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A possible hybrid zone in the *Mytilus edulis* complex in Japan revealed by PCR markers

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Abstract The adhesive protein allele of mussels collected at 13 points in Japan from Hokkaido to Kyushu was analyzed by the polymerase chain reaction using a set of primers which amplifies a part of the nonrepetitive region of the adhesive protein gene. While most mussels exhibited a 126 bp fragment, characteristic of the pure *Mytilus galloprovincialis*, 55 of 64 mussels sampled at Hiura and 1 of 14 mussels at Hakodate Port exhibited 168 and 126 bp fragments. Sequence analysis of the two fragments indicated that the 168 and 126 bp fragments are almost identical to previously reported sequences in *M. trossulus* and *M. galloprovincialis*, respectively. Since the frequency of heterozygous individuals in Hiura is very high, it is unlikely that they are simple hybrids. However, it is evident that mixing of genes occurred between the two species off Hokkaido.

Introduction

The three mussel species *Mytilus edulis*, *M. galloprovincialis* and *M. trossulus* are widely distributed in temperate intertidal zones of the northern and southern hemispheres (Gosling 1992). These species are closely related to one another and are referred to as the "*M. edulis* complex". The taxonomy of the three species has historically been confusing (Gosling 1984; Gardner 1992; Seed 1992). It is difficult, even at present, to identify the three species by morphological characteristics alone because their shell shapes are highly similar and often influenced by local environment. In addition, interspecific hybridization, commonly observed in regions where codistribution of species occurs (Koehn 1991), makes taxonomy even more complicated. Mussel populations have been characterized using allozyme characters instead of morphological characters (Koehn et al. 1984; McDonald and Koehn 1988; Varvio et al. 1988; Coustau et al. 1991; McDonald et al. 1991; Viard et al. 1994) or using mitochondrial or nuclear DNA sequences (Edwards and Skibinski 1987; Blot et al. 1990; Geller et al. 1993, 1994; Côrte-Real et al. 1994a, b) in recent years.

Mussels attach to wet surfaces using the byssus, an adhesive holdfast. Two major components of the byssus have been found in *Mytilus edulis*: Foot Protein 1, the adhesive protein (Waite and Tanzer 1981; Waite 1983, 1992; Waite et al. 1985), and Foot Protein 2, the plaque matrix protein (Rzepecki et al. 1992; Inoue et al. 1995a). Nucleotide sequences encoding the adhesive protein have been isolated and characterized in *M. edulis* and *M. galloprovincialis* (Strausberg et al. 1989; Filpula et al. 1990; Laursen 1992; Inoue and Odo 1994; Inoue et al. 1995b). It was found that the sequences consisted of a short nonrepetitive region followed by a long repetitive region. The sequences of the adhesive protein gene of the two species are essentially similar, but the remarkable difference is a 54 bp insertion in the nonrepetitive region of *M. edulis* (Inoue and Odo 1994). We designed a set of primers to amplify a part of the nonrepetitive region, including the point of the insertion. By polymerase chain reaction (PCR) using these primers, 180 and 126 bp fragments were amplified from *M. edulis* and *M. galloprovincialis* genomic DNA, respectively (Inoue et al. 1995c). It was also found that a 168 bp fragment was amplified from *M. trossulus*. Thus, sizes of fragments amplified using the PCR system are different among the three species. Since

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this system correctly identified *M. edulis* from Norway, Brittany (France) and Delaware (USA), *M. galloprovincialis* from Japan and the Mediterranean coast (France) and *M. trosulus* from Alaska, we considered it to be a suitable diagnostic system for the three species. In the present study, we analyzed mussel populations from Japan using this PCR system. It was found that most mussels from Japan have only the *M. galloprovincialis*-type sequence, but some mussels collected at two sites on Hokkaido Island have both *M. galloprovincialis*- and *M. trossulus*-type sequences. From these results, the origin of mussels in Japan is discussed.

Materials and methods

Mussels

Mussel samples were collected from January 1994 to February 1996 at sites shown in Fig. 1 and described in Table 1. Samples from Mutsu Bay (A), Kamaishi Bay and Saiki Bay were obtained from rafts and floats for aquaculture. Those from Matoya Bay and Hirota Bay were cultured mussels. Samples from Hiura, Mutsu Bay (B) and Ozaki-Shirahama were collected on natural rocks along a turbulent shoreline. They were usually clustered with *Septifer virgatus* (Mytilidae). Mussels from Hiura were also clustered with *Mytilus coruscus*, which can be distinguished from the *M. edulis*-complex species by morphology. All other samples were collected at wharves of ports. At Tokyo Port and Osaka Port, mussels are clustered with *Limnoperna fortunei kikuchii* (Mytilidae). Pure *M. trossulus* was collected at Juneau, Alaska, USA.

DNA extraction

Total DNA was purified from a piece of the gill of each mussel by Proteinase K–SDS treatment followed by phenol extraction and precipitation with ethanol (Inoue et al. 1995c). Isolated DNA was dissolved in 50 µl TE (10 m*M* Tris, 1 m*M* EDTA).

PCR amplification

About 100 ng DNA was dissolved in 100 µl 1 × Tth buffer (TOYOBO, Osaka, Japan) containing 4.5 µ*M* sense primer Me15, 4.5 µ*M* antisense primer Me16 and 200 µ*M* dNTP (Inoue et al. 1995c). After preheating to 94 °C, 1 unit of Tth DNA polymerase (TOYOBO, Osaka, Japan) was added and 30 cycles of amplification were performed. Each cycle consisted of 30 s at 94 °C, 30 s at 56 °C and 90 s at 70 °C (Inoue et al. 1995c). PCR products were electrophoresed on a 3% NuSieve 3:1 agarose (FMC, Rockland, Me.) gel.

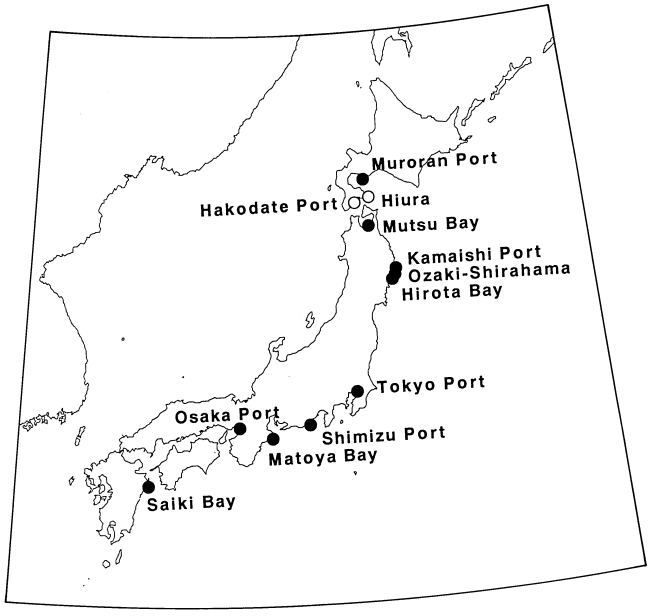

Fig. 1 *Mytilus galloprovincialis* and *M. galloprovincialis*/*M. trossulus* hybrid. Collection sites of mussel samples analyzed in the present study. *Open circles* indicate points where the *M. galloprovincialis*/*M. trossulus* hybrid type and *M. galloprovincialis* type were found. *Solid circles* indicate points where only *M. galloprovincialis* haplotype was found

Sequencing

Fragments amplified from the genome of a mussel collected at Hiura were inserted into the pCR-script SK(+) vector using the

Table 1 Mussel samples collected in present study and their adhesive protein haplotypes (*Mg*, *Mytilus galloprovincialis* haplotype; *Mg*/*Mt*, *M. galloprovincialis*/*M. trossulus* hybrid haplotype)

Region	Sampling point	Sampling date	Types of habitat	Number of samples	*Mg*	*Mg*/*Mt*	Coexisting mussel species
Hokkaido	Muroran Port	Mar 1995	Concrete wall	16	16	0	
	Hiura	May 1994, Feb 1996	Natural rock	64	9	55	*Septifer virgatus*, *Mytilus coruscus*
	Hakodate Port	Feb 1995	Concrete wall	14	13	1	
Honshu	Mutsu Bay (A)	Mar 1995	Raft	16	16	0	
	Mutsu Bay (B)	Mar 1995	Natural rock	32	32	0	
	Kamaishi Bay	Aug 1994	Raft	16	16	0	
	Ozaki-Shirahama	Aug 1994	Natural rock	16	16	0	*Septifer virgatus*
	Hirota Bay	Jan 1995	Raft[a]	8	8	0	
	Tokyo Port	Jul 1994	Concrete wall	8	8	0	*Limnoperna fortunei kikuchii*
	Shimizu Port	Jun 1994	Concrete wall	16	16	0	
	Matoya Bay	Jan 1994	Raft[a]	8	8	0	
	Osaka Port	Aug 1994	Concrete wall	8	8	0	*Limnoperna fortunei kikuchii*
Kyushu	Saiki Bay	Jun 1994	Raft	8	8	0	

[a]Cultured mussels

pCR-script Cloning Kit (Stratagene, La Jolla, Calif.). The sequences of both strands of three independent clones were determined using a 373A DNA sequencer (Applied Biosystems Inc., Foster City, Calif.) and a PRISM Dyeterminator Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, Calif.).

Results

Adhesive protein haplotypes

The adhesive protein allele of mussels was determined by PCR amplification of the variable region of the adhesive protein gene (Inoue et al. 1995c). All the mussels collected in Honshu and Kyushu exhibited a 126 bp fragment (126 bp) (Fig. 2; Table 1). Although a minor band sometimes appeared at the position of about 300 bp, it was disregarded as a nonspecific band (Fig. 2). Mussels from Muroran Port also exhibited a 126 bp fragment (Table 1). These mussels were considered to be pure *Mytilus galloprovincialis*. Mussels collected at Hiura and Hakodate Port were, in contrast, not uniform. While nine specimens from Hiura exhibited only a 126 bp band, as those from other sites, the 55 remaining samples exhibited two fragments, 168 and 126 bp, likely to correspond to the *M. trossulus*- and *M. galloprovincialis*-type sequences, respectively (Fig. 2; Table 1). The specimens from Hakodate Port also included one individual revealing two fragments.

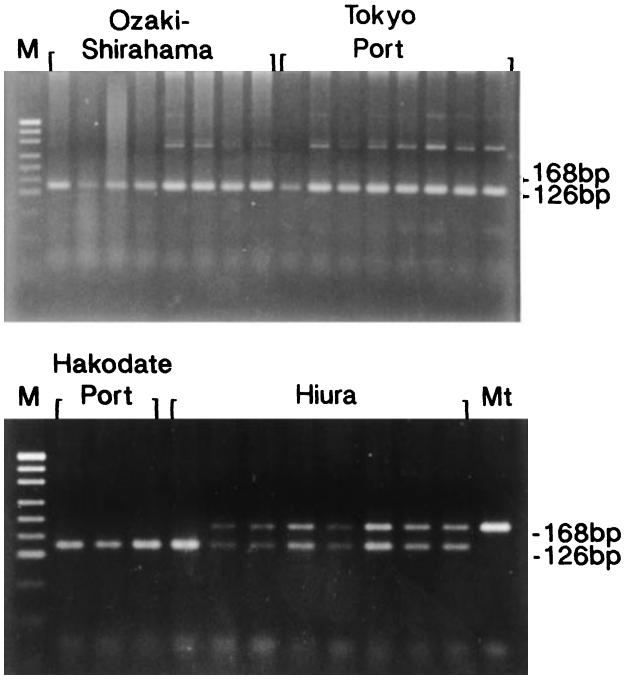

Fig. 2 *Mytilus galloprovincialis*, *M. trossulus* and their hybrid. Typical results of analysis of Foot Protein 1 haplotypes by PCR, exemplified with results on mussels collected at Tokyo Port, Ozaki-Shirahama, Hakodate Bay and Hiura. Results for pure *M. trossulus* collected at Juneau, Alaska, shown for comparison (*Mt*) (*M* pUC19/*Sty*I marker)

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A
CCAGTATACAAACCTGTGAAGACAAGTTATTCGTCACCATATAAACCACCAACATACCAA  60
P  V  Y  K  P  V  K  T  S  Y  S  S  P  Y  K  P  P  T  Y  Q    20

CCACTCAAAAAGAAACCaATGGACTATAATAGTTCTCCGCCAACATATGGATCAAAGACA 120
P  L  K  K  K  P  M  D  Y  N  S  S  P  P  T  Y  G  S  K  T    40

AACTATCTTGCAAAGAAGCTGTCATCTTACAAACCTATTAAGACAACA              168
N  Y  L  A  K  K  L  S  S  Y  K  P  I  K  T  T                 56

B
CCAGTATACAAACCTGTGAAGACAAGTTATCATCCTACGAATAGTTATCCGCCAACATAT  60
P  V  Y  K  P  V  K  T  S  Y  H  P  T  N  S  Y  P  P  T  Y    20

GGATCAAAGACAAACTATCTGCCACTTGCAAAGAAGCTGTCATCTTACAAACCTATTAAG 120
G  S  K  T  N  Y  L  P  L  A  K  K  L  S  S  Y  K  P  I  K    40

ACAACA                                                        126
T  T                                                           42
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Fig. 3 *Mytilus galloprovincialis*/*M. trossulus* hybrid. Nucleotide and deduced amino acid sequences of the two fragments amplified from the genomic DNA of a mussel collected at Hiura. **A** Sequence of the 168 bp fragment, which is almost identical to that of *M. trossulus* previously described (Inoue et al. 1995c) except for the 78th nucleotide indicated by *lower case*. **B** Sequence of the 126 bp fragment, which is identical to the corresponding part of *M. galloprovincialis* described by Inoue and Odo (1994). Sequences corresponding to primers *underlined*

Sequences of amplified fragments

Two fragments amplified from a mussel collected at Hiura (Fig. 2, Lane 8) were isolated and sequenced to confirm results (Fig. 3). The sequence of the 168 bp fragment was almost identical with that reported previously from pure *Mytilus trossulus* of Alaska (Inoue et al. 1995c) except for a single silent base substitution. The sequence of the 126 bp fragment was perfectly identical with that previously reported for *M. galloprovincialis* (Inoue and Odo 1994). Thus, most mussels collected at Hiura were shown to have sequences of both *M. galloprovincialis* and *M. trossulus* types. No pure *M. trossulus* haplotype was found at any site surveyed in the present study.

Discussion

Mussels have been well studied ecologically, genetically and physiologically. They have also received much attention as marine foulers as well as indicators of marine environmental quality in recent years (Kajihara 1991; Widdows and Donkin 1992). In addition, mussels are important as food in many countries. Since the three species of the *Mytilus edulis* complex are at least independent populations and have different phenotypic properties, it is important to identify them whatever the purpose for studying them.

Smooth-shelled mussels in Japan are often identified as *Mytilus edulis* (Kajihara 1991, for a review). In the present study, we showed that most mussels in Japan are *M. galloprovincialis* by using sequences of the variable region of the adhesive protein gene as a marker. The result is consistent with the report by Wilkins et al. (1983) in which mussels collected at Sanriku Bay were

identified as *M. galloprovincialis* by morphological and isozyme characters. *M. galloprovincialis* is supposed to have been introduced to Japan before 1935 from Europe (Wilkins et al. 1983), because no smooth-shelled mussel had been found in Honshu prior to the observation by Kanamaru (1935). It has also been suggested that *M. galloprovincialis* probably originated in the South Pacific, and introduction into Japan may have been from this area instead of the Mediterranean Sea (Koehn 1991). Wherever it originated *M. galloprovincialis* was likely introduced as a result of the increase in transoceanic traffic in the early twentieth century and spread immediately along Japanese coasts where few competitors were encountered. Now it is one of the most common animals in the intertidal zone of Honshu, regardless of the type of habitat (natural rock, artificial concrete wall, raft, etc.). Even cultured mussels have the same type of gene, which is not surprising since they are derived from the wild population. *M. galloprovincialis* was found even off Hokkaido and the northern part of Honshu in the present study. This result is interesting because the distribution of *M. galloprovincialis* in Europe is confined to southern Europe, including the Mediterranean Sea, while *M. edulis* is distributed in colder waters of the North Atlantic (McDonald et al. 1991). Results obtained in the present study indicate that *M. galloprovincialis* also has a potential to adapt to colder temperatures.

In contrast to Honshu, occurrences of native smooth-shelled mussels were recorded prior to 1919 off Hokkaido and in more northern areas (see Wilkins et al. 1983). Although originally identified as *Mytilus edulis*, they are now considered to be *M. trossulus* on the basis of the shell morphology of specimens preserved at the Hokkaido Wakkanai Fisheries Experimental Station (Kuwahara 1993). *M. trossulus* has also been recorded at several sites off Hokkaido, such as Hunka Bay and the Tsugaru Channel including Hakodate Port, Hiura and Muroran Port (Kuwahara 1993). It is unknown whether they are native to Hokkaido or invaders from the eastern North Pacific. In either case, it is evident that they appeared off Hokkaido prior to the occurrence of *M. galloprovincialis*. Since they did not occur off Honshu despite the proximity of Hokkaido and Honshu (the nearest distance is less than 20 km) and the enormous amount of marine traffic between the two islands, it appears that *M. trossulus* was not able to adapt to the environmental conditions of Honshu. Thus, Hokkaido seems to be a southern limit of the North Pacific *M. trossulus.* We also found mussels having both *M. galloprovincialis*- and *M. trossulus*-type sequences at Hiura and Hakodate Port, which are presumed to be caused by hybridization and introgression between the two species. Such mussels predominate in the Hiura population, and no pure *M. trossulus*-type sequence was found there. These are very puzzling results because pure *M. trossulus* should also be present if they are simple hybrids. Several explanations are possible: they may be true hybrids, more suited to the environmental conditions of Hiura than pure *M. trossulus* and *M. galloprovincialis*. It is also possible that the two-banded genotype was caused by introgression. They may have both types of sequences in their haploid genome, and both sequences may be thus always inherited to offspring. Pure *M. galloprovincialis* observed in the Hiura population may be invaders from proximate areas. Anyway, it is evident that genetic mixing is occurring at Hokkaido between *M. trossulus* from the North Pacific and *M. galloprovincialis* from the South Pacific or Europe. Further research and experiments are required, including detailed analysis of the distribution of *M. trossulus* off Hokkaido and artificial hybridization between the two species and their hybrids.

Although *Mytilus galloprovincialis* seems dominant in the intertidal zones of most of Japan at present, it is unknown whether this dominancy is stable. For example, at Harumi Wharf of Tokyo Port and Tenpozan Wharf of Osaka Port, *M. galloprovincialis* is rather rare, while *Limnoperna fortunei kikuchii* is dominant. In addition, it has been reported that the green mussel, *Perna virdis* (Mytilidae), is also increasing there (Kajihara 1991). These phenomena may be correlated with changes in environmental conditions, including water temperature, salinity and the extent of water pollution. Continuous research on the dynamic redistribution of mussel populations may be useful to reveal changes in environmental conditions.

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