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Multiplex PCR Diagnosis for *Crassostrea* Oyster Discrimination of *C. sikamea* and *C. gigas*

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Abstract: Kumamoto oyster *Crassostrea sikamea* (Amemiya, 1928) is a small, deep-cupped and threatened oyster species that limitedly occurs in Ariake Bay, Japan. Due to the morphological plasticity, *C. sikamea* and closely related Pacific oyster *C. gigas* (Thunberg, 1793) are hardly identifiable by shell morphology but genetically separable by mitochondrial DNA analysis. This study demonstrated the survey of *C. sikamea* in Yatsushiro Bay, where no occurrence record of *C. sikamea* had been available, by multiplex PCR analysis for species discrimination between *C. sikamea* and sympatric *C. gigas*. At the northeastern recesses of Yatsushiro Bay, which is neighboring south on Ariake Bay, 163 oyster specimens were collected and 28 *sikamea*-like oysters were then preferentially selected. Multiplex PCR amplification of the mitochondrial 16S ribosomal RNA gene using universal primers and additional *sikamea*-specific primer enabled to diagnose 18 *C. sikamea* and 10 *C. gigas* oysters. Meticulous screening of *C. sikamea* stocks in Yatsushiro Bay is imperative for the conservation of its biodiversity under the existing conditions that this species becomes endangered in Ariake Bay as its native habitat.

Key words: *Crassostrea sikamea*, *Crassostrea gigas*, oyster, threatened species, Yatsushiro Bay, multiplex PCR

INTRODUCTION

Kumamoto oyster *Crassostrea sikamea* (Amemiya, 1928) is the threatened oyster species that limitedly occurs in Ariake Bay, Kumamoto Prefecture, Kyushu, Japan (Sato, 2000). This species was first identified in 1928 as a distinct variety of Pacific oyster *C. gigas* (Thunberg, 1793) on the basis of ecobiological and morphological differences (Amemiya, 1928). Although several subsequent studies had so far been performed on genetics, e.g., crossbreeding (Imai and Sakai, 1961; Numachi, 1978), serology (Numachi, 1962), chromosomes (Armed, 1975) and allozymes (Buroker *et al*., 1979; Ozaki and Fujio, 1985), the taxonomical status for *C. sikamea* became more confused. Banks *et al*. (1994) finally affirmed the genetic separation of *C. sikamea* from *C. gigas* by PCR-RFLP analysis using a mitochondrial DNA fragment encoding the 16S ribosomal RNA (rRNA) gene.

Banks *et al*. (1994) also reported several failures to find *C. sikamea* in Ariake Bay known as its native and limited habitat, speculating that *C. sikamea* might be almost extinct in Japan. In September 1996, the detailed survey of *C. sikamea* was performed on 13 local sites in Ariake Bay and 1 site in Yatsushiro Bay, which is neighboring south of Ariake Bay (Fig. 1) and its dominant colonies were found at only 4 sites along the eastern to northern shores of Ariake Bay (Hedgecock *et al*., 1999). This result corresponded to the previous observation that *C. sikamea* colonies occurred at the recesses of Ariake Bay (Amemiya, 1928), but there has been no report available on an occurrence of *C. sikamea*

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Fig. 1: Map of Ariake Bay and Yatsushiro Bay, Kumamoto Prefecture, Kyushu, Japan, showing the sampling sites of *Crassostrea* oysters

in Yatsushiro Bay. The present study was undertaken to survey *C. sikamea* at the northeastern recesses of Yatsushiro Bay by multiplex PCR analysis for species discrimination between *C. sikamea* and sympatric *C. gigas*.

MATERIALS AND METHODS

DNA Preparation

Small cupped oysters with the morphology of *Crassostrea* oysters were sought in May 2006 at the northeastern recesses of Yatsushiro Bay, Kumamoto Prefecture, Kyushu, Japan (Fig. 1). Our collaborator who has engaged in oyster farming for more than 40 years collected 163 oyster specimens and 28 *sikamea*-like oysters that were morphologically somewhat different from *C. gigas* were then preferentially selected. They were transported alive to our laboratory in Miyazaki Prefecture, Kyushu, Japan and then their adductor muscle was collected followed by immediate store at -20°C until DNA extraction. Total genomic DNA was prepared from frozen adductor muscle of 28 *sikamea*-like oysters by the modified Urea-SDS-Proteinase K method (Aranishi and Okimoto, 2004, 2005; Aranishi, 2006) and the amount and quality of obtained genomic DNA were evaluated in a BioPhotometer (Eppendorf).

PCR Diagnosis

Multiplex PCR amplification of the partial 16S rRNA gene was carried out in 10 μL of PCR reaction mixture containing GoTaq Green Master Mix (Promega) supplemented with $MgCl_2$ to a final concentration of 2.0 mM, 0.5 μM universal forward primer 5'-CGCCT GTTTA TCAAA AACAT-3' and 0.05 μM universal reverse primer 5'-CCGGT CTGAA CTCAG ATCAC GT-3' (Kessing *et al*., 1989), 0.45 μM *sikamea*-specific reverse primer 5'-ACTCA GAAAG GTTAG GCTTA C-3' (Banks *et al*., 1993) and genomic DNA. PCR amplification protocol consisted of an initial denaturation

at 94°C for 2 min, followed by 35 cycles of 10 sec at 94°C, 10 sec at 54°C and 40 sec at 72°C and a final extension at 72°C for 5 min in a Techgene thermal cycler (Techne). A 5 μL portion of PCR amplicon was migrated onto 2.0% agarose gel at 15 V cm^{-1} for 40 min and then visualized under UV illumination in an EDAS290 Gel Documentation System (Invitrogen).

RESULTS

A simple diagnostic protocol for *C. sikamea* and *C. gigas* was developed on the basis of the previous method by Banks *et al*. (1993). Using the standard specimens of *C. sikamea* and *C. gigas*, modified multiplex PCR amplification of the mitochondrial 16S rRNA gene demonstrated a common 530 bp product from both species and additional 321 bp product from *C. sikamea* alone (Fig. 2). These PCR fingerprints enabled to rapidly and reliably discriminate *C. sikamea* from sympatric *C. gigas* in the field oyster specimens.

Out of 163 *Crassostrea* oysters collected at the northeastern recesses of Yatsushiro Bay, 28 morphologically *sikamea*-like oysters having wrinkled left valves and smooth right valves were then analyzed. Our multiplex PCR amplification was successfully performed to obtain the common PCR product from all oysters analyzed and the *C. sikamea*-specific PCR product from 18 of 28 oysters (64.3%). This result shows a new record for an occurrence of *C. sikamea* at the local site out of Ariake Bay.

DISCUSSION

C. sikamea is one of the most unique but least investigated cupped oyster species due to its limited distribution in Ariake Bay (Amemiya, 1928; Armed, 1975; Numachi, 1978; Hedgecock *et al*., 1999). There has been a well-known mystery why *C. sikamea* does not occur in other sea areas, e.g., Yatsushiro Bay (Sato, 2000), because *C. sikamea* spats are supposed to migrate by water circulation through several straits between the Uto Peninsula and Amakusa Islands from Ariake Bay to Yatsushiro Bay (Fig. 1). As *C. sikamea* was documented as the vulnerable species in Ariake Bay as its native habitat (Kumamoto Prefecture, 2004), a new occurrence record for *C. sikamea* in Yatsushiro Bay, which we ascertained in this study, is surely valuable information in its immediate conservation.

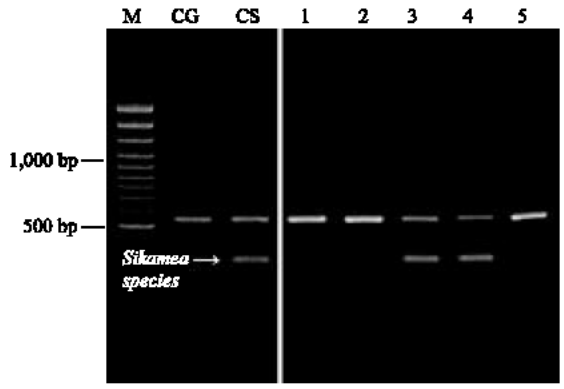

Fig. 2: Multiplex PCR diagnosis of the standard specimens of *C. gigas* and *C. sikamea* along with 5 *sikamea*-like oysters collected in Yatsushiro Bay using universal and *sikamea*-specific primers for mitochondrial 16S rRNA gene. CG and CS indicate the standard specimens of *C. gigas* and *C. sikamea*, respectively. M, 100 bp ladder marker

Oyster classification remains problematic and taxonomic status for many species has not been satisfactorily resolved because of the morphological plasticity (Wang *et al*., 2004). Of 28 *sikamea*-like oysters analyzed in this study, the shell length of most specimens measured <4 cm and some of them showed the shell morphology completely corresponding to that of *C. sikamea* recorded by Amemiya (1928). However, the shell morphology of at least 7 specimens, whose shell length measured >6 cm, was relatively similar to that of *C. gigas*. Nevertheless, not only the former specimens (e.g., Specimen 4 in Fig. 2) but also the latter specimens (e.g., Specimen 3 in Fig. 2) were genetically diagnosed as *C. sikamea* by mitochondrial DNA analysis. This result substantiates the use of molecular diagnosis to discriminate morphologically plastic *C. sikamea* and sympatric *C. gigas* in the field oyster specimens.

Several rivers flow from east into the northeastern recesses of Yatsushiro Bay and waters of these estuaries are less saline than those of the southwestern mouse of Yatsushiro Bay. The occurrence of *C. sikamea* at such less saline sites in Yatsushiro Bay is due to its low salinity tolerance (Amemiya, 1928), as evidenced during the recent survey of *C. sikamea* in Ariake Bay (Hedgecock *et al*., 1999). Meticulous screening of *C. sikamea* stocks at estuaries in Yatsushiro Bay is imperative for the conservation of its biodiversity under the existing conditions that this species becomes endangered in Ariake Bay as its native habitat (Kumamoto Prefecture, 2004).

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