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Research Article

PCR-RFLP typing reveals a new invasion of Taiwanese *Meretrix* (Bivalvia: Veneridae) to Japan

Ayako Yashiki Yamakawa[1]* and Hideyuki Imai[2]

1 *Department of Regional Economics and Environmental Policy, College of Economics and Environmental Policy, Okinawa International University, 2-6-1 Ginowan, Ginowan, Okinawa, 901-2701, Japan*
2 *Lab. of Marine Biology and Coral Reef Studies, Faculty of Science, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan*

E-mail: a.yamakawa@okiu.ac.jp (AYY), imai@sci.u-ryukyu.ac.jp (HI)

**Corresponding author*

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Abstract

Samples of three hard clam *Meretrix* spp. (*M. lusoria*, *M. petechialis*, and Taiwanese *Meretrix*) were collected from 12 localities in Japan, China, Korea, and Taiwan between 2004 and 2013. PCR-RFLP analysis and nucleotide sequence analysis for the mtDNA COI region was performed on these samples. *Hin*cII and *Ase*I restriction assay discriminated *M. lusoria, M. petechialis*, and Taiwanese *Meretrix*, corresponding to the relatively large nucleotide substitution (6.35–8.20 %). In Taiwan, *M. lusoria* was introduced from Japan in the 1920s; however, our results suggest that Taiwanese *Meretrix* is genetically differentiated from *M. lusoria*. As well, the exotic Taiwanese *Meretrix* was found from Nishinagisa on the north coast of Tokyo Bay, demonstrating the Taiwanese *Meretrix* now occurs with *M. lusoria* in its native habitat. *Meretrix* seedlings (Kumamoto origin) from a Taiwanese aquaculture facility has been released into Nishinagisa since 2008 suggesting the Taiwanese *Meretrix* is inadvertently mixed with the *M. lusoria* produced in the aquaculture facility. In contrast, all samples from Kisarazu, on the east coast of Tokyo Bay, were identified as *M. lusoria* despite there being mass releases of *M. lusoria* (Kumamoto origin) cultured in Taiwan since 2007. Quality control procedures are needed for future *Meretrix* spp. releases to prevent further spread of the Taiwanese *Meretrix*.

Key words: Species identification; PCR-RFLP; *Hin*cII; *Ase*I; *Meretrix lusoria*; Taiwanese *Meretrix*

Introduction

Asian hard clams, genus *Meretrix* (Veneridae) are commercially important bivalves in East and Southeast Asia and East Africa (Yoosukh and Matsukuma 2001). These clams inhabit tidal flats, estuaries, and sandy beaches of the Indian Ocean, including east Africa, Southeast Asia, and along the Chinese continent, Korean Peninsula, and Japanese Archipelago in the west Pacific. There are nine recognized species: *M. meretrix* (Linnaeus, 1758), *M. casta* (Chemnitz, 1782), *M. lusoria* (Röding, 1798), *M. petechialis* (Lamarck, 1818), *M. ovum* (Hanley, 1845), *M. planisulcata* (Sowerby, 1851), *M. lyrata* (Sowerby, 1851), *M. lamarckii* Gray, 1853, and *M. attenuata* Dunker, 1862 (OBIS Indo-Pacific Molluscan Database 2006). Most species are important fishery resources. Asian hard clams have been consumed since ancient times, and *Meretrix* shells are one of the most abundant molluscs found in shell middens in Japan and the Middle East (Kanamaru 1932; Charpentier et al. 2004).

Because of the economic importance of *Meretrix,* previous research has mainly focused on its production in aquaculture (Yoshida 1941; Wu and Liu 1992; Tuan and Phung 1998), effects of organotin compounds (Midorikawa et al. 2004; Harino et al. 2006), and incidences of shellfish poisoning (Nguyen et al. 2006). Seedlings of a few *Meretrix* species are mass-produced in several countries, and nearly all hard clams sold in Taiwanese markets originate from aquaculture (Wu and Liu 1989). Past taxonomic studies on *Meretrix* considered only shell morphology (Fischer-Piette and Fischer 1940–1941; Yoosukh and Matsukuma 2001), although shell shape and color patterns often show marked intraspecific variability (Hamai 1934, 1935; Kosuge 2003).

Systematic descriptions of *Meretrix* species are confusing and the specific name *M. meretrix* has apparently been used for various species (Yoosukh and Matsukuma 2001; Chen et al. 2011a). In recent years, genetic studies of *Meretrix* have been increasing, with inter- and intraspecific genetic variation being demonstrated (Yamakawa et al. 2008; Torii et al. 2010; Yamakawa and Imai 2012), especially on *Meretrix* species in China (Deng et al. 2008; Chen et al. 2009; Ren et al. 2009; Li et al. 2010; Wang et al. 2010; Li et al. 2011; Lu et al. 2011a,b; Chen et al. 2011b; Wang et al. 2011; Kang et al. 2012; Lu et al. 2013).

Two species of *Meretrix*, *M. lusoria* and *M. lamarckii*, occur in tidal flats and shallow waters in Japan. *M. lusoria* is distributed in sheltered sandy tidal flats in Japan (except for Hokkaido and the Ryukyu Archipelago) and along the southern coast of Korea (Yamashita et al. 2004). *M. lusoria* once provided an important commercial and recreational fishery resource; however, landings have decreased significantly since the mid-1960s, and the commercial catch remains low (Higano 2004; Fishbase 2013). *M. lusoria* was designated an endangered species by the Japanese Ministry of the Environment in 2012 (Ministry of the Environment 2012) and is now considered to be locally extinct in Chiba Prefecture in Tokyo Bay (Chiba Prefecture 2011). In response to a decrease in the supply of *M. lusoria*, *M. petechialis* was recently imported from China and the Korean Peninsula.

In 2008, media reports indicated that the commercial harvest of *M. lusoria*, under the brand name of *Edomae hamaguri*, resumed on the east coast of Tokyo Bay after a 40-year closure (Japanese Daily, The Asahi Shimbun 2008; Yomiuri Shimbun 2008; Tokyo Shimbun 2008; Minato Daily 2008). Since 2007, local fishery co-operatives have released large quantities of *M. lusoria* seedlings (Kumamoto, Japan origin) along the east coast of Tokyo Bay (Minato Daily 2008) and, since 2008, on the Nishinagisa artificial tidal flat near Tokyo Disneyland on the north coast of Tokyo Bay (NPO Executive Committee of Furusato, Tokyo 2011). These *Meretrix* seedlings (Kumamoto origin) were mass-cultured in an aquaculture facility in Changhua Prefecture in Taiwan (Minato Daily 2008). *Meretrix* aqua-culture in Taiwan has a long history, starting in the 1930s (Chen 1990). The dominant *Meretrix* species in aquaculture was generally regarded to be *M. lusoria* that originated from Japan (Chen 1984; Chen 1990; Shao and Chiu 2003). Yamakawa et al. (2008) suggested that cultured Taiwanese *Meretrix* might be a distinct species from *M. lusoria* due to a high degree of genetic differentiation. Moreover, Taiwanese *Meretrix* is indistinguishable from Japanese *M. lusoria* by shell appearance (Yamakawa unpublished data) and, therefore, can occur undetected with *M. lusoria* on shallow tidal flats in Tokyo Bay.

In the current study, we examined genetic variation within *M. lusoria*, *M. petechialis*, and Taiwanese *Meretrix* populations in Japan, China, Korea, and Taiwan. We conducted species identification and detected introduced specimens in Japan using genetic markers developed from mitochondrial DNA.

Materials and methods

Specimens and DNA samples

Meretrix samples were collected from 12 localities in Japan, China, Korea, and Taiwan between 2004 and 2013 (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 for DNA extraction (Asahida et al. 1996). For tissue digestion, 10 µl of proteinase K (Wako Chemicals, Osaka, 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 (Imai et al. 2004).

Mitochondrial DNA sequence and restriction assay

A 743-base fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene was amplified by polymerase chain reaction (PCR) using a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). PCR reactions were performed in a 50-µl volume containing 0.2 µl of ExTaq^{TM} (Takara Bio, Kyoto, 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'-TAAACTTCAGGGTGACCAAA AAATCA-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 15–30 s, annealing at 45–50°C for 15–30 s, extension at 72°C for 30–60 s, and then a post-cycle extension at 72°C for 7 min. We selected one *M. lusoria* from Mutsu Bay, Aomori Prefecture, Japan, one *M. petechialis*

Table 1. Collection information of 12 locations where samples of *Meretrix* spp. were collected in Japan, China, Korea, and Taiwan.

Country	No.	Locality	Abbreviation	Geographic coordinates	Sampling date	N
Japan	1	Mutsu Bay, Aomori Pref.	MUT	41°21', 140°11'	Apr. 2005	22
	2	Kisarazu, east of Tokyo Bay	KSZ	Tsukiji fish market	Nov. 2012	48
	3	Nishinagisa, north of Tokyo Bay	NNS	35°38', 139°51'	Apr. 2013	62
	4	Kuwana, Mie Pref.	KWN	35°03', 136°42'	Mar. 2005	21
	5	Kitsuki, Oita Pref.	KTK	33°24', 131°38'	Apr. 2005	47
	6	Yanagawa, Fukuoka Pref.	YNG	33°38', 131°22'	Dec. 2004	48
	7	Midorikawa, Kumamoto Pref.	MDR	32°42', 130°36'	Jun. 2006	45
China	8	Bo-hai Sea	BOH	via Fishing company	Feb. 2005	47
Korea	9	Gunsan	GUN	Local fish market	Sep. 2005	48
	10	Buan	BUA	Local fish market	Sep. 2005	39
Taiwan	11	Tanshui River	TAN	25°09', 121°26'	Sep. 2006	48
	12	Cultured	CUL	Local fish market	May. 2005	48

imported from Bohai Sea, China, and one Taiwanese *Meretrix* from Tanshui, Taiwan, in which the amplified fragments of these three typical *Meretrix* species were subjected to nucleotide sequence analysis using an ABI 3700 genetic analyzer (Applied Biosystems) with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). Obtained sequences were deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers AB853864 (*M. lusoria*, Mutsu), AB853867 (Taiwanese *Meretrix*, Tanshui) and AB853869 (*M. petechialis*, Bohai) after alignment using CLUSTAL X ver. 1.83.1 (Thompson et al. 1997). Number of substitutions was counted and substitution percentage between *Meretrix* spp. COI partial sequences was calculated. Network of *Meretrix* spp. was constructed using NETWORK ver. 4.6.1.1 (Bandelt et al. 1999). Nucleotide sequence data were examined to identify restriction site differences between *M. lusoria, M. petechialis*, and Taiwanese *Meretrix* (GENETYX-MAC Ver. 8.0). We selected two restriction enzymes, *Hin*cII and *Ase*I (Toyobo, Tokyo, Japan), which provided diagnostic restriction patterns among three *Meretrix* spp.. RFLP analysis was performed in a 10 µl volume containing 1 µl of buffer H (Toyobo), 3–5 µl of PCR product, and 5 units of restriction enzyme (*Hin*cII and *Ase*I) at 37°C for 2 h. A 10-µl sample of the reactant was examined using electrophoresis on a 1% agarose gel (Trevi Gel^{TM}500; Trevigen, Gaithersburg, MD, USA) in TAE buffer at 100 V. After electrophoresis, gels were stained with ethidium bromide, visualized under ultraviolet (UV) light, and photographed.

Table 2. Number of substitutions and substitution percentage (bracket) between three *Meretrix* spp. from Japan, China and Taiwan (Length 634 bp).

	M. lusoria	*M. petechialis*	Taiwanese *Meretrix*
	MUT	BOH	TAN
MUT	-	48 (7.43)	53 (8.20)
BOH		-	41 (6.35)
TAN			-

Results

Mitochondrial DNA typing

A 743bp COI fragment was successfully amplified and sequenced from all *Meretrix* specimens used in this study. The number of nucleotide substitutions and substitution percentage between *Meretrix* spp. COI partial sequences (length 646 bp) were: 53 (8.20 %) between *M. lusoria* (MUT) and Taiwanese *Meretrix* (TAN); 48 (7.43 %) between *M. lusoria* (MUT) and *M. petechialis* (BOH) and 41 (6.35 %) between *M. petechialis* (BOH) and Taiwanese *Meretrix* (TAN) (Table 2). The network diagram (Figure 1) for of *M. lusoria* (MUT), *M. petechialis* (BOH) and Taiwanese *Meretrix* (TAN) based on substitution of mtDNA COI partial region indicated the Taiwanese *Meretrix* was genetically differentiated from *M. lusoria* and *M. petechialis*. *Hin*cII and *Ase*I digestions detected two and four restriction types, respectively (Figure 2), and six composite haplotypes were found for 12 local samples (Table 3 and Figure 3).

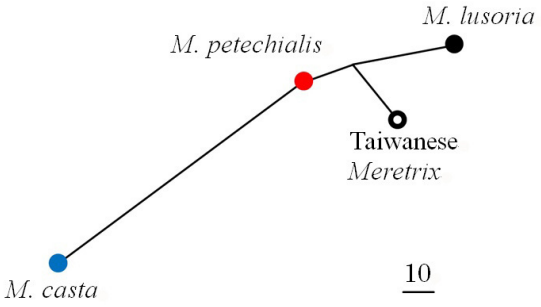

Figure 1. Network diagram of *M. lusoria* (Mutsu, Aomori Pref.), *M. petechialis* (Bohai, China), Taiwanese *Meretrix* (Tanshui, Taipei) constructed using the NETWORK ver. 4.6.1.1. with *M. casta* included as outgroup species. The scale bar indicates 10 substitutions.

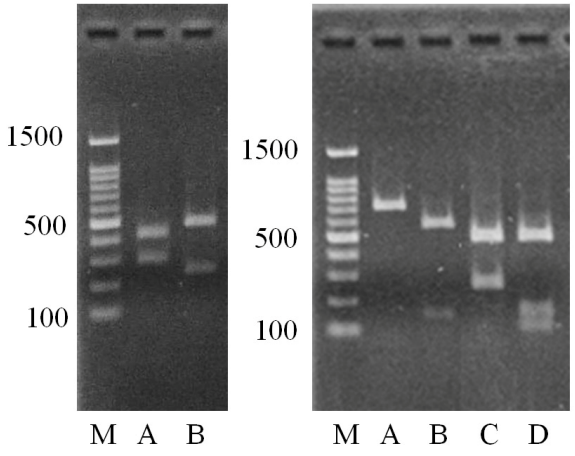

Figure 2. Agarose gel electrophoresis of *Hin*cII (left) and *Ase*I restriction types (right) of the mtDNA COI gene. Left, *Hin*cII A (427 and 307 bp), B (495 and 243 bp), which was able to distinguish *M. lusoria* from *M. petechialis* and Taiwanese *Meretrix*. Right, AseI A (743 bp), B (579 and 151 bp), C (465 and 265 bp), D (465, 153 and 125 bp) which was able to distinguish Taiwanese *Meretrix* from *M. lusoria* and *M. petechialis*. M, 100-bp molecular marker.

No composite haplotype was shared by the different species, except for the Nishinagisa samples, indicating that the three *Meretrix* spp. examined in this study could be readily discriminated using *Hin*cII and *Ase*I. Six individuals from Nishinagisa, on the north coast of Tokyo Bay, showed Taiwanese *Meretrix* haplotypes (Table 3 and Figures 3–4).

Discussion

This study revealed that both natural and cultured Taiwanese *Meretrix* samples were genetically distinct, in terms of mtDNA COI, from *M. lusoria* and *M. petechialis*. *M. lusoria* seedlings were introduced to the Tanshui River in Taiwan from Midorikawa, Kumamoto Prefecture and Saga Prefecture, Japan, in 1920s (Ministry of Agriculture and Forestry, Fisheries Research Institute 1931, Shao and Chiu 2003). Since then, *Meretrix* aquaculture has been very successful and spread rapidly around the west coast of Taiwan, specifically in Changhua and Yunlin prefectures (Chen 1984; Kuo 2005; Chien and Hsu 2006). The percentage of aquaculture-produced clams on the market reached 98.8% in 1986 (Wu and Liu 1989). The dominant species in aquaculture is generally regarded as *M. lusoria* (Chen 1990). Introduced Japanese *M. lusoria* are assumed to have become established as a cultured stock in Taiwan, and both cultured and natural *Meretrix* clams are thought to be artificially introduced species (Shao and Chiu 2003). However, our PCR-RFLP results indicate Taiwanese *Meretrix* to be genetically different from *M. lusoria* and *M. petechialis*. Our results suggest that a native population of Taiwanese *Meretrix* has existed since before the introduction of Japanese *M. lusoria* in the 1920s. Our previous allozyme analysis on 12 loci (Yamakawa et al. 2008) discovered that genetic distance between the Taiwanese *Meretrix* (aquacultured) and Japanese *M. lusoria* populations showed a high degree of genetic differentiation ($D > 0.386$), and genetic distance was large enough indicate separate species (based on Nei 1975). It is difficult to conclude that Taiwanese *Meretrix* is a different species from *M. lusoria* on the basis of RFLP analysis; however, we have a stronger case to suggest this by combining our RFLP and allozyme results. Further study using multiple genetic markers and suitable phylogenetic and morphological analysis would be needed to confirm whether Taiwanese *Meretrix* is a distinct species or not.

Six of 62 individuals collected in the artificial tidal flat at Nishinagisa on the north coast of Tokyo Bay possessed genetic characteristics of Taiwanese *Meretrix* (Table 3 and Figure 3) presumably were introduced from Taiwan. This is the first study to demonstrate that Taiwanese *Meretrix* individuals co-occur with local *M. lusoria*

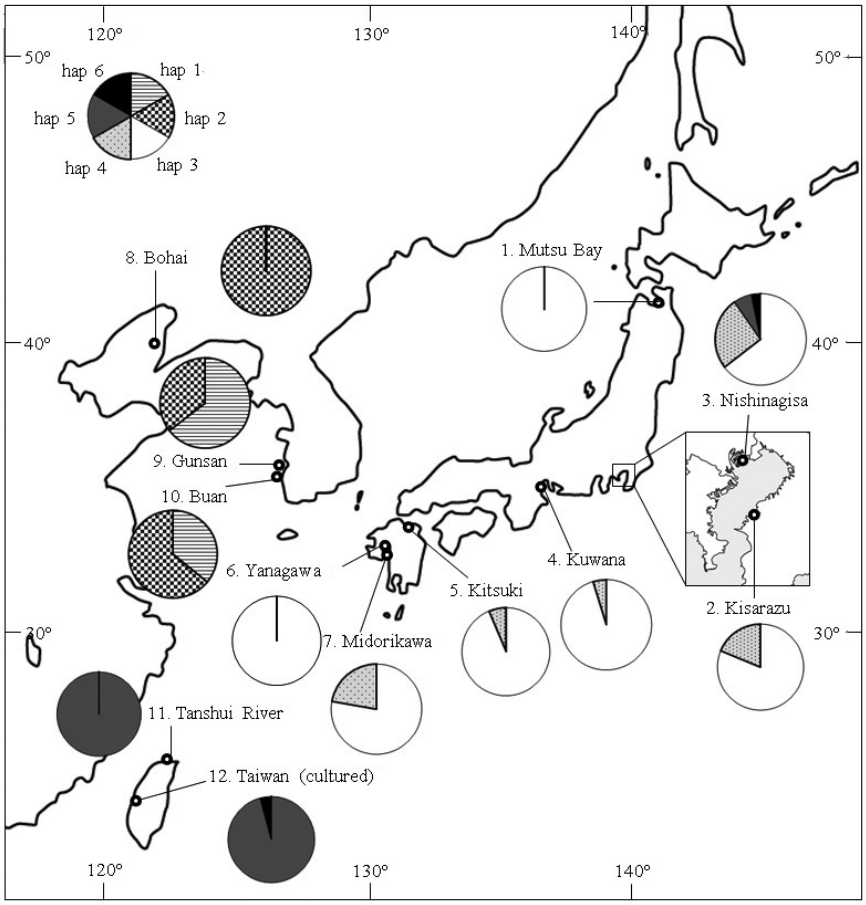

Figure 3. Haplotype frequencies for samples of *Meretrix* spp. collected from 12 locations in Japan, China, Korea, and Taiwan.

Table 3. Composite haplotype frequencies for samples of *Meretrix* spp. collected from 12 locations in Japan, China, Korea, and Taiwan. A–D indicates restriction types refer to Figure 2.

			M. lusoria							*M. petechialis*			Taiwanese	
Hap	*Hin*cII	*Ase*I	MUT	KSZ	NNS	KWN	KTK	YNG	MDR	BOH	GUN	BUA	TAN	CUL
1	A	A									0.65	0.36		
2	A	B								1.00	0.35	0.64		
3	B	B	1.00	0.81	0.65	0.95	0.94	1.00	0.78					
4	B	A		0.19	0.26	0.05	0.06		0.22					
5	A	C			0.06								1.00	0.96
6	A	D			0.03									0.04

in Japan. While once one of the most popular and valuable clams in Tokyo Bay, native *M. lusoria* nearly disappeared in the 1960s to 1970s due to massive reclamation and water pollution (Higano 2004; Yamashita et al. 2004; Henmi 2009). At present, *M. lusoria* is now considered to be extirpated in Chiba Prefecture in Tokyo Bay (Chiba Prefecture 2011). Taiwanese *Meretrix* may have been mixed with Japanese *M. lusoria* in the aquaculture facility by mishandling and/or misidentification (Figures 4). On the other hand, all individuals from Kisarazu, on the east coast of Tokyo Bay, were identified as *M. lusoria* (Figure 3), even though the local fishery cooperatives in Chiba Prefecture have conducted mass releases of *M. lusoria* aquacultured in Taiwan since 2007 (Fisheries Research Agency 2009–2012). It may be that number of analyzed

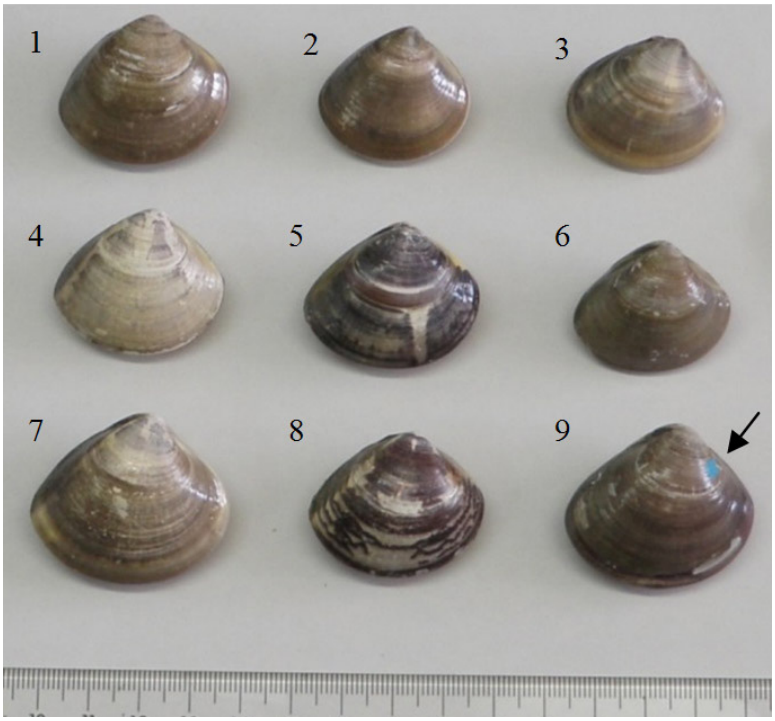

Figure 4. Examples of Taiwanese *Meretrix* and Japanese *M. lusoria* specimens from Nishinagisa, on the north coast of Tokyo Bay. No. 1–6. Taiwanese *Meretrix,* No. 7–9. Japanese *M. lusoria*. Arrow indicates blue paint as released marker.

samples (48) was too small to detect Taiwanese *Meretrix* in Kisarazu. A larger sample from other sites should be tested to see whether the Taiwanese *Meretrix* was simply missed due to chance and/or the small sample size.

A molecular-based study reported that *M. petechialis* that originated from China and Korea were observed in Japan as introduced species (Yamakawa and Imai 2012), and hybrids between *M. lusoria* and *M. petechialis* were found in natural habitats. The spawning season of *M. lusoria* in Tokyo Bay is from June to September (Taki 1950; Nakamura et al. 2010) while the spawning season of Taiwanese *Meretrix* is from April to November (Chen 1990; Wu and Liu 1992). Thus at the Nishinagisa site where the two co-occur, it is possible that hybridization could occur, if it has not already done so.

The impacts of introduced species can be diverse, including genetic disturbance through hybridization, exclusion and predation on native species, and economic damage to fisheries (Iwasaki 2006). For example, the Manila clam *Ruditapes philippinarum* (Adams and Reeve, 1850) is one of the most important commercial clams in Japan. Since the mid-1980s, the abundance of the Manila clam has declined considerably in Japan and large quantities of clams have been imported from China and Korea to provide seed for stocking and recreational shellfish gathering (Okoshi 2004). Kitada et al. (2013) reported that mass introductions of Chinese *Ruditapes,* which are genetically and morphologically different from Japanese *R. philippinarum*, were repeated in Japan. The *Ruditapes* population in the Ariake Sea includes hybrids between the alien Chinese *Ruditapes* and the native *R. philippinarum*. Furthermore, infections of the protozoan *Perkinsus* on *R. philippinarum* have been reported from various localities in Japan. Hamaguchi et al. (2002) reported that imported and introduced clams from China and Korea were infected with *Perkinsus* sp., that caused high mortality of *R. philippinarum* juveniles (Shimokawa et al. 2010) and contributed to drastic decreases in Japanese Manila clam resources (Waki et al. 2012). In Taiwan, several viruses have been isolated from cultured hard clams (*Meretrix* spp.); one birnavirus is associated with high juvenile mortality (Chou et al. 1994, 1998). Birnavirus-infected hard clams experience a higher predation pressure than noninfected clams (Liao et al. 2008). Avoiding clam-seedling releases from foreign countries is advisable until they can be given careful consideration because they can result in unexpected hybridization, new diseases, and high mortality in Japan.

Approximately 12 alien marine bivalve species have been introduced to Japan, either intentionally or accidentally (Iwasaki 2006). Most have successfully emigrated and undergone rapid nationwide expansion. Examples include *Mytilus galloprovincialis* Lamarck, 1819, *Perna viridis* (Linnaeus, 1758), *Xenostrobus securis* (Lamarck, 1819), and *Corbicula fluminea* (Müller, 1774). Our results suggest that Taiwanese *Meretrix* has genetically different characteristics from *M. lusoria* and *M. petechialis*, although further study would be needed to evaluate whether the Taiwanese *Meretrix* is a discrete species. The occurrence of exotic Taiwanese *Meretrix* is currently very limited in the north of Tokyo Bay. If Taiwanese *Meretrix* becomes established and expands its range within Tokyo Bay, it may cause the exclusion of native species and genetic disturbance *via* hybridization with *M. lusoria*. To conserve the local, endangered, *M. lusoria* resources, the identification of introduced Taiwanese *Meretrix* and *M. petechialis* by PCR-RFLP analysis is very effective because this method is much easier and cheaper than DNA sequencing methods. Rapid species identification at the local research institute is essential for monitoring introduced *Meretrix* species. While the Japanese *Meretrix* resource has almost collapsed in Tokyo Bay (Chiba Prefecture 2011), mass releases of *M. lusoria* seedlings from Taiwan are to be discouraged because of the possibility of introducing alien species.

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