiculate (two localities – Sendai River in Kagoshima Pref. and Yabe River in Fukuoka Pref.), from sparse globular to loose vermiculate for *I. reinianus* (two localities of Biwa Lake), and beaded for *I. jokohamensis* (Nagara River basin in Gifu Pref.) (Fig. 5).

Molecular genetic analysis and taxonomy

Among analysed specimens of *Middendorffinaia ussuriensis* and *M. sujfunensis*, three different haplotypes were identified (Table 1), showing a small variation between them (0.3%). Such a low level of genetic diversity suggests that these sequences belong to one species. The most frequent haplotype (H1) appeared four times in Arsenievka River and the other two, H2 and H3, were unique and occurred only once in Arsenievka and Gladkaya Rivers, respectively. All *COI* sequences had a total length of 644 bp and 3 polymorphic sites in the third position in the codon with a lack of amino acid substitutions. The mean divergence between the obtained sequences of *Middendorffinaia* mussels from two different sites (Arsenievka and Gladkaya Rivers) was 0.2%.

The results were compared with GenBank sequences of five *Middendorffinaia mongolica* (accession numbers MH974547–51) from three rivers in the Russian Far East (Shilka, Gladkaya, and Komarovka Rivers) (Klishko et al., 2019). These data represent three haplotypes: the most frequent is identical to our haplotype H3, and two unique ones are called here H4 and H5. Each of these haplotypes is characteristic of one locality where the specimens were collected: H3 for Gladkaya River, H4 for Komarovka River, and H5 for Shilka River. Analysis of all five genetically different haplotypes (H1–H5) showed sex polymorphic sites with one amino acid substitution; the mean divergence between them was 0.4% and this value fluctuated from 0.2 to 0.9%.

Specimens of *M. mongolica* collected from four Russian rivers represented five *COI* haplotypes: H1 and H2 from Arsenievka River are the most genetically similar (one polymorphic site), H3 from Gladkaya River, H4 from Komarovka River, and H5 from Shilka River is the most divergent one (compared with H3 and H4).

Based on the fragment of *COI* gene for *Nodularia* specimens collected from two sites (Arsenievka River and Khanka Lake) four haplotypes with an average length of 644 bp were distinguished (Table 1). The haplotypes show high genetic diversity (a level of genetic variation from 0.3 to 2.8%, with an average value 1.8%, Table 2) where each sequence represents a separate haplotype (h1–h4) and geographic site with the exception of h1, which appeared in both localities. Haplotypes differed at 22 polymorphic sites, with silent mutations in the third position of the codon, which does not change the encoded amino acids. The haplotype h4 (from Arsenievka River) was associated with the greatest diversity (it constitutes a separate MOTU in Fig. S4) and, with its exclusion, the above parameters become as follows: intraspecific variation from 0.3–1.2%, mean value 0.8% and eight polymorphic sites.

For the genera *Nodularia* there are many sequences for *COI* gene in GenBank described as *N. douglasiae* (from Russia, China, Japan, and South Korea), *N. sinuolata* Martens, 1905 (GQ451864, from South Korea), *N. nipponensis* (Martens, 1877) (MF975691, from Japan), *N. nuxpersicae* (Dunker, 1848) (KX822654, from Vietnam), and *N. jourdyi* (MH248376, from Vietnam). *Nodularia nipponensis* and *N. sinuolata* are considered as separate species and in the light of recent scientific studies the name *N. sinuolata* is invalid and the replacement name *Nodularia breviconcha* Lee, Kim, Bogan & Kondo, 2020 has been given (Lopes-Lima et al., 2020). However, *N. douglasiae* shows high genetic diversity, significant genetic differentiation and could be considered either as a single species or as multiple cryptic species (Klishko et al., 2017; Liu et al., 2017; Lopes-Lima et al., 2020). Our *COI* phylogenetic tree confirms that *N. breviconcha* (former *N. sinuolata*), *N. nipponensis* and *N. nuxpersicae* are valid taxa, constituting different clades and MOTUs. The tree also shows that the variation of three sequences from the Yangtze River (MG210527, MG210516, MG210536) forming separated MOTU is above 3% (Fig. S4). The

Table 3. Range of genetic *p*-distances for *COI* sequences for *Inversiunio* species divided into groups according to Fig. S5 (Gp 1–4) (the bold values indicate mean values). Data for *I. verrucosus* was obtained from GenBank (accession number MT020660).

Comparison	*COI* (%)
Within *I. jokohamensis* (Gp 4)	0.0
Within *I. reinianus* (Gp 1)	0.6–0.7 (**0.6**)
Within *I. yanagawensis* (Gp 2 and Gp 3)	0.0–3.1 (**1.6**)
I. yanagawensis, Gp 2 vs. *I. yanagawensis*, Gp 3	**2.5**
I. jokohamensis vs. *I. reinianus*	**8.9**
I. jokohamensis vs. *I. yanagawensis*, Gp 2	**8.0**
I. jokohamensis vs. *I. yanagawensis*, Gp 3	**6.8**
I. jokohamensis vs. *I. verrucosus*	**6.8**
I. reinianus vs. *I yanagawensis*, Gp 2	**1.8**
I. reinianus vs. *I yanagawensis*, Gp 3	**3.4**
I. reinianus vs. *I. verrucosus*	**3.0**
I. verrucosus vs. *I yanagawensis*, Gp 2	**2.0**
I. verrucosus vs. *I yanagawensis*, Gp 3	**0.5**

designation of these sequences from the Yangtze River as *N. douglasiae* is most likely erroneous.

The three haplotypes that we obtained formed a single large clade with *N. douglasiae* from Russia, China (Yangtze River), Japan and South Korea and with *N. jourdyi* from Vietnam, divided into four MOTUs (Fig. S4). The mean genetic diversity of this largest group amounts to 1.6%, with a wide range of between 0.0 and 4.0%. The most divergent sequences are MG210530 and MG210498, both of which come from the Yangtze River, China (data not shown), but only the former belongs to a separate MOTU (ADR9194, Fig. S4). Our haplotype h4 from the Arsenievka River (Amur River basin) forms a freestanding group (MOTU) with differentiation ranging from 2.4% (for MOTU ACH2241) to 4.3% (for BIN ADR9194) and higher for other species with MOTU ADJ6275 (6.9%), which are identified in the GenBank database as *N. douglasiae*.

Specimens representing three species of the genus *Inversiunio* from five localities in Japan were sequenced (Table 1), and four genetically different groups were distinguished that match the MOTUs (Fig. S5). The intraspecific variation, expressed as *p*-distances, was not detected for *I. jokohamensis* and was low for *I. reinianus* (0.6%), where the five polymorphic sites had neutral characteristics. Regarding the four sequences initially classified as *I. yanagawensis* from three localities, very high genetic differentiation (up to 3.1%, with the mean value 1.6%) was observed without variability for single localities (Table 3). Such high variability is due to the presence of 20 polymorphic sites that do not cause amino acid substitutions except one (TAT/AAT and Y/N). Both amino acids (tyrosine and asparagine) are polar. Among all three species of *Inversiunio*, *I. yanagawensis* is characterized by the greatest genetic diversity (up to 3.1%) divided into two groups with no variation within them (Table 3). It is necessary to point out that four types of microsculpture of *Inversiunio* glochidial shells correspond to four genetic groups of this genus, confirming the possibility of using the differences in glochidial microsculpture as an additional systematic feature.

BLAST analysis, available in the National Centre for Biotechnology Information, reveals that two specimens of *I. yanagawensis* from Fukuoka and Kagoshima Prefectures in Kyushu Island, Japan, have more than 99% identity with *I. verrucosus* from South Korea. On the other hand, BLAST analysis confirms 100% similarity of *I. yanagawensis* from Hiroshima Prefecture, Honshu Island (our data) to *I. yanagawensis* from GenBank (accession numbers MT020654 and LC518986).

The phylogenetic relations between all analysed genera are presented in Fig. 6. To maintain the clarity of the tree, the genus *Nodularia* is represented by a smaller number of taxa than in Fig. S4 maintaining the main topology within this clade. All analysed genera formed clades with high statistical support. *Nodularia* and *Middendorffinaia* are phylogenetically more similar to each other than to *Inversiunio*. The most diverse clade corresponds to the genus *Nodularia*, which most likely contains five, or even more species based on the BIN system. Clade *Middendorffinaia* is the most homogeneous and represents only one species, *M. mongolica* (Fig. 6).

Discussion

Taxonomy of unionids presents many challenges due to high variability of the shell shape (Graf, 2007). Variation of shell morphology can be explained based on genetic factors and/or environmental influences, and the interaction between them (Aldridge, 1999). An additional factor affecting the mussel shell shape is the lifestyle of Unionidae, when females store very large numbers of developing larvae (glochidia) in the gills, transformed into structures called marsupia (some use all four gills as marsupia, some use only the outer gills, other taxa use only the ventral portion of the outer gills, or in some genera the females use a restricted portion of the outer gills as the marsupia). The comparatory method (CM), proposed by Logvinenko and Starobogatov (1971) and based on the description and comparison of the contours of the frontal section of the shell valves of bivalve molluscs, does not explain the influence of different factors (genetic, environment, or sex) on the shell shape. As a result, the CM and its modifications (Bogatov, 2014, 2015; Shikov & Zatravkin, 1991) have been repeatedly criticized

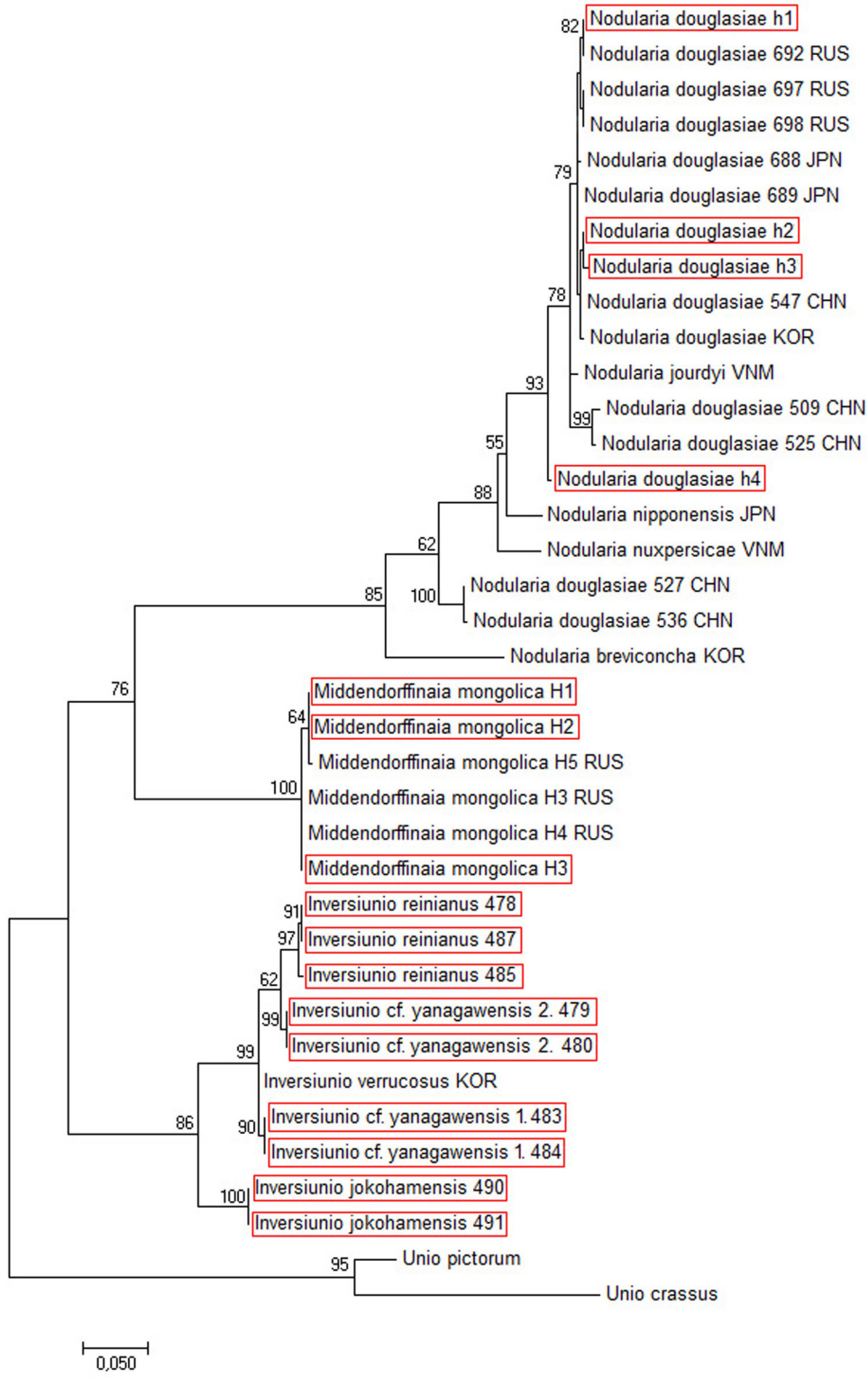

Fig. 6. Molecular phylogenetic analysis by Maximum likelihood method for three analysed genera using the Tamura–Nei model. The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 69.71% sites). The values above the branches are bootstrap support. To distinguish the different sequences from one country, the last three digits of GenBank accession numbers are used. Newly analysed sequences are marked by red. Country abbreviations are given in accordance with the ISO codes.

(Bolotov et al., 2013; Graf, 2007; Voroshilova, 2013; Zieritz & Aldridge, 2009, 2011). Often a specific factor associated with the change in shell shape could not be identified, but such differences in shape could induce species misidentification.

The implicit assumption that mussels have a large range of phenotypic variability of morphological traits has changed the approach to mussel taxonomy and reduced the number of species, but the ubiquity of certain shapes across taxa, as we observed with the investigated mussel taxa, has hindered the task of correct species determination. Our data prove that no single morphological trait can be used for species determination. Moreover, it is very difficult to distinguish *Nodularia* from *Middendorffinaia* based only on morphological parameters.

Previous investigations of the shell morphometric variability of *Middendorffinaia* showed the heterogeneity of the comparatory species (Sayenko & Kholin, 2007a). The analysis of morphological characters of seven comparatory species of *Nodularia* from the Middle Amur River (Russian Far East) made by Klishko (2008) showed a significant overlap of the morphological characteristics among the studied populations. Klishko et al. (2019) also demonstrated the inadequacy of using contours of the frontal section of shell valves for distinguishing between *Middendorffinaia* and *Nodularia*.

Shell sculpture of freshwater mussels has long been used for species identification. Genus *Middendorffinaia* was distinguished based on differences of shell umbo sculpture (Moskvicheva & Starobogatov, 1973). The differences of the beak sculpture between *Nodularia* and *Middendorffinaia* were first noted by Zatravkin and Bogatov (1987) and later validated by several investigations. Zatravkin and Bogatov (1987) described undulate rollers and coarse chevrons for *Nodularia* and *Middendorffinaia*, respectively. Later Bogatov (2012) clarified the description using a larger collection of specimens. He noticed that shells of *Nodularia* from the Russian Far East often have an invisible beak sculpture, and if it is noticeable, then near the umbo it is usually represented by broad, sometimes discrete W-shaped rollers, going down into narrow and repeatedly interrupted wavy ridges and/or tubercles; whereas species from South Asia have beak sculpture with short but large undulate rollers that cover most of the shell surfaces. The same description of the beak sculpture of *Nodularia* was made by Klishko et al. (2018) who also noted large chevrons or undulate elevations, or scarce W-shaped ridges, being clearly visible in young animals and weakly marked or absent in adults. According to Bogatov (2012) the beak sculpture of *Middendorffinaia* is represented by narrow straightened or short undulate rollers and/or small tubercles, usually arranged in staggered rows and often covering most of the shell surface. Klishko et al. (2018) also described the sculpture of the umbo and shell as short or elongated wavy elevations and/or small knobs. Kondo (1998, 2008) described the beak and shells sculpture of *Inversiunio* as corrugated nodules. When compared with the above-mentioned genera, the sculpture of adult *Inversiunio*, arranged in staggered rows and often covering most of the shell surfaces, resembles *Middendorffinaia* rather than *Nodularia* (Figs 1, 2).

A brief description of *Nodularia* glochidia from the Amur River basin (Russian Far East) was first made using light microscopy (Antonova & Starobogatov, 1988; Antonova et al., 1990). Later an investigation was broadened using both light and scanning electron microscopes (Sayenko, 2008, 2014; Sayenko & Kholin, 2007b). Glochidia of *Nodularia douglasiae* from Korea (Inaba, 1941, 1964; Kwon et al., 1993; Park & Kwon, 1993), Japan (Habe, 1973), and China (Wu et al., 1999) were observed using both light and scanning electron microscopes. First comparison of *Nodularia* and *Middendorffinaia* glochidia didn't provide any features that would allow one to distinguish these two genera (Sayenko, 2015). Measurements of glochidial shells exhibit remarkable variation among habitats in freshwater mussels and often do not provide distinctive features for identification at the generic level. However, differences in external glochidial valve sculpture are thought to help with taxonomic revisions and can be used as a diagnostic feature (Hoggarth, 1999; Panha & Eongprakornkeaw, 1995; Sayenko, 2016). Our data on external glochidial valve microsculpture agree with the molecular data, thus confirming the presence of three genera and emphasize the taxonomic complexity of *Nodularia* and *Inversiunio* (Figs S4–S5).

Molecular taxonomy uses the principle of a molecular clock, estimating the chronology of separation of individual evolutionary lines based on the differences observed in nucleotide (or amino acid) sequences in different groups of organisms. A very important methodological issue is the use of an appropriate molecular marker for the studied group of organisms and the taxonomic level. The use of a specific marker is also conditional on the availability of specific bioinformatic data about oligonucleotide primers or comparative sequence libraries. The most common genes used in systematics and phylogeny of animals are mitochondrial (12*S rDNA*, 16*S rDNA*, *COI*, *Cytb*, Control Region) and nuclear (*ITS*, 18*S rDNA*, 28*S rDNA*) markers, depending on the taxonomic rank (Wrzesińska et al., 2018). Since the 1990s, there has been an intense development of molecular methods and techniques used in systematics

and phylogeny of plants, animals, and bacteria. Among various molecular markers, the most widely applied is the mitochondrial *COI* gene as a species-specific marker in animals (DNA barcoding), which has been used for a number of years to resolve the complex systematic situation of Asian freshwater mussels (Bespalaya et al., 2018; Bolotov et al., 2016, 2017, 2018; Klishko et al., 2014, 2017, 2019; Sayenko et al., 2017; Vikhrev et al., 2017).

In the literature concerning *Nodularia*, *Middendorffinaia*, and *Inversiunio*, various mitochondrial markers have been applied, making it difficult to directly compare data from different publications. In this study, a single mitochondrial marker (considered to be a species-specific marker in animals) has been used for characterization of these three genera of molluscs. Prior studies of the *COI* gene have established that its intraspecific divergences rarely exceed 2%, but members of different species typically show a higher degree of divergence (Hebert et al., 2003; Pieńkowska & Lesicki, 2018). Most animal species accumulate more than 4% *COI* divergence from their nearest relative, and in the case of *Homo sapiens*, this parameter amounts to 11% (Ratnasingham & Hebert, 2013).

The investigation of *Nodularia* from the Yangtze River suggested the presence of three or five species. The three species hypothesis is supported by microsatellite analyses which treat *N. douglasiae* as a substantially divergent lineage, so *N. nipponensis* and *N. nuxpersicae* could be synonymized into *N. douglasiae* (Liu et al., 2017). Nuclear markers, such as the microsatelite loci, evolve more slowly (5–10%) than mitochondrial markers, and therefore at the beginning of speciation we can observe differentiation at the mitochondrial DNA level, but not yet on the nuclear DNA level. This has been confirmed by subsequent studies that identified four species of *Nodularia* (Klishko et al., 2018).

Klishko et al. (2018) used *COI* sequences data to demonstrate the existence of a single *N. douglasiae* species in the Russian Amur River basin instead of seven species in the genus *Nodularia* recognized in eastern Russia by using the Comparatory Method. Moreover, three other distinct *Nodularia* species were confirmed: *N. nipponensis* from Japan (GenBank number MF975691), *Nodularia breviconcha* (=*N. sinuolata*) from South Korea (GQ451864, deposited in GenBank as *N. douglasiae sinuolatus*), and an unnamed *Nodularia* sp. from China (KJ434520, sequence mislabelled in GenBank as *N. douglasiae*). The interspecific divergence between these four *Nodularia* species is from 5.1–8.8% and intraspecific differentiation of widespread *N. douglasiae* is up to 1.5% for specimens obtained from distinct countries (China, Japan, Russia, and South Korea). The species *N. nuxpersicae* from Vietnam was not included in these studies (Klishko et al., 2018).

Our research confirms the presence of five species of *Nodularia* (Fig. S4). In light of the current evidence, characterization of *N. jourdyi* as a separate species (Bolotov et al., 2018; Liu et al., 2017) cannot be justified. In our comparative analysis, *N. jourdyi* shows a genetic divergence of 1.1–2.1%, compared with the studied *N. douglasiae* sequences, 1.1–1.9% with other Russian *N. douglasiae* sequences, and 1.0–1.1% with Japanese *N. douglasiae* sequences. All of the above parameters reflect intraspecific differentiation within one species and one BIN number (ACH2241, Table 2, Fig. S4). In contrast, *N. jourdyi* shows a divergence of 4.8% compared with *N. nipponensis* and 7.4% compared with three sequences from the Yangtze River (MG210516, MG210527, and MG210536), which suggests a distinct species of *Nodularia* that is so far unidentified but forms a separate BIN (Table 2, Figs 6, S4).

Sano et al. (2017) found that *I. yanagawensis* was paraphyletic, forming a common clade with *I. reinianus*, and genetic distances between *I. yanagawensis* and *I. reinianus* were as low as the intraspecific genetic distances within *I. jokohamensis*. The marker, 16*S* ribosomal DNA, applied by Sano et al. (2017) evolves slower than the *COI* gene commonly used as a species-specific marker and therefore this marker is more valuable for inferring relationships between genera, rather than species (Brower, 1994; Pesole et al., 1999).

In accordance with findings of Sano et al. (2017) and Lopes-Lima et al. (2020), *COI* sequences of *I. yanagawensis* in our research did not form a single clade, but some of them from Honshu Island (here named *I.* cf. *yanagawensis* 2) formed a well-supported clade together with *I. reinianus*, and the remaining sequences of *I. yanagawensis* from Kyushu Island (here named *I.* cf. *yanagawensis* 1) formed a genetically distinguishable clade. We identify these specimens of *I.* cf. *yanagawensis* 1 (including a specimen from a canal of the Yabe River basin indirectly connected with and close to Futatsukawa Creek, the type locality of I. yanagawensis) as identical with *I. verrucosus*, which was thought to be restricted to South Korea only (Table 3, Fig. S5). All distinguished clades correspond to MOTUs supporting separation at the species level (Fig. S5).

Until recently, three *Inversiunio* species had been recorded in Japan – *I. jokohamensis* from Hokkaido and eastern Honshu, *I. reinianus*, endemic to the Biwa Lake basin, and *I. yanagawensis* from western Honshu, Shikoku, and Kyushu. The fourth species, *I. verrucosus*, was distinguished as endemic to the Seomjingang River basin in South Korea (Kondo et al., 2007; Kondo, 2008). Lopes-Lima et al. (2020) recognized the validity

of all *Inversiunio* species, based on morphological differences (Kondo et al., 2007; Kondo, 2008); however, *I. yanagawensis* mussels from Kyushu were not analysed. First genetic comparison of *I. verrucosus* from South Korea and *I. yanagawensis* from Kyushu reveals its similarity (Lee, 2017). Then, it was shown that mussels with the same haplotype as *I. yanagawensis* in the type locality (Futatsukawa Creek, Fukuoka Pref., Kyushu) are broadly distributed in Kyushu, moreover the populations of *I. yanagawensis* between Kyushu and Honshu are genetically distinct (Seo, 2019). In the present study we found a similarity between the *COI* molecular data of *I. yanagawensis* from Kyushu (including a specimen from a canal of the Yabe River basin indirectly connected with and close to Futatsukawa Creek, the type locality of I. yanagawensis) and *I. verrucosus* from the southern Korean Peninsula, thus, these populations can be regarded as the same species, with *I. verrucosus* as a junior synonym of *I. yanagawensis*. The taxonomic position of *I. yanagawensis* from Honshu Island (shown to be a separate, most probably new for science species) remains to be resolved.

The molecular identification of species in the new area is not an isolated case in the research world. The molecular methods used in species identification are very reliable, support morphological traits, and are often ahead of them. In 1992, as part of genetic-population research on the invasive species *Dreissena polymorpha* Pallas, 1771 in the Great Lakes of North America, another species of *D. bugensis* Andrusov, 1897 was identified (May & Marsden, 1992; Spidle et al., 1994). Both species occur sympatrically in the Great Lakes, exhibiting morphological diversity in the shape of shells, which was observed only after finding their high genetic diversity. In the case of rare or secretive species, molecular methods enable their reliable identification, while the analysis of morphological features, mainly mammal excrements, is subject to errors even when conducted by experienced researchers. Such mistakes most often occur in the case of very low populations and have been described for pine martens *Martes martes* (Linnaeus, 1758) and foxes *Vulpes vulpes* Linnaeus, 1758 (Davison et al., 2002), wolves, dogs, and coyotes (Pilgrim et al., 1998), and *Puma concolor* (Linnaeus, 1771) and *Lynx canadensis* Kerr, 1792 (Ernest et al., 2000).

If there are no taxonomically significant genetic differences between mussel populations, all the differences in shell morphology between habitats should rather be attributed to adaptive phenotypic plasticity. This is exactly what we found for the investigated so-called comparatory species of *Nodularia* and *Middendorffinaia*. However, for the *Inversiunio* populations, we revealed genetically separated clades that coincide with the separate localities of the four species in Japan.

Conclusions

An integrative taxonomic approach helps to assess the status of three nominal freshwater mussel taxa in the Russian Far East and Japan. Results indicate that the six morphological indices are not useful for the three mussel generic identifications. However, molecular data, umbo sculpture, and morphological differences of glochidia among the genera were important for the generic identifications. Three genetically distinct and highly divergent lineages were identified, confirming the existence of three distinct genera, notably *Nodularia*, *Middendorffinaia*, and *Inversiunio*.

Following Klishko et al. (2018) we confirm the complexity of the problem regarding the number of *Nodularia* taxa in China, as well as the erroneous designation of some sequences in GenBank. The existence of five *Nodularia* species in Southeast Asia is shown. The assignment of one sequence as *N. jourdyi* is not justified, instead, it represents *N. douglasiae* species. Our research using a larger sample of specimens supports the existence of a single *Nodularia* species, *N. douglasiae*, and a single *Middendorffinaia* species, *M. mongolica*, in the Russian Far East. Based on a complex number of features, we conclude that the comparatory species *Nodularia amurensis*, *N. schrencki*, and *N. vladivostokensis* are junior synonyms of the species *Nodularia douglasiae*; the comparatory species *Middendorffinaia ussuriensis* and *M. dulkeitiana* are junior synonyms of the species *Middendorffinaia mongolica*.

Four distinct species of *Inversiunio* are detected in Japan, wherein *Inversiunio verrucosus* from South Korea is regarded as a junior synonym of *Inversiunio yanagawensis* from Kyushu Island in Japan. The population of *I. yanagawensis* from Honshu Island is shown to be a separate, most probably new for science species, however now the taxonomic status of mussels referred to *I. yanagawensis* from Honshu is still in question and needs to be resolved in the future based on expanded sampling techniques and methods.

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No potential conflict of interest was reported by the author(s).

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