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M. A. Banks · D. J. McGoldrick · W. Borgeson
D. Hedgecock

Gametic incompatibility and genetic divergence of Pacific and Kumamoto oysters, *Crassostrea gigas* and *C. sikamea*

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Abstract The biological and taxonomic separation of the Pacific oyster *Crassostrea gigas* (Thunberg, 1793) from the Kumamoto oyster *C. sikamea* (Amemiya, 1928) is affirmed by three concordant lines of evidence: (1) fixed differences in 2% of a mtDNA sequence coding for large subunit rRNA; (2) a genetic distance of 0.440 based on 19 allozyme loci, including 5 diagnostic loci (*Aat-1*, *Idh-1*, *Idh-2*, *Mpi-1*, *Mdh-2*); (3) one-way gametic incompatibility resulting in partial reproductive isolation in interspecific crosses. *C. gigas* sperm×*C. sikamea* egg fertilizations form viable hybrid offspring, but *C. sikamea* sperm do not fertilize *C. gigas* eggs. Divergence between these two species is mediated by differing peaks in the periods for gamete release and by one-way sperm/egg incompatibility. Two attempts to recover *C. sikamea* from its place of origin in southern Japan have yielded only individuals with the mitochondrial haplotype that characterizes *C. gigas*. We thus identify a crucial need for careful screening, management, and conservation of the cultivated populations of *C. sikamea* on the US west coast.

Introduction

Amemiya (1928) identified a distinct variety of the Japanese or Pacific oyster, *Ostrea gigas* var. *sikamea* (from the Japanese "shikame", meaning "wrinkled"), in Ariake Bay, Kumamoto Prefecture, Kyushu, Japan. Differences in shell morphology, salinity tolerance, growth rate and egg size were the basis of Amemiya's characterization of the variety. The genus name for cupped oysters was subsequently changed from *Ostrea* to *Crassostrea* (Sacco, 1897) in 1955 by ruling of the International Commission on Zoological Nomenclature.

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M. A. Banks (✉) · D. J. McGoldrick · W. Borgeson · D. Hedgecock
Bodega Marine Laboratory, The University of California at Davis, P.O. Box 247, Bodega Bay, California 94923, USA

Later, Numachi (1958 *in* Numachi 1978) described two cupped oysters from Ariake Bay on the basis of gamete compatibility. Type B was fully interfertile with Pacific oysters from throughout Japan. Sperm from Type A could fertilize neither Type B eggs nor the eggs of Pacific oysters from the Hiroshima, Miyagi or Hokkaido Prefectures (locations in Fig. 1). On the other hand, Type A eggs could be fertilized by sperm from all populations of *Crassostrea gigas*, although concentrated solutions of sperm from the sympatric Type B Kumamoto oysters were required for fertilization of Type A eggs. Unfortunately, this distinction of two sympatric oyster species, based on definitive although largely one-way reproductive isolation, was not appreciated by subsequent workers. Continued reference to these oysters by the name Kumamoto, rather than reference to two separate species, has been a root cause of much confusion.

A second report of experimental crosses among Japanese oysters (Imai and Sakai 1961) yielded results opposite to those of Numachi. This long-term inbreeding and crossbreeding study reported that oysters from Kumamoto, Hiroshima, Miyagi and Hokkaido Prefectures were fully interfertile. These authors did not cite Numachi's work and continued to use Kumamoto as a single variety name; presumably they used only Numachi's Type B Kumamoto oysters in their experimental crosses. Imai and Sakai's paper, written in English, may have been responsible for spreading the notion to western biologists that variation among the geographical populations of Japanese oysters was a continuum of racial differences (Quayle 1988). The apparent morphological similarity of Type A and Type B Kumamoto oysters suggests that racial variation and disjunct species differences were confounded in the early literature on Japanese oysters.

Despite Imai and Sakai's work, Ahmed (1975) was sufficiently impressed with the magnitude of morphological differences among Japanese oyster populations to propose that Amemiya's Kumamoto oyster be elevated to full species, *Crassostrea sikamea*. Buroker et al. (1979 a) acknowledged and affirmed this distinction with allozyme frequency data for native oyster populations from Kumamoto, Hiroshima and Miyagi. Ozaki and Fujio (1985),

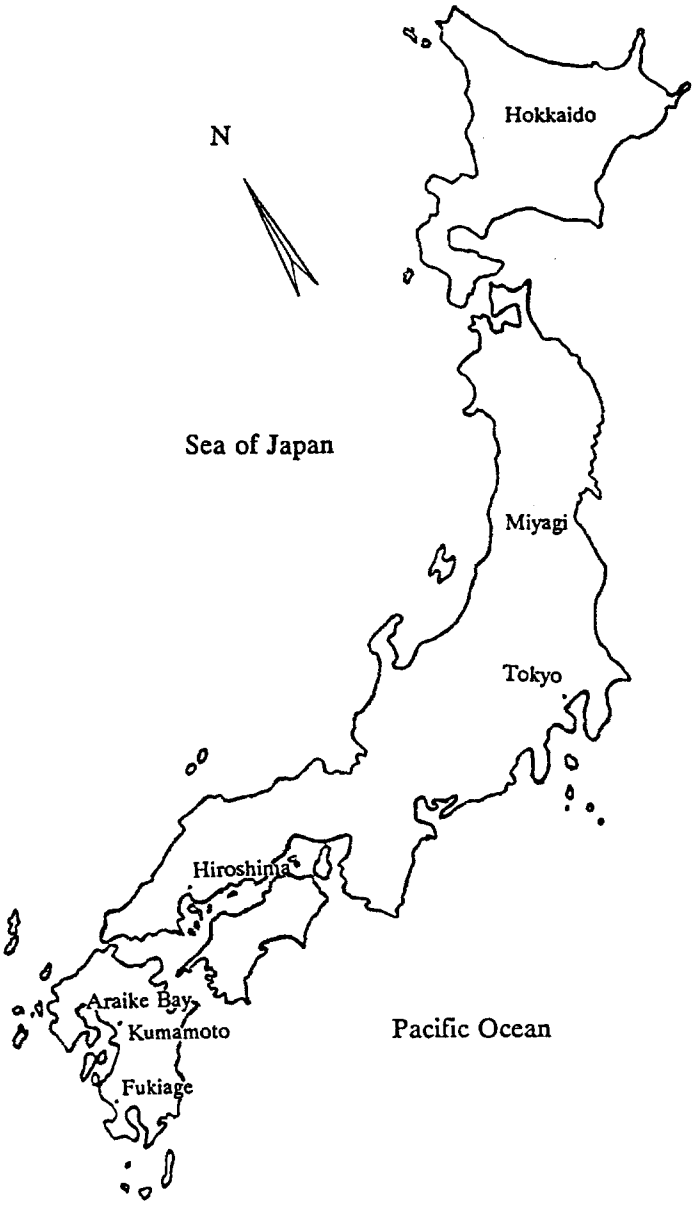

Fig. 1 Map of Japan showing location of sample sites for *Crassostrea sikamea* recovery and Prefectures mentioned in text

however, in a survey of protein variation in natural and hatchery-propagated oyster stocks, found no evidence for any oyster but *C. gigas*. They attributed this result to the widespread distribution of *C. gigas* seed among the prefectures.

Unfortunately, judging from failures to recover *Crassostrea sikamea* by our collaborators and others (T. Seki, Oyster Research Institute, Karakuwa, Miyagi, personal communication), the Kumamoto oyster may be extinct in Japan. Historically, the Japanese did not regard the Kumamoto oyster as commercially valuable owing to its "dwarf or stunted form" (Amemiya 1928), which may in part account for the demise of natural stocks.

This paper addresses the confusion in the taxonomic status of the Kumamoto oyster by making use of three different means of evaluating genetic divergence and reproductive isolation. We have employed (1) allozyme electrophoresis, (2) mitochondrial DNA sequence analysis, and (3) inter-specific hybridization experiments to analyze Kumamoto and Pacific oysters from North American commercial stocks. These stocks were initiated and have been supplemented by introductions from the Kumamoto and the Miyagi prefectures since the late 1940s (Woelke 1955) and early 1910s (Quayle 1988), respectively. Our findings agree with those of Ahmed (1975) and Buroker et al. (1979 a) in supporting a specific status for the Kumamoto oyster, *Crassostrea sikamea* (Amemiya 1928). We examined oyster specimens imported from southern Japan in an attempt to identify *C. sikamea* from its place of origin. Finally, we speculate on the mechanism of speciation between these closely related species based on their present differences and on the nature of the partial reproductive isolation between them.

Materials and methods

Samples

Adult Pacific oysters, *Crassostrea gigas* (Thunberg, 1793) were obtained in June 1991 from the naturalized population in Dabob Bay, Puget Sound, Washington (USA), which had been established by massive importations of seed from the Miyagi Prefecture (Chew 1979). Adult Kumamoto oysters, *C. sikamea* (Amemiya, 1928) were obtained from the Coast Oyster Company (now Coast Seafoods) in Humbolt Bay, California (USA) in June 1991. Oyster samples were obtained from Ariake Bay, Kumamoto (November 1991) and Fukiage, Kagoshima (August 1992), Kyushu, Japan.

Allozyme electrophoresis

Adult *Crassostrea gigas* from a mass spawn of Dabob Bay wild stock and adult *C. sikam*ea from production stock (Coast Oyster Company, 1987 year-class harvested in 1991) were sampled as base-line populations for assessing typical allelic frequencies for each species. Sampling, horizontal starch-gel electrophoresis, specific enzyme-staining procedures, and zymogram interpretation were performed according to methods described by Hedgecock and Okazaki (1984) and Hedgecock and Sly (1990). Specifically, enzyme staining procedures were used to visualize: aspartate amino transferase [*Aat-1*, (EC) 2.6.1.1), aconitase hydratase (*Acon-1*, *Acon-2*, EC 4.2.1.3), adenylate kinase (*Adkin*, EC 2.7.4.3), glycerol-3-phosphate dehydrogenase (*G3pdh*, EC 1.1.1.8), glucose phosphate isomerase (*Gpi*, EC 5.3.1.9), isocitrate dehydrogenase (*Ihd-1*, *Idh-2*, EC 1.1.1.42), leucine aminopeptidase (*Lap-2*, EC 3.4.11.1), malate dehydrogenase (*Mdh-1*, *Mdh-2*, EC 1.1.1.37), mannose-6-phosphate isomerase (*Mpi-1*, EC 5.3.1.8), 6-phosphogluconate dehydrogenase (*6pgdh*, EC 1.1.1.44), phosphoglucomutase (*Pgm*, EC 2.7.5.1), superoxidase dismutase (*Sod*, EC 1.15.1.1)]. General protein (*Pt*) was visualized using Coomassie Brilliant Blue stain. Three diagnostic enzymes (sensu Ayala and Powell 1972) for *C. gigas* and *C. sikamea*, *Aat-1*, *Idh-1*, and *Mdh-2* (Buroker et al. 1979 a and present paper) were used to diagnose *C. gigas* and *C. sikamea* parents used in hybridization experiments.

Mitochondrial DNA characterization

Banks et al. (1993) describe nucleotide differences in the DNA sequences of the large subunit rRNA (lrDNA) mitochondrial gene that

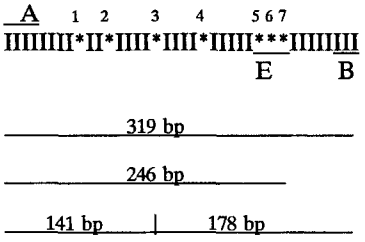

Fig. 2 *Crassostrea gigas* and *C. sikamea*. Schematic map of oyster large subunit rRNA coding gene [*A, B* generic, oyster-specific polymerase chain reaction (PCR) primer sites, *E C. sikamea*-specific PCR primer site; *1–7* sites of substitutions discriminating *C. gigas* from *C. sikamea*, *3* being a *Dra I* recognition site in *C. gigas*]

enable discrimination between *Crassostrea gigas* and *C. sikamea* (Fig. 2). DNA sequence-specific techniques, including species-specific polymerase chain reaction (PCR) priming (Saiki et al. 1986) and restriction endonuclease digestion, were used to diagnose *C. gigas* and *C. sikamea* parents used in the hybridization experiments as well as putative *C. sikamea* collected from Ariake Bay and Fukiage, Japan. Genomic DNA was extracted by digestion in a buffered proteinase-K solution to provide template for the amplification of mitochondrial lrDNA following methods described by Banks et al. Multiplex PCR involved the use of three primers, two conserved oyster-specific primers, 5′-CCGGCCGCCCTAGCGTGAGGG-3′ and 5′-ATTAGCCTGTTATCCCCGGCG-3′, and a *C. sikamea*-specific primer, 5′-ACTCAGAAAGGTTAGGCTTAC-3′ (Positions A, B and E in Fig. 2). Alternate haplotypes for each species were further characterized using *Dra I* restriction endonuclease digestion following the methods of Banks et al. PCR products and restriction endonuclease digest fragments were electrophoresed in a 3% NuSieve agarose gel in 1×TBE buffer, stained with ethidium bromide, and visualized via UV transillumination.

Interspecific hybridization experiments

Overlap in the seasons of reproductive maturity for Pacific and Kumamoto oysters (Berg 1969; Robinson 1992; own personal observations) allows a brief opportunity for performing hybridization experiments in the fall. Gonadal tissue samples from approximately a dozen adult *Crassostrea gigas* and a dozen *C. sikamea* were smeared on glass slides and examined by phase-contrast light microscopy (400x) to identify individuals with active sperm or mature oocytes. Tissue samples were taken from several areas of the gonad to ensure exclusion of hermaphroditic individuals; none were found.

Two experiments were made to evaluate gametic compatibility. In both experiments, we made three types of crosses: (1) within-species fertilizations (*Crassostrea gigas* ×*C. gigas* and *C. sikamea C. sikamea*), (2) *C. gigas*-paternal hybrids (*C. gigas* ♂×*C. sikamea* ♀), and (3) *C. sikamea*-paternal hybrids (*C. sikamea* ♂×*C. gigas* ♀). Control treatments consisted of eggs for each species incubated in separate containers, identical to those in which the fertilizations were performed but without any sperm. In both experiments also, gametes were stripped directly from gonads as described by Stephano and Gould (1988). Stripping of gametes has become a standard and recommended procedure for making controlled crosses of oysters (Allen et al. 1989; Allen and Bushek 1992), and we have successfully employed gamete stripping for large experimental crosses (Hedgecock et al. 1991). Moreover, the same results as described below were repeatedly obtained in interspecific crosses using gametes derived from thermal induction of spawning (Robinson and Lannan personal communication). Eggs were aged for 1 h in aerated ambient seawater before fertilization in order to reduce the likelihood of polyspermy (Stephano and Gould 1988). General procedures for gamete screening, fertilization and embryo treatment were according to Breese and Malouf (1975).

The first experiment involved single parents for each species. Fertilization success was assessed for each of the crosses at 1 h post-fertilization by counting numbers of cleaved versus uncleaved eggs in samples of 100 or more.

In the second experiment we grouped gametes from three conspecific individuals before making pure-species and hybrid crosses. Eggs were divided for fertilization at two sperm concentrations: 500 (normal) and 50 000 (excess) sperm/single egg. Aliquots of 1200 eggs in 1 ml of ambient filtered seawater were inoculated with appropriate amounts of sperm. Sperm concentration was determined using a hemacytometer. Excess sperm were removed by rinsing zygotes on a 20 μm screen at 2 min post-fertilization. Zygotes were then fixed 10 min after fertilization using paraformaldehyde (1% final conc) and stained with a DNA-specific fluorescent stain, Hoechst 33342 (Sigma Chemical Co., St. Louis, USA), before examination with an Olympus (Tokyo, Japan) BH 2 epifluorescence microscope equipped with ultraviolet filters. Fertilization in this experiment was assessed by counting the number of eggs containing male pronuclei.

Results

Allozyme electrophoresis

The allozyme frequencies for 19 loci in North American base-line populations for both *Crassostrea gigas* and *C. sikamea* (Table 1) are very similar to those reported for native Japanese population samples in Buroker et al. (1979 a). Nei's (1978) unbiased genetic distance (*D*) and identity (*I*) for the comparison of *C. gigas* with *C. sikamea* are $D=0.440$ and $I=0.644$, respectively, in agreement with the $D=0.4521$ and $I=0.6363$ values for native Japanese oysters given by Buroker et al. (1979 a). The parity of these values demonstrates the consistency of allozyme methods, particularly given that Buroker's study was completed more than a decade ago and involved native rather than North American hatchery oyster stocks.

Genotypes for five loci enabled discrimination between the two species (Table 2). Samples of Kumamoto and Pacific oysters have no *Idh-1* genotypes in common (Tables 1 and 2). Based on these samples, there is zero probability that two *Idh-1* genotypes, one drawn from each of the two species, are identical, so that this locus provides an unambiguous species-diagnosis. For the remaining loci, overlaps in the frequencies for genotypes that are common to both species are sufficiently low to be used to diagnose the species (Ayala and Powell 1972); i.e., the error in assigning an individual to the species in which the common genotype is most frequent would be <1 in a 100 (1%). This error is calculated as one-half the overlap in genotype frequencies assuming two species to be equally frequent in a hypothetical, mixed population. For example at the *Aat-1* locus, the $Aat\text{-}1^{92/92}$ genotype occurs almost exclusively in *Crassostrea sikamea*; however, 2 out of 259 (or 0.8%) *C. gigas* individuals share this genotype. Classifying all oysters with the $Aat\text{-}1^{92/92}$ as *C. sikamea* would entail an error rate of 0.4% in the hypothetical mixed population. Likewise, the $Aat\text{-}1^{92/100}$ heterozygote occurs in 10% of *C. gigas* but in only 1% of *C. sikamea*; classifying all $Aat\text{-}1^{92/100}$ genotypes as *C. gigas* would entail an error rate of 0.5%. The sum of these error rates yields an overall error rate of 0.9% for the *Aat-1* locus. *Idh-2*, *Mdh-2* and *Mpi-1*

Table 1 *Crassostrea* spp. Allelic frequencies for 19 loci in comparison of Pacific oysters *C. gigas* (*C.g.*) from Dabob Bay, Washington (USA) [data pooled from Hedgecock and Sly (1990), Hedgecock 1994, and present study], with Kumamoto oysters *C. sikamea* (*C.s.*) from a commercial hatchery stock grown in Humboldt Bay, California (USA) (*N* number of individuals studied; – zero)

Locus allele	Population		Locus allele	Population		Locus allele	Population	
	C.g.	*C.s.*		*C.g.*	*C.s.*		*C.g.*	*C.s.*
Aat			*Idh-1*			*6pgdh*		
(*N*)	(178)	(24)	(*N*)	(99)	(30)	(*N*)	(116)	(34)
112	0.003	–	103	0.040	–	110	–	0.015
108	0.301	–	100	0.949	–	108	0.022	–
106	0.003	–	97	0.010	–	106	0.069	0.074
100	0.612	0.021	95	–	0.983	103	–	0.574
92	0.081	0.979	92	–	0.017	102	0.060	–
						100	0.823	0.338
Acon-1			*Idh-2*			98	0.017	–
(*N*)	(165)	(25)	(*N*)	(118)	(30)	96	0.004	–
105	0.009	–	105	0.047	–	93	0.004	–
103	0.176	–	100	0.729	–			
100	0.691	0.980	97	0.093	–	*Pgm*		
97	0.121	0.020	95	0.131	0.800	(*N*)	(119)	(34)
95	0.003	–	93	–	0.200	107	0.013	–
						105	0.076	–
Acon-2			*Lap-2*			103	0.059	0.044
(*N*)	(177)	(22)	(*N*)	(177)	(18)	102	0.046	–
109	–	0.045	105	0.020	–	100	0.517	0.206
105	0.003	–	103	0.096	0.250	96	0.164	0.456
103	0.059	–	101	–	0.139	93	0.101	–
100	0.853	0.545	100	0.771	0.611	90	0.025	0.118
97	0.073	0.364	98	0.023	–	86	–	0.088
95	0.011	0.045	96	0.076	–	82	–	0.088
			94	0.003	–			
Adkin			92	0.011	–	*Pt-1*		
(*N*)	(170)	(34)				(*N*)	(59)	(34)
105	0.006	0.044	*Tap-2*			100	1.000	1.000
103	0.076	0.235	(*N*)	(178)	(18)			
100	0.744	0.603	104	0.011	–	*Pt-2*		
97	0.135	0.088	102	0.152	0.111	(*N*)	(59)	(34)
95	0.038	0.029	100	0.747	0.806	100	1.000	1.000
			98	0.084	0.083			
G3pdh			96	0.006	–	*Sod-1*		
(*N*)	(110)	(34)				(*N*)	(119)	(33)
105	0.005	–	*Mdh-1*			114	0.004	–
100	0.682	0.044	(*N*)	(178)	(34)	100	0.996	0.803
95	0.314	0.956	108	–	0.029	90	–	0.197
			100	0.997	0.971			
Gpi			97	0.003	–	*Sod-2*		
(*N*)	(176)	(18)				(*N*)	(119)	(34)
110	0.006	–	*Mdh-2*			100	0.966	1.000
105	0.026	0.306	(*N*)	(178)	(34)	95	0.029	–
102	0.009	–	107	0.003	0.985	90	0.004	–
100	0.906	0.639	100	0.997	0.015			
98	0.003	–						
95	0.045	–	*Mpi*					
94	–	0.056	(*N*)	(145)	(32)			
80	0.006	–	105	0.007	–			
			100	0.969	–			
			97	–	0.938			
			95	0.024	0.063			

likewise have individual error rates of <1%, permitting 99% probability of assigning an individual to the correct species using any one of these loci. Probabilities of incorrect assignments at independent markers can be multiplied together so that, for example, concordant results from three loci provide virtually certain species-diagnosis of parents used in the hybridization experiments (Table 3).

Mitochondrial DNA characterization

Parents used in the hybridization experiments and specimens from Ariake Bay and Fukiage, Japan, were diagnosed via multiplex-PCR (Fig. 3). Alternative *Crassostrea gigas* and *C. sikamea* PCR-products differ in size and susceptibility to *Dra I* digestion. For *C. gigas*, only a single 319

Table 2 *Crassostrea* spp. Diagnostic differences in allozyme phenotypes between Pacific and Kumamoto oysters. Phenotypic frequencies are those expected under Hardy–Weinberg–Castle equilibria. Data of Buroker et al. (1979 a) are pooled with data from Table 1 of present paper for *C. gigas* and *C. sikamea* except for *Mdh-2* locus (*N* total number of individuals)

Phenotype	*C. gigas*	*C. sikamea*	
		USA	Japan
Aspartate amino transferase (*Aat-1*)	(*N* = 259)	(*N* = 102)	
92	2	101	
92/100	26	1	
100	97	0	
100/108	97	0	
108	24	0	
92/108	13	0	
Isocitrate dehydrogenase (*Idh-1*)	(*N* = 196)	(*N* = 107)	
92/95	0	4	
95	0	103	
97/100	2	0	
100	178	0	
100/103	15	0	
Isocitrate dehydrogenase (*Idh-2*)	(*N* = 214)	(*N* = 107)	
92	0	1	
92/95	0	13	
95	4	93	
95/97	7	0	
95/100	42	0	
97/100	33	0	
100	101	0	
other	27	0	
Malate dehydrogenase (*Mdh-2*)	(*N* = 262)	(*N* = 34)	(*N* = 78)
100	261	0	69
100/107	1	1	9
107	0	33	0
Mannose phosphate isomerase (*Mpi-1*)	(*N* = 256)	(*N* = 110)	
95/97	0	10	
97	0	96	
97/100	5	4	
95/100	7	0	
100	225	0	
100/105	18	0	
105	1	0	

base-pair (bp) PCR-product is synthesized (Primers A and B, Fig. 2), and it is then cleaved into fragments of 141 and 178 bp in length by *Dra 1* digestion. For *C. sikamea*, PCR-products of two sizes are synthesized: a 319 bp fragment (generated by Primers A and B, Fig. 2) and a 246 bp fragment (from Primer A and *C. sikamea*-specific Primer E, Fig. 2); both products remain uncleaved after *Dra 1* exposure. Sequence-specific PCR and *Dra 1* digestion (Figs. 2 and 3) use 4 out of 7 nucleotide differences that characterize lrDNA mitochondrial haplotypes of the two *Crassostrea* species in Banks et al. (1993). These results, combined with those from three diagnostic allozyme-coding loci, confirm the genetic identity of the *C. gigas* and *C. sikamea* parents used in the hybridization experiments (Table 3). Oyster samples from Ariake Bay and Fukiage contained only *C . gigas*-specific mitochondrial genetic material (Fig. 3).

Fertilization competency

The proportions of cleaved eggs resulting from crosses made in the first hybridization experiment (Table 4) revealed >90% fertilization in the *Crassostrea gigas*×*C. gigas*, *C. sikamea*×*C. sikamea* and *C. gigas*×*C. sikamea* crosses but a complete failure of *C. sikamea* sperm to fertilize *C. gigas* eggs. In the second experiment, for crosses made at normal sperm concentrations epifluorescent staining revealed internal sperm pronuclei in all crosses save *C. sikamea* ♂×*C. gigas* ♀ (Table 4, Fig. 4). The same results were obtained in the high-sperm concentration treatment (data not shown). Furthermore, although *C. sikamea* sperm are obviously attracted to *C. gigas* eggs in seawater, they are easily removed by washing; in contrast, sperm remain bound to eggs after washing in all other crosses (Fig. 4). This absence of sperm–egg binding characterized

Table 3 *Crassostrea gigas* (*C.g*) and *C. sikamea* (*C.s*). Concordant diagnosis of genetic identity for individuals used in fertilization and gametic compatibility tests (– no genotyping scored)

Individual	mtDNA PCR	*Dra 1*	*Aat-1*	*Idh-1*	*Mdh-2*
Inter-specific Hybridization Experiment I					
C.g ♀	G	G	100/108	100	100
C.g ♂	G	G	100/108	–	100
C.s ♀	S	S	92	–	107
C.s ♂	S	S	92	95	107
Inter-specific Hybridization Experiment 2					
C. gigas female parents					
C.g 1 ♀	G	G	100/108	100	100
C.g 2 ♀	G	G	100/108	100	100
C.g 3 ♀	G	G	100	100	100
C. gigas male parents					
C.g 4 ♂	G	G	100/108	100	100
C.g 5 ♂	G	G	100/108	100	100
C.g 6 ♂	G	G	100/108	100	100
C. sikamea female parents					
C.s 1 ♀	S	S	92	95	107
C.s 2 ♀	S	S	92	95	107
C.s 3 ♀	S	S	92	93/95	100/107
C. sikamea male parents					
C.s 4 ♂	S	S	92	95	107
C.s 5 ♂	S	S	92	–	100/107
C.s 6 ♂	S	S	92	95	107

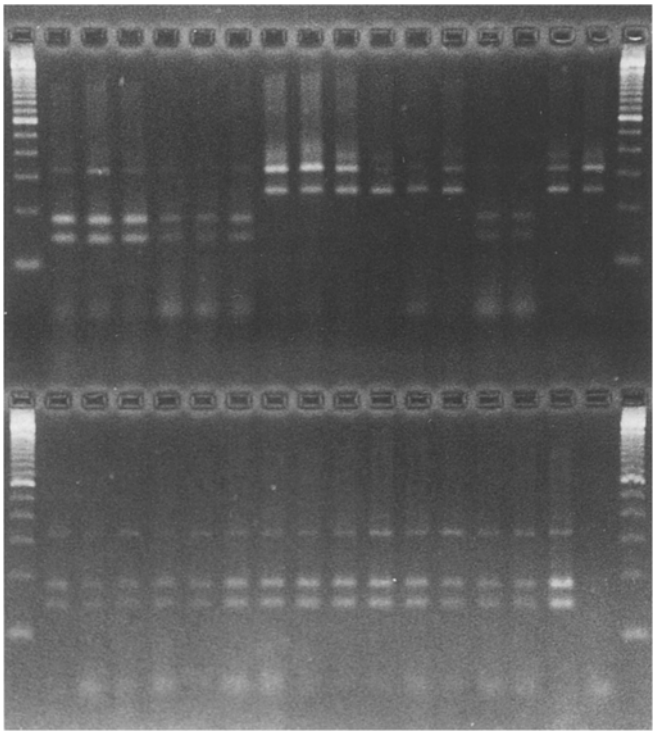

Fig. 3 *Crassotrea gigas* and *C. sikamea*. Electrophoresis of *Dra I* restriction endonuclease digests of lrDNA polymerase chain reaction (PCR) product from parent oysters used in hybridization experiments and oysters from Japan. *C. gigas* haplotype yields 141 and 178 bp bands with some of the undigested 319 bp band visible, while *C. sikamea* haplotype yields 319 and 246 bp bands; small bands, ±10 to 30 bp are PCR-primers. Samples were loaded as follows: Lanes 1 and 18 (upper panel), 19 and 36 (lower panel) = DNA size-standard ladder with 100 bp increments. Lanes 2 to 13 = parents for Hybridization Experiment 2, Lanes 2 to 4 = *C. gigas* male parents, Lanes 5–7 = *C. gigas* female parents; likewise, Lanes 8–13 = *C. sikamea* parents, Lanes 14 to 17 = parents for Hybridization Experiment 1, Lanes 14 and 15 = male and female *C. gigas*, Lanes 16 and 17 = male and female *C. sikamea*, Lanes 20 to 24 = oysters from Ariake Bay shown to have *C. gigas* mtDNA, Lanes 25 to 34 = oysters from Fukiage also shown to have *C. gigas* mtDNA, and Lane 35 = no-template PCR negative control for all amplifications

C. sikamea ♂×*C. gigas* ♀ crosses at both normal and excess sperm concentrations.

Discussion

Our results fully support the classification of *Crasssostrea sikamea* (Amemiya, 1928) as a species distinct from the closely related Pacific oyster *C. gigas* (Thunberg, 1793), based on the concordance of gametic incompatibility, fixed alternate mitochondrial haplotypes, and nuclear-encoded, allozyme-frequency differences, including five diagnostic loci. The congruence of these findings for North American populations with findings for Japanese native oyster populations (Numachi 1958 *in* Numachi 1978; Buroker et al. 1979 a) demonstrates first the repeatability and reliability of these techniques for discrimination between the species, and second that the North American populations maintain the diagnostic features which characterized the Japanese native populations of the past. Other features distinguishing the two species include differences in peak

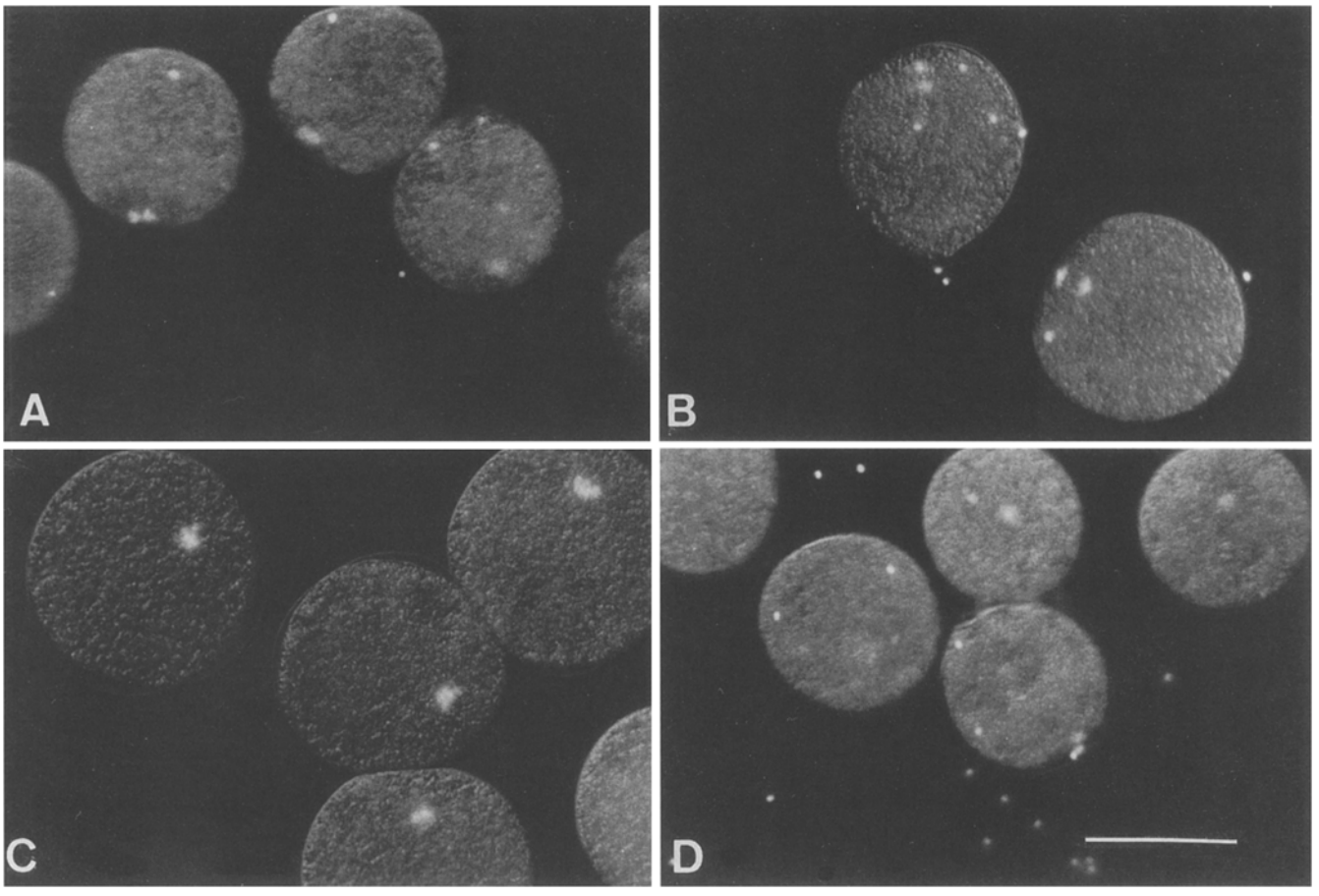

Fig. 4 *Crassostrea gigas* and *C. sikamea*. Photomicrographs of epifluorescence microscopy (400×) for sperm-exposed eggs fixed at 10 min post-fertilization and stained with Hoechst 33342. **A**, *C. sikamea* × *C. sikamea*; **B**, *C. gigas* × *C. gigas*; **C**, *C. sikamea* ♂ × *C. gigas* ♀; **D**, *C. gigas* ♂ × *C. sikamea* ♀. Sperm pronuclei are visible as small fluorescent bodies within eggs in **A, B** and **D**. External sperm is visible as much smaller fluorescent bodies bound to surface of eggs. Maternal genetic material is visible as large fluorescent bodies in all egss. (Scale bar = 40 μm)

Table 4 *Crassostrea gigas* (*C.g*) and *C. sikamea* (*C.s*). Fertilization success in intra- and interspecific crosses. For Experiment 1, success is evaluated as percent of eggs cleaved 1 h after fertilization; for Experiment 2, success is percent of eggs with sperm pronuclei 10 min after fertilization (Numbers in parentheses are total sample size)

Experiment	× *C.g* ♀	× *C.s* ♀
Inter-specific Hybridization Experiment 1 (single parents)	% embryos	% embryos
C.g ♂	96 (101)	94.5 (109)
C.s ♂	0 (135)	91.3 (104)
Inter-specific Hybridization Experiment 2 (grouped parents)	% sperm pronuclei	% sperm pronuclei
C.g ♂	90.9 (11)	80 (10)
C.s ♂	0 (14)	45.6 (11)

spawning seasons (see below), salinity tolerances, growth rates, egg sizes (Amemiya 1928) and shell morphologies (Amemiya 1928; Ahmed 1975).

The genetic distance $D=0.440$, calculated from allozyme data (Table 1), indicates that Pacific and Kumamoto oysters diverged from each other almost 2 million years ago (mya), according to the relationship $D=2\alpha t$, in which α is assumed to be 10^{-7} electrophoretically detectable amino acid substitutions per locus per year (Nei 1987). This estimate is in adequate agreement with a divergence time of 1.4 million years ago for these species, which was based on lrDNA mtDNA sequence data (Banks et al. 1993). For comparison, genetic distances at various stages of evolutionary divergence in *Drosophila* spp. average 0.226 ± 0.033 for subspecies, 0.581 ± 0.039 for sibling species and 1.056 ± 0.068 for morphologically different species (Ayala et al. 1973). For *Crassostrea* spp., average genetic distances of 0.678, 0.537 and 0.548 have been reported for *C. virginica–C. corteziensis, C. virginica–C. rhizophorae* and *C. rhizophorae–C. corteziensis* comparisons, respectively (Hedgecock and Okazaki 1984). Thus, the genetic distance between *C. gigas* and *C. sikamea* is at the low end of the range for interspecific comparisons; indeed, these congeners are the most closely related oyster species known.

The failure of repeated attempts to find *Crassostrea sikamea* in its natural habitat in southern Japan is of great concern. Samples from two independent and well-in-

formed collaborators have yielded only individuals with mitochondrial haplotypes of *C. gigas*. It presently appears that the only remaining bona fide *C. sikamea* are those under commercial culture along the west coast of the USA. Some of these few hatchery-propagated stocks, however, are contaminated by admixture of pure *C. gigas* and by first-generation hybrids from *C. gigas*♂×*C. sikamea*♀ crosses (Hedgecock et al. 1993). Hybridization by commercial breeders has been both inadvertent and intentional, for production of the so-called "Gigamoto oyster" (Quayle 1988). In addition, an estimate of 5.4 for the effective population number of one commercial Kumamoto brood stock (Hedgecock et al. 1993) indicates that long-term survival of this species may be at great risk. Meticulous screening and management of remaining stocks is imperative for the ongoing viability of the Kumamoto oyster.

Complete fertilization failure for *Crassostrea sikamea*♂×*C. gigas*♀ crosses, which agrees with previous observations (Numachi 1958 in Numachi 1978; Robinson and Lannan personal communication; Allen and Hedgecock personal communication), indicates a discrete block in the molecular mechanism of sperm–egg interaction and fertilization. This block appears to be the failure of *C. sikamea* sperm to undergo the acrosome reaction at the *C. gigas* egg surface; artificial stimulation of the acrosome reaction, through use of the ionophore Ionomycin (Sigma Chemical Co., St. Louis, USA), allows *C. sikamea* sperm to enter *C. gigas* eggs, as observed in normal crosses (M. Pillai and G. Cherr unpublished data and personal observations). Mechanisms of fertilization, however, are incompletely understood for marine invertebrates. Extensive research into the molecular mechanisms mediating sperm–egg interaction for sea urchins has yet to identify the receptor on the sperm cell-surface that triggers the acrosome reaction (Foltz and Lennarz 1993). For the mouse, however, Leyton et al. (1992) and Miller et al. (1992) have shown the acrosome reaction to be triggered by an interaction between a 95 kdaltons sperm protein and a glycoprotein in oocyte-specific extracellular matrix. Co-evolution of genes encoding such specific protein components of sperm and egg surfaces in either *C. gigas* or *C. sikamea* or both is a likely explanation for the observed gametic incompatibility.

Palumbi (1992) notes that molecular findings from interspecific crosses for urchins and abalones imply that an "... interaction of a small number of gene products on the surfaces of eggs and sperm may play a disproportionate role in reproductive isolation of free-spawning marine invertebrates.". Such a mechanism appears suited to the marine environment, given that other means of establishing pre-zygotic reproductive isolation, such as courtship behavior, are not available to sedentary broadcast spawners (Palumbi 1992). The discrete molecular block implicated for *Crassostrea sikamea*–paternal interspecific crosses makes it tempting to add *C. gigas/C. sikamea* speciation to the list of examples for which reproductive isolation was primarily achieved by the relatively rapid co-evolution of gene products on the surfaces of sperm and eggs. Given that it is possible to force hybrid fertilization via artificially acrosome-reacted *C. sikamea* sperm and that fertilization occurs for *C. gigas* paternal hybrids without any manipulation, we suggest that this pair of oyster species would facilitate study of the molecular mechanisms which mediate reproductive isolation at the gamete level.

Alternatively, we should consider the classical view of speciation mediated by a gradual accumulation of genomic incompatibility in allopatric populations. Of critical importance in this regard is evidence for temporal reproductive isolation for the two species. Did divergence in peak spawning seasons arise before or after the gametic barrier evolved? Woelke (1955) identified two periods for oyster seed-set in the Kumamoto Prefecture, an early set in May and one later in August or September, implying a bimodal distribution of spawning with peaks in spring and summer. Imai and Sakai (1961) were able to form viable hybrids between Kumamoto and Hokkaido oysters in July, implying that summer-ripening Kumamoto oysters were Numachi's Type B *Crassostrea gigas*. Imai and Sakai also reported that Kumamoto oysters resumed maturation in the fall and carried ripe gametes in November and December; part of them even retained ripe gametes until early spring, presumably accounting for the May seed-set described by Woelke (1955). We infer, therefore, that spring spatfall in Ariake Bay resulted from the spawning of Numachi's Type A oyster, *C. sikamea*. This inference is reinforced by the characteristic winter-maturation of oocytes in North American cultured populations of *C. sikamea* (Robinson 1992; Hedgecock et al. 1993).

While the peak oocyte maturation months for *Crassostrea sikamea* in Yaquina Bay, Oregon, are from August until the end of December, most *C. sikamea* males are reproductively fully mature as early as March and remain so during the peak spawning months for *C. gigas*, from June until the end of August (Lannan et al. 1980; Robinson 1992). However, any potential for *C. sikamea* sperm to fertilize *C. gigas* eggs is removed by the one-way gametic incompatibility barrier. Coincidence of the direction of the barrier with the overlap in maturity of *C. sikamea* males during the *C.gigas* spawning peak, together with the development of weak gametic incompatibility in the reciprocal direction for sympatric but not allopatric crosses (Numachi 1958 *in* Numachi 1978), implies that gametic incompatibility evolved to reinforce the primary reproductive, seasonal isolation ("the Wallace effect"; Grant 1966). Were this the case, we would in principle expect to see evidence for hybrid breakdown in artificial *C. sikamea* ♂×*C. gigas* ♀ crosses. Absence of any evidence for hybrid breakdown would favor the hypothesis that co-evolution of discrete molecular mechanisms of gamete interaction was the primary reproductive isolating barrier.

In support of the classical view of speciation, Buroker et al. (1979 a, b) noted that geographic isolation between *Crassostrea. sikamea* and other Japanese populations may have been caused by land bridges formed as sea levels receded during glaciation periods. This could have allowed the initial divergence in the reproductive seasons of *C. gigas* and *C. sikamea* which now serves as a temporal reproductive isolating barrier. Our estimate that these two

species diverged about 2 million years ago places their separation in the Pleistocene. The generation of new species appears to have been prevalent in Japan during this time, as evidenced by naticid snails which increased in species number from 4 to 26 in the Pliocene and early Pleistocene (Majima 1989).

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