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Occurrence of the Kumamoto oyster *Crassostrea sikamea* in the Ariake Sea, Japan

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Abstract The Kumamoto oyster *Crassostrea sikamea* is distinguished from the closely related Pacific oyster *C. gigas* by concordant differences in 16S rDNA, allozymes, and a one-way gametic incompatibility. After repeated failures to find this oyster in its native habitat, we speculated in 1994 that "the Kumamoto oyster may be extinct in Japan". In September 1996, we sampled small, deep-cupped oysters from the Ariake Sea and typed these for 16S rDNA and ITS-1 DNA markers previously shown to be diagnostic for the three most common oysters in the Ariake Sea, *C. gigas*, *C. sikamea* and *C. ariakensis*. Our earlier suggestion of the demise of *C. sikamea* proved incorrect. Of the 256 oysters sampled, 181 (71%) were *C. gigas*, 53 (21%) were *C. sikamea*, and 22 (9%) were *C. ariakensis*; no interspecific hybrids were observed. The distributions of *C. sikamea* and *C. ariakensis* are clumped in the Ariake Sea: *C. sikamea* occurs on the eastern and northern shores, *C. ariakensis* occurs only in the northern part. These results emphasize the value of molecular markers for discriminating these morphologically plastic species both in the field and in aquaculture.

Introduction

The Kumamoto oyster *Crassostrea sikamea* was introduced from Japan to the west coast of the USA in the late 1940s (Woelke 1955). Unfortunately, by the 1980s, the Kumamoto oyster had been both inadvertantly and purposefully hybridized with the more common Pacific oyster *C. gigas* (Quayle 1988). Interest in the specific status and conservation of this species was revived after Banks et al. (1993) showed that, compared to *C. gigas*, *C. sikamea* had fixed substitutions at 7 of 319 nucleotides of the mitochondrial gene coding for large-subunit rRNA. Banks et al. (1994) provided concordant molecular and biological evidence for the specific status of *C. sikamea*, and Hedgecock et al. (1993) and P.M. Gaffney et al. (unpublished data) subsequently used molecular markers to identify pure Kumamoto oyster brood stocks for commercial breeders.

Banks et al. (1994) reported several failures to find Kumamoto oysters in their native Japan. They also studied 5 oysters from the Ariake Sea and 10 from Fukiage, Kyushu, Japan, which the mtDNA test revealed to be C*rassostrea gigas*, and suggested that "the Kumamoto oyster may be extinct in Japan." As previous sampling was limited and might have missed *C. sikamea*, an expedition to the Ariake Sea was undertaken in September 1996 to collect small deep-cupped oysters from estuaries where the more mesohaline *C. sikamea* might reside. We subsequently typed 256 specimens for the mitochondrial DNA (mtDNA) marker described by Banks et al. (1993, 1994) and for the first internal transcribed spacer (ITS-1) region of the nuclear rRNA gene family (Wilbur and Gaffney 1997); both markers are diagnostic for three species of oyster native to the Ariake Sea (Numachi 1978): *C. gigas*, *C. sikamea* and *C. ariakensis*.

Materials and methods

Samples

Oysters with the morphology of *Crassostrea sikamea*, < 4 cm shell height, with deep cupped, wrinkled left valves and smooth right valves, were sought from 24 to 26 September 1996 from 14 localities around the Ariake Sea (Fig. 1; Table 1). However, considerable variation in shell morphology was encountered among oysters of the desired size, both within and among sites; flat shapes and foliose ornamentations on right valves suggested the presence of small *C. gigas* or *C. ariakensis*. Sites 3 to 6, 8, 10, 11 and 14 appeared to be dominated by *sikamea*-like oysters, while Sites 1, 2,

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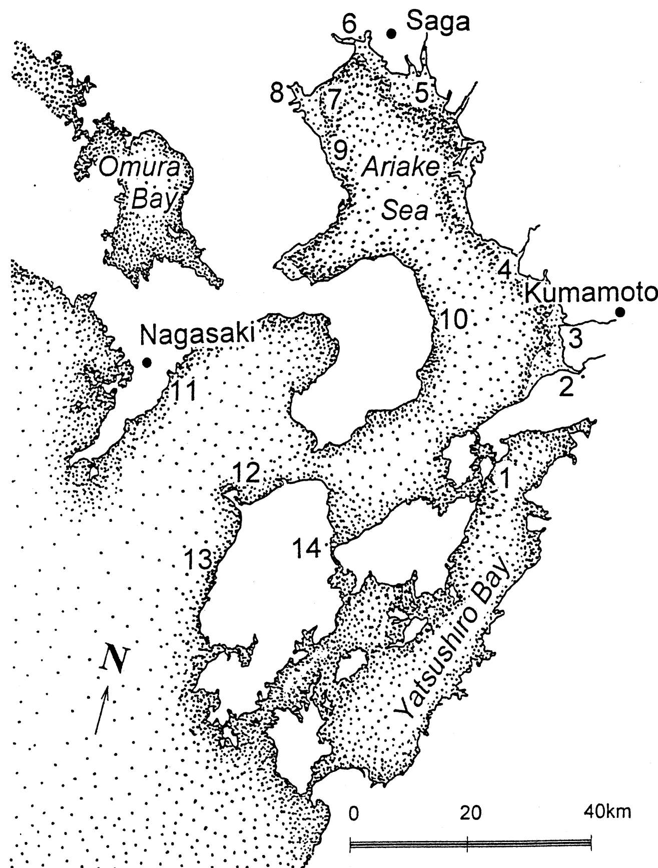

Fig. 1 Map of Ariake Sea, Kyushu, Japan, showing 14 localities at which *Crassostrea* spp. were collected

7, 12 and 13 appeared to be dominated by *gigas*-like oysters. Collections from Sites 3, 12 and 13 appeared to be mixtures of these two species, while Site 9 appeared to have all three types. The *sikamea* morph was preferentially selected where it was in the minority. Small pieces of mantle tissue were taken from each specimen and placed into a 1.5 ml Eppendorf tube with ~1 ml 100 m*M* NaCl, 10 m*M* Tris-Cl (pH8) and 25 m*M* EDTA (pH 8). No gonadal development was observed in any specimen.

Molecular methods

DNA was extracted from each tissue sample, as described by Banks et al. (1993). Methods for amplifying a 319 base-pair (bp) segment of mitochondrial large-subunit rDNA were described by Banks et al. (1993); we used two conserved *Crassostrea* primers, 5′-CCGGCCGCCCTAGCGTGAGGG-3′ and 5′-ATTAGCCTGTT ATCCCCGGCG-3′, and cut the resulting polymerase chain reaction (PCR) product with *Dra I* restriction endonuclease. *Dra I* does not cleave the *C. sikamea* sequence but does cleave the *C. gigas* sequence into two fragments of 141 and 178 bp. *Dra I* is also expected to cleave the *C. ariakensis* sequence into two fragments of 103 and 217 bp, according to the nucleotide sequence of O'Foighil et al. (1995).

We assayed the ITS-1 phenotypes of specimens, to confirm the mtDNA test and to check for interspecific hybrids. Primers (P. M Gaffney unpublished data), were ITS-A: 5′-GGTTTCTGTAGG TGAACCTGC-3′ and ITS-B: 5′-CTGCGTTCTTCATCGACCC-3′. Samples were amplified in 20 μl volumes with 2.0 μl of proteinase-K digestion as template; 1x PCR buffer (Promega); 1.6 μl 25 m*M* $MgCl_2$; 0.4 μl 10 μ*M* dNTP; 0.4 μl of each primer (10 μ*M* stock solutions) and 0.12 unit *Taq* polymerase (Promega). PCRs were performed for one cycle of 3 min at 94 °C, 15 s at 52 °C, and 20 s at 72 °C; and 35 cycles of 30 s at 94 °C, 15 s at 52 °C, and 20 s at 72 °C with a last extension of 2 min at 72 °C. Products were digested with *TaqI* restriction endonuclease at 65 °C for 3 h and the resulting fragments were separated on 4% agarose gels. The three species have different size products and digest patterns, but exact fragment sizes are not yet known (P. M. Gaffney personal communication).

Results

PCR products and restriction digests were obtained for all specimens except one from Site 10, which was not scored for the 16S rDNA marker. Each individual had a restriction enzyme digest pattern consistent with *Crassostrea gigas*, *C. sikamea* or *C. ariakensis* (Fig. 2). Species diagnoses by mitochondrial and nuclear DNA restriction digest patterns were concordant for the 255 individuals scored for both markers.

Table 1 *Crassostrea* spp. Three species, diagnosed by restriction digest patterns of 16S rDNA and ITS-1 PCR products (Fig. 2), among 256 oysters collected from 14 sites around Ariake Sea, Kyushu, Japan (Fig. 1)

Site	*C. gigas*	*C. sikamea*	*C. ariakensis*	(Total)
1: Iwa Jima	15	0	0	(15)
2: Midori River, north of Uto	3	11	1	(15)
3: Kumamoto Port and Shirakawa River	30	0	0	(30)
4: Mouth of Kikuchi River	6	9	0	(15)
5: Between Hayatsue and Chikugo Rivers	1	14	0	(15)
6: Between Rokkaku and Kasegana River	2	7	6	(15)
7: East of Kashima River	1	0	14	(15)
8: Mouth of Kashima River	13	1	1	(15)
9: Tara River	35	10	0	(45)
10: Shimabara Port	15	0	0	(15)
11: Mogi Port	15[a]	0	0	(15)
12: Tomioka	14	0	0	(14)
13: Shinoda	16	0	0	(16)
14: Hondo	15	1	0	(16)
(Total):	(181)	(53)	(22)	(256)

[a] One individual in this sample was classified by ITS-1 alone

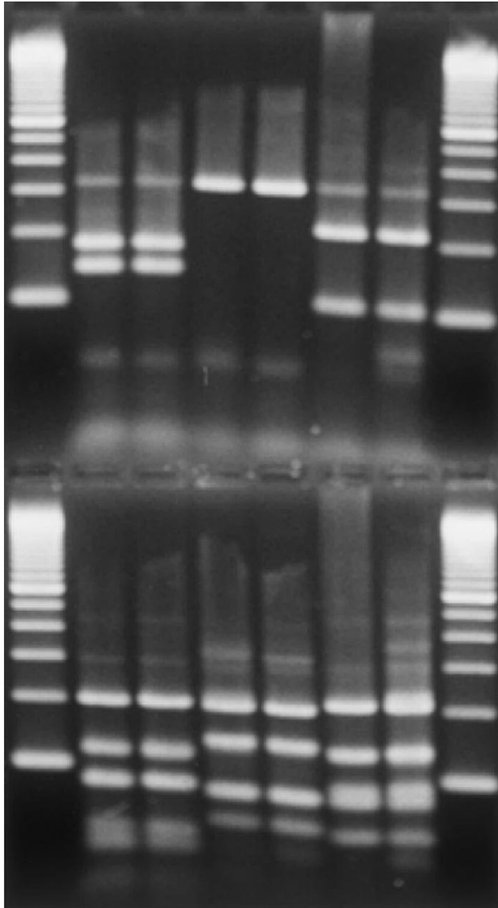

Fig. 2 *Crassostrea* spp. Restriction endonuclease digest patterns of 16S rDNA (*top panel*) and ITS-1 (*bottom panel*) in specimens of *C. gigas* (Lanes 2 and 3), *C. sikamea* (Lanes 4 and 5) and *C. ariakensis* (Lanes 6 and 7) from Ariake Sea, Kyushu, Japan. Lanes 1 and 8 of each panel contain a standard DNA 100 base-pair (bp) ladder, starting with a 100 bp fragment

Despite preferential collection, where possible, of small deep-cupped oysters from the Ariake Sea, 181 (70.7%) of the 256 oysters were classified as *Crassostrea gigas* by molecular markers (Table 1). *C. sikamea* comprised only 53 (20.7%) of the total sample, and *C. ariakensis*, a still smaller fraction – 22 (8.6%). The distributions of *C. sikamea* and *C. ariakensis* were clumped. *C. sikamea* dominated four sites (Sites 2, 4 to 6) on the eastern and northern shores of the Ariake Sea, and constituted a substantial minority of samples from the Tara River. Almost all the *C. ariakensis* were collected in the northern part of the Ariake Sea, near the Kasegana and Kashima Rivers.

Discussion and conclusions

Clearly, the Kumamoto oyster *Crassostrea sikamea* (Amemiya, 1928) is not extinct in its native habitat, the Ariake Sea, Kyushu, Japan, as feared by Banks et al. (1994). However, it is not possible to distinguish it from other native oysters on the basis of shell morphology. For example, most of the *gigas*-like oysters taken from the Midori River (Site 2) were later diagnosed as *C. sikamea* by DNA analyses, and only half of the eight sites dominated by *sikamea*-like oysters proved to be occupied by them. Molecular diagnosis revealed most sites to be dominated by *C. gigas* (e.g. Sites 1, 3, 8 to 13, 14); this probably accounts for previous failures to find *C. sikamea*. These results emphasize the necessity of using molecular markers to discriminate these morphologically plastic species in the field and in aquaculture (Hedgecock et al. 1993). Although the morphological collecting bias appears to have been weak, its affect on inferences about the distribution and abundance of the *C. gigas, C. sikamea* and *C. ariakensis* is unknown. The DNA results suggest, nevertheless, that *C. sikamea* dominates certain estuaries on the eastern and northern shores of the Ariake Sea, in accord with the known salinity tolerances of the mesohaline *C. sikamea* (Amemiya 1928). Waters of the eastern and northern shores of the Ariake Sea are, on average, less saline and more turbid than those of the western and southern shores (Coastal Oceanography Research Committee, The Oceanographical Society of Japan 1985). The apparently patchy distribution of these oysters also fits Numachi's description (1978) that they "...form colonies alternately...in the tidal belt, or they mix with other types grown in Ariake-Kai."

Numachi (1958; as cited in Numachi 1978) and Banks et al. (1994) both demonstrated that the sperm of *Crassostrea sikamea* cannot fertilize the eggs of *C. gigas*. The reverse cross, fertilization of *C. sikamea* eggs by *C. gigas* sperm, yielded viable interspecific hybrids in these studies, although Numachi (1958; in Numachi 1978) noted that greater concentrations of sperm were required when sympatric *C. gigas* were used. Concordance of species assignment by both mitochondrial and nuclear DNA markers suggests that hybridization between male *C. gigas* and female *C. sikamea* does not occur or occurs only rarely in the Ariake Sea. Of the 53 oysters diagnosed as *C. sikamea* by the mtDNA marker, none were heterozygous at the nuclear ITS-1 marker. Nevertheless, the upper 95% confidence limit on a relative frequency of 0 in 53 cases, is ~0.06, so a low frequency of hybridization cannot be excluded. Alternatively, hybrids may have been excluded by the collection bias for *C. sikamea* morphology, although, again, the abundance of *C. gigas* in samples suggests that this bias was weak.

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