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 endemicity 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 suggest 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 (Hamanaka & Tosha 1985; Torii et al. 1985; Jolivet 1992). 2) After 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
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 planorbid, 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; Kaptes & 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
| # | Family | Species | Sample Ab. | Locality | Accession No. of mitochondrial COI DNA | Accession No. of mitochondrial 16S rDNA | Accession No. of nuclear 28S rDNA |
|---|---|---|---|---|---|---|---|
| U1 | Margaritiferaeae | Margaritifera laevis | Ma14-1SUTO | Gujo, Gifu, Japan | LC518956 | LC223972* |  |
| U2 | | | Ma14-2SUTO | Gujo, Gifu, Japan | LC518957 | LC223973* | LC519050 |
| U3 | | | Ma14-3SUTO | Gujo, Gifu, Japan | LC518958 | LC223974* |  |
| U4 | | | Ma-u2 | Teeshingawa River, Hokkaido, Japan | LC518959 | LC519029 | LC519051 |
| U5 | | — | — | Iwaiizumi, Japan | KU763223* | EU590915* |  |
| U6 | | — | — | Iwaiizumi, Honshu, Japan | KU763221* | EU590914* |  |
| U7 | | — | — | Iwaiizumi, Town, Honshu, Japan | KU763222* | KU763192* |  |
| U8 | Margaritifera togakushiensis | Mt32-04 | Togakushi, Nagano, Japan | | LC518960 | LC519030 | LC519052 |
| U9 | | Mt-k | Togakushi, Nagano, Japan | | LC518961 | LC224020* | LC519053 |
| U10 | | — | Togakushi, Nagano, Japan | | KU763244* | KU763215* |  |
| U11 | | — | Togakushi, Nagano, Japan | | KU763245* | KU763216* |  |
| U12 | Margaritifera marviana | — | Hunter, Creek, Alabama, USA | | KU763243* | KU763214* |  |
| U13 | Margaritifera hembeli | — | Valentine, Creek, Louisiana, USA | | KU763218* | KU763189* |  |
| U14 | | — | Brown, Creek, Louisiana, USA | | KU763219* | KU763190* |  |
| U15 | Margaritifera dahurica | — | Komissarivka River, Primorye, Terr, Russia | | AY579123* | KF514426* |  |
| U16 | Margaritifera margaritifera | — | Barabassetse 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* | AF303320* |  |
| U21 | | — | Nore River, Ireland | | AF303342* | AF303361* |  |
| U22 | | — | Dereen River, Hacketstown, Ireland | | KU763237* | AF303293* |  |
| U23 | | — | Varraga River, Kola Peninsula, Russia | | KU763238* | KU763208* |  |
| U24 | | — | Varraga River, Kola Peninsula, Russia | | KU763239* | KU763209* |  |
| U25 | | — | Suomojoki, Finnlnd | | KU763230* | KU763199* |  |
| U26 | | — | Salmon Stream, Maine, USA | | KU763232* | KU763201* |  |
| U27 | | — | Salmon Stream, Maine, USA | | KU763233* | KU763202* |  |
| U28 | Margaritifera rochechouartii | — | 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 | | MG595548* |  |  |
| U34 | Margaritifera laosensis | — | Mun River, Thailand | | KU763224* | KU763193* |  |
| U35 | | — | Luang Prabang, Laos | | KU763225* | KU763194* |  |
| U36 | Margaritifera auriculata | — | Canal Imperial Zaragoza, Spain | | AF303309* | AF303274* |  |
| U37 | | — | Ebro River, Tarragona, Spain | | AY579125* | AY579083* |  |
| U38 | | — | Canal Imperial Zaragoza, Spain | | AF303313* | AF303278* |  |
| U39 | Margaritifera marocana | — | Ouat Er Rbia River, Dange Bradia, Morocco | | EU429677* | EU429687* |  |
| U40 | | — | Ouat Er Rbia River, Dange Bradia, Morocco | | EU429678* | EU429689* |  |
| U41 | | — | Abid River, Imnadahine Oued Abid, Morocco | | EU429679* | EU429691* |  |
| U42 | Margaritifera falcata | — | Idaho, USA | | AY579128* | AY579085* |  |
| U43 | Cumberlandia monodonta | — | Missouri, USA | | AY579131* | AY579089* |  |
| U44 | Unionidae | Scabia crispa | — | Southeast Asia | KF795023* | KX713253* |  |
| U45 | Nodularia douglasiae | — | Bwatake 3 | Lake Biwa, Shiga, Japan | LC518962 | LC223961* | LC519054 |
| U46 | | — | Lake Kawaguchiko, Yamanashi, Japan | | LC518963 | LC223964* |  |
| U47 | | — | Lake Kawaguchiko, Yamanashi, Japan | | LC518964 | LC223965* |  |
| U48 | Un-n0-01 | — | Wakayama, Japan | | LC518965 | LC223975* |  |
| U49 | Un-n0-02 | — | Wakayama, Japan | | LC518966 | LC223976* |  |
| U50 | Un-n3-06f | — | Nakama, Fukuoka, Japan | | LC518967 | LC223977* | LC519055 |
| U51 | Un-n3-07f | — | Nakama, Fukuoka, Japan | | LC518968 | LC223978* |  |
| U52 | Un-u5 | — | Yodo River, Osaka, Japan | | LC518969 | LC519031 |  |
| U53 | Un-u6 | — | Mukogawa River, Hyogo, Japan | | LC518970 | LC519032 |  |
| U54 | | — | China | | NCO26111* | NCO26111* |  |
| U55 | | — | China | | KM657954* | KM657954* |  |
| U56 | | — | Gan River, Jiangxi, China | |  | MG595555* |  |
| U57 | Union delphinius | — | Portugal | | NCO33854* | NCO33854* |  |
| U58 | Union pictorum | — | England, UK | | K429109* | K429296* |  |
| U59 | Union crassus | — | Europe | | NCO33976* | NCO33976* |  |
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 | Cuneopsis pisciculus     |                          |              |                                 | NC026306*                             |                                       |                                  |
| U61 | Cuneopsis celiformis     |                          |              |                                 | NC026306*                             |                                       |                                  |
| U62 | Lamprotula gottschei     |                          |              |                                 | NC023806*                             |                                       |                                  |
| U63 | Inversiunio reinianus    | Inr07-01                 | Lake Biwa, Shiga, Japan |                                | LC158971                              | LC223979*                              |                                  |
| U64 | Inversiunio jokohamensis | Ij25-01                  | Sakai, Yamagata, Japan |                                | LC158977                              | LC223980*                              | LC519058 |
| U70 | Inversiunio yanagawensis | Iy09-10                  | Gion, Okayama, Japan |                                | LC158986                              | LC223987*                              | LC519060 |
| U79 | Arcoma lanceolata        |                          |              |                                 | NC023955*                             |                                       |                                  |
| U88 | Lanceolaria grayana      |                          |              |                                 | NC026886*                             |                                       |                                  |
| U90 | Lanceolaria gladiola     |                          |              |                                 | NC026886*                             |                                       |                                  |
| U91 | Lepidodesma languilati   |                          |              |                                 | NC029491*                             |                                       |                                  |
| U92 | Aculamprotula tientinensis |                          |              |                                 | NC029491*                             |                                       |                                  |
| U93 | Lamprotula caveata       |                          |              |                                 | NC029491*                             |                                       |                                  |
| U94 | Obovalis omiensis        |                          |              |                                 | NC029491*                             |                                       |                                  |
| U99 | Ptychorhynchus pfisteri  |                          |              |                                 | NC029491*                             |                                       |                                  |
| U103| Pronodularia japonensis  | Pj25-06                  | Sakai, Yamagata, Japan |                                | LC158998                              | LC223996*                              |                                  |
| U104| Pj14-02f                 | Gifu, Japan               |              |                                 | LC158999                              | LC223997*                              |                                  |
| U105| Pj14-05f                 | Gifu, Japan               |              |                                 | LC159000                              | LC223998*                              | LC519066 |
| U106| Pj04-01SUTO              | Hiroshima, Japan          |              |                                 | LC159001                              | LC224001*                              | LC519067 |
| U107| Pj04-03SUTO              | Hiroshima, Japan          |              |                                 | LC159002                              | LC224002*                              |                                  |
| U117| Sinohyriopsis schlegeli  | Hsz21-02f                | Lake Anemura, Aomori, Japan |                            | LC159006                              | LC224005*                              | LC519070 |
| U118| Hsz21-05f                | Lake Anemura, Aomori, Japan |            |                                 | LC159007                              | LC224006*                              | LC519071 |

* Data obtained from GenBank.
Table 1. Continued.

| # | Family | Species | Sample Ab. | Locality | Accession No. of mitochondrial COI DNA | Accession No. of mitochondrial 16S rDNA | Accession No. of mtDNA 28S rDNA |
|---|--------|---------|------------|----------|--------------------------------------|----------------------------------------|-----------------------------------|
| U119 | Sinohyriopsis cumingii | — | China | | HQ641406* | HQ641406* | |
| U120 | — | China | | | HM347668* | HM347668* | |
| U121 | — | Poyang Lake, Jiangxi, China | | | | | |
| U122 | Lampsis cardium | — | Illinois, USA | | KX713142* | KX713142* | MG95611* |
| U123 | Cristaria plicata | Cp21-10f | Lake Anenuma, Aomori, Japan | LC519008 | LC224007* | LC519072 |
| U124 | Cp21-11f | Lake Anenuma, Aomori, Japan | LC519009 | LC224008* | |
| 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* | |
| U129 | YAMAKARA 6 | Lake Yamanakako, Yamanashi, Japan | LC519014 | LC223970* | |
| U130 | — | Zhejiang, China | FJ986302* | FJ986302* | |
| U131 | — | Gan River, Jiangxi, China | | | MG955485* | |
| U132 | Sinonodonta lauta | fk168 | Ishikawa, Japan | LC519015 | LC224010* | LC519074 |
| U133 | KONZAIYSU E | Lake Biwa, Shiga, Japan | LC519016 | LC223967* | |
| U134 | FUKUNUMA 22 | Minamisoma, Fukushima, Japan | LC519017 | LC223966* | |
| U135 | Sinonodonta japonica | fk20f | Kyoto, Japan | LC519018 | LC224011* | |
| U136 | fk35f | Kushiro, Hokkaido, Japan | LC519019 | LC224012* | |
| U137 | fk59f | Kagawa, Japan | LC519020 | LC519046 | |
| U138 | Sinonodonta calypygus | bk221 | Lake Biwa, Shiga, Japan | LC519021 | LC224013* | |
| U139 | bk222 | Lake Biwa, Shiga, Japan | LC519022 | LC519047 | |
| U140 | Sinonodonta oguare | bk156 | Yodo River, Osaka, Japan | LC519023 | LC224015* | |
| U141 | bk-01 | Osaka, Japan | LC519024 | LC519048 | |
| U142 | Sinonodonta woodiana | — | China | NCO24943* | NCO24943* | |
| U143 | Sinonodonta lucida | — | Asian continent | NCO26673* | NCO26673* | |
| U144 | Sinonodonta angula | — | Anren County, Hunan, China | | | MG95575* |
| U145 | Anemina arcusiformis | fk36f | Kagawa, Japan | LC519025 | LC224014* | |
| U146 | fk90f | Ishikari, Hokkaido, Japan | LC519026 | LC519049 | |
| U147 | — | Asian continent | KF667530* | KF667530* | |
| U148 | — | Qinglan Lake, Jiangxi, China | | | MG95464* | |
| U149 | Anemina eucosphys | — | China | NCO26792* | NCO26792* | |
| U150 | Pletholobus tenais | P43-02 | Munakata, Fukuoka, Japan | LC519027 | LC224017* | |
| U151 | P43-03 | Munakata, Fukuoka, Japan | LC519028 | LC224018* | |
| U152 | Alasmidonta varicosa | — | Connecticut River, Croyden, Brook, USA | MG938673* | MG938673* | |
| U153 | Acuticosta chinensis | — | Gan River, Jiangxi, China | | | MG95450* |
| U154 | Acalamprodula tortuosa | — | Qinglan Lake, Jiangxi, China | | | MG95443* |
| U155 | Alasmidonta heterodon | — | USA | NCO37431* | NCO37431* | |
| U156 | Lasmigona compressa | — | USA | HM856638* | HM856638* | |
| U157 | Pyganodon grandis | — | USA | FJ809754* | FJ809754* | |
| U158 | Utterbackia imbecilla | — | USA | HM856637* | HM856637* | |
| U159 | Utterbackia peninsularis | — | USA | HM856636* | HM856636* | |
| U160 | Anodonta anatina | — | Poland | NCO22803* | NCO22803* | |
| U161 | Anodonta cygnea | — | Germany | NCO36488* | NCO36488* | |
| U162 | Schistodesmus lampeyanus | — | Gan River, Jiangxi, China | | | MG95569* |
| U163 | Solenaia olivora | — | Poyang Lake, Jiangxi, China | | | MG95618* |
| U164 | Hyriidae | Hyridella australis | BivAToL-378 | Australia, New South Wales | KX713467* | KX713224* | |
| U165 | Velenusinio ambiguus | — | Australia, New South Wales | KC429106* | KC429263* | |
| U166 | Tripodon corrugatus | BivAToL-380 | Peru | KX713505* | KX713262* | |
| U167 | Hyridae | Mycetopodidae | Anodontites elongata | BivAToL-323 | Peru | KX713444* | KX713190* |
| U168 | Lamproscapha ensiformis | BivAToL-382 | BivAToL-382 | KX713471* | KX713225* | |
| U169 | Etheriidae | Etheria elliptica | BivAToL-404 | Zambia | KX713462* | KX713219* | |
| U170 | Iriridae | Aspasharia pfeifferiana | BivAToL-330 | Zambia | KC429107* | KC429264* | |
| U171 | Chambradaria wahlbergii | BivAToL-405 | Zambia | KX713448* | KX713202* | |
| U172 | Muteidae | Muteidula harkeri | BivAToL-401 | Zambia | KX713482* | KX713237* | |
| U173 | Trigonidae | Neotrigonia margaritacea | — | Australia | KX713243* | KX713243* | |
| U174 | Neotrigonia lamarkii | — | 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′-GTT GAT TGT GTT CTA CTA ATC ATA AGG ATA TTG G-3′ under the following conditions: initial denaturation at 95°C for 3 min; 30 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 et al. 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, respectively. The program Tracer v. 1.6 (Rambaut et al. 2013) was used to evaluate MCMC chain 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=3.6×10⁶; 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=5.4×10⁵; 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±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

| 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                         |

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.
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. dahuica_–_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. 2. Phylogeny of the Japanese unionoid mussels based on mitochondrial COI and 16S rDNA. This phylogenetic tree was constructed based on a total of 937 bp of mitochondrial COI and 16S genes. *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 rRNA. 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 *Oncorhynus* 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 lower 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. reiniana*). 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. reiniana* 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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