

Hybridization between *Meretrix lusoria* and the alien congeneric species *M. petechialis* in Japan as demonstrated using DNA markers

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Abstract

Meretrix lusoria supports an important shellfish fishery distributed around Japan and the southern coast of Korea. The species is considered to be either local extinct or endangered in Japan and in response to a decrease in availability in Japan, *M. petechialis*, a closely related species, has recently been imported from China and the Korean Peninsula. *M. petechialis* is currently defined as an “alien species” in Japan, has been released into habitats where the local species *M. lusoria* occurs. We conducted a survey and detected natural hybrids between *M. lusoria* and *M. petechialis*. Nineteen *Meretrix* spp. population samples were collected in Japan, China, and Korea from 2004 to 2006 (15 samples from Japan, two from China, and two from South Korea). Polymerase chain reaction-restriction fragment length polymorphisms in the cytochrome c oxidase subunit I region after *HincII* digestion and amplification of the nuclear gene *ANT* enabled identification of the two species. The exotic *M. petechialis* was found in Yoshinogawa and Kanonji, where periodically, large numbers of *M. petechialis* seedlings have been released. Combined results of RFLP and *ANT* individual genotypes suggest the existence of at least one hybrid individual and two putative hybrids between the Japanese local species *M. lusoria* and the continental alien species *M. petechialis* found in Chichibugahama and Kanonji. This is the first record demonstrating the existence of the alien species *M. petechialis* and of hybrids co-occurring with local *M. lusoria* in a natural habitat. Our results show that the alien species *M. petechialis* is currently relatively rare in Japan. If however, *M. petechialis* populations expand in the wild, they could replace the local *M. lusoria* and cause genetic disturbance via hybridization, as observed at one site (Kanonji, Kagawa Pref.). To conserve the endangered local *M. lusoria* resource, immediate controls of the release of *M. petechialis* will be necessary to prevent this species spreading and hybridizing with the native *M. lusoria* population.

Key words: Species identification, PCR-RFLP, *HincII*, Nuclear gene *ANT*, Kanonji, Yoshinogawa

Introduction

Asian hard clams, genus *Meretrix* (Bivalvia: Veneridae), are commercially important bivalves in East and Southeast Asia and East Africa (Yoosukh and Matsukuma 2001). The two *Meretrix* species, *M. lusoria* (Röding, 1798) and *M. lamarckii* Deshayes, 1853 are local species of tidal flats and shallow waters in Japan. *M. lusoria* is distributed in sheltered sandy tidal flats in Japan (except for the Hokkaido and the Ryukyu Archipelago) and the southern coast of Korea (Yamashita et al. 2004). *M. lusoria* once provided an important fishery resource and recreational shell-gathering species. Shells of *M. lusoria* are one of the most abundant mollusks found in shell middens in Japan

(Kanamaru 1932; Matsushima 1982; Yamazaki and Oba 2009). The annual catch of *M. lusoria* however, has decreased significantly since the mid-1960s, and the commercial catch remains low (Higano 2004; Fishbase 2011). *M. lusoria* is now considered to be local extinct in Chiba and endangered in the Aichi, Mie, Hyogo, Ehime and Kumamoto prefectures in Japan (Search System of Japanese Red Data 2011).

In response to a decrease in the national supply of *M. lusoria*, *M. petechialis* (Lamarck, 1818), a closely related exotic species, was recently imported from China and the Korean Peninsula. *M. petechialis* occurs naturally along the west coast of the Korean Peninsula and China (Henmi 2009) and where it lives in sheltered muddy and sandy tidal flats of similar

habitat to *M. lusoria*. Imported *M. petechialis* have been released into local *M. lusoria* habitat areas for commercial and recreational fishing purposes. The species appears to have settled well into its new habitats, and was designated as an “alien species” by the Japanese Ministry of the Environment (Ministry of the Environment 2011). Kosuge (2002) suggested that exotic *M. petechialis* could potentially replace local *M. lusoria* and cause genetic disturbance if the two species crossbreed. Kawase (2002) reported that a suspected hybrid individual with intermediate morphological characters between *M. lusoria* and *M. petechialis* was found in a natural tidal flat in Japan. Despite increasing concern about introductions of alien species, no molecular-based studies have been conducted to verify if populations of *M. petechialis* have established in the wild in Japan. Additionally, no studies have tested for the existence of hybrids between *M. lusoria* and *M. petechialis* individuals in Japan.

Generally, *Meretrix petechialis* can be easily distinguished from *M. lusoria* by morphology, with the posterior dorsal margin of *M. lusoria* being straight, while that of *M. petechialis* is quite swollen, and the apex position of *M. lusoria* is skewed to the anterior side relative to that of *M. petechialis* (Henmi 2009). Both species however, sometimes show intraspecific shell color polymorphisms and their shell form can vary from these patterns due to differential additive growth between shell parts as is the case with many bivalves (Huxley 1932; Wilbur and Owen 1964; Eager 1978). Juveniles of *M. lusoria* and *M. petechialis* have very similar morphologies and shell colors, making species identification difficult at the juvenile stage. Many cases of misidentification of *M. petechialis* as *M. lusoria* or *M. meretrix* (Linnaeus, 1758) exist in the scientific literature. Recently DNA-based techniques for detecting interspecific differentiation have been developed and applied to some bivalve species, including closely related species belonging to the same genus. Restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA) and nuclear DNA markers have been used to identify fish and shellfish and to provide evidence for interspecific hybridization (e.g., Inoue et al. 1997; Rawson et al. 1999; Imai and Takeda 2005; Masaoka and Kobayashi 2005; Nakadate and Chow 2008; Imai et al. 2009).

In the current study, we examined genetic variation within *M. lusoria* and *M. petechialis*

populations collected from natural habitats in Japan, China, and Korea and conducted species identification and detection of hybrids between *M. lusoria* and *M. petechialis* using genetic markers developed from mitochondrial and nuclear DNA.

Methods

Specimens and DNA samples

In total, 19 *Meretrix* spp. population samples were collected in Japan, China, and Korea between 2004 and 2006 (Table 1). Foot muscle tissue was dissected from fresh specimens for DNA analysis. A small amount of muscle tissue was maintained in 500 µl of TNES (10 mM Tris-HCl, 0.3 M NaCl, 10 mM EDTA, 2% sodium dodecyl sulfate (SDS))/8M urea buffer (Asahida et al. 1996) for DNA extraction. For tissue digestion, 10 µl of proteinase K (Wako Chemicals, Japan) was added to the TNES mixture, which was then incubated for 2 h at 37°C. Total DNA was extracted using a phenol-chloroform-isoamyl alcohol method.

Mitochondrial DNA typing

An approximate 720-base-pair fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI) was amplified by polymerase chain reaction (PCR) using a thermal cycler GeneAmp 9700 (Applied Biosystems, USA). PCR reactions were performed in a 50-µl volume containing 0.2 µl of ExTaq™ (Takara Bio, Japan), 5 µl of 10× ExTaq buffer, 5 µl of a 2.5 mM dNTP mixture, 0.5 µl each of 25 pM primers (LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198: 5'-TAAACTTCAGGGTGACCAAAA AATCA-3'; Folmer et al. 1994), and 0.5 µl of template DNA. PCR conditions included preheating at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 50°C for 15 s, and extension at 72°C for 30 s, and then a post-cycle extension at 72°C for 7 min. Nucleotide sequence data from the two species were analyzed to identify restriction site differences between *M. lusoria* and *M. petechialis*. Sequenced individuals included typical *M. lusoria* and *M. petechialis* identified by shell shape and color (*M. lusoria* from Mutsu Bay, Aomori Prefecture, Japan, and *M. petechialis* imported from Bohai, China). Sequencing was conducted using an ABI 3700 genetic analyzer (Applied Biosystems) with the

Table 1. Collected *Meretrix* samples. Frequencies of *HincII* restriction types of the mitochondrial DNA cytochrome *c* oxidase subunit I and nuclear DNA *ANT* genotypes.

Country	No.	Location (geographic coordinates)	N	Sampling date	<i>HincII</i> restriction type		<i>ANT</i> genotype		
					A	B	A/A	A/B	B/B
Japan	1	Mutsu Bay (41°21', 140°11')	20	Apr. 2005	0	20	0	0	20
	2	Hamagurihama (38°20', 141°08')	2	Sept. 2004	0	2	0	0	2
	3	Hamana (34°40', 137°36')	8	July 2005	0	8	0	0	8
	4	Kisogawa (35°02', 136°44')	27	Mar. 2006	0	27	0	0	27
	5	Kuwana (35°03', 136°42')	26	Mar. 2005	0	26	0	0	26
	6	Yoshinogawa (34°05', 134°34')	27	Mar. 2006	7	20	4	3	20
	7	Chichibugahama (34°11', 133°38')	41	May 2006	1	40	0	1	40
	8	Kanonji-0606 (34°07', 133°38')	48	June 2006	1	47	0	2	46
	9	Kanonji-0706 (Festival)	25	July 2006	25	0	22	3	0
	10	Kanonji-1006	44	Oct. 2006	1	43	0	1	43
	11	Ohji (34°02', 131°01')	20	Feb. 2005	0	20	0	0	20
	12	Kafuri (33°33', 130°09')	22	Nov. 2004	0	22	0	0	22
	13	Yanagawa (33°38', 131°22')	21	Dec. 2004	0	21	0	0	21
	14	Morie (33°24', 131°38')	21	Apr. 2005	0	21	0	0	21
	15	Midorikawa (32°42', 130°36')	23	June 2006	0	23	0	0	23
China	16	Bo-hai	27	Feb. 2005	27	0	23	4	0
	17	Yellow River mouth	18	Apr. 2005	18	0	13	5	0
Korea	18	Gunsan	27	Sept. 2005	27	0	18	9	0
	19	Buan	38	Sept. 2005	38	0	32	6	0

Geographic coordinates are not available for foreign samples. This is because Chinese samples were collected via a fishing company and Korean samples were purchased in the local market.

Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). Preliminary RFLP surveys of the COI region indicated that *HincII* (Toyobo, Japan) digestion yielded heterogeneous distributions of restriction types among samples. RFLP analysis was performed in a 10 µl volume containing 1 µl of buffer H (Toyobo, Japan), 3 µl of PCR product, and 5 units of *HincII* at 37°C for 2 h. A 10-µl portion of the reactant was examined using electrophoresis on a 1% agarose gel (TreviGelTM500; Trevigen, USA) in TAE buffer at 100 V. After electrophoresis, gels were stained with ethidium bromide, visualized under ultraviolet (UV) light, and photographed.

Nuclear DNA typing

Adenine nucleotide transporter/ADP-ATP Translocase (*ANT*) is a nuclear gene that encodes for the protein adenine nucleotide (ADP/ATP) translocase, a component of mitochondrial permeability transition pores (Audzijonyte and

Vrijenhoek 2010). *ANT* is an antiport that carries ADP into the inner mitochondrial space (Alberts et al. 1994). The *ANT* locus was amplified using the primer set ANTf1 (5'-TGCTTCG TNTACCCVCTKGACTTTGC-3') and ANTr1 (5'-CDGCRGCKTACTACTACGTCAGACC-3') (Jarman et al. 2002). PCR reactions were performed in a 50 µl volume containing 2.5 units of ExTaqTM polymerase (Takara Bio), 5 µl of 10× Extaq buffer, 5 µl of a 2.5 mM dNTP mixture, 1 µl each of 25 pM primers, and 2.5 µl of template DNA. PCR conditions included preheating at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 46°C for 40 s, extension at 72°C for 1 min 30 s, and a post-cycle extension at 72°C for 7 min. PCR products were examined using electrophoresis on a 1% agarose gel (TreviGelTM500) in TAE buffer at 100 V. After electrophoresis, gels were stained with ethidium bromide, visualized under UV light, and photographed. Amplification products were

Table 2. Species identification and hybrid individuals between the Japanese local species *M. lusoria* and alien species *M. petechialis* inferred from *HincII* and *ANT*.

<i>HincII</i> Restriction type	<i>ANT</i> Genotype	N	Species Identification
A	A/A	112	<i>M. petechialis</i>
A	A/B	31	<i>M. petechialis</i>
A	A/B	2	Putative hybrid and/or <i>M. petechialis</i>
A	B/B	0	-
B	A/A	0	-
B	A/B	1	Hybrid
B	B/B	339	<i>M. lusoria</i>

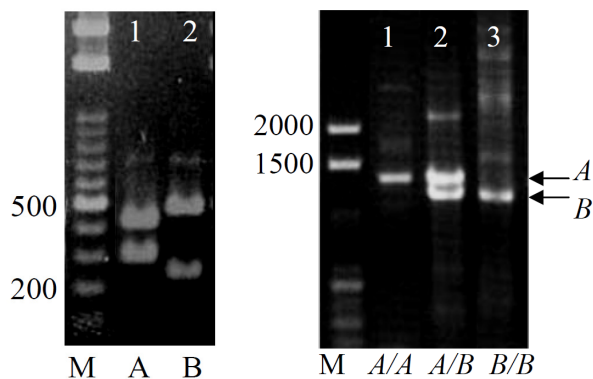


Figure 1. Agarose gel electrophoresis of *HincII* restriction types of mitochondrial DNA cytochrome *c* oxidase subunit I (Left) and *ANT* genotypes (Right). M, molecular marker. Size (bp) is shown along the left margin. Left: lane 1, *Meretrix petechialis*; lane 2, *M. lusoria*. Right: lane 1, *M. petechialis*; lane 2, Hybrid; lane 3, *M. lusoria*.

compared to detect species-specific fragment lengths. Combined results of *ANT* and COI digestion phenotypes were used to test for presence of hybrid individuals between the Japanese local species, *M. lusoria* and the continental alien species, *M. petechialis* (Table 2).

Results

Mitochondrial DNA typing

Amplification of a single COI fragment (approximately 720 bp) was visualised, and no apparent size differences were detected in amplified fragments between *Meretrix lusoria* and *M. petechialis* individuals. *HincII* digestion of PCR products revealed two unique restriction

patterns designated type A (418- and 306-bp fragments) and type B (479 and 247 bp), that were completely diagnostic to distinguish *M. lusoria* from *M. petechialis* individuals (Tables 1, 2 and Figures 1, 2). Five (no. 6-10, Table 1) of the 15 populations sampled in Japan contained *M. petechialis* restriction type A phenotypes.

Nuclear DNA typing

Using the *ANT* PCR products, *M. lusoria* could be clearly distinguished from *M. petechialis* based on species-specific fragment lengths. Pure species populations would be expected to be fixed for species-specific genotypes, i.e., *M. petechialis* in China and Korea for the A/A (1,360 bp) and A/B (1,360 bp, 1,177 bp) genotypes and *M. lusoria* for the B/B (1,177 bp) genotype in Japan (Tables 1, 2 and Figures 1, 3). All sampled individuals could be classified as A/A, A/B, or B/B genotypes (no. 6-10, Table 1). Five of the 15 sampled populations in Japan contained A/A and A/B genotypes, a result which is in accordance with the COI results described above.

Hybridization between *Meretrix lusoria* and *M. petechialis*

Based on combined COI and *ANT* results, we confirmed 143 pure of *Meretrix petechialis* individuals, a single hybrid, two putative hybrids and 339 *M. lusoria* individuals (Table 2). The single conformed hybrid individual was found at Kanonji (Kagawa Prefecture). Kanonji-0606 #27 exhibited the *M. lusoria* COI type B phenotype with the *M. petechialis* *ANT* A/B genotype; it was classified accordingly as a hybrid and appeared to be an offspring of a cross between a female *M. lusoria* and a male *M. petechialis* (Table 3 and Figure 4). Moreover, Chichibugahama #14 and Kanonji-0606 #18 were considered to be the suspected hybrids (offspring of a female *M. petechialis* and male *M. lusoria*) or *M. petechialis* based on the results of *M. petechialis* COI type A and the *M. petechialis* *ANT* type A/B (Table 3 and Figure 4).

Discussion

The alien bivalve species, *Meretrix petechialis* has been observed in the wild at two locations in Japan (Yoshinogawa and Kanonji; Table 3 and Figure 4). The current study is the first study to

Figure 2. *HincII* restriction type (**A**, **B**) frequencies of 19 populations. **A** indicates *Meretrix petechialis* individuals and **B** indicates *M. lusoria* individuals. Numbers alongside the pie graph represent population numbers.

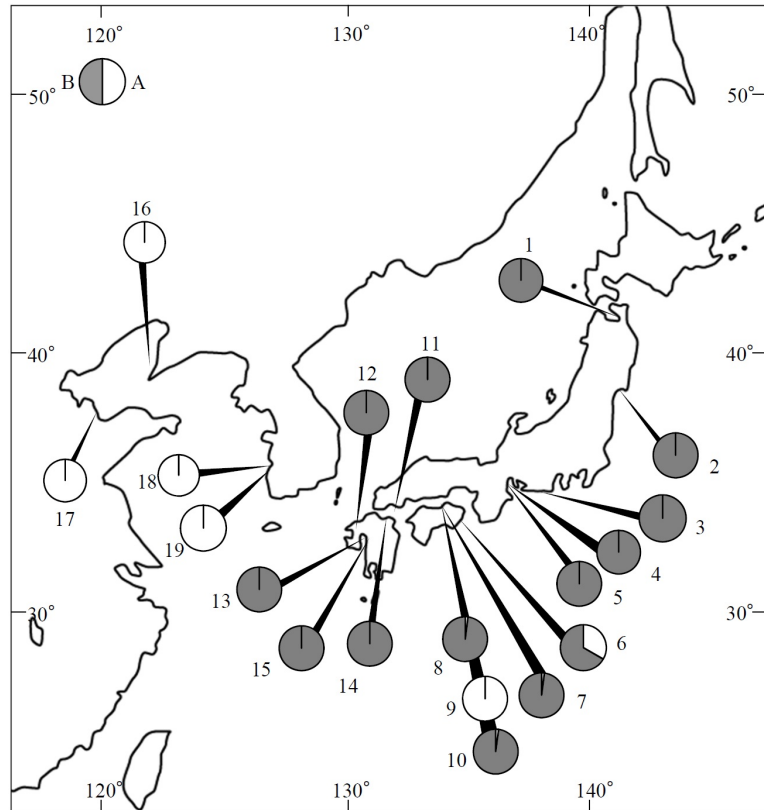


Figure 3. The *ANT* genotype (*A/A*, *A/B*, and *B/B*) frequencies of 19 populations. *A/A* indicates *Meretrix petechialis* individuals, *A/B* indicates *M. petechialis* or hybrid individuals, and *B/B* indicates *M. lusoria* individuals. Numbers alongside the pie graph represent population numbers.

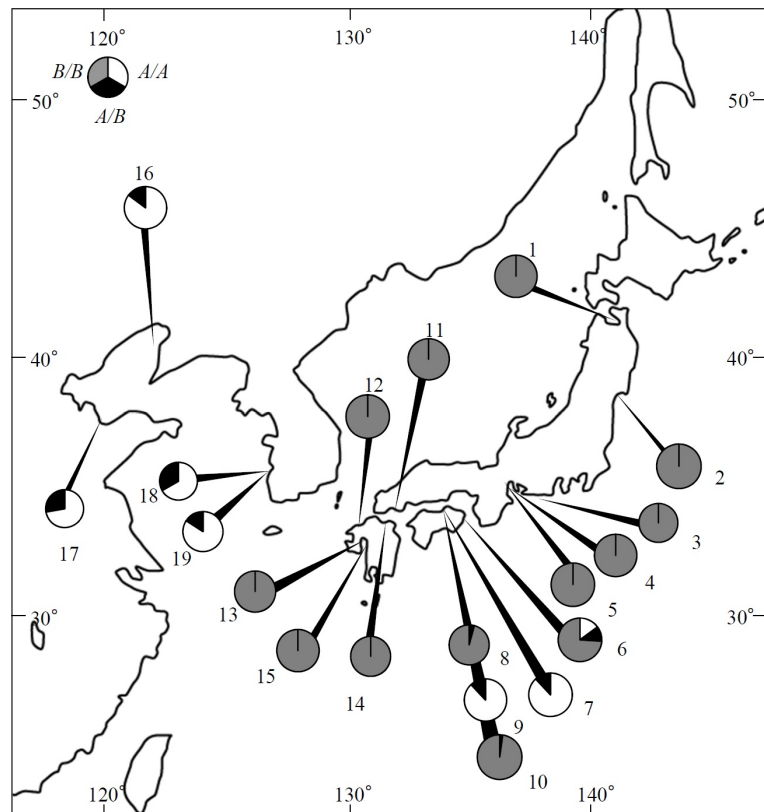


Table 3. Alien species *Meretrix petechialis* and hybrid individuals found in Japanese populations (F: female, M: male).

No.	Location	ID#	<i>HincII</i> Restriction type	<i>ANT</i> Geno- type	Species Identification	Note
6	Yoshinogawa	13, 15, 17, 18, 19, 20, 21	A	<i>A/A, A/B</i>	<i>M. petechialis</i>	Released individual imported from China by fisherman
7	Chichibugahama	14	A	<i>A/B</i>	Putative hybrid or <i>M. petechialis</i>	Putative hybrid individual (F: <i>M. petechialis</i> × M: <i>M. lusoria</i>)
8	Kanonji-0606	18	A	<i>A/B</i>	Putative hybrid or <i>M. petechialis</i>	Putative hybrid individual (F: <i>M. petechialis</i> × M: <i>M. lusoria</i>)
		27	B	<i>A/B</i>	Hybrid	Hybrid individual (F: <i>M. lusoria</i> × M: <i>M. petechialis</i>)
9	Kanonji-0706 (Festival)	All	A	<i>A/A, A/B</i>	<i>M. petechialis</i>	Released individual imported from China for shell gathering event at festival
10	Kanonji-1006	2	A	<i>A/B</i>	<i>M. petechialis</i>	Stranded individual imported from China for shell gathering event at festival at least three months ago.

demonstrate that exotic *M. petechialis* individuals co-occur with the local *M. lusoria* in a natural habitat in Japan. At the Yoshinogawa River mouth, many Chinese *M. petechialis* were released into local *M. lusoria* habitats by a local fishery cooperative more than 15 years ago (N. Azuma, personal communication). Local fishermen however, did not recognize them as a different species, although they could distinguish the two species by shell appearance. Given that a high percentage of *M. petechialis* individuals were collected, introduced *M. petechialis* individuals seem to have settled successfully and may be replacing *M. lusoria* in areas of the Yoshinogawa River mouth where once they were common (Table 3).

At Kanonji beach, a large number of Chinese *M. petechialis* (>100,000 individuals per year) were released annually at a local shell-gathering event called “Zeniasari” since 1996 (Japan Sea Farming Association (1986-2002) and the National Center for Stock Enhancement (2003-2008)). *M. petechialis* individuals were claimed to have been released in a designated area and that all released individuals had been gathered at the event (Henmi 2009). Certainly all individuals obtained from the Zeniasari event were *M. petechialis* (Kanonji-0706, Table 3 and Figures 2, 3, 4); however, a single *M. petechialis* individual was observed three months after the event (Kanonji-1006; Figure 4). Thus, stocked exotic *M. petechialis* individuals can apparently

survive longer term, at least in Japanese tidal flat environments.

We also identified a single hybrid between Japanese *M. lusoria* and the exotic *M. petechialis*, Kanonji-0606 #27 showing *M. lusoria* COI type B and the *M. petechialis* *ANT* type *A/B* (Table 3 and Figure 4) in Japan. Torii et al. (2010) reported that natural hybridization between *M. lusoria* and *M. petechialis* may occur around Gangjin Bay in South Korea, and *M. lusoria* and *M. petechialis* are currently considered to be subspecies with only weak natural geographical isolation. The border between *M. lusoria* and *M. petechialis* distribution on the Korean Peninsula is located along the southwestern coast of Korea from Gangjin Bay to Baeksu (Yamashita et al. 2004). Natural hybridization between *M. lusoria* and *M. petechialis* may be occurring in the area of natural habitat overlap.

M. lusoria like *M. petechialis* matures during summer (Chung 2007), and mass spawning occurs from August to September (Nakamura et al. 2010). In Kanonji, *M. lusoria* and *M. petechialis* occur in the same shallow tidal flat areas. Additionally, based on phylogenetic studies that employed allozyme markers, taxonomically, *M. petechialis* is considered to be the closest relative of *M. lusoria* (Yamakawa et al. 2008). Thus, genetic similarity as well as overlapping habitat use and similar reproductive seasons has likely facilitated hybridization between

Figure 4. Shell morphologies of *Meretrix lusoria*, *M. petechialis*, and a putative interspecific hybrid between *M. lusoria* and *M. petechialis*. **A.** *M. lusoria*, 1. Kanonji-0606 #21, 2. Kanonji-0606 #23, 3. Chichibugahama #13. **B.** Hybrid or *M. petechialis*; 1. Kanonji-0606 #18, 2. Kanonji-0606 #27 (hybrid), 3. Chichibugahama #14. **C.** *M. petechialis* 1. Kanonji-0706 #23, 2. Kanonji-1006 #2, 3. Yoshinogawa #15 (Photographs by A. Yamakawa and M. Yamaguchi).

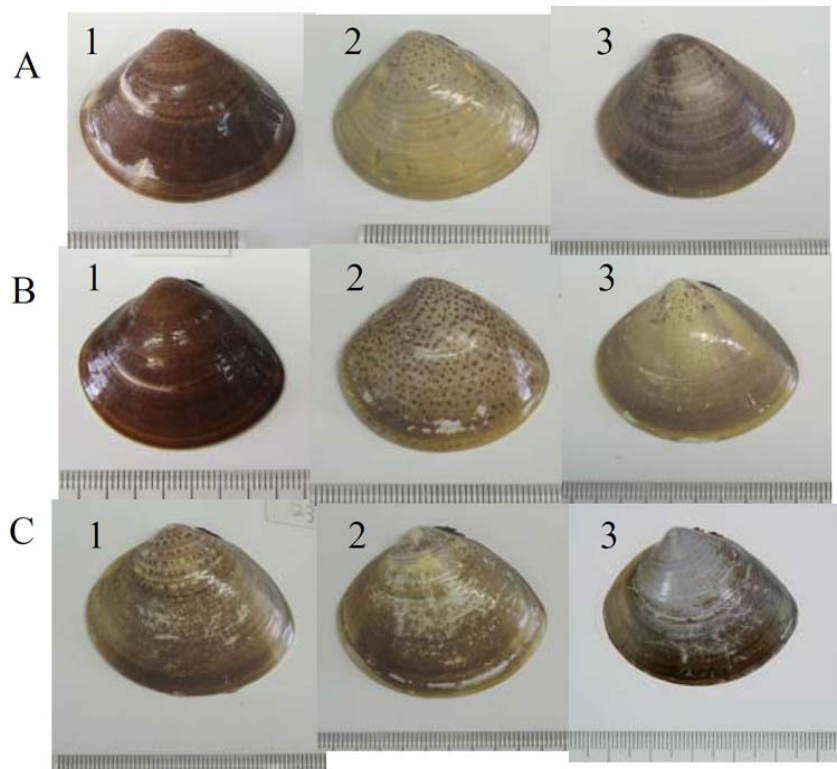


Figure 5. A live photograph of local *Meretrix lusoria* and released *M. petechialis* from Yoshinogawa, Tokushima Prefecture. 1, 2: local *M. lusoria*, 3-9: imported *M. petechialis* from China, 10: dead *M. petechialis* (Photographs by H. Imai).



M. lusoria and *M. petechialis* in Kanonji. The shell length of the hybrid measured 42 mm (Kanonji-0606 #27; Figure 4), and a 3-year-old *M. lusoria* can reach approximately 45 mm in shell length (Henmi 2009). Thus, the hybrid individual may have been produced in 2003.

An additional suspected hybrid individual (Chichibugahama #14) was observed in Chichibugahama (Table 3 and Figure 4). This individual showed a *M. petechialis* COI mitochondrial A phenotype combined with a *M. petechialis* nuclear *ANT A/B* genotype. This combination of genotypes can potentially be present in either a pure *M. petechialis* or a hybrid individual (offspring of female *M. petechialis* and male *M. lusoria*). There are no release records however, of Chinese *M. petechialis* in this area (Japan Sea Farming Association (1986-2002) and the National Center for Stock Enhancement (2003-2008)). Chichibugahama is very close to Kanonji, where a large number of Chinese *M. petechialis* are released every year and the distance between the two tidal flats is only 5 km. Most marine bivalve species, including the genus *Meretrix*, possess a pelagic larval dispersal phase of various lengths that allows movement away from home habitats. The length of the pelagic veliger larval phase in this species is 2 to 3 weeks (Henmi 2009) and this would in theory allow *Meretrix* hybrid larvae born in Kanonji to disperse to Chichibugahama if an appropriate ocean current was available during the larval dispersal phase. Therefore, the Chichibugahama #14 showing *M. petechialis* COI type A and *M. petechialis* *ANT* type *A/B* is likely to be a true hybrid and not a pure *M. petechialis* individual based on circumstantial evidence. Since, *M. petechialis* can possess either *A/A* or *A/B* genotype at the *ANT* locus, this may not be the most appropriate nuclear marker to use for unambiguous identification of hybrid. Specific nuclear DNA markers can improve the accuracy of hybrid individual identification where they are available.

Released *M. petechialis* (Yoshinogawa and Kanonji-0706) could be easily distinguished from *M. lusoria* individuals based on shell morphology. Most *M. petechialis* are imported to Japan from the northern part of China, Bohai Sea (Liaoning, Hebei, and Shandong) (F. Ito, personal communication) or from the western part of North Korea (Namp'o and Haeju) (Nagayama 2006). The periostracum (i.e., the outermost thin layer of the shell) of imported

M. petechialis often flakes off and is dull in color because transshipped *M. petechialis* individuals are packed tightly and can rub against each other during long-distance transport from the Chinese mainland (Figure 5). Only three individuals suspected of being hybrids in the current study however, could not be identified using morphological shell appearance. Thus, Chichibugahama #14 and Kanonji-0606 #18, exhibited the *M. petechialis* type in COI restriction type and *ANT* type, are likely to be putative hybrids rather than pure *M. petechialis* because the external shell appearance (i.e., periostracum) of these two were as smooth as that for *M. lusoria*.

Generally, the adult shell shape of *M. lusoria* is a rounded-triangle, while that of *M. petechialis* is sub-oval. The posterior dorsal margin of *M. lusoria* is straight, whereas that of *M. petechialis* is quite swollen, and the apex position of *M. lusoria* is skewed to the anterior side relative to that of *M. petechialis* (Okutani 2000; Henmi 2009). The shells of the putative hybrid individuals identified here were much more oval-shaped than that of *M. lusoria*, and the posterior dorsal margin was slightly swollen compared with that of *M. lusoria*. The periostracum condition of putative hybrids was more similar to that of *M. lusoria* than to *M. petechialis*. Identifying hybrids based only on morphological shell appearance can be difficult while judgments based on a combination of morphology and genetic information is generally more reliable.

Approximately 12 alien marine bivalve species have been introduced to Japan, both intentionally and accidentally (Iwasaki et al. 2004). Most have established natural populations that have expanded their ranges rapidly across many prefectures, example include; *Mytilus galloprovincialis* Lamarck, 1819, *Perna viridis* (Linnaeus, 1758), *Xenostrobus securis* (Lamarck, 1819), and *Corbicula fluminea* (Müller, 1774). *M. petechialis* seedlings were stocked in Mie Prefecture in 1969 and in Kagawa Prefecture in 1975, events that were followed by release of *M. petechialis* seedlings as reported by Ministry of the Environment (2011). Our results suggest that occurrence of the alien *M. petechialis* currently is limited. If *M. petechialis* establishes successfully and expands its range in Japan, genetic disturbance via hybridization with *M. lusoria* is a likely outcome in Kanonji, Kagawa Prefecture. To conserve the endangered

local *M. lusoria* resource, it will be important to control any future releases of *M. petechialis* to prevent this alien species from spreading across Japan more widely.

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