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Phylogenetic Relationships of Japanese Unionoida (Mollusca: Bivalvia) Based on Mitochondrial 16S rDNA Sequences

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Abstract

Japanese unionoid mussels are classified into 2 families (Margaritiferidae and Unionidae), 12 genera, and 18 species based on the morphological characteristics of both adults and larvae; however, there are some debates regarding their systematics. In this study, we determined mitochondrial 16S ribosomal DNA sequences (347-bp) for 60 specimens belonging to 18 species and constructed trees to elucidate phylogenetic relationships and evaluate the current systematics of Japanese unionoid mussels. Almost all species formed clades, except for *Inversiunio yanagawensis*, *Sinanodonta lauta*, *S. japonica*, and *Margaritifera laevis*, even though two or more specimens were collected from distant localities. All genera formed highly supported clades with the exception of the genus *Sinanodonta*. Phylogenetic relationships obtained in this study supported systematics based on morphological and larval traits. Therefore, the current phylogenetic relationships and systematics of Japanese unionoid mussels are stronger than they were before; now that they are corroborated by genetic data.

Keywords

Systematics, Molecular Barcoding, Endangered Species, Conservation, East Asian Mussels

1. Introduction

The order Unionoida includes more than 850 species and is more diverse than any other group of freshwater bivalves [1] [2]. Unionoid mussels are widely distributed in all continents except Antarctica and are divided into 6 families (the

Unionidae, Margaritiferidae, Etheriidae, Hyriidae, Iridinidae, and Mycetopodidae). In Japan, two families, the Unionidae and Margaritiferidae, have been recognized; however, there are some debates regarding the systematics of Japanese unionoid mussels. Kondo [3] classified them into 2 families, 3 subfamilies, 12 genera, 17 species, and 1 subspecies, but later revised his classification [4] to include 2 families, 2 subfamilies, 12 genera, and 18 species (**Table 1**). In the Unionidae, Kihira *et al.* [5] included one additional species, *Lanceolaria oxyrhyncha* (Sasanohagai in Japanese), and founded two subspecies, *Nodularia douglasiae biwae* (Tateboshigai in Japanese) and *N. d. nipponensis* (Ishigai in Japanese), for *N. douglasiae* in Kondo [4] and also two subspecies, *Cristaria plicata clessini* (Menkarasugai in Japanese) and *C. p. plicata* (Karasugai in Japanese), for *C. plicata* in Kondo [4]. The taxa classified by Kihira *et al.* are endemic to Lake Biwa

Table 1. Systematics of Japanese Unionoida based mainly on adult morphological characteristics and larval forms [4].

Family	Subfamily	Genus	Species
Margaritiferidae Henderson, 1929		*Margaritifera* Schumacher, 1816	*Margaritifera laevis* Haas, 1910
			Margaritifera togakushiensis Kondo & Kobayashi, 2005
Unionidae Rafinesque, 1820	Unioninae Rafinesque, 1820	*Nodularia*	*Nodularia douglasiae* Gray in Griffith & Pidgeon, 1833
		Inversiunio	*Inversiunio reinianus* Kobelt, 1879
			Inversiunio jokohamensis Ihering, 1893
			Inversiunio yanagawensis Kondo, 1982
		Lanceolaria	*Lanceolaria grayii* Gray in Griffith & Pidgeon, 1833
		Sinanodonta	*Sinanodonta japonica* Clessin, 1874
			Sinanodonta lauta Martens, 1877
			Sinanodonta calipygos Kobelt, 1879
			Sinanodonta ogurae Kuroda & Habe, 1987
		Anemina	*Anemina arcaeformis* Heude, 1977
		Cristaria	*Cristaria plicata* Leach, 1815
		Plethlophus	*Plethlophus tenuis* Gray in Griffith & Pidgeon, 1833
	Gonideinae Ortmann, 1916	*Hyriopsis*	*Hyriopsis schlegeli* Martens, 1861
		Inversidens	*Inversidens brandti* Kobelt, 1879
		Obovalis	*Obovalis omiensis* Heimburg, 1884
		Pronodularia	*Pronodularia japanensis* Lea, 1859

and/or the Yodo River. Although Graf and Cummings [2] reviewed the worldwide unionoids and proposed classification, their classification was still tentative as mentioned by themselves. At present, there is not enough information to deduce relationships between Japanese species and similar species in other parts of the world.

The classification of unionoid mussels has mainly been based on the morphological characteristics of the adults and/or the larvae such as beak sculpture, hinge teeth, shell length and larval hook. Shell characters in particular have attracted a great deal of attention for the classification of unionoid mussels because they can be used to classify fossils. However, Heard *et al.* [6] suggested that morphological similarities among unionoid species were caused in part by convergent or parallel evolution and not by their ancestry. They also insisted that unionoid systematics based on reproductive aspects more accurately reflected natural, evolutionary affinities than those based on morphological characteristics, and they thus revised the classification of North American unionoid mussels.

Studies using genetic information can objectively provide accurate phylogenetic relationships, reflecting morphological and reproductive differences, and are less influenced by convergent or parallel evolution [7] [8] [9] [10]. Recently, Lopes-Lima *et al.* [11] investigated the phylogenetic relationships of 70 unionoid species using the mitochondrial cytochrome oxidase subunit I (COI) and nuclear 28S rRNA sequences (1032-bp) and showed that unionid mussels formed three clades. They assigned three subfamilies, the Unioninae, Anodontinae, and Gonideinae, to the clades. However, they used only 1 of the 18 Japanese unionoid species identified by Kondo [4]. Takeuchi *et al.* [12] analyzed the mitochondrial COI and nuclear 18S rRNA and 28S rRNA genes to deduce relationships between the morphologically distinguished *Margaritifera togakushiensis* and *M. laevis*, and confirmed that *M. togakushiensis* was genetically distinct from *M. laevis*. Their results were further supported from an ecological viewpoint; however, they used only 2 of the 18 Japanese unionoid species. Therefore, the phylogenetic relationships of Japanese Unionoida have not been resolved. To elucidate the phylogenetic relationships of Japanese unionoid mussels, genetic studies using more species are needed.

The Japanese Ministry of the Environment [13] has designated 13 of the 18 Japanese unionoid species as endangered due to deterioration of freshwater systems by human activities [14] [15] [16] [17]. These filter-feeding mussels greatly influence ecological systems and play an important role in purifying water. Negishi *et al.* [18] unraveled the processes of degradation of unionoid habitats and tried to restore them. Genetic and ecological information is indispensable for preserving wildlife, and one can estimate how long a population is likely to survive by estimating its genetic diversity and population size. Phylogenetic relationships and classification corroborated by genetic analyses are also needed to transplant endangered mussels whose habitats seem to disappear in the near future and to rear them in institutions with genetic contamination avoided.

In this study, we determined mitochondrial 16S ribosomal DNA sequences

(347-bp) and constructed trees to elucidate phylogenetic relationships and establish the systematics of Japanese Unionoida. We also constructed trees to reveal relationships of East Asian unionoid mussels using their 16S rDNA sequences (256-bp). We demonstrated that the phylogenetic relationships of Japanese Unionoida obtained in this study supported the systematics proposed by Kondo [4]. Therefore, the phylogenetic relationships and systematics of Japanese unionoid mussels based on genetic and morphological data were in agreement.

2. Materials and Methods

2.1. Materials

In total, 60 unionoid specimens were collected in Japan and preserved in 99.5% ethanol. Prior to our genetic study, we identified specimens according to Kondo [4] and assigned them to the Margaritiferidae (2 species) and Unionidae (16 species). However, *Nodularia douglasiae* was divided into two subspecies, *N. d. biwae* and *N. d. nipponensis*, according to Kihira *et al.* [5], to evaluate the validity of these subspecies. Lopes-Lima *et al.* [11] reassigned *Hyriopsis cumingii* to *Sinohyriopsis cumingii* because this species did not cluster with the other three *Hyriopsis* species, and Shirai *et al.* [19] analyzed mitochondrial DNA (COII-COI) and nuclear DNA (ITS1) and showed that *H. cumingii* and *H. schlegeli* were closely related. Therefore, *Hyriopsis schlegeli* used in this study may be renamed *Sinohyriopsis schlegeli* in the future. Detailed information about the specimens is listed in **Table 2**.

2.2. DNA Sequencing

We dissected out the foot muscle from each unionoid mussel and boiled them at 100˚C. Then, total DNA was extracted using DNeasy® Blood & Tissue Kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's protocol. To amplify the mitochondrial 16S rDNA gene, PCR was performed using KOD dash (Toyobo Co., Ltd., Osaka, Japan) under the following conditions: initial denaturation at 94˚C for 2 min, 40 cycles of denaturation at 94˚C for 1 min, annealing at 60˚C for 30 sec, and extension at 72˚C for 1 min, followed by a final extension at 72˚C for 8 min. We designed the primers (sense 16S-FW1F, 5'-GTTAGCGTGAGCGTGCTAAG-3' and antisense 16S-FW1R, 5'-CGGTCTTAACTCAGCTCGTG-3') to amplify female-type mitochondrial DNA sequences because unionoid mussels have the unique hereditary system named "DUI: doubly uniparental inheritance" [20]. In this system, mitochondria are transmitted from both male and female parents to progeny. The male-type mitochondria, which are highly divergent in DNA sequences from the female-type mitochondria, are localized exclusively in the testis, whereas the female-type mitochondria are in the whole body except for the testis. When we could not amplify DNA well, we designed two additional antisense primers (16S-FW2R, 5'-TCTTTGGGTCCTTTCGTACAA-3' and 16S-FW3R, 5'-TTGGGGTCCTTTCGTACAA-3') and used them for PCR. PCR products

Table 2. Specimen details for Japanese unionoids samples used in phylogenetic analyses.

#	Order	Family	Species	Sample Ab.	Locality	Accession No.
1	Unionoida	Margaritiferidae	*Margaritifera laevis*	Ma14-1SUTO	Gujo, Gifu, Japan	LC223972
2				Ma14-2SUTO	Gujo, Gifu, Japan	LC223973
3				Ma14-3SUTO	Gujo, Gifu, Japan	LC223974
4					Iwaizumi, Iwate, Japan	EU590914*
5			*Margaritifera togakushiensis*	Mt-k	Togakushi, Nagano, Japan	LC224020
6		Unionidae	*Nodularia douglasiae biwae*	BIWATATE 2	Lake Biwa, Shiga, Japan	LC223962
7				BIWATATE 3	Lake Biwa, Shiga, Japan	LC223961
8				KAWATATE 2	Lake Kawaguchiko, Yamanashi, Japan	LC223964
9				KAWATATE 6	Lake Kawaguchiko, Yamanashi, Japan	LC223965
10				YAMATATE 8	Lake Yamanakako, Yamanashi, Japan	LC223963
11			*Nodularia douglasiae nipponensis*	Un40-01	Wakayama, Japan	LC223975
12				Un40-02	Wakayama, Japan	LC223976
13				Un43-06f	Nakama, Fukuoka, Japan	LC223977
14				Un43-07f	Nakama, Fukuoka, Japan	LC223978
15					South Korea	GQ451850*
16					South Korea	GQ451851*
17					Jiangxi, China	AF389406*
18			*Inversiunio reinianus*	Ir07-02	Lake Biwa, Shiga, Japan	LC223979
19			*Inversiunio jokohamensis*	Ij25-01	Sakai, Yamagata, Japan	LC223980
20				Ij25-09	Sakai, Yamagata, Japan	LC223981
21				Ij21-28f	Lake Anenuma, Aomori, Japan	LC223982
22				Ij21-30f	Lake Anenuma, Aomori, Japan	LC223983
23				Ij08-01	Lake Kitaura, Ibaraki, Japan	LC223984
24				Ij08-03	Lake Kitaura, Ibaraki, Japan	LC223985
25			*Inversiunio yanagawensis*	Iy09-08	Gion, Okayama, Japan	LC223986
26				Iy09-10	Gion, Okayama, Japan	LC223987
27				Iy43-01	Fukuoka, Japan	LC223988
28				Iy43-05f	Fukuoka, Japan	LC223989
29			*Lanceolaria grayii*	Lg04-01SUTO	Hiroshima, Japan	LC223990
30				Lg04-02SUTO	Hiroshima, Japan	LC223991
31				Lg14-01SUTO	Gifu, Japan	LC223992
32				Lg14-2f	Gifu, Japan	LC223993
33					Jiangxi, China	AF389408*
34			*Obovalis omiensis*	Oo14-01m	Gifu, Japan	LC223994
35				Oo16-01SUTO	Kyoto, Japan	LC223995
36			*Pronodularia japanensis*	Pj25-06	Sakai, Yamagata, Japan	LC223996
37				Pj14-02f	Gifu, Japan	LC223997
38				Pj14-05f	Gifu, Japan	LC223998
39				Pj08-01	Lake Kitaura, Ibaraki, Japan	LC223999

Continued

40				Pj08-02	Lake Kitaura, Ibaraki, Japan	LC224000
41				Pj04-01SUTO	Hiroshima, Japan	LC224001
42				Pj04-03SUTO	Hiroshima, Japan	LC224002
43				Pj-k	Sakurai, Nara, Japan	LC224019
44			*Inversidens brandti*	Ib14-01f	Gifu, Japan	LC224003
45				Ib14-02f	Gifu, Japan	LC224004
46			*Hyriopsis schlegeli*	Hs21-02f	Lake Anenuma, Aomori, Japan	LC224005
47				Hs21-05f	Lake Anenuma, Aomori, Japan	LC224006
48			*Cristaria plicata*	Cp21-10f	Lake Anenuma, Aomori, Japan	LC224007
49				Cp21-11f	Lake Anenuma, Aomori, Japan	LC224008
50				Cp31-01fmg	Joetsu, Niigata, Japan	LC224009
51				YAMAKARA 1	Lake Yamanakako, Yamanashi, Japan	LC223968
52				YAMAKARA 2	Lake Yamanakako, Yamanashi, Japan	LC223969
53				YAMAKARA 5	Lake Yamanakako, Yamanashi, Japan	LC223971
54				YAMAKARA 6	Lake Yamanakako, Yamanashi, Japan	LC223970
55					Zhejiang, China	FJ986302*
56					Jiangxi, China	AF389414*
57			*Sinanodonta lauta*	fk168	Ishikawa, Japan	LC224010
58				KONZAISYU E	Lake Biwa, Shiga, Japan	LC223967
59				FUKUNUMA 22	Minamisoma, Fukushima, Japan	LC223966
60			*Sinanodonta japonica*	fk20f	Kyoto, Japan	LC224011
61				fk35f	Kushiro, Hokkaido, Japan	LC224012
62			*Sinanodonta calipygos*	fk221	Lake Biwa, Shiga, Japan	LC224013
63			*Sinanodonta ogurae*	fk156	Yodo River, Japan	LC224015
64			*Anemina arcaeformis*	fk63f	Kagawa, Japan	LC224014
65			*Plethobophus tenuis*	Pt43-01	Munakata, Fukuoka, Japan	LC224016
66				Pt43-02	Munakata, Fukuoka, Japan	LC224017
67				Pt43-03	Munakata, Fukuoka, Japan	LC224018
68			*Acuticosta ovata*		Jiangxi, China	AF389412*
69			*Arconaia lanceolata*		Jiangxi, China	AF389409*
70			*Cuneopsis pisciculus*		Jiangxi, China	AF389407*
71			*Hyriopsis cumingii*		Jiangxi, China	AF389418*
72			*Lamprotula leai*		Jiangxi, China	AF389415*
73			*Lepidodesma languilati*		Jiangxi, China	AF389411*
74			*Ptychorhynchus ptisteri*		Jiangxi, China	AF389416*
75			*Schistodesmus lampreyanus*		Jiangxi, China	AF389410*
76			*Sinanodonta woodiana*		Jiangxi, China	AF389413*
77			*Solenaia oleivora*		Jiangxi, China	AF389417*
78	**Trigoniida**	**Trigoniidae**	*Neotrigonia lamarckii*		data not available	KC429262*
79			*Neotrigonia margaritacea*		data not available	DQ093489*
80					data not available	DQ280034*

*Obtained from the DDBJ.

were purified using QIAquick® PCR Purification Kit (QIAGEN GmbH, Hilden, Germany). Sequence reactions were performed using GenomeLab™ DTCS-Quick Start Kit (Beckman Coulter Inc., California, USA), and the same primers for PCR under the following conditions: 30 cycles of denaturation at 96˚C for 20 sec, annealing at 50˚C for 20 sec, and extension at 60˚C for 4 min. Direct sequencing of the double-stranded PCR products was performed using a CEQ™ 2000XL DNA Analysis system (Beckman Coulter Inc., California, USA) following the manufacturer's instructions. Sequences were deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers LC223961-LC224020. The length of the sequences obtained ranged from 444 bp in *Hyriopsis schlegeli* (Hs21-05f) to 525 bp in *Margaritifera togakushiensis* (Mt-k). We used seventeen 16S rDNA sequences of Unionoida registered in the DDBJ (EU590914, GQ451850, GQ451851, AF389406, FJ986302, AF389414, AF389412, AF389409, AF389407, AF389418, AF389415, AF389408, AF389411, AF389416, AF389410, AF389413, AF389417), and we used *Neotrigonia lamarckii* (KC429262) and *Neotrigonia margaritacea* (DQ093489, DQ280034) sequences as the outgroup (**Table 2**).

2.3. Phylogenetic Analysis

DNA sequences of mitochondrial 16S rDNA were edited and aligned using DNASIS (Hitachi Software Engineering Co., Ltd., Tokyo, Japan) and MEGA 6.0 [21] and confirmed by visual inspection. No saturation was observed via analysis of nucleotide substitution patterns in mitochondrial 16S rDNA [9]. We used 347-bp sequences for tree construction including only Japanese unionoid species as the ingroup. On the other hand, we used 256-bp sequences for tree construction of East Asian unionoid species. Unfortunately, sequences for Chinese mussels deposited in the DDBJ [22] were shorter than those determined in this study. A neighbor-joining (NJ) tree was constructed using MEGA 6.0, and genetic distances were computed using Kimura's two-parameter model [23]. Tree reliability was evaluated by generating 1000 bootstrap replicates. Using PAUP*4.0 beta10 [24], a majority-rule consensus maximum parsimony (MP) tree was constructed by conducting a heuristic search based on the 1000 bootstrap replicates with an unweighted transition/transversion ratio. A Bayesian (BI) tree was constructed using MrBayes version 3.2.6 [25] based on model evaluation done with MrModeltest 2.3 [26]. The best model for both trees was GTR + G. The Monte Carlo Markov chain (MCMC) length was 5×10^6 generations, and we sampled the chain every 100 generations. MCMC convergence was assessed by calculating the potential scale reduction factor, and the first 2.5×10^4 generations were discarded.

3. Results

Phylogenetic relationships of Japanese unionoids based on 347 bp of the 16S rRNA gene are shown in **Figure 1**. There were 183 variable sites and 176 informative sites. Topologies depicted by MP and Bayesian trees were essentially

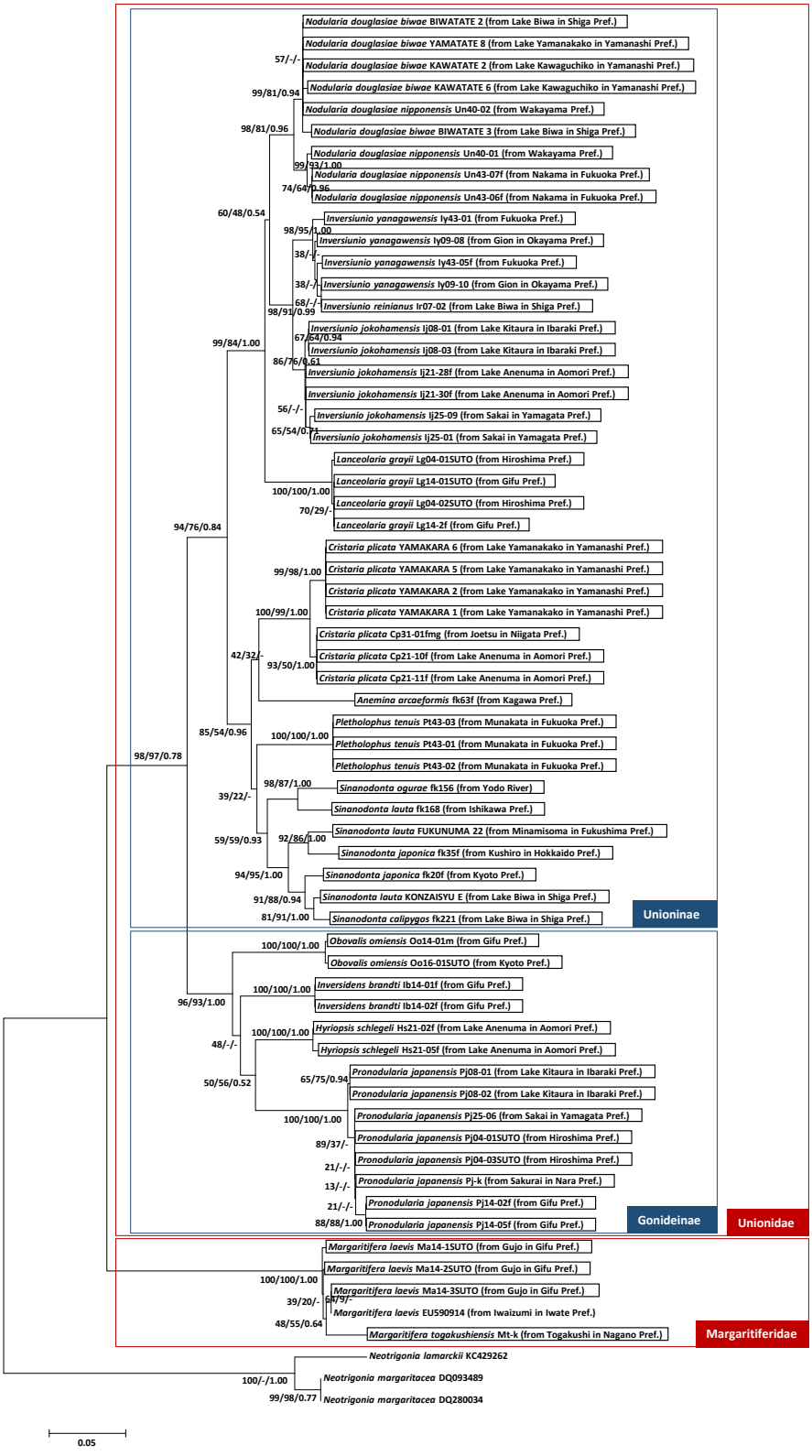

Figure 1. Japanese unionoid mussel NJ tree based on 347-bp 16S rDNA sequences from 64 specimens including three outgroup *Neotrigonia* specimens. The specimens, the sequences of which were newly determined in this study, are enclosed in squares. The scale bar indicates 0.05 substitutions per site. NJ (left) and MP (middle) bootstrap values and Bayesian posterior probabilities (right) are specified near the relevant nodes. *Nodularia douglasiae* was divided into two subspecies, *N. d. biwae* and *N. d. nipponensis* according to Kihira *et al.* [5]. The classifications depicted here are mainly based on Kondo [4].

identical to that of the NJ tree. Based on our analyses, Japanese unionoid mussels were divided into two well-supported clades (98/97/0.78 and 100/100/1.00, NJ/MP/Bayesian, respectively) corresponding to two families, the Margaritiferidae and Unionidae. In the Unionidae, there were two well-supported clades (94/76/0.84 and 96/93/1.00) corresponding to the subfamilies Unioninae and Gonideinae. Most genera formed clades with high statistical supports. The only exception was the genus *Sinanodonta*, which formed a poorly supported clade (59/59/0.93). When two or more specimens were used, most species formed clades with robust statistical supports, even though the specimens were collected in distant localities. For example, *Obovalis omiensis* specimens were obtained from Gifu and Kyoto Prefectures, those of *Lanceolaria grayii* from Gifu and Hiroshima Prefectures, and those of *Pronodularia japanensis* from Ibaraki, Hiroshima, Nara, Gifu, and Yamagata Prefectures. However, *Inversiunio jokohamensis* formed a marginally supported clade (86/76/0.61). *Inversiunio yanagawensis* and *Margaritifera laevis* did not form their own species clades, and two species, *Sinanodonta lauta* and *S. japonica*, exhibited complicated relationships with *S. ogurae and S. calipygos*. The two subspecies described by Kihira *et al.* [5], *Nodularia douglasiae biwae* and *N. d. nipponensis*, did not form their own subspecies clades.

Phylogenetic relationships of East Asian unionoids based on 256 bp of the 16S rRNA gene are shown in **Figure 2**. There were 138 variable sites and 130 informative sites. Topologies depicted by MP and Bayesian trees were essentially identical to that of the NJ tree. Two robustly supported clades corresponding to the Margaritiferidae and Unionidae were recognized (100/100/1.00 and 100/99/1.00, respectively). Most genera and species formed clades; however, *Sinanodonta* did not form its own clade. The *Inversiunio* clade was well supported in the Japanese unionoid tree (**Figure 1**), but was only marginally supported in the East Asian unionoid tree (**Figure 2**) likely due to the shorter sequences used to make the latter tree. On the other hand, *Margaritifera laevis* was paraphyletic in the Japanese unionoid tree (**Figure 1**), but it formed a clade in the East Asian unionoid tree (**Figure 2**).

4. Discussion

Japanese unionoid mussels were basally divided into two clades corresponding to the two families described by Kondo [4] who showed that the Margaritiferidae had interlamellar gill junctions arranged in diagonal rows, while the Unionidae had interlamellar gill junctions combined into vertical septa. Rosenberg *et al.* [27] presented preliminarily the molecular phylogeny of invertebrate animals, including the Unionoida, by analyzing the D6 region (about 150-bp) of the nuclear 28S rRNA gene. They showed that margaritiferid species could be distinguished from other unionoid species, which supported our results.

Kondo [4] reported that there were two subfamilies, the Unioninae and Gonideinae, in the family Unionidae. He regarded mussels having subtriangular and hooked glochidia as Unioninae and those having essentially semi-elliptical

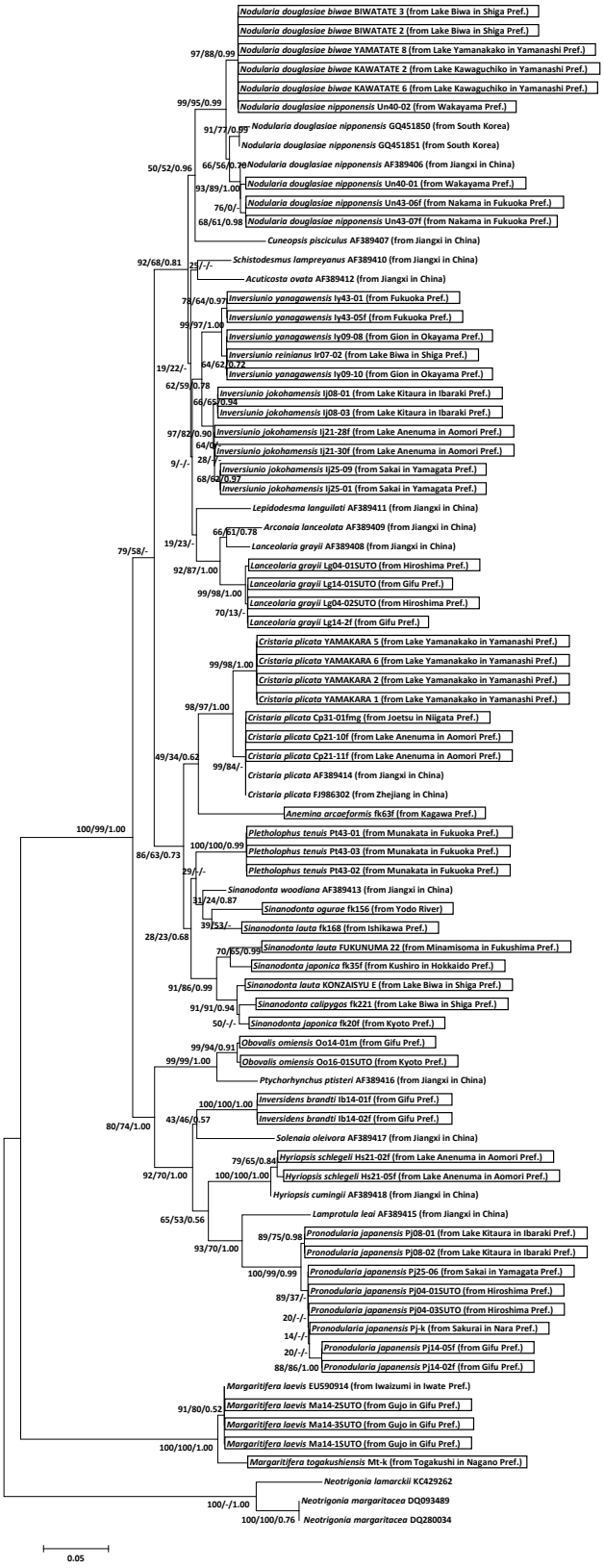

Figure 2. East Asian unionoid mussel NJ tree based on 256-bp 16S rDNA sequences from 80 specimens including three outgroup *Neotrigonia* specimens. The specimens, the sequences of which were newly determined in this study, are enclosed in squares. The scale bar indicates 0.05 substitutions per site. NJ (left) and MP (middle) bootstrap values and Bayesian posterior probabilities (right) are specified near the relevant nodes.

and unhooked glochidia as Gonideinae. Our results supported Kondo's subfamily classification. Kondo [4] further classified unionoid mussels into 12 genera and 18 species, mainly based on their shell morphology. In this study, most genera formed well supported clades, although the genus *Sinanodonta* was poorly supported. Most species also formed well supported clades, but one species formed a marginally supported clade or some did not form clades at all. Takeuchi *et al.* [12] showed that a phylogenetic tree based on mitochondrial COI sequences distinguished *M. laevis* from *M. togakushiensis*. Our results supported their results in the East Asian unionoid tree (**Figure 2**), but not in the Japanese unionoid tree (**Figure 1**). Of the three species of *Inversiunio*, *I. jokohamensis* was marginally supported and *I. yanagawensis* was paraphyletic, but *I. yanagawensis* and *I. reinianus* formed a clade together. Genetic distances between *I. yanagawensis* and *I. reinianus* (0.00581 in **Table 3** and 0.00394 in **Table 4**) were as low as the intraspecific genetic distances within *I. jokohamensis* (0.00405 in **Table 3** and 0.00419 in **Table 4**). Therefore, in this study, *I. yanagawensis* could not be genetically well-distinguished from *I. reinianus*. Complicated relationships between *Sinanodonta* species seem to be derived from large morphological variation within the genus, which sometimes confuses species identification [5] [28]. To obtain more robust phylogenetic relationships of those species, we need to collect and analyze more specimens and use another gene such as the mitochondrial cytochrome oxidase subunit I (COI) gene for molecular barcording.

Kihira *et al.* [5] recognized *Lanceolaria oxyrhyncha* as a valid species different from *L. grayii*, which is widely distributed in Japan. However, Kondo [3] claimed that *L. oxyrhyncha* could not be separated morphologically from *L. grayii*, and Shirai [29] showed that *L. oxyrhyncha* was not genetically distinct from *L. grayii*. Kihira *et al.* [5] recognized *Cristaria plicata clessini* as a valid subspecies. However, Kondo [3] showed that *C. p. clessini* seems to be a lacustrine type of *C. plicata* and considered it doubtful that *C. p. clessini* is a subspecies. Hence, we did not use *L. oxyrhyncha* or *C. p. clessini* in this study. *Nodularia douglasiae* was also problematic. Kihira *et al.* [5] established two subspecies, *N. d. biwae* and *N. d. nipponensis*, and Kondo [30] regarded the individuals with milky-white glochidia as *N. d. biwae* and the others with buff glochidia as *N. d. nipponensis*. Later, Kondo [4] resolved that glochidium colors did not seem to be sufficiently diagnostic to identify the subspecies because there was substantial variation in color as is also often the case with *Inversidens brandti* and *Lanceolaria grayii*. We showed in this study that one specimen of *N. d. nipponensis* (Un40-02) was more closely related to specimens of *N. d. biwae* than to the other specimens of *N. d. nipponensis*. Shirai [29] showed, based on mitochondrial DNA (COI + II), that *Nodularia douglasiae* is divided into two clades (east and west), but each subspecies did not form its own clade. Taken together, these results suggest that *N. d. biwae* and *N. d. nipponensis* are not valid as subspecies.

The phylogenetic relationships obtained in this study essentially supported the systematics proposed by Kondo [4] that are primarily based on morphological and larval traits. Never before have any published studies comprehensively in-

Table 3. Genetic distances between Japanese unionoid mussel species computed using Kimura's two-parameter model based on 347-bp 16S rDNA sequences.

	Margaritifera laevis	*Margaritifera togakushiensis*	*Nodularia douglasiae biwae*	*Nodularia douglasiae nipponensis*	*Inversiunio reinianus*	*Inversiunio jokohamensis*	*Inversiunio yanagawensis*	*Lanceolaria grayii*	*Obovalis omiensis*	*Pronodularia japanensis*	*Inversidens brandti*	*Hyriopsis schlegeli*	*Cristaria plicata*	*Sinanodonta lauta*	*Sinanodonta japonica*	*Sinanodonta calipygos*	*Anemina arcaeformis*	*Sinanodonta ogurae*	*Plethlophus tenuis*	*Neotrigonia* (Outgroup)
Margaritifera laevis	0.00337																			
Margaritifera togakushiensis	0.03175																			
Nodularia douglasiae biwae	0.27765	0.31127	0.00347																	
Nodularia douglasiae nipponensis	0.26598	0.29910	0.01489	0.01068																
Inversiunio reinianus	0.27895	0.30401	0.05889	0.06170																
Inversiunio jokohamensis	0.27874	0.30402	0.04906	0.05028	0.02803	0.00405														
Inversiunio yanagawensis	0.28018	0.30633	0.05798	0.06055	0.00581	0.02803	0.00871													
Lanceolaria grayii	0.30202	0.33735	0.07087	0.07927	0.07527	0.07547	0.07779	0.00145												
Obovalis omiensis	0.29051	0.31404	0.16243	0.15761	0.18577	0.17538	0.18489	0.17504	0.00289											
Pronodularia japanensis	0.29441	0.31377	0.18982	0.18770	0.19103	0.18821	0.19017	0.19986	0.12765	0.00498										
Inversidens brandti	0.28129	0.30486	0.16635	0.16705	0.17106	0.16697	0.17392	0.19025	0.11810	0.10834	0.00000									
Hyriopsis schlegeli	0.28521	0.30925	0.16120	0.15991	0.17059	0.16414	0.16977	0.17111	0.12581	0.10294	0.11110	0.00289								
Cristaria plicata	0.27309	0.29778	0.11326	0.11246	0.12844	0.11396	0.13125	0.11646	0.18333	0.20032	0.17072	0.16450	0.00833							
Sinanodonta lauta	0.28914	0.31065	0.11189	0.11188	0.12919	0.12225	0.13105	0.13574	0.19320	0.22070	0.17394	0.20233	0.10300	0.07219						
Sinanodonta japonica	0.28452	0.30233	0.11213	0.11308	0.13417	0.12856	0.13603	0.13298	0.18976	0.22731	0.17622	0.20748	0.11200	0.05265	0.05070					
Sinanodonta calipygos	0.28061	0.30401	0.11057	0.11116	0.13232	0.12495	0.13326	0.13644	0.17202	0.21432	0.16453	0.19456	0.10684	0.05461	0.04645					
Anemina arcaeformis	0.30386	0.32986	0.13532	0.14667	0.14366	0.13262	0.14286	0.15149	0.21056	0.22873	0.18734	0.19214	0.10363	0.11672	0.11993	0.11284				
Sinanodonta ogurae	0.29290	0.31803	0.12115	0.11832	0.13610	0.13225	0.13888	0.14004	0.20719	0.21633	0.18015	0.18338	0.09981	0.07571	0.08436	0.09602	0.10976			
Plethlophus tenuis	0.29744	0.31331	0.11798	0.11840	0.12932	0.12317	0.13387	0.14411	0.21125	0.22456	0.17248	0.17171	0.10178	0.09864	0.10279	0.10284	0.11729	0.09638	0.00000	
Neotrigonia (Outgroup)	0.43253	0.44513	0.42109	0.42100	0.42160	0.41089	0.42603	0.44404	0.43731	0.42422	0.41543	0.43080	0.41028	0.42064	0.41379	0.44262	0.42797	0.39093	0.42767	0.04255

Table 4. Genetic distances between East Asian unionoid species computed using Kimura's two-parameter model based on 256-bp 16S rDNA sequences.

	Margaritifera laevis	*Margaritifera togakushiensis*	*Nodularia douglasiae biwae*	*Nodularia douglasiae nipponensis***	*Inversiunio reinianus*	*Inversiunio jokohamensis*	*Inversiunio yanagawensis*	*Lanceolaria grayii***	*Obovalis omiensis*	*Pronodularia japanensis*	*Inversidens brandti*	*Hyriopsis schlegeli*	*Cristaria plicata***	*Sinanodonta lauta*	*Sinanodonta japonica*	*Sinanodonta calipygos*	*Anemina arcaeformis*	*Sinanodonta ogurae*	*Plethlophus tenuis*	*Acuticosta ovata**	*Arconaia lanceolata**	*Cuneopsis pisciculus**	*Hyriopsis cumingii**	*Lamprotula leai**	*Lepidodesma languilati**	*Ptychorhynchus ptisteri**	*Schistodesmus lampreyanus**	*Sinanodonta woodiana**	*Solenaia oleivora**	*Neotrigonia* (Outgroup)*
Margaritifera laevis	0.00000																													
Margaritifera togakushiensis	0.02794																													
Nodularia douglasiae biwae	0.32541	0.35876	0.00000																											
*Nodularia douglasiae nipponensis***	0.31623	0.34920	0.02046	0.01817																										
Inversiunio reinianus	0.31267	0.34515	0.06535	0.07282																										
Inversiunio jokohamensis	0.31059	0.34293	0.05964	0.06539	0.03089	0.00419																								
Inversiunio yanagawensis	0.31267	0.34515	0.06535	0.07251	0.00394	0.03089	0.00525																							
*Lanceolaria grayii***	0.33617	0.37097	0.07651	0.08852	0.06908	0.07497	0.07261	0.01951																						
Obovalis omiensis	0.31409	0.33296	0.16590	0.17104	0.15634	0.15636	0.15634	0.15942	0.00392																					
Pronodularia japanensis	0.35591	0.35591	0.20675	0.21553	0.21652	0.21655	0.21652	0.21104	0.17744	0.00677																				
Inversidens brandti	0.32541	0.33191	0.17803	0.17669	0.17722	0.18071	0.18249	0.19738	0.16586	0.12846	0.00000																			
Hyriopsis schlegeli	0.34595	0.35289	0.20289	0.20949	0.18554	0.18555	0.18554	0.20165	0.16726	0.12510	0.11862	0.00392																		
*Cristaria plicata***	0.32811	0.33477	0.14065	0.14806	0.14327	0.13052	0.14832	0.14399	0.19317	0.24828	0.18144	0.19677	0.01552																	
Sinanodonta lauta	0.33801	0.34475	0.13342	0.13981	0.13991	0.13555	0.14406	0.14838	0.19727	0.26037	0.20164	0.24804	0.11072	0.07193																
Sinanodonta japonica	0.33330	0.33997	0.12844	0.13617	0.14524	0.14113	0.14897	0.14749	0.19312	0.26534	0.20175	0.25472	0.11456	0.05107	0.05693															
Sinanodonta calipygos	0.34445	0.35117	0.13258	0.14025	0.14715	0.14063	0.14962	0.15661	0.19721	0.27829	0.22014	0.26528	0.11772	0.05762	0.04475															
Anemina arcaeformis	0.33570	0.34250	0.16286	0.18099	0.16802	0.16116	0.16802	0.17946	0.19629	0.25831	0.18919	0.21209	0.10630	0.11683	0.12404	0.12364														
Sinanodonta ogurae	0.35191	0.35876	0.15239	0.15753	0.14741	0.14577	0.15241	0.14847	0.20949	0.23530	0.20397	0.21852	0.10688	0.08975	0.09901	0.11489	0.10973													
Plethlophus tenuis	0.33404	0.32741	0.13845	0.13857	0.13845	0.12871	0.14343	0.14482	0.19357	0.23015	0.15776	0.17633	0.09623	0.07801	0.08361	0.08778	0.11517	0.08347	0.00000											
*Acuticosta ovata**	0.30869	0.34160	0.08253	0.08888	0.07877	0.06846	0.07877	0.07937	0.16184	0.22350	0.18867	0.20848	0.13410	0.12379	0.12343	0.12310	0.14276	0.14249	0.13355											
*Arconaia lanceolata**	0.34670	0.36754	0.10038	0.10628	0.07877	0.07581	0.07877	0.05062	0.16634	0.22792	0.21958	0.20075	0.12661	0.14018	0.14031	0.14224	0.17323	0.14306	0.13845	0.08290										
*Cuneopsis pisciculus**	0.36050	0.38193	0.08738	0.09258	0.10160	0.08176	0.10160	0.12375	0.19684	0.22670	0.17722	0.19088	0.13696	0.16104	0.17118	0.16286	0.15947	0.17803	0.14840	0.10529	0.11517									
*Hyriopsis cumingii**	0.33570	0.34250	0.19462	0.20110	0.17761	0.17763	0.17761	0.19348	0.15962	0.11796	0.11138	0.00590	0.18849	0.23898	0.24559	0.25604	0.20348	0.21567	0.16845	0.20011	0.19266	0.18288								
*Lamprotula leai**	0.37507	0.38201	0.20152	0.20077	0.23449	0.21775	0.23449	0.22447	0.14990	0.10162	0.13761	0.13945	0.25523	0.25326	0.25442	0.26196	0.26311	0.26850	0.19652	0.20760	0.23449	0.22250	0.13228							
*Lepidodesma languilati**	0.32088	0.35445	0.08723	0.09631	0.06569	0.05990	0.06569	0.08310	0.16661	0.20592	0.20450	0.18068	0.13867	0.14703	0.15309	0.15741	0.15387	0.13786	0.13459	0.08306	0.08757	0.10190	0.17280	0.22468						
*Ptychorhynchus ptisteri**	0.35432	0.36094	0.18089	0.18837	0.17626	0.17117	0.17626	0.18148	0.04873	0.18924	0.18820	0.18435	0.20421	0.20421	0.20130	0.19226	0.21958	0.22582	0.19310	0.17687	0.18141	0.18089	0.17655	0.15710	0.18173					
*Schistodesmus lampreyanus**	0.30122	0.33328	0.06968	0.07722	0.05316	0.04320	0.05316	0.08783	0.17142	0.21092	0.20949	0.21287	0.14327	0.14820	0.15271	0.15210	0.14840	0.14249	0.16371	0.06122	0.09695	0.09289	0.20450	0.22884	0.07047	0.17626				
*Sinanodonta woodiana**	0.33995	0.36050	0.10994	0.12500	0.11937	0.11779	0.12418	0.12086	0.17803	0.24253	0.17898	0.20417	0.08685	0.06670	0.07695	0.08347	0.11548	0.07470	0.07096	0.10529	0.11937	0.13879	0.19580	0.24021	0.12446	0.17761	0.13387			
*Solenaia oleivora**	0.34983	0.36335	0.19721	0.19428	0.19189	0.18664	0.19189	0.20468	0.14031	0.13845	0.10600	0.13633	0.20093	0.21033	0.21588	0.22326	0.22644	0.20258	0.18173	0.19266	0.20183	0.21351	0.13387	0.15656	0.20847	0.17204	0.21351	0.20897		
Neotrigonia (Outgroup)*	0.41040	0.41993	0.41742	0.42205	0.42098	0.40632	0.42473	0.41451	0.42375	0.45120	0.43340	0.44160	0.41118	0.40707	0.39479	0.43364	0.42828	0.38279	0.41218	0.40439	0.43702	0.45701	0.44552	0.43891	0.39180	0.45134	0.38632	0.41656	0.45639	0.05565

*indicates specimens, DNA sequences of which were obtained from the DDBJ. **indicates specimens, DNA sequences of which were determined in this study and obtained from the DDBJ.

vestigated the molecular phylogeny of Japanese unionoid taxa; thus, this study is the first to do so and the first to evaluate the current classification of Japanese Unionoida. Since our genetic data and Kondo's morphological data present consistent phylogenetic relationships, we conclude that mitochondrial 16S rDNA is useful for assessing relationships among invertebrate animals, including unionoid mussels, as has been described before [9] [31].

Since Japanese unionoid mussels seem to have evolutionary origins and common ancestors in the East Asian continent, we investigated the phylogenetic relationships between Japanese and East Asian unionoids. Huang *et al.* [22] investigated the phylogenetic relationships of Chinese unionoids using 16S rDNA sequences and presented similar results to ours despite the addition of Japanese unionoids in our study, confirming reliability of our sequences. However, they insisted that Chinese unionids, formerly classified into two subfamilies, should be divided into three: the Unioninae comprising seven species (*Nodularia douglasiae nipponensis*, *Cuneopsis pisciculus*, *Lepidodesma languilati*, *Schistodesmus lampreyanus*, *Arconaia lanceolate*, *Lanceolaria grayii*, and *Acuticosta ovata*), the Anodontinae comprising two species (*Sinanodonta woodiana* and *Cristaria plicata*), and the Ambleminae comprising four species (*Ptychorhynchus ptisteri*, *Hyriopsis cumingii*, *Lamprotula leai*, and *Solenaia oleivora*). The subfamily Ambleminae corresponded to the subfamily Gonideinae by Kondo, and the subfamilies Unioninae and Anodontinae together corresponded to the subfamily Unioninae by Kondo [4].

Recently, Lopes-Lima *et al.* [11] analyzed a combined dataset of mitochondrial COI + nuclear 28S rDNA sequences and classified global unionoids. According to their classification, the mussels in Kondo's Unioninae were separated into two subfamilies, the Unioninae and Anodontinae. In this study, mussels included in Kondo's Unioninae were genetically divided into two clades. However, the clades were not assigned to the two subfamilies of Lopes-Lima *et al.* because the position of the genus *Lanceolaria* was inconsistent between their study and ours.

Many unionoid mussels are facing extinction [13] [14] [15] [16], and freshwater mussels have close associations with other freshwater organisms thus offering important information for identifying hotspots where biodiversity is high but is being destroyed due to human activities [32]. However, it is only when classification is well established and genetic diversity has been sufficiently investigated that this important information can be obtained. Therefore, the precise classification and evaluation of genetic diversity is indispensable for conserving Japanese unionoid mussels [33] [34]. The present study provides useful information that can be used for the conservation of endangered mussels and for promoting their protection.

This study is a first report presenting phylogenetic relationships of all Japanese unionoid species. We summarized our important findings concerning the phylogeny and classification as follows: (1) the order Unionoida formed a clade, (2) unionoid mussels were divided into two clades corresponding to the two families, Margaritiferidae and Unionidae, (3) unionid mussels were divided into

two clades corresponding to the two subfamilies, Unioninae and Gonideinae, (4) unionine mussels were further divided into two clades, (5) most genera and species are monophyletic, (6) the phylogenetic relationships obtained in this study fundamentally supported the systematics of Japanese unionoids proposed by Kondo [4] that are based on morphological and larval traits. Some uncertainties were detected among the phylogenetic relationships of Japanese unionoid mussels, although our study demonstrated that 16S rDNA was useful for deducing these relationships. Therefore, further studies are needed using more specimens obtained from different localities and using other genes in addition to the 16S rDNA gene. More refined morphological and physiological studies are also necessary. Sequencing of the COI gene is in progress to obtain more robust phylogenetic relationships and to enable molecular barcoding of the species, and we have been obtaining promising data fundamentally consistent with those presented in this study.

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