Evolutionary History and Diversity of Unionoid Mussels (Mollusca: Bivalvia) in the Japanese Archipelago

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Abstract: The evolutionary history and diversity of unionoid mussels in East Asia need to be clarified and would shed light on the formation process of the unique fauna of Japan. Unionoid mussels (Mollusca: Bivalvia) are unique models for understanding the process by which organisms have diversified before and after the formation of the Japanese archipelago. Unionoid mussels have poor dispersal ability, so it is thought that they would have been strongly influenced by the archipelago's formation. Therefore, the speciation and diversification processes of mussels before and after the archipelago's formation were investigated by analyzing the nuclear and mitochondrial DNA of a wide range of species, particularly those inhabiting East Asia. The evolutionary history and divergence time of these mussels were examined. Unionoid mussels were found to have higher endemism than other freshwater organisms. Although most of the endemic unionoid mussels of Japan are likely to have diverged before the formation of the Japanese archipelago, some other Japanese unionoid mussel species, including species endemic to Lake Biwa, an ancient lake in Japan, potentially diverged after the Japanese archipelago began to separate from the continent. This suggests that adaptation to the unique habitat of the ancient lake has caused diversification in the mussels endemic to it.

Key words: divergence time, Margaritiferidae, nuclear DNA, phylogeny, Unionidae

Introduction

Located at the eastern end of the Eurasian continent, the Japanese archipelago is a biodiversity hotspot (Ceballos & Brown 1995; Kerswell 2006; Fonseca 2009). The high biodiversity of the Japanese archipelago is seemingly due to the complex formation history of the Japanese fauna (Motokawa & Kajihara 2016). Japanese freshwater fauna also have a complex formation history (Watanabe et al. 2006; Tojo et al. 2017). 1) The formation of the Japanese archipelago dates back to about 15 million years ago (Hamano & Tosha 1985; Torii et al. 1985; Jolivet 1992). 2) Af-

ter the formation of the archipelago, the freshwater fauna appeared to have interacted with continental fauna several times owing to the glacial-interglacial cycle (Cronin et al. 1994). 3) The Japanese archipelago has been historically affected by various climatic and environmental conditions because it is located in a wide latitude range spanning more than 3,000 km from north to south and has climate zones ranging from subarctic to subtropical (Kubota et al. 2017; Tojo et al. 2017). 4) Some regions and taxa in the archipelago are thought to have gone through large-scale extinctions (Nakajima 1986; Nakajima 1987; Watanabe & Uyeno 1999).

In contrast, the histories of the geographical formation and the regional fauna of each taxon are not always consistent. It is important to understand whether the formation of

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freshwater fauna has been influenced mainly by common factors such as geographic isolations, or whether the dispersal capacity, interspecific relationships, and randomness of individual taxa have shaped freshwater fauna beyond such commonalities (Arbogast & Kenagy 2001).

To clarify this, it is important to construct and compare the molecular phylogenies of various taxa collected from both the Asian continent and the Japanese archipelago. However, reports of comparative studies that consider the historical relationship between organisms living on the Asian continent and those living in the Japanese archipelago have not been fully accumulated. Many studies using several freshwater organisms (Insect: Suzuki et al. 2014; Saito et al. 2016, 2018. Fish: Miyazaki et al. 2011; Gao et al. 2012; Tominaga et al. 2016; Tsao et al. 2016; Kano et al. 2018. Mollusca: Park & Kim 2003; Yamada et al. 2010; Hirano et al. 2015, 2019; Saito et al. 2018a, 2018c) have been conducted to verify the diversity of fauna in East Asia, mainly that of fauna in the Japanese archipelago. However, the evolutionary relationship between the fauna of the Asian continent and the Japanese archipelago has not been substantially clarified, with most studies having mainly used samples from the Japanese archipelago and only a few samples from the Asian continent. In recent years, studies using cyprinid fishes and planorbid snails have examined the process of faunal diversification using sufficient continental samples as well as samples from the Japanese archipelago (Saito et al. 2018b; Jang-Liaw et al. 2019). Small freshwater snails, such as planorbids, are passive dispersers that disperse across water systems via birds and within water systems via fishes (Rees 1965; Boag 1986; Wesselingh et al. 1999; Green & Figuerola 2005; Kappes & Haase 2012; van Leeuwen & van der Velde 2012; van Leeuwen et al. 2012), whereas fishes are active dispersers that do not move outside water systems. Unionoid mussels are also passive dispersers that migrate within water systems; their larvae can disperse by attaching to fishes, though they do not move after larval settlement to the bottom of a water body. Levels of gene flow among populations of unionoid mussels are likely to reflect their passive dispersal ability. In addition, bitterling fishes have a unique trait of laying eggs in the gills of unionoid mussels, whereas unionoid larvae need to parasitize mainly freshwater fishes other than bitterlings (Smith et al. 2000a, 2000b, 2001; Mills & Reynolds 2002a, 2002b; Itoh et al. 2003; Kitamura 2005, 2006a, 2006b; Mills et al. 2005; Reichard et al. 2006, 2007, 2010). Thus, unionoid mussels, unlike cyprinid fishes and planorbid snails, form close symbiotic relationships with other freshwater organisms and are central to biological interactions in freshwater. The evolutionary history of these mussels, therefore, appears to reflect more strongly the history of geographic formation than that of other freshwater organisms and is therefore a good model for examining the relationship between the formations of fauna and geography.

There are many freshwater-mussel species that have not

been analyzed or have been studied only piecemeal, and there is an increasing need for more accurate research on individual taxa. Kano et al. (2019) reported a phylogenetic tree of specimens in Japan based on mitochondrial 16S rDNA, but the positions of almost all clades on the tree, except those of species-level clades, were poorly supported. Furthermore, the samples they used were limited to those from Kyushu and the Ryukyu Islands, making it difficult to understand the diversification process of unionoid mussels in the Japanese archipelago.

In contrast, Sano et al. (2017) constructed phylogenetic trees using species living across the Japanese archipelago and evaluated the current systematics of Japanese unionoid mussels. However, the evolutionary relationships between mussels living in continental East Asia and those living in the Japanese archipelago remain unclear, as do the changes in unionoid divergence before and after the archipelago's formation. Therefore, it is necessary to analyze nuclear and mitochondrial DNA using a comprehensive set of species inhabiting the Asian continent and the archipelago.

In this study, we estimated phylogenetic relationships based on nuclear and mitochondrial sequences. In addition, we estimated the divergence times of East Asian unionoid mussel species to elucidate how unionoid mussels diversified before and after the formation of the Japanese archipelago.

Materials and Methods

Materials

Seventy-three unionoid specimens were collected in Japan and preserved in 99.5% ethanol. Prior to our genetic study, we identified specimens based on the morphological features of the shell and larvae described by Kondo (2008, 2015) and Lopes-Lima et al. (2017) and assigned them to Margaritiferidae (2 species) and Unionidae (16 species). In addition, we selected specimens from each species belonging to the same families as those living in the Japanese archipelago and obtained their data from the GenBank database. Detailed information on the specimens and sample abbreviations, including those of samples used by Sano et al. (2017), are given in Table 1.

DNA sequencing

To prepare samples for sequencing, we removed the foot muscle from each unionoid mussel and boiled it at 100°C. Then, total DNA was extracted using a DNeasy® Blood & Tissue Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol. PCRs for nuclear 28S rDNA using primers, 28S-RD1.3f and 28S-rD4b (Whiting 2002), were conducted under the following condition: initial denaturation at 95°C for 3 min; 35 cycles of denaturation at 95°C for 45 sec, annealing at 60°C for 3 min, and extension at 72°C for 30 sec; followed by a final extension at 72°C for 7 min. Mitochondrial COI DNA was amplified

Table 1. Specimens used in phylogenetic analyses (*: data obtained from GenBank).

#	Family	Species	Sample Ab.	Locality	Accession No. of mitochondrial COI DNA	Accession No. of mitochondrial 16S rDNA	Accession No. of nuclear 28S rDNA
U1	Margaritiferidae	<i>Margaritifera laevis</i>	Ma14-1SUTO	Gujo, Gifu, Japan	LC518956	LC223972*	LC519050
U2			Ma14-2SUTO	Gujo, Gifu, Japan	LC518957	LC223973*	
U3			Ma14-3SUTO	Gujo, Gifu, Japan	LC518958	LC223974*	
U4			Ma-u2	Teshiogawa River, Hokkaido, Japan	LC518959	LC519029	LC519051
U5			—	Iwaizumi, Japan	KU763223*	EU590915*	
U6			—	Iwaizumi, Honshu, Japan	KU763221*	EU590914*	
U7		<i>Margaritifera togakushiensis</i>	—	Iwaizumi, Town, Honshu, Japan	KU763222*	KU763192*	LC519052
U8			Mt32-04	Togakushi, Nagano, Japan	LC518960	LC519030	
U9			Mt-k	Togakushi, Nagano, Japan	LC518961	LC224020*	
U10			—	Togakushi, Nagano, Japan	KU763244*	KU763215*	LC519053
U11		<i>Margaritifera marrianae</i>	—	Togakushi, Nagano, Japan	KU763245*	KU763216*	
U12			—	Hunter, Creek, Alabama, USA	KU763243*	KU763214*	
U13		<i>Margaritifera hembeli</i>	—	Valentine, Creek, Louisiana, USA	KU763218*	KU763189*	MG595548*
U14		—	—	Brown, Creek, Louisiana, USA	KU763219*	KU763190*	
U15		<i>Margaritifera dahurica</i>	—	Komissarovka River, Primorye, Terr, Russia	AY579123*	KF514426*	
U16		<i>Margaritifera margaritifera</i>	—	Carrabassett River, Maine, USA	KU763234*	KU763203*	
U17		—	—	Thurma River, Kola Peninsula, Russia	AF303334*	AF303296*	
U18		—	—	Locust Creek, Pennsylvania, USA	KU763227*	KU763196*	
U19		—	—	Regen River, Germany	KU763235*	KU763204*	
U20		—	—	Nore River, Ireland	AF303343*	AF303302*	
U21		—	—	Nore River, Ireland	AF303342*	AF303301*	
U22		—	—	Dereen River, Hacketstown, Ireland	KU763237*	AF303293*	
U23		—	—	Varzuga River, Kola Peninsula, Russia	KU763238*	KU763208*	
U24		—	—	Varzuga River, Kola Peninsula, Russia	KU763239*	KU763209*	
U25		—	—	Suomujoki, Finland	KU763230*	KU763199*	
U26		—	—	Salmon Stream, Maine, USA	KU763232*	KU763201*	
U27		—	—	Salmon Stream, Maine, USA	KU763233*	KU763202*	
U28		<i>Margaritifera rochechouartii</i>	—	Nanxinxiang, Gan River, Jiangxi, China	MF072499*	MF072506*	
U29			—	Nanxinxiang, Gan River, Jiangxi, China	MF072498*	MF072505*	
U30			—	Nanxinxiang, Gan River, Jiangxi, China	MF072500*	MF072507*	
U31			—	Nanxinxiang, Gan River, Jiangxi, China	MF072501*	MF072508*	
U32			—	Nanxinxiang, Gan River, Jiangxi, China	MF072502*	MF072509*	
U33			—	Nanxinxiang, Gan River, Jiangxi, China	—	—	
U34		<i>Margaritifera laosensis</i>	—	Mun River, Thailand	KU763224*	KU763193*	
U35		—	—	Luang Prabang, Laos	KU763225*	KU763194*	
U36		<i>Margaritifera auricularia</i>	—	Canal Imperial Zaragoza, Spain	AF303309*	AF303274*	
U37			—	Ebro River, Tarragona, Spain	AY579125*	AY579083*	
U38			—	Canal Imperial Zaragoza, Spain	AF303313*	AF303278*	
U39		<i>Margaritifera marocana</i>	—	Oum Er Rbia River, Dange Bradia, Morocco	EU429677*	EU429687*	
U40			—	Oum Er Rbia River, Dange Bradia, Morocco	EU429678*	EU429689*	
U41			—	Abid River, Imadahine Oued Abid, Morocco	EU429679*	EU429691*	
U42		<i>Margaritifera falcata</i>	—	Idaho, USA	AY579128*	AY579085*	
U43		<i>Cumberlandia monodonta</i>	—	Missouri, USA	AY579131*	AY579089*	
U44	Unionidae	<i>Scabies crispata</i>	—	Southeast Asia	KP795023*	KX713253*	
U45		<i>Nodularia douglasiae</i>	BIWATATE 3	Lake Biwa, Shiga, Japan	LC518962	LC223961*	LC519054
U46			KAWATATE 2	Lake Kawaguchiko, Yamanashi, Japan	LC518963	LC223964*	
U47			KAWATATE 6	Lake Kawaguchiko, Yamanashi, Japan	LC518964	LC223965*	
U48		—	Un40-01	Wakayama, Japan	LC518965	LC223975*	LC519055
U49		—	Un40-02	Wakayama, Japan	LC518966	LC223976*	
U50		—	Un43-06f	Nakama, Fukuoka, Japan	LC518967	LC223977*	
U51		—	Un43-07f	Nakama, Fukuoka, Japan	LC518968	LC223978*	LC519055
U52		—	Un-u5	Yodo River, Osaka, Japan	LC518969	LC519031	
U53		—	Un-u6	Mukogawa River, Hyogo, Japan	LC518970	LC519032	
U54		—	—	China	NC026111*	NC026111*	MG595555*
U55		—	—	China	KM657954*	KM657954*	
U56		—	—	Gan River, Jiangxi, China	—	—	
U57		<i>Unio delphinus</i>	—	Portugal	NC033854*	NC033854*	MG595555*
U58		<i>Unio pictorum</i>	—	England, UK	KC429109*	KC429266*	
U59		<i>Unio crassus</i>	—	Europe	NC033976*	NC033976*	

Table 1. Continued.

#	Family	Species	Sample Ab.	Locality	Accession No. of mitochondrial COI DNA	Accession No. of mitochondrial 16S rDNA	Accession No. of nuclear 28S rDNA
U60		<i>Cuneopsis pisciculus</i>	—	China	NC026306*	NC026306*	
U61		<i>Cuneopsis celtiformis</i>	—	Gan River, Jiangxi, China			MG595492*
U62		<i>Lamprotula gottschei</i>	—	China	NC023806*	NC023806*	
U63		<i>Inversiunio reinianus</i>	Ir07-01	Lake Biwa, Shiga, Japan	LC518971	LC223979*	
U64			Ir07-k10	Lake Biwa, Shiga, Japan	LC518972	LC519033	LC519056
U65			Ir07-k11	Lake Biwa, Shiga, Japan	LC518973	LC519034	LC519057
U66			Ir07-k12	Lake Biwa, Shiga, Japan	LC518974	LC519035	
U67			Ir07-k13	Lake Biwa, Shiga, Japan	LC518975	LC519036	
U68			Ir07-k14	Lake Biwa, Shiga, Japan	LC518976	LC519037	
U69		<i>Inversiunio jokohamensis</i>	Ij25-01	Sakai, Yamagata, Japan	LC518977	LC223980*	
U70			Ij25-09	Sakai, Yamagata, Japan	LC518978	LC223981*	LC519058
U71			Ij21-28f	Lake Anenuma, Aomori, Japan	LC518979	LC223982*	LC519059
U72			Ij08-01	Lake Kitaura, Ibaraki, Japan	LC518980	LC223984*	
U73			NiiYoko1	Niigata, Japan	LC518981	LC519038	
U74			NiiYoko2	Niigata, Japan	LC518982	LC519039	
U75			NiiYoko3	Niigata, Japan	LC518983	LC519040	
U76			NiiYoko4	Niigata, Japan	LC518984	LC519041	
U77			NiiYoko6	Niigata, Japan	LC518985	LC519042	
U78		<i>Inversiunio yanagawensis</i>	Iy09-10	Gion, Okayama, Japan	LC518986	LC223987*	LC519060
U79			Iy43-01	Fukuoka, Japan	LC518987	LC223988*	
U80			Iy43-05f	Fukuoka, Japan	LC518988	LC223989*	LC519061
U81		<i>Lanceolaria grayii</i>	Lg04-01SUTO	Hiroshima, Japan	LC518989	LC223990*	LC519062
U82			Lg04-02SUTO	Hiroshima, Japan	LC518990	LC223991*	
U83			Lg14-01SUTO	Gifu, Japan	LC518991	LC223992*	
U84			Lg14-2f	Gifu, Japan	LC518992	LC223993*	LC519063
U85			Lg-u3	Shiga, Japan	LC518993	LC519043	
U86			Lg-u4	Shiga, Japan	LC518994	LC519044	
U87		<i>Arconaia lanceolata</i>	—	China	NC023955*	NC023955*	
U88			—	Dongting Lake, Hunan, China			MG595478*
U89		<i>Lanceolaria grayana</i>	—	China	NC026686*	NC026686*	
U90		<i>Lanceolaria gladiola</i>	—	Gan River, Jiangxi, China			MG595534*
U91		<i>Lepidodesma languilati</i>	—	China	NC029491*	NC029491*	
U92			—	Gan River, Jiangxi, China			MG595544*
U93		<i>Aculamprotula tientsinensis</i>	—	China	NC029210*	NC029210*	
U94			—	Nanxinxiang Gan River, Jiangxi, China	MF072504*	MF072511*	
U95		<i>Lamprotula coreana</i>	—	Korea	NC026035*	NC026035*	
U96		<i>Lamprotula tortuosa</i>	—	China	KC109779*	KC109779*	
U97		<i>Lamprotula caveata</i>	—	Qinglan Lake, Jiangxi, China			MG595513*
U98		<i>Obovalis omiensis</i>	Oo14-01 m	Gifu, Japan	LC518995	LC223994*	
U99			Oo16-01SUTO	Kyoto, Japan	LC518996	LC223995*	LC519064
U100			Oo-u1	Shiga, Japan	LC518997	LC519045	LC519065
U101		<i>Ptychorhynchus pfisteri</i>	—	China	KY067440*	KY067440*	
U102			—	Xiangyin, Hunan, China			MG595562*
U103		<i>Pronodularia japonensis</i>	Pj25-06	Sakai, Yamagata, Japan	LC518998	LC223996*	
U104			Pj14-02f	Gifu, Japan	LC518999	LC223997*	
U105			Pj14-05f	Gifu, Japan	LC519000	LC223998*	LC519066
U106			Pj04-01SUTO	Hiroshima, Japan	LC519001	LC224001*	LC519067
U107			Pj04-03SUTO	Hiroshima, Japan	LC519002	LC224002*	
U108			Pj-k	Sakurai, Nara, Japan	LC519003	LC224019*	
U109		<i>Potomida littoralis</i>	—	Mirandela Abreiro, Portugal	NC030073*	NC030073*	
U110			—	Cadiz, Spain	KP217871*	KP217981*	
U111			—	Cadiz, Spain	KP217873*	KP217983*	
U112			—	Cadiz, Spain	KP217872*	KP217982*	
U113		<i>Lamprotula leai</i>	—	China	NC023346*	NC023346*	
U114			—	Qinglan Lake, Jiangxi, China	MF072503*	MF072510*	
U115		<i>Inversidens brandti</i>	Ib14-01f	Gifu, Japan	LC519004	LC224003*	LC519068
U116			Ib14-02f	Gifu, Japan	LC519005	LC224004*	LC519069
U117		<i>Sinohyriopsis schlegeli</i>	Hs21-02f	Lake Anenuma, Aomori, Japan	LC519006	LC224005*	LC519070
U118			Hs21-05f	Lake Anenuma, Aomori, Japan	LC519007	LC224006*	LC519071

Table 1. Continued.

#	Family	Species	Sample Ab.	Locality	Accession No. of mitochondrial COI DNA	Accession No. of mitochondrial 16S rDNA	Accession No. of nuclear 28S rDNA
U119		<i>Sinohyriopsis cumingii</i>	—	China	HQ641406*	HQ641406*	
U120			—	China	HM347668*	HM347668*	
U121			—	Poyang Lake, Jiangxi, China			MG595611*
U122		<i>Lampsilis cardium</i>	—	Illinois, USA	KX713472*	KX713226*	
U123		<i>Cristaria plicata</i>	Cp21-10f	Lake Anenuma, Aomori, Japan	LC519008	LC224007*	
U124			Cp21-11f	Lake Anenuma, Aomori, Japan	LC519009	LC224008*	LC519072
U125			Cp31-01fmg	Joetsu, Niigata, Japan	LC519010	LC224009*	
U126			YAMAKARA 1	Lake Yamanakako, Yamanashi, Japan	LC519011	LC223968*	
U127			YAMAKARA 2	Lake Yamanakako, Yamanashi, Japan	LC519012	LC223969*	
U128			YAMAKARA 5	Lake Yamanakako, Yamanashi, Japan	LC519013	LC223971*	LC519073
U129			YAMAKARA 6	Lake Yamanakako, Yamanashi, Japan	LC519014	LC223970*	
U130			—	Zhejiang, China	FJ986302*	FJ986302*	
U131			—	Gan River, Jiangxi, China			MG595485*
U132		<i>Sinanodonta lauta</i>	fk168	Ishikawa, Japan	LC519015	LC224010*	LC519074
U133			KONZAISYU E	Lake Biwa, Shiga, Japan	LC519016	LC223967*	
U134			FUKUNUMA 22	Minamisoma, Fukushima, Japan	LC519017	LC223966*	LC519075
U135		<i>Sinanodonta japonica</i>	fk20f	Kyoto, Japan	LC519018	LC224011*	LC519076
U136			fk35f	Kushiro, Hokkaido, Japan	LC519019	LC224012*	LC519077
U137			fk59f	Kagawa, Japan	LC519020	LC519046	
U138		<i>Sinanodonta calipygos</i>	fk221	Lake Biwa, Shiga, Japan	LC519021	LC224013*	LC519078
U139			fk222	Lake Biwa, Shiga, Japan	LC519022	LC519047	LC519079
U140		<i>Sinanodonta ogurae</i>	fk156	Yodo River, Osaka, Japan	LC519023	LC224015*	LC519080
U141			fk-o1	Osaka, Japan	LC519024	LC519048	LC519081
U142		<i>Sinanodonta woodiana</i>	—	China	NC024943*	NC024943*	
U143		<i>Sinanodonta lucida</i>	—	Asian continent	NC026673*	NC026673*	
U144		<i>Sinanodonta angula</i>	—	Anren County, Hunan, China			MG595576*
U145		<i>Anemina arcaeiformis</i>	fk63f	Kagawa, Japan	LC519025	LC224014*	LC519082
U146			fk90f	Ishikari, Hokkaido, Japan	LC519026	LC519049	LC519083
U147			—	Asian continent	KF667530*	KF667530*	
U148			—	Qinglan Lake, Jiangxi, China			MG595464*
U149		<i>Anemina euscaphys</i>	—	China	NC026792*	NC026792*	
U150		<i>Pletholophus tenuis</i>	Pt43-02	Munakata, Fukuoka, Japan	LC519027	LC224017*	LC519084
U151			Pt43-03	Munakata, Fukuoka, Japan	LC519028	LC224018*	LC519085
U152		<i>Alasmidonta varicosa</i>	—	Connecticut River, Croyden, Brook, USA	MG938673*	MG938673*	
U153		<i>Acuticosta chinensis</i>	—	Gan River, Jiangxi, China			MG595450*
U154		<i>Aculamprotula tortuosa</i>	—	Qinglan Lake, Jiangxi, China			MG595443*
U155		<i>Alasmidonta heterodon</i>	—	USA	NC037431*	NC037431*	
U156		<i>Lasmigona compressa</i>	—	USA	HM856638*	HM856638*	
U157		<i>Pyganodon grandis</i>	—	USA	FJ809754*	FJ809754*	
U158		<i>Utterbackia imbecillis</i>	—	USA	HM856637*	HM856637*	
U159		<i>Utterbackia peninsularis</i>	—	USA	HM856636*	HM856636*	
U160		<i>Anodonta anatina</i>	—	Poland	NC022803*	NC022803*	
U161		<i>Anodonta cygnea</i>	—	Germany	NC036488*	NC036488*	
U162		<i>Schistodesmus lampreyanus</i>	—	Gan River, Jiangxi, China			MG595569*
U163		<i>Solenia oleivora</i>	—	Poyang Lake, Jiangxi, China			MG595618*
U164	Hyridae	<i>Hyridella australis</i>	BivAToL-378	Australia, New South Wales	KX713467*	KX713224*	
U165		<i>Velesunio ambiguus</i>	—	Australia, New South Wales	KC429106*	KC429263*	
U166		<i>Triplodon corrugatus</i>	BivAToL-380	Peru	KX713505*	KX713262*	
U167	Mycetopodidae	<i>Anodontites elongata</i>	BivAToL-323	Peru	KX713444*	KX713190*	
U168		<i>Lamproscapha ensiformis</i>	BivAToL-382	Peru	KX713471*	KX713225*	
U169	Etheriidae	<i>Etheria elliptica</i>	BivAToL-404	Zambia	KX713462*	KX713219*	
U170	Iridinidae	<i>Aspatharia pfeifferiana</i>	BivAToL-330	Zambia	KC429107*	KC429264*	
U171		<i>Chambardia wahlbergi</i>	BivAToL-405	Zambia	KX713448*	KX713202*	
U172		<i>Mutela hargeri</i>	BivAToL-401	Zambia	KX713482*	KX713237*	
U173	Trigoniidae	<i>Neotrigonia margaritacea</i>	—	Australia	KX713243*	KX713243*	
U174		<i>Neotrigonia lamarckii</i>	—	Queensland, Australia	KC429262*	KC429262*	
U175			—	Queensland, Australia			AM779652*

using nested PCR. The first set of PCR reactions was conducted using the sense and antisense primers FWCO1-2F, 5'-CAA ACC TAT CTG GAT AAT CAG AAT ACC GAC GAG G-3' and FWCO2-2R, 5'-TGA GCT TTT GGG GTC AAT TAG GGT TTC A-3' under the following conditions: initial denaturation at 95°C for 3 min; 45 cycles of denaturation at 95°C for 45 sec, annealing at 60°C for 10 min, and extension at 72°C for 1 min; followed by a final extension at 72°C for 7 min. The second set of PCR reactions was performed using the sense and antisense primers HCO2198-1F, 5'-TAC ACT TCA GGA TGA CCA AAA AAC CA-3' and LCO1490-1R, 5'-GGT GAT TGT GTT CTA CTA ATC ATA AGG ATA TTG G-3' under the following conditions: initial denaturation at 95°C for 3 min; 35 cycles of denaturation at 95°C for 45 sec, annealing at 60°C for 3 min, and extension at 72°C for 30 sec; followed by a final extension at 72°C for 7 min.

PCR products for the nuclear 28S and mitochondrial COI were purified using a QIAquick® PCR Purification Kit (QIAGEN GmbH, Hilden, Germany). Sequence reactions were performed using a GenomeLab™DTCS-Quick Start Kit (Beckman Coulter Inc., California, USA) and the same primers for the final 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) according to the manufacturer's instructions. Mitochondrial 16S rDNA was amplified and sequenced as described previously (Sano et al. 2017).

Phylogenetic analysis

Alignments of nuclear 28S rDNA, mitochondrial COI, and 16S rDNA sequences were performed using MUSCLE v3.8 (Edgar 2004). The results were confirmed by visual inspection using MEGA 6.0 (Tamura et al. 2013). Then, trimAl v1.4 (Capella-Gutiérrez et al. 2009) was used to remove regions of the 16S rDNA and 28S rDNA aligned sequences unsuitable for phylogenetic analysis. We constructed trees using 413-bp 28S rDNA. We also used 937-bp concatenated COI DNA+16S rDNA sequences, including 532-bp COI DNA and 405-bp 16S rDNA, for the construction of trees.

Bayesian (BI) trees were constructed using MrBayes5d version 3.1.2.2012.12.13 (Ronquist et al. 2012; Tanabe 2008), based on a model evaluation performed using PartitionFinder v2.1.1 (Lanfear et al. 2016) (Table 2). The Markov chain Monte Carlo (MCMC) lengths for nuclear and mitochondrial DNA were 6×10^6 and 3×10^7 generations, respectively, and we sampled the chain every 1,000 generations. MCMC convergence was assessed by calculating the potential scale reduction factor, and the first 601 and 3,001 generations were discarded for nuclear and mitochondrial DNA, respectively. The program Tracer v. 1.6 (Rambaut et al. 2013) was used to evaluate MCMC chain

Table 2. Partitioning schemes and best fit models identified using PartitionFinder for the nuclear and combined mitochondrial dataset. "+X" was added when base frequencies were estimated.

Partition contents	Model of sequencing evolution: MrBayes and IQ-TREE	Model of sequencing evolution: BEAST
28S	GTR+G	—
COI 1st	GTR+I+G	TRN+I+G+X
COI 2nd	TIM+I	GTR+I+X
COI 3rd	GTR+G	GTR+G+X
16S	GTR+I+G	GTR+I+G+X

convergence and to compute marginal posterior distributions of parameters, after the removal of 10% of the chain as burn-in. In the case of the mitochondrial DNA analysis, when the temperature was the initial setting, the likelihood did not cease, so the temperature was set to 0.15. A maximum-likelihood (ML) tree was constructed using IQ-TREE (Nguyen et al. 2014) based on a model evaluation performed using PartitionFinder v2.1.1 (Lanfear et al. 2016) (Table 2). The tree reliability was evaluated by generating 1,000 bootstrap replicates.

Recent studies have published evidence that could be useful for more accurately estimating the divergence times of unionoid mussels. Fossil records were reported (Huang et al. 2018) and molecular clock rates based on geographic events were estimated for unionoid mussels (Froufe et al. 2016). Fossil records are based primarily on shell morphology. However, because previous studies have shown that there are cases in which the shell morphology and molecular lineage of unionoid mussels are not consistent (Pfeiffer & Graf 2013), there is a possibility of overestimating the divergence time based on fossil records. Thus, we conducted divergence time estimation based on the molecular clock rates in addition to fossil record-based estimation. Both nuclear and mitochondrial sequences of the same individuals have rarely been published, and much more sequence data have been published for mitochondrial genes than for nuclear genes. Therefore, we estimated the divergence time using BEAST2 v2.4.4. (Bouckaert et al. 2014) without the nuclear sequence dataset. The Bayesian inference was based on the substitution rates estimated using geological events (tree prior=Yule process; ngen= 3.6×10^8 ; samplefreq=1,000; clock models=uncorrelated lognormal relaxed clock) and based on molecular clock node calibrations estimated by fossil records (tree prior=Yule process; ngen= 5.4×10^8 ; samplefreq=1,000; clock models=uncorrelated lognormal relaxed clock). When we used substitution rates estimated based on geological events, we made estimates using a substitution rate for COI of $0.265 \pm 0.06\%$ /million years, which was reported by Froufe et al. (2016). Froufe et al. (2016) examined the impact of the Strait of Gibraltar, which appeared around 5.33 Mya (Krijgsman et al. 1999), on the diversification of *Unio* species. Using the

results of this study, they calculated the substitution rates, which are currently the most reliable within Unionoida. In contrast, when we used molecular clock node calibrations estimated based on fossil records, the constraints, as indicated by Huang et al. (2018), were as follows: (1) The tree root height of Palaeoheterodonta (normal distribution prior, mean=475, stdev=2); (2) Margaritiferidae+Unionidae (exponential prior, min=230 Ma, lambda=30); (3) Unionidae (exponential prior, min=152 Ma, lambda=20); (4) The most recent common ancestor (MRCA) of *M. falcata*–*M. laevis* (exponential prior, min=46 Ma, lambda=12.5); (5) The MRCA of *M. dahurica*–*M. margaritifera* (exponential prior, min=34 Ma, lambda=9.3); and (6) The MRCA of *M. marocana*–*M. auricularia* (exponential prior, min=35 Ma, lambda=9.5). Huang et al. (2018) estimated the divergence time, mainly within Margaritiferidae, using fossils of the early Ordovician genus *Noradonta* and the oldest fossil *Shifangella* assigned to Margaritiferidae. We examined convergence and effective sample size (ESS) using Tracer

v. 1.6 (Rambaut et al. 2013). Substitution models of each partition were selected from the available evolutionary models of BEAST2 v2.4.4 (Bouckaert et al. 2014) using PartitionFinder v2.1.1 (Lanfear et al. 2016) (Table 2). We used *Neotrigonia* (KX713243, KC429262, and AM779652) sequences as the outgroup (Table 1).

Results

Fig. 1 shows the phylogenetic relationships among Japanese unionoid mussels based on the sequences of their nuclear 28S rDNA (413 bp). There were 145 variable and 103 informative sites in the 28S tree. The topologies depicted by the BI trees were essentially identical to those depicted by the ML tree. Japanese unionoid mussels were divided into two clades corresponding to the two families Margaritiferidae and Unionidae. In Unionidae, there were two clades corresponding to the subfamilies Unioninae, and Gonideinae. The resolution of distal clades, however,

Fig. 1. Phylogeny of Japanese unionoid mussels based on nuclear 28S rDNA. This phylogenetic tree was constructed based on 413 bp of the nuclear 28S gene. *Neotrigonia* was used as an outgroup. Bayesian inference (BI) posterior probabilities (left) and maximum-likelihood (ML) bootstrap values (right) are specified near the relevant nodes. The operational taxonomic units (OTUs) accompanied by "*" live in the Japanese archipelago. "●" indicates the endemic species in Japan. Nodes, supported by a probability of 1.00/100, were written as "+".

Fig. 3. Time estimation based on substitution rates based on geological events. This phylogenetic tree was constructed based on a total of 937 bp of mitochondrial COI and 16S genes. *Neotrigonia* was used as an outgroup. The gray node bar represents the 95% confidence interval for the divergence time. The operational taxonomic units (OTUs) accompanied by “*” live in the Japanese archipelago. “●” indicates the endemic species in Japan.

Fig. 4. Time estimation based on fossil records. This phylogenetic tree was constructed based on a total of 937 bp of mitochondrial COI and 16S genes. *Neotrigonia* was used as an outgroup. The gray node bar represents the 95% confidence interval for the divergence time. The operational taxonomic units (OTUs) accompanied by “*” live in the Japanese archipelago. “●” indicates the endemic species in Japan. The numbers in parentheses indicate the locations of the constraints used in the analysis.

was poor, and detailed phylogenetic relationships among species and among genera could not be obtained.

Fig. 2 shows the phylogenetic relationships of Japanese unionoids based on combined sequences (937 bp) of the 16S rRNA and COI genes. There were 264 variable and 249 informative sites in the COI and 283 variable and 227 informative sites in the 16S rDNA. The topologies depicted by the BI trees were essentially identical to those depicted by the ML tree. We presented the 16S rDNA tree in our previous study (Sano et al. 2017) and revised it here by adding the newly collected specimens of *Nodularia douglasiae*, *Inversiunio jokohamensis*, *I. reinianus*, *Lanceolaria grayii*, *Anemina arcaeformis*, *Sinanodonta ogurae*, *S. japonica*, *S. calipygos*, *Obovalis omiensis*, *Margaritifera laevis*, and *M. togakushiensis*.

Japanese unionoid mussels were divided into two clades corresponding to two families, the Margaritiferidae and Unionidae. In Unionidae, there were three clades corresponding to the subfamilies Parreysiinae, Unioninae, and Gonideinae. Most clades corresponding to each genus were formed with high statistical support. The only exception was *Sinanodonta* (0.44/79).

Most species living in the Japanese archipelago formed clades with robust statistical supports, even though the specimens of some species were collected from distant localities. Based on Kondo (2008, 2015), species living in the Japanese archipelago can be broadly divided into two types: a) species that are not endemic to the Japanese archipelago and are found on both the Asian continent and Japan, and b) species that are endemic to Japan and occur only in the Japanese archipelago. The above type a) species include *M. laevis*, *A. arcaeformis*, *Pletholophus tenuis*, *Cristaria plicata*, *L. grayii*, and *N. douglasiae*. All these species formed their own clade, except for *A. arcaeformis*. Type b) species include *M. togakushiensis*, *Pronodularia japonensis*, *O. omiensis*, *Inversidens brandti*, *Sinohyriopsis schlegeli*, *S. lauta*, *S. japonica*, *S. calipygos*, *S. ogurae*, *I. jokohamensis*, *I. yanagawensis*, and *I. reinianus*. All these species formed their own clade, except for *S. lauta*, *S. japonica*, *S. calipygos*, and *I. yanagawensis*. Three *Sinanodonta* species, *S. lauta*, *S. calipygos*, and *S. japonica*, did not form their own clades and exhibited complicated relationships with *S. ogurae*.

Two types of divergence time estimations were made: one based on evolutionary rates (Fig. 3) and another based on fossil records (Fig. 4). The divergence times among the major clades were older for those estimated by fossil records than for those by molecular clock rates, but their 95% confidence intervals were mostly overlapped. When we assumed that the Japanese archipelago started to develop at the time of the formation of the Sea of Japan (Figs. 3 and 4); 10 of the 12 endemic Japanese species were found to diverge before the formation of the Japanese archipelago (*M. togakushiensis*, *P. japonensis*, *O. omiensis*, *I. brandti*, *S. lauta*, *S. japonica*, *S. calipygos*, *S. ogurae*, *I. jokohamensis*, and *I. yanagawensis*). In contrast, two species, *S.*

schlegeli and *I. reinianus*, may have diverged after the formation of the Japanese archipelago.

Discussion

In this study, we sequenced all 18 species of the order Unionoida from Japan and examined the evolutionary history and species diversity of the order before and after the formation of the Japanese archipelago using published data for the Asian continent. The results revealed that nuclear sequences did not have as much phylogenetic information as mitochondrial sequences in the case of Unionoida. Nuclear 28S rDNA was suitable for estimating family-level and subfamily-level phylogenetic relationships, but not for estimating lower-order phylogenetic relationships (Fig. 1). Thus, the results based on mitochondrial DNA are discussed.

Unionoid mussels living in the Japanese archipelago were found to be highly endemic. Detailed genetic analysis revealed that most of the 12 species endemic to the Japanese archipelago formed monophyletic groups and differentiated independently (Fig. 2). In contrast, our results also confirmed that some species of the genera *Sinanodonta* and *Inversiunio* showed morphological and molecular phylogenetic inconsistencies, and *Sinanodonta lauta*, *S. japonica*, *S. calipygos*, and *Inversiunio yanagawensis* were not found to form their own clade upon molecular phylogenetic analysis. In general, diverse morphologies are found within several mollusk genera and species, often interfering with species recognition based on the concept of reproductive isolation (Pfeiffer & Graf 2013; Hirano et al. 2015). Furthermore, it is suggested that species within a genus may have undergone plastic changes in morphology as they adapted to their habitat, because each of these genera formed a separate monophyletic group. All species of the genus *Inversiunio* are endemic to Japan: i) *I. jokohamensis* is found mainly east of Lake Biwa; ii) *I. yanagawensis* is found mainly west of Lake Biwa; and iii) *I. reinianus* is found only in Lake Biwa (Kondo 2008, 2015). Judging from the phylogenetic trees in this study, it is suggested that the endemic species of Lake Biwa, *I. reinianus*, was possibly differentiated from *I. yanagawensis*. Furthermore, while *I. yanagawensis* formed a paraphyletic group, the clade of *I. reinianus*+*I. yanagawensis* was supported with high confidence, suggesting that a rapid evolution of morphology has occurred in Lake Biwa. Phylogenetic trees based on the nuclear 28S rDNA did not have sufficient information to elucidate the differentiation process within the genus *Inversiunio*. The results of this study indicate that morphological classification and phylogenetic relationships are not necessarily concordant, as is the case for *Sinanodonta* and *Inversiunio*. It is unclear whether this is due to a plastic change in morphology or because the molecular markers used in this study do not correctly reflect the differentiation among morphological species. Therefore, it is necessary to analyze a large amount of genomic informa-

tion in combination with morphological re-examination to clarify this issue.

The Japanese archipelago is geographically close to the Asian continent, and a lot of lineages of its endemic fauna are closely related to continental lineages. These Japanese lineages may have originated on the Asian continent. *Cristaria plicata* and *Nodularia douglasiae* are distributed widely in East Asia, while they could not be genetically separated between Japan and the Asian continent. This can be explained by “back dispersal” (Tojo et al. 2017) from the Japanese archipelago to the Asian continent. This may also be explained by artificial genetic contamination, as is observed between Japanese *Sinohyriopsis schlegeli* and Chinese *S. cumingii* (Shirai et al. 2010). However, to address this issue, further genetic research is needed.

The Japanese archipelago is geographically close to the Asian continent; however, the margaritiferid mussels in the archipelago are genetically close to species associated with the North American continent. The larvae of unionoid mussels are known to be salt-tolerant while being parasitic to host fishes (Itoh et al. 2017), suggesting that margaritiferid mussels may have invaded the Japanese archipelago from North America by dispersion through ocean currents. The parasitism period of glochidia on fishes has not yet been fully elucidated, but is reported to be approximately twenty five days for *S. schlegeli* (Hatano & Ishizaki 2016) and approximately ten days for *N. douglasiae* (Itoh 2013), and it is believed that unionoid mussels are often parasitic for more than one week. It is also known that the lower the water temperature, the longer the parasitism period (Hurukawa et al. 1965). Therefore, it is considered that the parasitism period becomes longer in environments with low water temperature, such as the Bering Sea, the Sea of Okhotsk, and the Sea of Japan. In future research, it is necessary to examine the parasitism period of glochidia on fishes in detail to investigate whether or not freshwater mussels can disperse across the sea. The larval hosts of the *Margaritifera* in Japan and western North America are the salmonids *Oncorhynchus* and *Salmo* (Taylor & Uyeno 1965). The distribution ranges of these mussels overlap with those of salmonids. Therefore, it is possible that the salmonids carried the glochidia of these mussels from North America to the Japanese archipelago.

The estimations of the divergence time suggest that the unionoid species that are now widely distributed in East Asia diversified on the Asian continent long before the formation of the Japanese archipelago (Figs. 3, 4). The diversification of most Japanese endemic species began before the Japanese archipelago separated from the Asian continent because the Sea of Japan was formed 15 million years ago. Although taxon sampling of continental species might be insufficient to estimate the exact age, this study provides the most reliable estimation for the divergence time of the East Asian unionoid mussels so far; all of the genera and their representative species of the East Asian region described by Lopes-Lima et al. (2017) are included

in this study. An alternative hypothesis is that continental species that are phylogenetically closely related to Japanese extant species have already gone extinct. Previous studies on insect fauna have shown that the formation of the Japanese archipelago caused high species diversity in Japan (Gamboa et al. 2019). Future surveys using fossil records from the Asian continent are needed to address this issue. However, unionoid mussels have proportionally many more endemic species than other organisms such as insects, suggesting that unionoid mussels have a lower dispersal ability than other organisms. Adult unionoid mussels have a lower dispersal ability than other freshwater organisms, and their passive dispersal ability in the larval stage is also limited. The limited passive dispersal ability of unionoid mussels may reflect the low dispersal ability of the fishes that carry parasitic larvae of unionoid mussels. Because of their low dispersal ability, it is most likely that the diversification of Japanese unionoid mussels had started long before the formation of the Japanese archipelago.

Likewise, mussels diverged at earlier times than freshwater fishes and snails. Japanese planorbid snails diverged 6.5 million years ago (mya), whereas Japanese cyprinid fishes diverged around 18 mya (Saito et al. 2018b; Jang-Liaw et al. 2019). In contrast, species of the unionoid genus endemic to Japan were found to have diverged earlier than approximately 60 mya (for example, 63.5 and 86.3 mya for *Inversidens*, as shown in Fig. 3 and in Fig. 4, respectively). This indicates that mussels are genetically more unique than other freshwater organisms.

In contrast, some species may have diverged after the formation of the Sea of Japan (*S. schlegeli* and *I. reinianus*). These species are endemic to Lake Biwa, one of the ancient lakes in Japan. Since Lake Biwa began to form about 4 mya (Satoguchi 2012), the values of the divergence time estimations obtained in this study are within the estimated range of time in which *S. schlegeli* and *I. reinianus* adaptively differentiated to the habitat of Lake Biwa. Two other endemic species of Lake Biwa are known: *S. calipygos* and *S. ogurae*. The results of this study showed that these two species diverged before the formation of the Japanese archipelago. However, *S. ogurae* may have had an excessively large divergence times because of the extinction of related species. In fact, fossils of *Kobiwakodonta nakajimai*, which is considered to be a closely related extinct species of *S. ogurae*, have been excavated from the Kobiwako Group (Nakano et al. 2003). In addition, it was shown that *S. calipygos* is a polyphyletic group (Fig. 2). This result suggests the parallel morphological evolution of *S. calipygos*; however, the cause for this is not yet fully understood and may be the standing variation or low resolution of the trees.

Most freshwater creatures face high risks of extinction (Strayer et al. 2004; Walker et al. 2014). Unionoid mussels are especially likely to suffer the impacts of anthropogenic activities because of their poor dispersal ability and symbiosis with freshwater fishes during their development. This

study could contribute to the promotion of future taxonomic revisions and morphological and molecular phylogenetic and phylogeographic studies, as well as the proposal of scientifically refined plans to conserve unionoids.

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