

Research Article

Molecular analysis of the distributions of the invasive Asian clam, *Corbicula fluminea* (O.F. Müller, 1774), and threatened native clam, *C. leana* Prime, 1867, on Kyushu Island, Japan

Takuya Okawa, Yoshihisa Kurita*†, Kazuki Kanno, Akihiko Koyama and Norio Onikura

Fishery Research Laboratory, Kyushu University, 4-46-24 Tsuyazaki, Fukuoka 811-3304, Japan

*Corresponding author

†Current address: Graduate School of Agricultural Science, Tohoku University, Onagawa 986-2242, Japan

E-mail: katachiya@gmail.com

Received: 13 July 2015 / Accepted: 27 November 2015 / Published online: 16 December 2015

Handling editor: Darren Yeo

Abstract

The invasion of the Asian clam, *Corbicula fluminea* (O.F. Müller, 1774), has caused serious ecological and economic problems worldwide. In the Japanese archipelago, *C. fluminea* has been identified using morphological characteristics in most distribution surveys. However, a recent study has shown that morphology alone cannot be used to identify *C. fluminea* due to its superficial similarity to the related native species *C. leana* Prime, 1867. Consequently, the present distribution of *C. fluminea* is still poorly understood. We surveyed the distribution of *C. fluminea* on Kyushu Island, Japan, by sequencing the cytochrome b region of the mitochondrial DNA, a region that can be used to distinguish the two clam species, and found that *C. fluminea* was already established throughout the entire island, except at a single site where *Corbicula* clams comprised solely of *C. leana*. In response to this serious situation, we suggest using PCR-RFLP method, which was employed here, to distinguish the two clam species for more effective and rapid surveys.

Key words: biological invasion, bivalve, PCR-RFLP, distributional survey

Introduction

Clams of the genus *Corbicula* Megerle von Mühlfeld, 1811 are native to Africa, Australia, Asia, and the Middle East; however, as invasive species, they are nowadays distributed worldwide (Araujo et al. 1993). *Corbicula* clams possess high fecundity and great adaptability allowing them to establish in new environments. For example, the genus was introduced into North America in the 1920s (Counts 1981) and spread rapidly throughout the continent, reaching South America in the 1970s (Ituarte 1994). The clams invaded Europe around 1980 (Mouthon 2001). These clams have caused serious economic and ecological problems worldwide. In Europe and America, *Corbicula* clams have reached high densities, competing with native bivalves (Fuller and Imlay 1976; Karatayev et al. 2003), reducing phytoplankton concentrations (Descy et al. 2003;

Pigneur et al. 2014b), and damaging industrial cooling systems (Rosa et al. 2011).

In Japan, there are two native freshwater *Corbicula* clams. *Corbicula leana* Prime, 1867 is distributed in rivers and ponds throughout the Japanese archipelago, except within the Ryukyu Chain and Hokkaido Islands (Yamada et al. 2010). *Corbicula sandai* Reinhardt, 1878 has a more restricted distribution; it is endemic to Lake Biwa in Honshu (Ishibashi and Komaru 2003). The populations of both these native species have been decreasing, and they are now treated as threatened species (VU) in the Red List of the Japan Ministry of the Environment (Japan Ministry of the Environment 2012). One of the causes of native clam population decline is the introduction of the alien clam *Corbicula fluminea* (O.F. Müller, 1774). *C. fluminea* was imported into Japan for food trade from Korea and China around the 1980s (Tamai et al. 2008). Owing to *C. fluminea*

brooding its larvae in the inner demibranch, larvae probably accidentally escaped and/or were discarded into drainage ditches in the process of preparation or washing such as removing sand from their body (Japan Wild Research Center 2008). In addition, *C. fluminea* were also released as prey for a freshwater snail, *Semisulcospira libertine* (Gould, 1851), in Japan (Japan Wild Research Center 2008). Thus, it is now established in several freshwater environments in Japan. Invasions of *C. fluminea* brought risks of modification of abiotic conditions such as water clarity and dissolved oxygen levels (Ilarri and Sousa 2012), and resource competition with the native *Corbicula* clams (Japan Wild Research Center 2008). Therefore, the distribution of *C. fluminea* must be determined in order to evaluate ecological damage, and to construct a management program for this invasive clam.

Corbicula fluminea has been reported from several areas in Japan (Masuda and Habe 1988; Ishibashi and Komaru 2003; Sonohara et al. 2005; Yasuki 2014), in which species identification has been based on shell morphology and colors. However, recent studies suggested that it is impossible to distinguish *C. fluminea* and *C. leana* morphologically (Sousa et al. 2007; Hedtke et al. 2008; Yamada et al. 2010). Another biological aspect of *C. fluminea* and *C. leana* is that these species are hermaphrodites and have a very peculiar mode of reproduction—androgenesis (Komaru et al. 1998; Houki et al. 2011; Pigneur et al. 2012). At fertilization, DNA from an unreduced spermatozoon is incorporated into the egg, while during oogenesis all the chromosomes (maternal nuclear DNA) are extruded from the egg as two polar bodies. As a consequence, only the paternal pronucleus is kept in the zygote of androgenetic *Corbicula*, while the mitochondria of the egg are retained (Komaru et al. 1998; Pigneur et al. 2014a). Several studies intensively investigated the evolutionary origin of this unique reproductive mode in *Corbicula* clams using molecular phylogenetic approaches (e.g., Hedtke et al. 2008; Houki et al. 2011; Pigneur et al. 2014a). Yamada et al. (2010) found that the cytochrome b region in the mitochondrial DNA includes mutations that nevertheless may be used to distinguish *C. fluminea* and *C. leana*. Although Yamada et al. (2010) used molecular data to clarify the distribution of *C. fluminea* in central Honshu and some remote islands, the distribution of *C. fluminea* in many other regions of the Japanese archipelago remains unclear. A more complete survey using molecular data is required

to fully assess the current distributions of *C. fluminea* and *C. leana*.

In this study, we investigated the distributions of *C. fluminea* and *C. leana* on Kyushu, which is the third largest island in the Japanese archipelago, using DNA sequencing of the cytochrome b region. Since Komaru et al. (1997) published a collection record of *C. fluminea* from northwestern Kyushu, we focused on this geographical region. Furthermore, we used a method based on cytochrome b sequences that can distinguish *C. fluminea* and *C. leana* by using PCR-restriction fragment length polymorphism (RFLP).

Materials and methods

Sampling was conducted between May 2014 and January 2015. *Corbicula fluminea* and *C. leana* were collected from 21 sites on Kyushu and the surrounding area by hand-net and hand (Figure 1). The specimens were kept in 100% ethyl alcohol after boiling for 1 min in the laboratory. Clam DNA was extracted from a piece of mantle or foot using SimplePrep™ DNA reagent (Takara, Japan).

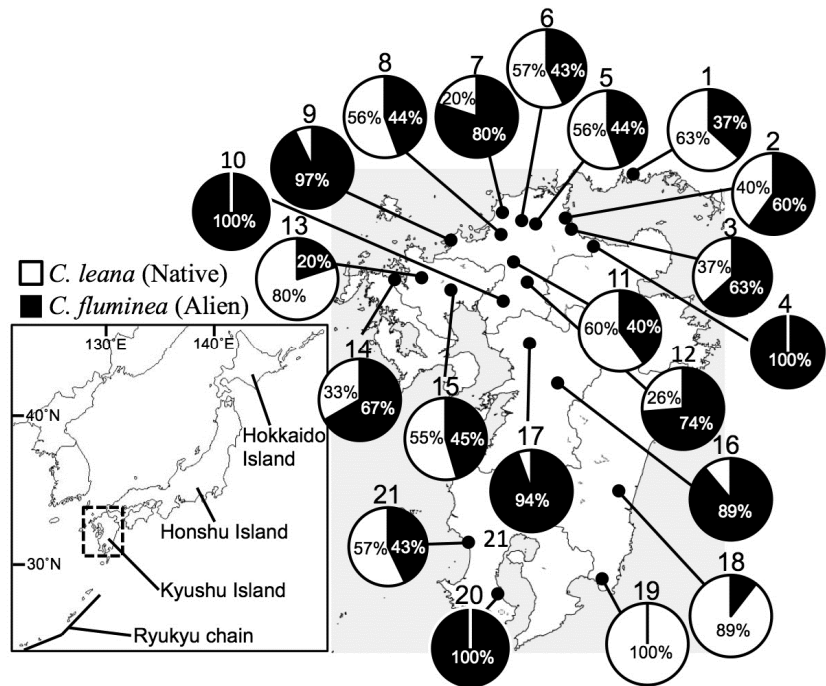
Yamada et al. (2010) revealed the haplotypes of mitochondrial cytochrome b (cyt-b) of *C. leana* and *C. fluminea*, collected from several part of Japan, and suggested the cyt-b region includes mutations which can distinguish between two species. Therefore, we used the cyt-b region for this survey. Cyt-b DNA was PCR amplified using the primer sets and protocol described by Houki et al. (2011). DNA was sequenced using an outsourced service (Fasmac, Japan).

The reference cyt-b sequences of *C. fluminea* and *C. leana* were analyzed using GENETYX-MAC ver. 14.0.6 to identify specific restriction sites for each species. The PCR product was digested in a solution of 0.5 µL of restriction enzyme (10 U), 0.5 µL of buffer (New England Biolabs, Japan), and 4 µL of PCR product. The solution was incubated at 37°C for 120 min. The DNA fragments were separated on a 1% agarose gel at 100 V for 25 min. Fragment size was determined using a 2-log DNA ladder (New England Biolabs, Japan).

Results and discussion

The study examined 306 individuals [registered as KUME-PI-00003077(No.1) ~ KUME-PI-00003097 (19) in the Kyushu University museum]. Cyt-b partial sequences (621 bp) were amplified and

Figure 1. Sampling sites and composition of *C. leana* and *C. fluminea* at each site. For details see supplementary material Table S1.



seven haplotypes were found (supplementary material Table S1). Five haplotypes were identified as haplotypes of *C. fluminea* cyt-b (CB6–CB9; DDBJ accession no. AB551542–AB551549, H3; accession no. AB598630) and two haplotypes were matched with haplotypes of *C. leana* (CB1; accession no. AB551535, CB5; accession no. AB551541), as reported previously (Yamada et al. 2010; Houki et al. 2011).

DNA sequencing identified 184 *C. fluminea* and 122 *C. leana*. In *C. fluminea*, haplotypes CB7 (n=90) and CB9 (n=76) were the most common, representing 49% and 41% of the collection, respectively; the minor haplotypes H3 (n=13), CB8 (n=4), and CB6 (n=1) accounted for approximately 7%, 2%, and 1%, respectively. In *C. leana*, haplotype CB1 was the most common (n=104), representing approximately 85% of the collection, while CB5 (n=18) accounting for about 15% of the *C. leana* we collected.

Regarding distribution, *C. fluminea* and *C. leana* occurred at 20 and 18 sites, respectively (Figure 1, Table S1). Three sites (sites 4, 10, and 20) were occupied only by *C. fluminea* and three other sites (sites 9, 16, and 17) were more than 80% occupied by *C. fluminea*. Site 19 consisted only of *C. leana* and site 18 was more than 80% *C. leana*.

For the restriction site assessment, we used 12 haplotype sequences of partial cyt-b DNA of *C. fluminea* and *C. leana* (7 haplotypes found in this study, 5 haplotypes reported in Yamada et al. 2010). This showed that *MspI* cleaves the fragment into two fragments (436 and 185 bp) in *C. fluminea*, and into three fragments (436, 114, and 71 bp) in *C. leana*. Using *MspI*, we performed restriction enzyme digests of six haplotype sequences to confirm the lengths of the restricted fragments, which were as expected for the two species (Figure 2).

This study is the first distribution study of *Corbicula* clams on Kyushu to use molecular methods to identify clam species. Our study has shed light on the serious state of the invasion by *C. fluminea*, demonstrating that almost all of Kyushu has already been invaded by *C. fluminea* (20/21 sites). We identified only one site, the Fukushima River, entirely occupied by *C. leana*; since the Oyodogawa River is also occupied by a high proportion of *C. leana*, this suggests that southeast Kyushu has experienced only minor effects of the *C. fluminea* invasion.

A major problem for mapping the distribution of *Corbicula* has been the difficulty of species identification using morphology (Sousa et al. 2007; Hedtko et al. 2008; Yamada et al. 2010).

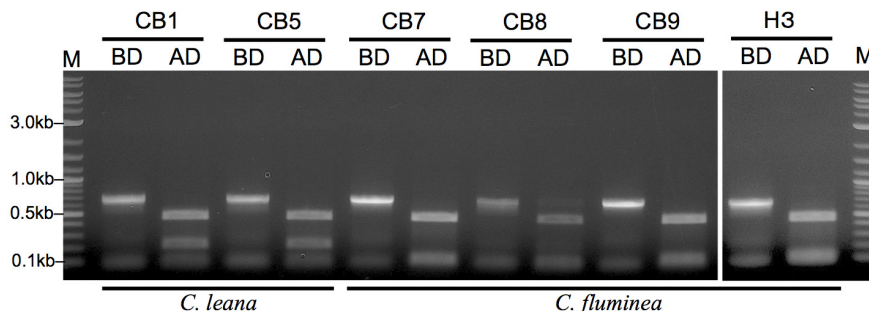


Figure 2. Digestion patterns for each haplotype of *C. leana* and *C. fluminea* using the restriction enzyme *Msp*I. M, the 2-log ladder marker; BD, before digestion; AD, after digestion.

To address this issue and assist future studies, we employed PCR-RFLP to distinguish *C. fluminea* and *C. leana*. Application of this method should facilitate rapid, more effective surveys of the distributions of *C. fluminea* and *C. leana*. It is important to note, however, that because the present study was conducted by using only mitochondrial DNA (cyt-b region), we could not evaluate the species hybridization between *C. fluminea* and *C. leana*. Androgenetic *Corbicula* species including *C. leana* and *C. fluminea*, show a phenomenon called “egg parasitism” in which the DNA content of spermatozoa is identical to that of somatic cells, but mtDNA is maternal (Houki et al. 2011). Because *C. fluminea* releases larger amount of sperm than *C. leana*, a *C. leana* population could potentially be replaced with a population possessing nuclear DNA of *C. fluminea* via unilateral hybridization caused by certation (Sonohara 2005). The problem then is the underestimation of *C. fluminea* distributions due to the retention of *C. leana* lineage mtDNA even after being completely replaced with *C. fluminea* nuclear DNA. Therefore, further study using both mtDNA genes and nuclear genes is essential to clarify the state of species hybridization between the two *Corbicula* species.

Acknowledgements

The authors thank Dr. Darren Yeo Chong Jinn and three anonymous reviewers for their helpful comments on the manuscript. Dr. Yuichi Kano provided *Corbicula* clams for this study. This work was partly supported by JSPS KAKENHI Grant no.15K14561.

References

- Araujo R, Moreno D, Ramos M (1993) The Asiatic clam *Corbicula fluminea* (Müller, 1774) (Bivalvia: Corbiculidae) in Europe. *American Malacological Bulletin* 10: 39–49
- Counts CL (1981) *Corbicula fluminea* (Bivalvia: Sphaeriacea) in British Columbia. *Nautilus* 95: 12–13
- Descy JP, Everbecq E, Gosselain V, Viroux L, Smits J (2003) Modelling the impact of benthic filter feeders on the composition and biomass of river plankton. *Freshwater Biology* 48: 404–417, <http://dx.doi.org/10.1046/j.1365-2427.2003.01017.x>
- Fuller SL, Imlay MJ (1976) Spatial competition between *Corbicula manilensis* (Philippi), the Chinese clam (Corbiculidae), and fresh-water mussels (Unionidae) in the Waccamaw River basin of the Carolinas (Mollusca: Bivalvia). *Association of Southeastern Biologists Bulletin* 23: 60
- Hedtke SM, Stanger Hall K, Baker RJ, Hillis DM (2008) All-male asexuality: Origin and maintenance of androgenesis in the Asian clam *Corbicula*. *Evolution* 62: 119–1136, <http://dx.doi.org/10.1111/j.1558-5646.2008.00344.x>
- Houki S, Yamada M, Honda T, Komaru A (2011) Origin and possible role of males in hermaphroditic androgenetic *Corbicula* clams. *Zoological Science* 28: 526–531, <http://dx.doi.org/10.2108/zsj.28.526>
- Ilari M, Sousa R (2012) *Corbicula fluminea* Müller (Asian clam). In: Francis RA (ed), *A Handbook of Global Freshwater Invasive Species*. Routledge, UK, pp 173–183
- Ishibashi R, Komaru A (2003) Invasion of *Corbicula fluminea* into the Lake Biwa-Yodo River System. *Venus: Journal of the Malacological Society of Japan* 62: 65–70
- Ituarte CF (1994) *Corbicula* and *Neocorbicula* (Bivalvia: Corbiculidae) in the Paraná, Uruguay and Río de la Plata basins. *The Nautilus* 107: 129–135
- Japan Ministry of the Environment (2012) The 4th version of the Japanese Red List of molluscs. <https://www.env.go.jp/press/files/jp/20555.pdf> (Accessed 7 July 2015)
- Japan Wild Research Center (ed) (2008) *A photographic guide to the invasive alien species in Japan*. Heibonsha, Tokyo, Japan, pp 242–243
- Karatayev AY, Burlakova LE, Kesterson T, Padilla DK (2003) Dominance of the Asiatic clam, *Corbicula fluminea* (Müller), in the benthic community of a reservoir. *Journal of Shellfish Research* 22: 487–494

- Komaru A, Konishi K, Nakayama I, Kobayashi T, Sakai H, Kawamura K (1997) Hermaphroditic freshwater clams in the genus *Corbicula* produce non-reductional spermatozoa with somatic DNA content. *The Biological Bulletin* 193: 320–323, <http://dx.doi.org/10.2307/1542934>
- Komaru A, Kawagishi T, Konishi K (1998) Cytological evidence of spontaneous androgenesis in the freshwater clam *Corbicula leana*. *Development Genes and Evolution* 208: 46–50, <http://dx.doi.org/10.1007/s004270050152>
- Masuda O, Habe T (1988) *Corbicula insularis*. Prime established in Kurashiki City, Okayama Pref., Honshu. *Chiribotan* 19: 39–40
- Mouthon J (2001) Life cycle and population dynamics of the Asian clam *Corbicula fluminea* (Bivalvia: Corbiculidae) in the Saone River at Lyon (France). *Hydrobiologia* 452: 109–119, <http://dx.doi.org/10.1023/A:1011980011889>
- Pigneur LM, Hedtke SM, Etoundi E, Van Doninck K (2012). Androgenesis: a review through the study of the selfish shellfish *Corbicula* spp. *Heredity* 108: 581–591, <http://dx.doi.org/10.1038/hdy.2012.3>
- Pigneur LM, Etoundi E, Aldridge DC, Marescaux J, Yasuda N, Van Doninck K (2014a) Genetic uniformity and long-distance clonal dispersal in the invasive androgenetic *Corbicula* clams. *Molecular Ecology* 23: 5102–5116, <http://dx.doi.org/10.1111/mec.12912>
- Pigneur LM, Falisse E, Roland K, Everbecq E, Deliège JF, Smitz JS, Descy JP (2014b) Impact of invasive Asian clams, *Corbicula* spp., on a large river ecosystem. *Freshwater Biology* 59: 573–583, <http://dx.doi.org/10.1111/fwb.12286>
- Rosa IC, Pereira JL, Gomes J, Saraiva PM, Gonçalves F, Costa R (2011) The Asian clam *Corbicula fluminea* in the European freshwater-dependent industry: A latent threat or a friendly enemy? *Ecological Economics* 70: 1805–1813, <http://dx.doi.org/10.1016/j.ecolecon.2011.05.006>
- Sonohara T (2005) Invasion of *Corbicula fluminea* into the Sagami river system and taxonomical change of Subfamily Corbiculinae. *Actinia: bulletin of the Manazuru Marine Laboratory for Science Education* 16: 11–19
- Sonohara T, Fujiwara Y, Harigai M, Yoshida T (2005) Invasion of *Corbicula fluminea* (Bivalvia: Corbiculidae) into the Sagami River and Kaname River systems, Kanagawa Prefecture, Japan. *Chiribotan* 36: 18–25
- Sousa R, Freire R, Rufino M, Méndez J, Gaspar M, Antunes C, Guilhermino L (2007) Genetic and shell morphological variability of the invasive bivalve *Corbicula fluminea* (Müller, 1774) in two Portuguese estuaries. *Estuarine, Coastal and Shelf Science* 74: 166–174, <http://dx.doi.org/10.1016/j.ecss.2007.04.011>
- Tamai N, Arai N, Inamoto Y, Shibabe Y, Shibata F, Ieyama H (2008) An attempt to use fresh water bivalve *Corbicula* for teaching materials. *Bulletin of the Faculty of Education Ehime University* 55: 97–103
- Yamada M, Ishibashi R, Kawamura K, Komaru A (2010) Interrelationships of the freshwater clams *Corbicula leana* Prime, 1864 and *C. fluminea* (Muller, 1774) distributed in Japan inferred from shell type and mitochondrial DNA Cyt b region. *Bulletin of the Japanese Society of Scientific Fisheries* 76: 926–932, <http://dx.doi.org/10.2331/suisan.76.926>
- Yasuki S (2014) Invasion of Seika Cho, Kyoto Prefecture by Asiatic Clams (*Corbicula fluminea*). *Bulletin of the Osaka International University* 27: 71–74

Supplementary material

The following supplementary material is available for this article:

Table S1. Summary of the sampling sites, sample size, and number of haplotypes of each clam species in this study.

This material is available as part of online article from:

http://www.reabic.net/journals/bir/2016/Supplements/BIR_2016_Okawa_etal_Supplement.xls