

Rapid Communication**First record of the non-indigenous freshwater snail *Galba humilis* (Say, 1822) (Mollusca: Hygrophila: Lymnaeidae) in Japan**

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OPEN ACCESS**Abstract**

Galba humilis (Say, 1822) is a freshwater mollusc belonging to the family Lymnaeidae, and it is distributed in North America. The present article documented the first record of this mollusc in Japan, which is outside the indigenous distribution range of the species. The species was identified using molecular phylogenetics. This is the first report of *G. humilis* introduction in the world, including Japan.

Key words: alien species, freshwater mollusc, Gastropoda, introduced species, Marsh Fossaria

Introduction

Freshwater molluscs are some of the dominant members of the freshwater ecosystems (Vaughn 2018). Unfortunately, many species are critically endangered because of several threats such as pollution, climate change, and habitat loss (Böhm et al. 2021). Meanwhile, many freshwater molluscs have been introduced and have spread worldwide (Global Invasive Species Database 2021), causing several problems such as biodiversity crises, agricultural damage, and economic loss (Dextrase and Mandrak 2006; Horgan et al. 2014; Sousa et al. 2014).

The superorder Hygrophila mainly comprises freshwater snails, including many cosmopolitan introduced species (Global Invasive Species Database 2021), such as *Physella acuta* (Draparnaud, 1805) – one of the most widespread introduced species (Vinarski 2017). However, these cosmopolitan hygrophilids have not garnered much attention due to their small size and relatively high taxonomic ambiguity, as opposed to major invasive molluscan species such as the golden apple snail *Pomacea canaliculata* (Lamarck, 1822) and the zebra mussel *Dreissena polymorpha* (Pallas, 1771) which have significant impacts on various areas. In recent decades, with the progress of molecular techniques, the dynamics of invasion of hygrophilids has been clarified (Walther et al. 2006; Bargues et al. 2011a; Marrone et al. 2011; Lounnas et al. 2017; Vecchioni et al. 2017; Schniebs et al. 2017; Saito et al. 2018). Furthermore, although recent studies have proposed the adverse

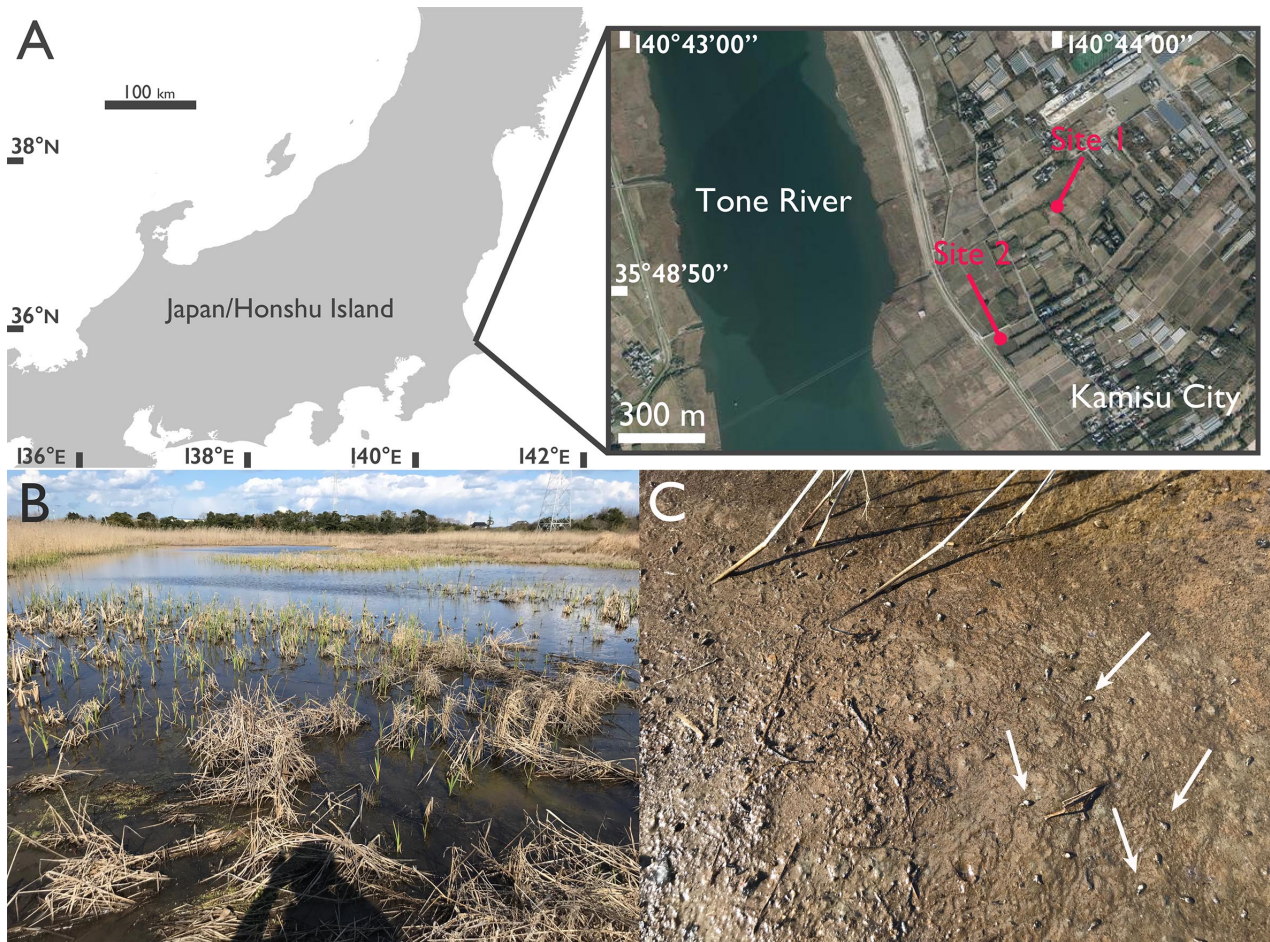


Figure 1. Map and photograph of the collection locality of *Galba humilis* in Japan. A: Map of the collection sites. The blank map was generated using the Global Self-consistent, Hierarchical, High-resolution Geography Database version 2.3.5 (Wessel and Smith 1996, 2016) in QGIS 2.18 (QGIS development team 2016). The aerial map was created using the maps and aerial photographs provided by the Geospatial Information Authority of Japan (2021). B: Habitat at site 1. C: *G. humilis* population at site 1. The arrows indicate *G. humilis* individuals. Photo by Takumi Saito.

effects of hygrophilid invaders on human health and biodiversity (Levy 2004; Vignoles et al. 2018), their specific impacts are not well documented as those of other well-studied major invasive species. Therefore, it is an important task for researchers to monitor the introduction of this often-overlooked taxonomic group. To this end, the present article describes the first record of the introduction of a non-indigenous species belonging to the family Lymnaeidae, identified using molecular phylogenetics, in Japan.

Materials and methods

A lymnaeid species was discovered during fieldwork in Kamisu City, Ibaraki Prefecture, Honshu Island, Japan, on 24 March 2020 (Figure 1A). Numerous individuals inhabiting a wetland and a paddy field were observed (Figure 1B, C). Based on shell morphology, these snails appeared to be different from any other lymnaeid species recorded in Japan (Masuda and Uchiyama 2004; Saito *pers. obs.*). However, the shell morphology of lymnaeid snails is highly variable because of intraspecific polymorphism and phenotypic plasticity (Ward et al. 1997; Pfenninger et al. 2006; Terry

and Duda Jr. 2021); therefore, samples were collected from the wetland (Site 1) for further accurate identification. The efficiency of molecular identification of Lymnaeidae species has been demonstrated previously (Pfenninger et al. 2006; Aksenova et al. 2018; Alda et al. 2018, 2021). Accordingly, three individuals were preserved in 99.5% ethanol for molecular identification after photography and shell measurement.

Molecular identification was performed based on mitochondrial cytochrome oxidase subunit 1 (*COI*), for which numerous reference sequences are available. Total DNA was isolated from the three specimens using the DNeasy Blood & Tissue Kit (Qiagen Inc., Germany), according to the manufacturer's standard protocol. The *COI* region was amplified using the primers and conditions described in Supplementary material Table S1. The polymerase chain reaction (PCR) products were purified using Exo-SAP-IT (Amersham Biosciences), sequenced using the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), and electrophoresed using the ABI 3130xl Genetic Analyzer (Applied Biosystems). The obtained sequences are deposited in GenBank (LC635515–LC635517).

For molecular identification, a phylogenetic tree was constructed based on the *COI* gene and reference sequences in GenBank. Upon preliminary examination, the collected lymnaeid snails appeared to belong to the genus *Galba*. Accordingly, reference sequences were selected as described by Alda et al. (2021) (Table 1) who previously generated a comprehensive molecular taxonomy of *Galba*. Molecular phylogenies were reconstructed using maximum likelihood (ML) and Bayesian inference (BI). Before phylogenetic analyses, each sequence was aligned using MUSCLE (Edgar 2004), and the same haplotypes were collapsed using FaBox (Villesen 2007). ML analysis was performed using IQ-TREE 1.6.7 (Nguyen et al. 2015) with ultrafast bootstrap approximation (UFBoot2) (Hoang et al. 2018), and BI analysis was performed using MrBayes5d 3.1.2 (Ronquist and Huelsenbeck 2003; Tanabe 2012) after model selection using PartitionFinder 2 (Lanfear et al. 2016). The detailed settings of each analysis are described elsewhere (Saito et al. 2020). The selected models and generations of BI analysis are shown in Table S2.

Finally, to examine the dynamics of introduction, a haplotype network was illustrated using POPART (Leigh and Bryant 2015) based on 23 *COI* sequences of identified species registered in GenBank (Table 1). The TCS method was used to illustrate the network (Templeton et al. 1992).

Results

Shell height was 10.1 mm in TUSC_L1605, 12.5 mm in TUSC_L1606, and 11.5 mm in TUSC_L1607, and aperture height was less than half of the shell height (4.76, 5.97, and 5.22 mm, respectively). All specimens showed very high shell spires (Figure 2A). The tentacles were round and unobtrusive, and the foot was yellowish to light olive (Figure 2B). The mantle was black

Table 1. The sample list used for CO1 phylogeny and illustrating of haplotype network. The treatment of taxon name followed Alda et al. (2021) instead of the original registered name. See also original references for further information of locality.

Taxon	Locality	GenBank Acc. No. CO1	Hap. No.	Reference
<i>Galba cousini</i>	Colombia, Bogotá	FN598162	–	Bargues et al. 2011b
	Ecuador, Chanchu-Yacu	FN598161	–	Bargues et al. 2011b
<i>G. cubensis</i>	Colombia, Barbosa (Antioquia)	JN614390	–	Correa et al. 2011
	Cuba, Vaqueria 21	AM494009	–	Bargues et al. 2007
	Uruguay, Paysandú	JN614396	–	Correa et al. 2011
	USA, Charleston County (South Carolina)	JN614395	–	Correa et al. 2011
	Venezuela, Mucura	FN182205	–	Bargues et al. 2011b
	Spain, Ebro Delta, Tarragona, Sant Carles de la Rápita	LT623604	–	Schniebs et al. 2018
<i>G. humilis</i>	USA, San Antonio, New Mexico	KT781340	7	Alda et al. 2021
	USA, Owego, New York	FN182197	1	Correa et al. 2011
	Canada, Parc de l'île de la Visitation	KT781330	6	Alda et al. 2021
	USA, Owego, New York	FN182198	2	Correa et al. 2011
	USA, Anderson, Tennessee	KY612886	2	Alda et al. 2021
	USA, Owego, New York	MG421721	2	GenBank
	USA, Owego, New York	FN182199	3	GenBank
	USA, Angel Fire, New Mexico	KT781293	5	GenBank
	USA, Angel Fire, New Mexico	KT781294	5	Alda et al. 2021
	USA, Angel Fire, New Mexico	KY612851	5	Alda et al. 2021
	USA, Angel Fire, New Mexico	KY612852	5	Alda et al. 2021
	USA, Angel Fire, New Mexico	KY612853	5	Alda et al. 2021
	USA, Beaver Dam, New Mexico	KT781338	7	Alda et al. 2021
	USA, Beaver Dam, New Mexico	KT781339	7	Alda et al. 2021
	USA, Randolph, North Carolina	KY612848	8	Alda et al. 2021
	USA, Randolph, North Carolina	KY612849	9	Alda et al. 2021
	USA, Kerzshaw, South Carolina	KY612861	9	Alda et al. 2021
	USA, Augusta, Virginia	KY612888	10	Alda et al. 2021
	USA, Enamet, Michigan	KY612890	11	Alda et al. 2021
	Canada, Ontario, Essex County, Oxley Swamps	MG422742	12	GenBank
	(TUSC_L1605) Japan, Ibaraki Pref., Kamisu City	LC635515	4	This study
(TUSC_L1606) Japan, Ibaraki Pref., Kamisu City	LC635516	4	This study	
(TUSC_L1607) Japan, Ibaraki Pref., Kamisu City	LC635517	4	This study	
<i>G. meridensis</i>	Venezuela	JN614389	–	Correa et al. 2011
	Venezuela, Laguna Mucubají (Mérida)	FN598164	–	Bargues et al. 2011b
<i>G. neotropica</i>	Argentina, Ipizca	KT215350	–	Bargues et al. 2016
	Peru, Lima	AM494008	–	Correa et al. 2011
	Uruguay, Tacuarembó	KX781342	–	Armúa-Fernandez et al. 2016
	Venezuela, Finca El Arenal	JF461486	–	Bargues et al. 2011b
<i>G. truncatula</i>	Argentina, Cerro Palauco	JN872455	–	Standley et al. 2013
	Bolivia, Viacha	JN614380	–	Correa et al. 2011
	Chile, Isla Teja	JN051372	–	Artigas et al. 2011
	Peru, Baños del Inca	HE610435	–	Bargues et al. 2012
	Venezuela, El Sapo	JN614382	–	Correa et al. 2011
	Bulgaria, Osogovo Mountains, karst spring	FR797873	–	Vinarski et al. 2011
	France, Limousin region	JN614384	–	Correa et al. 2011
	Italy, Val d'Inzino	LT623586	–	Schniebs et al. 2018
	Spain, Benicasim, Castellon	AM494011	–	Bargues et al. 2007
	Sweden, Rådetorp/Bjurtjärn near Storfors	LT623587	–	Schniebs et al. 2018
	Japan, Hokkaido (Gt 11)	LC360901	–	Ohari et al. 2020
	Japan, Hokkaido (Gt 50)	LC360940	–	Ohari et al. 2020
<i>G. schirazensis</i>	Spain, Albufera of Valencia, Valencia province	JF272607	–	Bargues et al. 2011a
	France	KP242685	–	Patel et al. 2015
	Reunion Island, Bras de Pontho	JN614377	–	Correa et al. 2011
	Venezuela, El Molino	JN614376	–	Correa et al. 2011
	Colombia, Finca Jocum Bucaramanga	KY198253	–	Lounnas et al. 2018
	Peru, Baños del Inca	JF272610	–	Bargues et al. 2011a
	USA, Louisiana Bedico	KT781332	–	Lounnas et al. 2018
	Japan, Hokkaido	LC360904	–	Ohari et al. 2020

Table 1. (continued).

Taxon	Locality	GenBank Acc. No. CO1	Hap. No.	Reference
<i>G. viator</i>	Argentina, Agua Escondida	JN872449	–	Standley et al. 2013
	Chile, Casa Blanca	JN051373	–	Artigas et al. 2011
<i>Galba</i> sp. A	Ethiopia	HQ660031	–	Dayrat et al. 2011
<i>Galba</i> sp. B	USA, Bosque del Apache, New Mexico	KT781290	–	Alda et al. 2021
	USA, Bosque del Apache, New Mexico	KT781292	–	Alda et al. 2021
Outgroups				
<i>Lymnaea stagnalis</i>		MH189887	–	Aksenova et al. 2018
<i>Radix auricularia</i>		KU950448	–	Aksenova et al. 2016

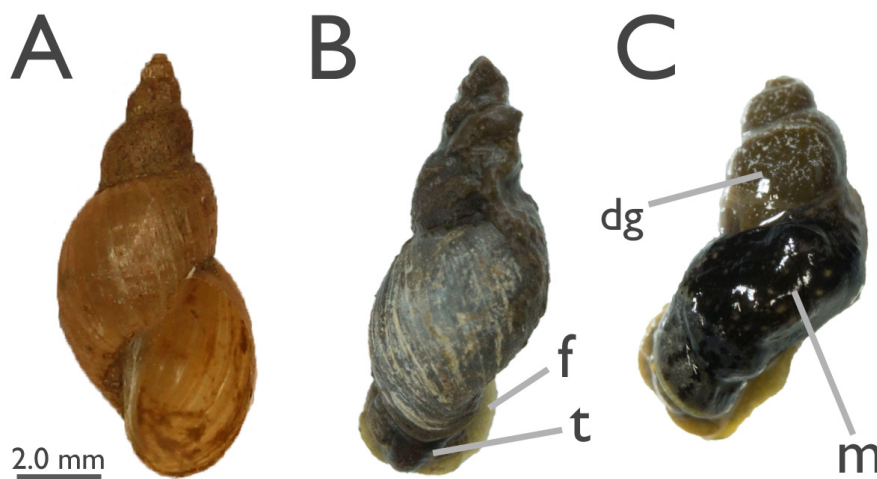


Figure 2. Shell morphology (A), external morphology (B), and soft body (C) of *Galba humilis* collected from Japan (TUSC_L1065). f: foot; t: tentacle; m: mantle; dg: digestive gland. Photo by Takumi Saito.

with small white dots, and the digestive gland was olive green with white (Figure 2C).

The phylogenetic tree constructed based on the *CO1* reference sequences of global *Galba* revealed that the collected individuals formed a well-supported clade (ML ultrafast bootstrap value [BV] = 98, BI posterior probability [BPP] = 1.00) and showed well-supported monophyly with North American *Galba humilis* (Say, 1822) (BV = 98, BPP = 1.00; Figure 3).

In the haplotype network based on the shorter region shared by all sequences of *G. humilis*, the three Japanese sequences shared a single haplotype (Table 1). No other sequence showed the same shortened haplotype as the Japanese sequences (Figure 4). The most closely related haplotypes with a single nucleotide substitution represented sequences from New York, Tennessee, and Canada (Figure 4).

Discussion

The lymnaeid snails collected from Japan in the present study were genetically similar to *G. humilis* from North America (Figure 3). In the genus *Galba*, morphological identification of species based on soft body characteristics is difficult and thus taxonomic confusion has occurred (Alda et al. 2021), although molecular identification is very effective and robust (Correa et al.

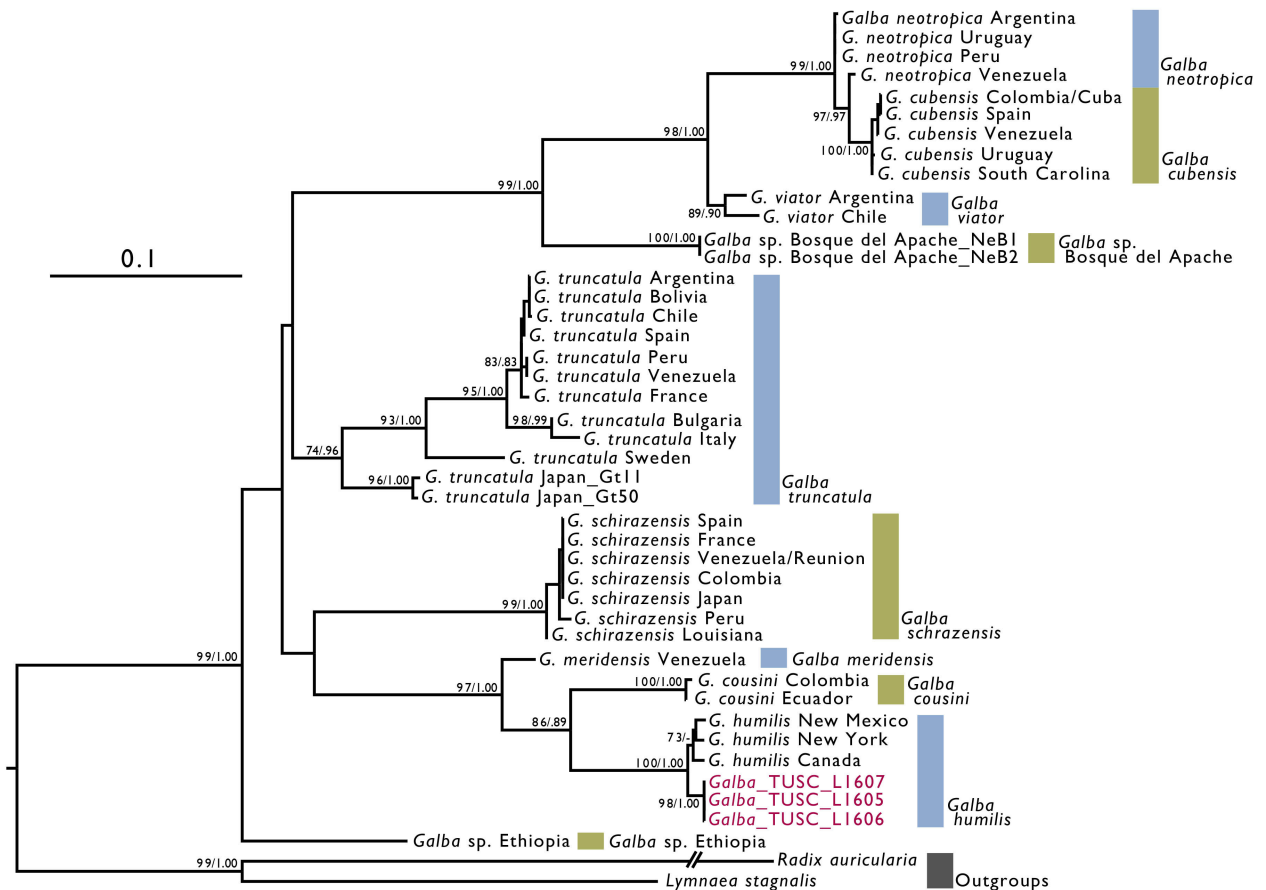


Figure 3. Maximum likelihood (ML) tree of the genus *Galba* estimated using IQ-TREE for molecular identification (mitochondrial cytochrome oxidase subunit 1: 672 bp). Numbers at the branch nodes indicate the ML ultrafast bootstrap values and Bayesian posterior probabilities. The values of terminal and uncertain nodes were omitted for visibility. Bars on the right indicate the species based on previous a molecular taxonomic study (Alda et al. 2021). Further details of each operational taxonomic unit are provided in Table 1.

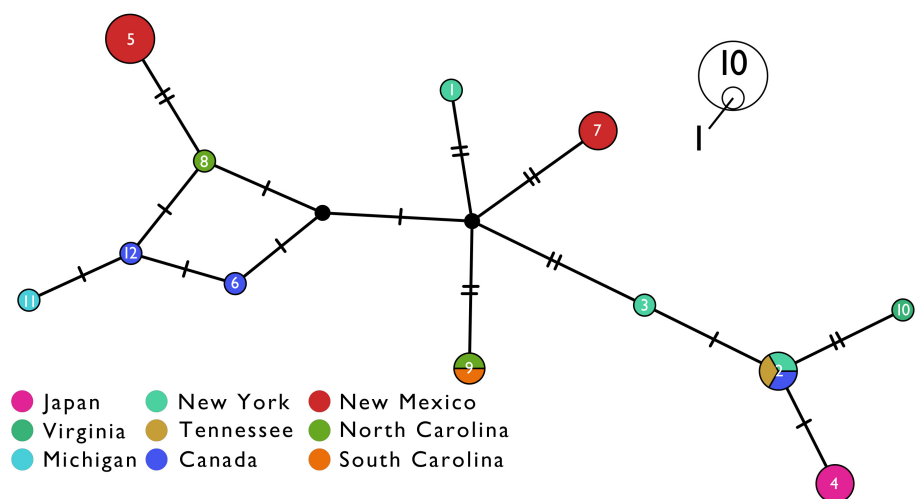


Figure 4. CO1 (537 bp) haplotype network of *Galba humilis* illustrated using POPART. The colour of each circle indicates the region of collection of each individual. The size of each haplotype represents the number of individuals presenting that haplotype. The white numbers within each circle indicate the haplotype number (see Table 1).

2011; Alda et al. 2018, 2021). Alda et al. (2021) showed that *Galba* comprises at least six genetically distinct species. The *CO1* haplotype sequence of the lymnaeid snails collected in this study was almost the same, with only a

single nucleotide substitution, as that of *G. humilis* (Figure 4). Accordingly, the collected lymnaeid snails were classified as *G. humilis* based on *COI* molecular phylogenetics. However, it should be noted that the possibility of the incongruence between mitochondrial and nuclear gene such as an introgressive hybridization cannot completely be rejected as no nuclear data were generated in this study. Nevertheless, the following facts support the robustness of this molecular identification: introgressive hybridizations between *G. humilis* and other lymnaeid snails do not seem to be recorded (Alda et al. 2021), all individuals had *COI* gene sequences consistent with *G. humilis*, and there are clear morphological differences from other *Galba* species in Japan (see below). Therefore it is likely that *G. humilis* is introduced to Japan and future nuclear gene data may strengthen the robustness of this identification.

Galba humilis was described from South Carolina, USA (Say 1822) and has a long list of synonyms (MolluscaBase 2021). It is distributed in North America, including the USA, Canada, and Mexico, and this species has not been recorded from any other region (Johnson et al. 2013; Czaja et al. 2020; Alda et al. 2021). I believe that this is the first report of a *G. humilis* introduction to a non-indigenous region and Japan. However, as the *COI* haplotype of this study did not match any published sequences from the indigenous region, the origin of the introduced population is uncertain. A thorough phylogeographic investigation of *G. humilis* of the indigenous region will be necessary to reveal this issue. In Japan, two non-indigenous lymnaeid species, *Pseudosuccinea columella* (Say, 1817) and *G. schirazensis* (Küster, 1862), are widespread (Masuda and Uchiyama 2004). *Galba humilis* represents the third non-indigenous lymnaeid species recorded in the wild in Japan. Nonetheless, the actual status of the Japanese lymnaeid fauna remains unclear, and there may be additional introduced species that have not yet been identified (Masuda and Uchiyama 2004; Kawase 2018).

Typically, morphological identification based on shell characteristics is difficult in the family Lymnaeidae, including the genus *Galba*. Nevertheless, morphological identification of lymnaeid species in Japan may be relatively easy, because the shell aperture height is more than half of the shell height in most Japanese lymnaeids, except for the two *Galba* species and *Stagnicola palustris* (O. F. Müller, 1774) (Itagaki and Imai 1960; Masuda and Uchiyama 2004; Ohari et al. 2020). In the non-indigenous *G. schirazensis* and the possibly indigenous *G. truncatula* (O. F. Müller, 1774), the shell height is less than 10 mm (Masuda and Uchiyama 2004; Ohari et al. 2020); therefore, these species can be distinguished from other Japanese lymnaeids based on their shell size. *S. palustris* has been recorded from only one locality on the Hokkaido Island—the northern part of Japan—but no illustrations, voucher specimens, and additional records are available (Itagaki and Imai 1960; Kurozumi et al. 2011). A recent phylogenetic study focused on lymnaeid

snails on the Hokkaido Island did not detect *S. palustris* (Ohari et al. 2020), and the actual status of this species in Japan remains ambiguous. The tentacles of *S. palustris* are elongated and triangular (U.S. Fish and Wildlife Service 2018), whereas those of *G. humilis* are round (Figure 2B); thus, these two species can also be easily distinguished based on their tentacle morphology.

The population of *G. humilis* discovered in this study comprised many individuals inhabiting two sites in Kamisu City (Figure 1A, C). Therefore, this population may have colonised some time before its recognition. On the other hand, the mechanism of this colonisation is not unclear at this stage. In general, the dominant vector of introductions of non-indigenous molluscs is considered to be aquarium-associated, whether intentional or unintentional (Padilla and Williams 2004; Duggan 2010; Patoka et al. 2017; Duggan et al. 2018). Kamisu City is an urbanized area (population: 95,372, density: 648.9/km²; Ibaraki Prefecture 2021), and so the possibility of an aquarium-associated introduction is possible; however, *G. humilis* have never been observed from aquariums in Japan (Saito *pers. obs.*). Kamisu City is the adjacent area to the Tone River and Lake Kasumigaura, two large drainages with a high number of invasive species (Ozaki and Miyabe 2007; Yamaguchi 2008; Yanai et al. 2008), which might have contributed to the introduction mechanisms. In any case, further research is needed to identify the mechanism of introduction. *Galba humilis* is a self-fertile hermaphrodite (Alda et al. 2021), and its population may therefore rapidly and easily increase from even a single or few individuals, facilitating the establishment of an introduced population. In addition, many freshwater organisms from North America have been introduced and have spread in Japan (e.g. largemouth bass, red swamp crayfish, and Florida crangonyctid) (Ecological Society of Japan 2002). Moreover, the hygrophilid freshwater snails *Physella acuta*, *Ferrissia californica* (Rowell, 1863), *Menetus dilatatus* (Gould, 1841), and *Pseudosuccinea columella* have been introduced from North America and have now spread across Japan (Masuda and Uchiyama 2004; Takakura 2008; Saito and Hirano 2017; Saito et al. 2018). Therefore, climatic and environmental factors may not prevent the expansion of this species. Despite the widespread invasion of non-indigenous hygrophilid species in Japan, their impacts on other organisms and the environment have not been well documented. Nevertheless, the possibility of introgressive hybridisation and competition between indigenous and non-indigenous species has been suggested (Takakura 2008; Saito et al. 2018). Negative interspecific interactions between the indigenous *G. truncatula* and the non-indigenous *G. humilis* are a concern. In any case, monitoring changes in the distribution of *G. humilis* is imperative for understanding the expansion dynamics of non-indigenous hygrophilid invaders and to address the impacts of their introduction.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Primers and PCR conditions used in this study.

Table S2. The estimated approximate evolution model for our CO1 phylogenetic analyses and the generation and sample frequency of Bayesian analysis.

This material is available as part of online article from:

http://www.reabic.net/journals/bir/2022/Supplements/BIR_2022_Saito_etal_SupplementaryMaterial.xlsx