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THE STATUS OF THE KUMAMOTO OYSTER *CRASSOSTREA SIKAMEA* (AMEMIYA 1928) IN U.S. COMMERCIAL BROOD STOCKS

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ABSTRACT Long-standing confusion about the taxonomic status of the Kumamoto oyster has recently been resolved by demonstration of concordant molecular and reproductive-trait differences between *Crassostrea sikamea* (Amemiya 1928) and the closely related Pacific oyster *C. gigas* (Thunberg). Concern for the status of Kumamoto oyster brood stocks in the U.S. oyster-culture industry stems from reported contamination of these stocks with Pacific oysters and failure to find native populations of *C. sikamea* in Japan. Two commercial brood stocks of Kumamoto oysters were surveyed for allozyme and mitochondrial DNA markers that allow discrimination of Kumamoto and Pacific oysters. Pacific oysters were detected in both Kumamoto stocks, in one case, even after careful culling on the basis of shell morphology. Interspecific hybridization was also detected. Inadvertent admixture of Pacific oysters in Kumamoto brood stocks can result in hybridization because Pacific oyster sperm can fertilize Kumamoto oyster eggs. Hybridization and introgression thus pose potential threats to the integrity of Kumamoto oyster stocks in North America. Another threat to these stocks is loss of genetic diversity owing to random genetic drift in very small hatchery-propagated populations. Random changes in allozyme frequencies between generations of one Kumamoto oyster stock imply an effective population size of only 5.4. Steps should be taken both to eradicate Pacific oysters and interspecific hybrids from North American Kumamoto brood stocks and to retard the erosion of genetic diversity within these brood stocks.

KEY WORDS: Kumamoto oysters, Pacific oysters, North American stocks, hybridization, species mixture, genetic drift, mitchondrial DNA, allozymes, gametic compatibility

INTRODUCTION

Three species of cupped oysters have been introduced from Japan to the west coast of North America for cultivation, the Pacific oyster *Crassostrea gigas* (Thunberg), the Kumamoto oyster *C. sikamea* (Amemiya 1928), and the Suminoe oyster *C. ariakensia* (Wakiya 1929), formerly *C. rivularis* (status currently being revised, Eugene V. Coan, pers. comm.). The Pacific oyster comprises the vast majority of west coast oyster production, while the Kumamoto oyster commands a sizeable share of the half-shell trade. Hatchery methods for breeding the Suminoe oyster have been determined (Robinson and Langdon 1993), but there is no commercial production yet. Recently, the Pacific and Kumamoto oyster were found to differ at seven nucleotide positions in a portion of the mitochondrial gene coding for the large subunit ribosomal RNA (Banks et al. 1993a). This observation, coupled with concern among U.S. oyster growers that Kumamoto oyster hatchery seed might be contaminated with Pacific oysters, prompted us to re-investigate the taxonomic status of the Kumamoto oyster (Hedgecock and Robinson 1992, Banks et al 1993b).

Previous evidence for specific distinction of the Kumamoto and Pacific oysters includes differences in shell morphology (Amemiya 1928, Ahmed 1975), salinity tolerance (Amemiya 1928), growth rate (Amemiya 1928, Numachi 1978), egg size (Amemiya 1928, Numachi 1978), reproductive season (Numachi 1978, Robinson 1992), allozymes (Buroker et al. 1979), and most critically, gamete compatibility (Numachi 1958, cited in Numachi 1978). On the basis of fertilization tests, Numachi defined two types of Kumamoto oysters, A and B, from Ariake Bay, Kyushu, Japan. Type B oysters were fully interfertile with all *C. gigas* populations tested. Sperm from Type A males, however, were unable to fertilize eggs from Type B or *C. gigas* oysters. On the other hand, eggs from Type A females could be fertilized by Pacific oyster sperm and by concentrated suspensions of Type B Kumamoto oyster sperm. We have recently confirmed Numachi's observations; sperm from Kumamoto oysters cultured on the U.S. west coast cannot fertilize eggs from the common, Miyagi variety, Pacific oysters cultivated in North America, but the reciprocal cross produces viable offspring (Banks et al. 1993b).

Numachi's finding that two morphologically similar but reproductively isolated Pacific oysters were sympatric in the Kumamoto region of Japan went largely unappreciated. Imai and Sakai (1961), for example, must have used Type B Kumamoto oysters in crossbreeding experiments showing complete interfertility of oysters from the Kumamoto, Hiroshima, Miyagi, Hokkaido Prefectures, but they make no mention of Numachi's work. North American malacologists and oyster culturists have likewise regarded the Kumamoto oyster as a geographical variety of the Pacific oyster (Woelke 1955, Quayle 1988). Previous confusion about the biological species status of *C. sikamea* in North American commercial oyster stocks has been resolved by the compelling concordance of diagnostic differences in mtDNA and allozymes with the one-way gametic segregation described by Numachi (Banks et al. 1993b).

More than correct taxonomy may be at stake. Recent attempts to find *C. sikamea* in Ariake Bay, Japan, have yielded only specimens with the allozyme or mtDNA profiles of *C. gigas* (Ozaki and Fujio 1985, Banks et al. 1993b). Of great concern, therefore, is the status of Kumamoto stocks in North America, which may be threatened by hybridization with Pacific oysters and by very small effective sizes of commercial brood stock populations. We report here the results of a genetic survey of two major independent lineages of Kumamoto oysters in the U.S. commercial oyster culture industry. Diagnostic allozyme and mtDNA markers are used to discriminate species, and changes in allozyme frequencies be-

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tween generations within a stock are used to measure genetic drift and estimate the effective population number.

MATERIALS AND METHODS

Stocks. Two distinct lineages of Kumamoto oysters that have been propagated in the U.S., but whose histories (numbers of founders, dates of introduction, etc.) are poorly documented, were sampled. One, represented by a stock belonging to Taylor United Co., Shelton, WA, was derived from stocks imported from Japan in the late 1970s by Oregon Oyster Co. and initially propagated by Oregon State University researchers at the Hatfield Marine Science Center, Newport, OR (A. Robinson, pers. comm.). By 1991, however, the Taylor United stock appeared, on the basis of shell morphology, to comprise "Kumamoto-like" and "*gigas*-like or hybrid" oysters (D. Robertson and K. Cooper, Taylor United Co., per. comm.). Four specimens from each group were shipped to the Bodega Marine Laboratory for molecular testing in July 1991; an additional 41 "Kumamoto-like" and 14 "*gigas*-like or hybrid" specimens from this same stock were subsequently sent to the BML in February, 1992. A second lineage of Kumamoto oysters is represented by stocks belonging to Coast Oyster Co. (now Coast Seafoods Co., Bellevue, WA), which were imported and propagated independently of the OSU stocks. Both four year-old adults (N = 34) and spat (N = 30), were obtained from Coast Oyster Company's facilities at Humboldt Bay, CA, in June, 1991. Allozyme data for the Coast adults and mtDNA data for selected individuals used in experimental crosses were previously reported by Banks et al. (1993b).

Allozyme Electrophoresis. Electrophoretic methods and nomenclature have been described previously (Hedgecock and Sly 1990, Banks et al. 1993b). Thirteen loci were scored for the Coast Oyster Co. 1991 spat: *Acon-1, Acon-2, Adkin, Gpi, Idh-1, Idh-2, Lap-2, Tap-2, Mdh-1, Mdh-2, Pgm, Sdh, Sod-1*. Four loci, *Aat, Idh-1, Idh-2,* and *Mpi* are diagnostic (*sensu* Ayala and Powell 1972) for the Kumamoto and Pacific oysters (Buroker et al. 1979, Banks et al. 1993b). These loci, together with *Mdh-2,* which appeared to be diagnostic for Coast Oyster Co. Kumamoto oysters (Banks et al. 1993b), were scored for 56 specimens in the Feb. 1992 sample from Taylor United. *Mpi* and *Idh-2* were not scored for the initial Taylor sample.

Mitochondrial DNA Typing. Methods for extracting DNA and typing mitochondrial haplotypes of the Kumamoto and Pacific oysters were described by Banks et al. 1993a. Polymerase chain reactions (PCR) using two primers, A and B, that amplify a 319 base-pair (bp) segment of oyster 16SrDNA were done on the first eight Taylor specimens. For 50 specimens in the second Taylor series, multiplex PCR reactions were made using primers A and B plus a third internal primer, E, which in combination with primer A specifically directs the amplification of a 246 bp DNA fragment from *C. sikamea* templates only. PCR products were incubated with the restriction endonuclease *Dra I*, according to manufacturer's directions (USBiochemical), electrophoresed in 3% NuSieve/agarose gels with 1X TBE buffer, stained with ethidium bromide, and photographed on a UV transilluminator. The resulting RFLP gel patterns are diagnostic because *Dra I* cleaves the 319 bp PCR product from *C. gigas* but not that from *C. sikamea* into two fragments of 141 and 178 bp (Banks et al. 1993a). For the 50 individuals from the second Taylor sample, PCR products were blotted on replicate nylon membranes in a vacuum, dot-blot apparatus, cross-linked to the membranes by exposure to UV light, and hybridized against species-specific oligonucleotide probes **Cg, Cs, Dg,** and **Ds,** where **C** and **D** denote two nucleotide sites in the 16S rDNA sequence at which the Pacific and Kumamoto oysters differ and **g** and **s** denote *C. gigas*-specific and *C. sikamea*-specific oligonucleotide probes, respectively, as described by Banks et al. (1993a). Redundancy of maternal-species diagnosis at seven nucleotides in the 16SrDNA gene, which is provided by the combination of multiplex PCR, restriction endonuclease digestion, and hybridization with oligonucleotide probes, eliminates any possibility that intra-specific polymorphism could result in false identification of any specimen (see Banks et al. 1993a).

Analysis of Temporal Change. The Coast Oyster Co. hatchery used their 1987 class of adult Kumamoto oysters to produce the 1991 spat (J. Donaldson, pers. comm.). Samples from these two year classes permit an analysis of genetic change between parent and offspring generations for this commercial stock by methods described in detail elsewhere (Hedgecock and Sly 1990, Hedgecock et al. 1992, and references therein). The analysis is based on the inverse relationship between observed temporal change in the frequencies of alleles and the effective size of an isolated population, N_e:

$$E(\hat{F}) \approx t/(2N_e) + 1/(2S_o) + 1/(2S_t),$$

where $E(\hat{F})$ is the expected variance, owing to random drift of allelic frequencies, between an initial sample (taken without replacement) of S_o individuals and a second sample of S_t individuals taken (without replacement) after an interval of t generations. Rearrangement of this equation yields an estimator of the effective population number:

$$\hat{N}_K = t/(2[\bar{F} - 1/(2S_o) - 1/(2S_t)]),$$

where $\hat{N}_K$ and $\bar{F}$ denote estimators of the parameters N_e *and* $E(\hat{F})$, respectively. In this study, $t = 1$, and $1/(2S_o)$ and $1/(2S_t)$ are harmonic mean sample sizes per locus, weighted by numbers of independent alleles per locus, for the 1987 adult and 1991 spat population samples, respectively. Variances of allelic frequencies between adult and spat samples were calculated for the 13 allozyme loci assayed in the Coast Oyster Co. stock (the $\hat{F}$s in Table 2). These variances are standardized to eliminate the effect of differences in initial allelic frequencies and then averaged across loci, weighting by the number of independent alleles at each locus, to yield an estimate, $\bar{F}$, of $E(\hat{F})$ (Hedgecock et al. 1992). If allozymes are selectively neutral, then $13\hat{F}/E(\hat{F})$ is distributed as a chi-square variable with 13 degrees of freedom corresponding to the number of independent loci sampled in this case. Agreement of the observed distribution with the chi-square distribution provides a test of the assumption of selective neutrality, as well as a means for calculating confidence limits on $\hat{N}_K$. An independent test of selective neutrality compares the actual loss of alleles over time to that predicted by population genetic theory assuming $N_e = \hat{N}_K$.

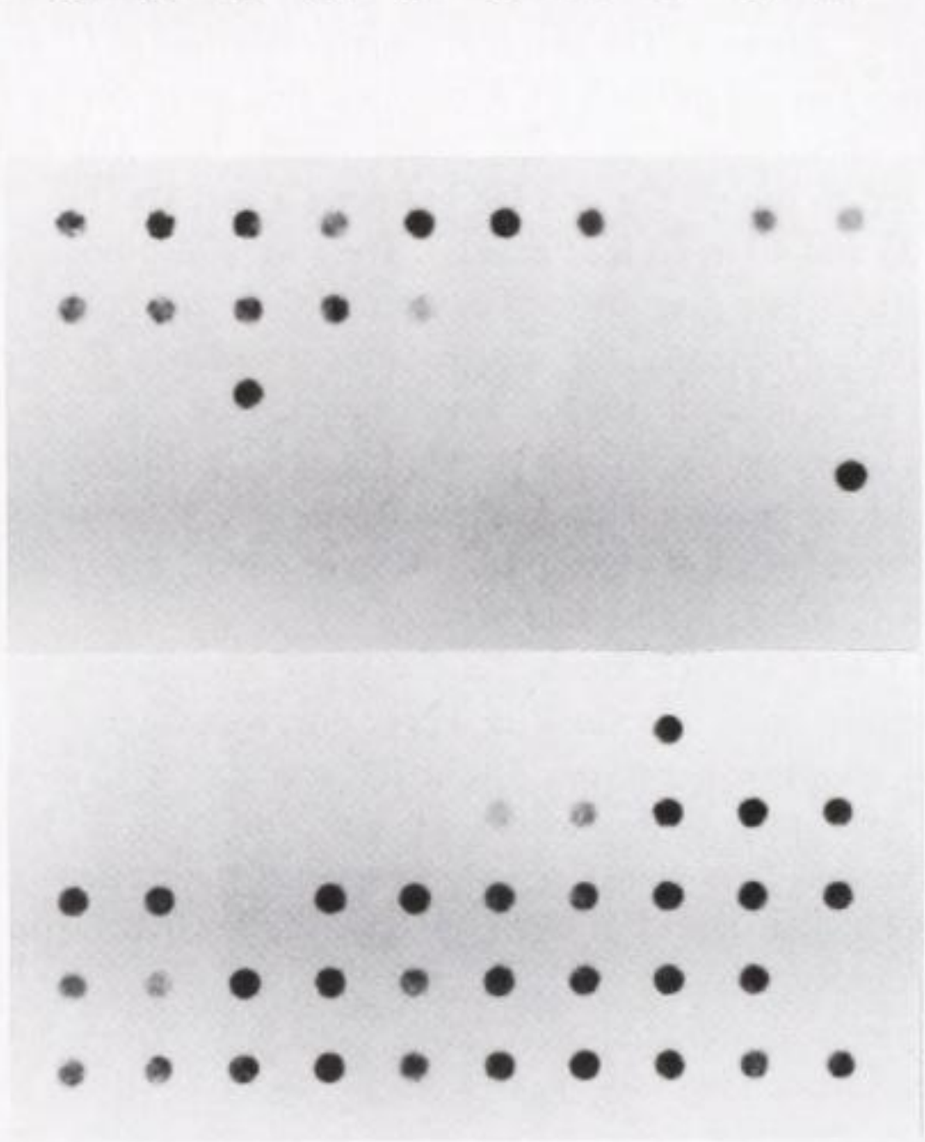
RESULTS

The Taylor United Stock. All four "Kumamoto-like" specimens in the first of two samples from this stock had molecular genetic markers diagnostic of *C. sikamea, i.e.* they were homozygous, $Aat^{92/92}$ and $Idh\text{-}1^{95/95}$, and yielded mtDNA PCR products that could not be cut with *Dra I*. Three of the individuals were homozygous, $Mdh\text{-}2^{107/107}$ like Coast Oyster Kumamoto oysters sampled previously (Banks et al. 1993b), but one was homozygous for the most common Pacific oyster allele, $Mdh\text{-}2^{100}$. The four specimens in the "*gigas*-like or hybrid" category appeared to be evenly divided between those two possibilities. Two were ho-

mozygous for the most common Pacific oyster alleles at *Aat, Mdh-2,* and in one case, *Idh-1;* both of these oysters also yielded mtDNA PCR products that were digested by *Dra I*. The other two were heterozygous for diagnostic alleles at the *Aat* and *Idh-1* loci and had *C. sikamea* mtDNA haplotypes (*i.e.*, PCR products uncleaved by *Dra I*) expected from successful fertilizations of *C. sikamea* eggs by *C. gigas* sperm. One of these individuals was homozygous, $Mdh\text{-}2^{100/100}$, while the other was heterozygous, $Mdh\text{-}2^{100/107}$.

In the second series of samples from this stock, 14 were classified morphologically as "*gigas*-like" and 41 were classified as "Kumamoto-like"; one small, unclassified oyster attached to a "*gigas*-like" oyster was also studied. All 14 "*gigas*-like" individuals had molecular markers diagnostic of *C. gigas*. They were each homozygous for the $Idh\text{-}1^{100}$ and Mpi^{100} alleles; 13 of 14 were homozygous for the $Mdh\text{-}2^{100}$ allele and one was a $Mdh\text{-}2^{100/107}$ heterozygote. Genotypes at the more polymorphic *Aat* locus were typical for *C. gigas* and none was homozygous for the Aat^{92} allele that is fixed in *C. sikamea*. The mtDNA typing results for these "*gigas*-like" individuals are given in Figs. 1 and 2 (Fig. 1, upper panel, samples in lanes 2–8, 10–16; positions 1–7, 9–15 in each of the four panels in Fig. 2). The small oyster attached to the shell of one of these Pacific oysters proved to have markers diagnostic of *C. sikamea* (Fig. 1, upper panel, sample in lane 9; position eight in each of the four panels in Fig. 2).

Of the 41 "Kumamoto-like" oysters in the second Taylor series, 38 had molecular marker diagnostic of *C. sikamea,* 2 had markers diagnostic of *C. gigas,* and one was an apparent hybrid. Specimens classified as *C. sikamea* yielded the Kumamoto-specific 246 bp product in multiplex PCR (Fig. 1, upper panel, lanes 17–23, 25, 26; lower panel, all lanes except 16 and 20) and PCR products that hybridized to probes **Cs** and **Ds**, but not **Cg** and



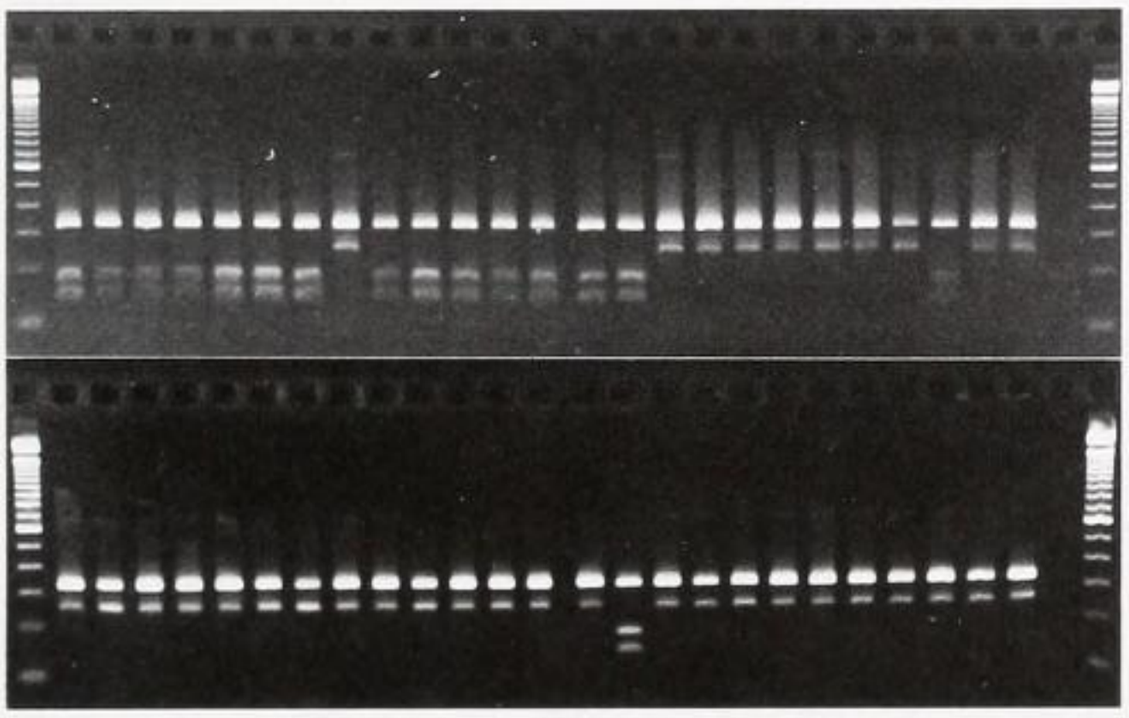


Figure 1. Photograph of two agarose gels containing products from 50 PCR amplifications of mitochondrial DNA (mtDNA) coding for large subunit ribosomal RNA from Pacific and Kumamoto oysters (*Crassostrea gigas* and *C. sikamea*, respectively). PCR products were partially digested with the restriction enzyme *Dra I* prior to electrophoresis, and the gels were stained with ethidium bromide. Lanes 1 and 28 in each gel contain a 100 base pair (bp) ladder of DNA standards; lane 27 in each gel contains a no-template PCR control reaction. The bright, 319 bp band in all other lanes corresponds to the full-length product obtained from oyster mtDNA with primers A and B (see Methods; Banks et al. 1993a). *Dra I* cleaves the Pacific, but not the Kumamoto oyster PCR products into 141 and 178 bp fragments (top gel: lanes 2–8, 10–16, 24; bottom gel: lane 16). A third primer in each PCR reaction (see Methods; Banks et al. 1993a) directs the synthesis of a 246 bp product from *C. sikamea* templates only (top gel: lanes 9, 17–23, 25, 26; bottom gel: lanes 2–15, 17–26).

Figure 2. Photographs of four dot-blot hybridizations of the same 50 PCR products as in Fig. 1 (arrayed in five rows of ten in each panel). Panels 1–4 (top to bottom) were hybridized with probes Cg, Cs, Dg, and Ds, respectively, where C and D denote two nucleotide sites in the 16SrDNA sequence at which Pacific and Kumamoto oysters differ and g and s denote *C. gigas*-specific and *C. sikamea*-specific oligonucleotide probes, respectively. PCR products from individuals 1–7, 9–15, 23 and 40 hybridize to Cg and Dg (panels 1 and 3), while PCR products from individuals 8, 16–22, 24–39, 41–40 hybridize to Cs and Ds (panels 2 and 4).

Dg (Fig. 2, positions 16–22, 24–39, 41–43, and 45–50 in all four panels). These same specimens were also homozygous for the following diagnostic allozymes: Aat^{92} (N = 38), $Idh\text{-}1^{96}$ (N = 38), $Idh\text{-}2^{95}$ (N = 25; 13 not scored), and Mpi^{96} (N = 38). At the *Mdh-2* locus, 18 individuals were homozygous for the *107* allele, 4 were homozygous for the *100* allele, and 17 were heterozygous for these two alleles (Table 1). Including the four individuals from the first sample, frequencies of the *100* and *107* alleles in the Taylor United stock are estimated to be about 0.3 and 0.7, respectively; the *Mdh-2* genotypic proportions are in agreement with Hardy-Weinberg-Castle expectations (goodness-of-fit chi-square

TABLE 1.

Frequencies of *Mdh-2* genotypes in wild and cultivated populations of *Crassostrea sikamea* and *C. gigas*. Data for Japanese *C. sikamea* are from Buroker et al. (1979); data for *C. gigas* are pooled from Buroker et al. (1979) and Banks et al. (1993b). Genotypic frequencies are those expected under Hardy-Weinberg-Castle equilibria, rounded to the nearest integer.

	Mdh-2 Genotypes			Total No. individuals
	100/100	100/107	107/107	
sikamea (Coast stock)	0	3	60	63
sikamea (Taylor stock)	4	17	21	42
sikamea (Japan)	69	9	0	78
gigas (pooled)	261	1	0	262

= 0.9, 1 d.f.). Table 1 compares *Mdh-2* genotypic frequencies for *C. gigas*, native *C. sikamea* and the Coast and Taylor hatchery stocks of *C. sikamea*. The two individuals classified as *C. gigas* in this group yielded mtDNA PCR products that were cut by *Dra 1* (Fig. 1, upper panel, lane 24; lower panel, lane 16) and that hybridized to probes **Cg** and **Dg** but not **Cs** and **Ds** (Fig. 2, positions 23 and 40 in all four panels). They were also homozygous for Pacific oyster diagnostic allozymes (*Idh-2* was not scored in one individual). Finally, the apparent hybrid yielded a *C. sikamea*-specific PCR product (Fig. 1, lower panel, lane 20), which hybridized to probes **Cs** and **Ds** but not **Cg** and **Dg** (Fig. 2, position 44 in all four panels). This individual was also heterozygous for the diagnostic or most common *C. sikamea* and *C. gigas* alleles at *Aat*, *Idh-1*, *Idh-2*, *Mdh-2* and *Mpi*.

The Coast Oyster Stock. Of the thirty Kumamoto hatchery spat sampled from this stock in 1991, one was diagnosed as *C. gigas* by allozyme genotype. Allelic frequencies for 13 allozymes for the remaining 29 individuals are given in Table 2. Together with allozyme frequencies reported previously for adults of this same stock (Banks et al. 1993b), these data allow calculation of a mean temporal variance of allelic frequencies, $\hat{F} = 0.1399$, which in turn yields an estimate of effective stock size, $\hat{N}_K = 5.4$. The 95% confidence range for $\hat{N}_K$ is from 2.5 to 11.8. Of the total of 40 alleles observed at these loci in the adult generation, only 30 remain in the sample of spat; numbers of alleles remaining and lost in this comparison of adults and offspring are not significantly different that those expected, 33.0 and 7.0, respectively, in a model of random genetic drift in a population of effective size equal to 5.4 (goodness-of-fit chi-square = 1.040, 1 d.f.). The distribution of standardized temporal variances at individual loci is not significantly different from the chi-square distribution expected under random genetic drift (Fig. 3).

DISCUSSION

The Kumamoto oyster *Crassostrea sikamea* (Amemiya 1928) can be unambiguously discriminated from the Pacific oyster *C. gigas* (Thunberg) on the basis of molecular markers and gametic compatibility. Although re-evaluation of the taxonomic status of the Kumamoto oyster has been carried out on stocks cultivated in North America, results are in accord with earlier studies made on Japanese native populations. Previously observed differences between the two oysters in reproductive traits (Amemiya 1928, Numachi, 1958 cited in Numachi, 1978) and allozymes (Buroker et al. 1979) are shown to be congruent for North American stocks

TABLE 2.

Variances in allelic frequencies, $\hat{F}$, for 13 allozyme-coding loci between two year classes of Coast Oyster Co. Kumamoto oysters.

Locus	Allele	1987	1991	$\hat{F}$
Acon-1	(N)	(25)	(29)	
	100	0.980	1.000	
	97	0.020	0.000	
				0.0400
Acon-2	(N)	(22)	(18)	
	109	0.045	0.000	
	103	0.045	0.000	
	100	0.545	0.861	
	97	0.364	0.139	
	95	0.045	0.000	
				0.1533
Adkin	(N)	(34)	(22)	
	105	0.029	0.000	
	103	0.235	0.068	
	100	0.603	0.591	
	97	0.088	0.250	
	95	0.029	0.091	
				0.1154
Gpi	(N)	(18)	(29)	
	105	0.306	0.069	
	100	0.639	0.879	
	94	0.056	0.052	
				0.1879
Idh-1	(N)	(30)	(29)	
	95	0.983	0.983	
	92	0.017	0.017	
				0.0
Idh-2	(N)	(30)	(29)	
	95	0.800	0.983	
	93	0.200	0.017	
				0.3186
Lap-2	(N)	(18)	(28)	
	103	0.250	0.071	
	101	0.139	0.000	
	100	0.611	0.857	
	98	0.000	0.07	
				0.2340
Mdh-1	(N)	(34)	(29)	
	108	0.029	0.121	
	100	0.971	0.879	
				0.1184
Mdh-2	(N)	(34)	(29)	
	107	0.985	0.966	
	100	0.015	0.034	
				0.0150
Tap-2	(N)	(18)	(13)	
	102	0.111	0.077	
	100	0.806	0.846	
	98	0.083	0.077	
				0.0073
Pgm	(N)	(34)	(9)	
	103	0.044	0.167	
	100	0.206	0.000	
	96	0.456	0.556	
	90	0.118	0.000	
	86	0.088	0.167	
	82	0.088	0.111	
				0.1731

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TABLE 2.

continued

Locus	Allele	1987	1991	$\hat{F}$
Sdh	(N)	(34)	(25)	
	106	0.000	0.020	
	104	0.000	0.080	
	100	0.897	0.900	
	97	0.088	0.000	
	93	0.015	0.000	0.1015
Sod-1	(N)	(33)	(18)	
	100	0.803	0.972	
	90	0.197	0.028	
				0.2670

(Banks et al. 1993b). Moreover, newly observed differences in mitochondrial DNA sequences, which are also congruent with allozyme and reproductive-trait differences, permit rapid and accurate diagnosis of maternal species lineage (Banks et al. 1993a).

Oysters collected from the Kumamoto area in recent times have all been *C. gigas* by molecular tests (Ozaki and Fujio 1985, Banks et al. 1993b). Interestingly, some of these had the shallow, fluted, purple-streaked, shells typical of the Miyagi-type Pacific oyster, while others resembled the smaller, deeper, wrinkled shell morphology of true *C. sikamea*. The latter may represent the endemic form of *C. gigas*, Numachi's (1978) Type B Kumamoto oyster. Until a more systematic search reveals relict native populations of *C. sikamea*, the only Kumamoto oysters known to exist are those cultivated in the U.S. Our survey of the two major lineages of Kumamoto oysters propagated in North America highlights two

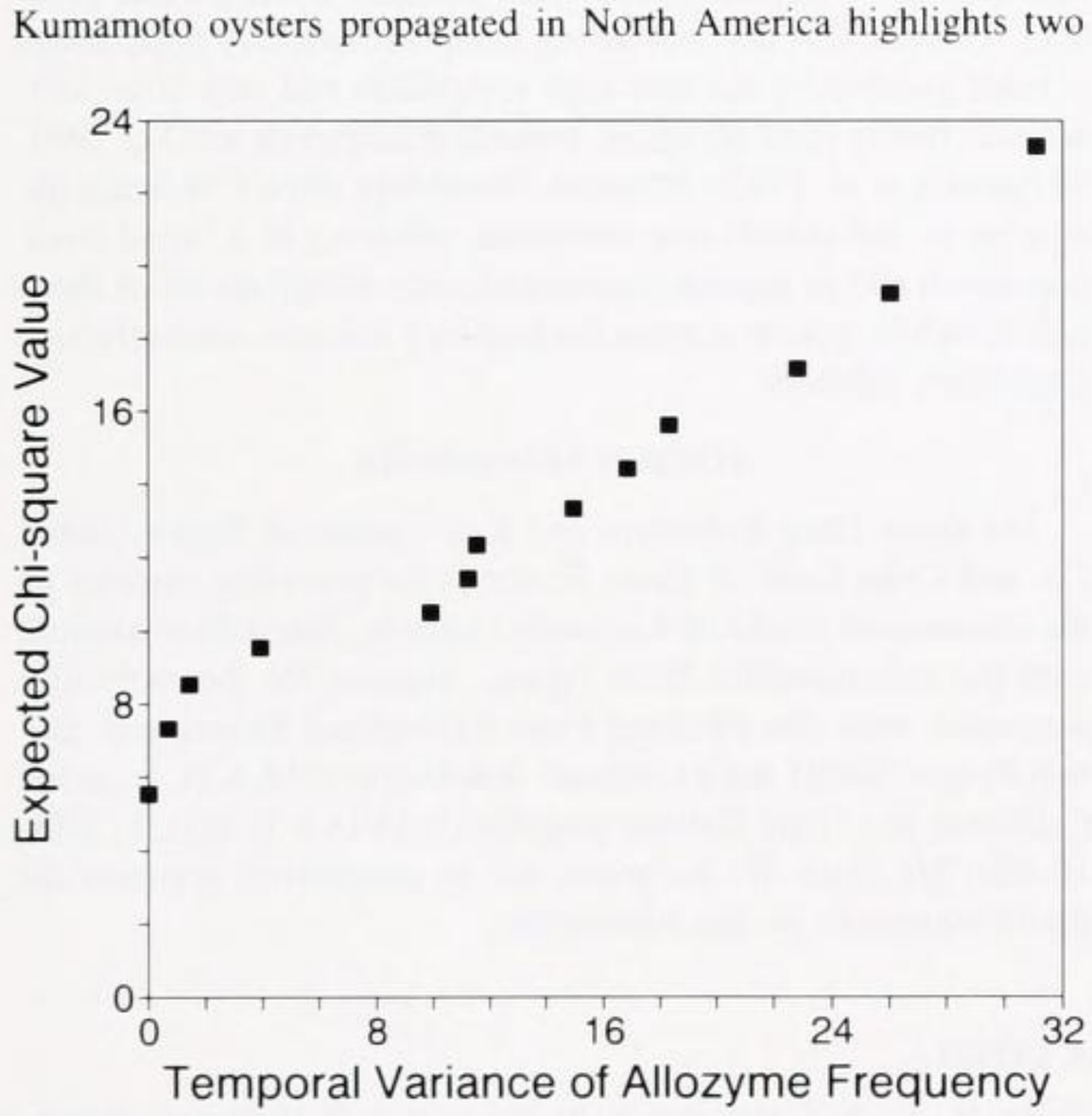

Figure 3. Probability plot of standardized variances of allelic frequencies at 13 allozyme-coding loci between two generations of a Kumamoto oyster commercial hatchery stock. Observed temporal variance on the x-axis is plotted against the corresponding expected value from the chi-square distribution with 13 d.f. on the y-axis (see Hedgecock et al. 1992 for details of the method). The linearity of the plot suggests that the observed variances are distributed as chi-square variables, as expected under random genetic drift in the absence of selection.

potential threats to the integrity of these genetic resources, perhaps to the survival of this oyster species. The first is admixture and hybridization with Pacific oysters, leading possibly to progressive introgression of Pacific oyster genes into Kumamoto oysters brood stocks. The second is erosion of genetic diversity from random genetic drift within small, hatchery-propagated populations.

Reports from several U.S. growers that Kumamoto oyster hatchery seed contained significant numbers of Pacific oyster "weeds" aroused concern about contamination of commercial brood stocks (Hedgecock and Robinson 1992). We document here admixture and/or hybridization of Pacific and Kumamoto oysters in the two commercial brood stock lineages. For one stock, admixture of Pacific oysters and possible hybrids was initially suggested by examination of shell morphology (D. Robertson and K. Cooper, Taylor United Co., pers. comm.). Molecular diagnoses subsequently showed that 42 of 45 individuals classified morphologically as Kumamoto oysters were indeed *C. sikamea*, but that one was a hybrid and two were Pacific oysters. Two additional hybrids were detected in a group classified morphologically as "*gigas*- or hybrid-like" and an unclassified *C. sikamea* was found attached to a larger "*gigas*-like" individual. Of the 69 Kumamoto oysters sampled from Coast Oyster Co. stocks, only one spat was diagnosed as *C. gigas;* the remaining adults and spat typed as pure *C. sikamea*.

Results for the Taylor United stock suggest that commercial breeders can, on the basis of shell morphology, fairly reliably discriminate the Kumamoto oyster *C. sikamea* from the Miyagi strain of Pacific oyster typically grown in North America. Yet, diagnosis of brood stock must be absolutely correct in order to preserve the specific integrity of Kumamoto oyster stocks. Sperm from *C. gigas* can fertilize eggs from *C. sikamea* so that accidental admixture of the two species in mass spawnings of commercial brood stock could lead to hybridization. We infer from our finding of three hybrid individuals that hybridization has indeed occurred in commercial spawns of supposed Kumamoto brood stocks. All three Pacific X Kumamoto hybrid oysters were heterozygous for species-diagnostic allozyme markers but had *C. sikamea* mitochondrial DNA haplotypes, the composite nuclear and mitochondrial hybrid genotype expected on the basis of the one-way gametic compatibility between these species.

The fate of hybrids in commercial stocks is presently unknown. Pacific X Kumamoto hybrids seem morphologically to be quite variable. Some are evidently indistinguishable from pure Kumamoto oysters. Unless hybrids can be excluded from Kumamoto brood stocks, there is potential for progressive introgression of Pacific oyster genes into Kumamoto stocks. In this regard, there is need to assess compatibilities of gametes from interspecific hybrids, both in crosses among themselves and in backcrosses to the parental species. If hybrid sperm is unable to fertilize the Pacific oyster egg, hybrids would be falsely diagnosed as pure Kumamoto oysters by the cross-fertilization test that otherwise unambiguously discriminates Kumamoto from Pacific oyster males (Numachi 1958 cited in Numachi 1978, Hedgecock and Robinson 1992, Banks et al. 1993b).

The diversity of genetic resources within commercial stocks of Kumamoto oysters may be threatened by random genetic drift, as shown for numerous aquaculture hatchery stocks (Waples and Teel 1990, Hedgecock et al. 1992). Substantial genetic change occurred between the '87 and the '91 year classes of Coast Oyster Co. Kumamoto oyster stocks, as estimated from allelic frequencies at 13 loci in samples taken from the Humboldt Bay, CA,

production grounds. Assuming that we randomly sampled parental and offspring generations, we estimate, from average temporal variance of allelic frequencies, that the effective size of this stock is only 5.4. That the genetic changes in this stock represent random genetic drift is evidenced by (1) agreement between observed and expected numbers of alleles lost and retained in the '91 year class and (2) agreement between the distribution of drift variances for individual loci and a chi-square distribution with 13 degrees of freedom (Fig. 3; Hedgecock et al. 1992). With an effective number of 5.4, the Coast stock of Kumamoto oysters is expected, from population genetics theory, to lose a little more than one-tenth of its genetic diversity each generation and to become rapidly inbred. Inbreeding in small, unpedigreed populations is likely to reduce greatly the long-term productivity and persistence of the stock, as appears to have happened in the very small lines of the American oyster *C. virginica* bred for resistance to MSX (Vrijenhoek et al. 1990, Hedgecock et al. 1992, P. Gaffney, pers. comm.). Loss of genetic diversity through random genetic drift is especially worrisome in the case of the Kumamoto oyster since it cannot be counteracted by importation of fresh brood stock from extant natural populations.

Divergence of the two major U.S. brood stocks of Kumamoto oysters at the *Mdh-2* locus, from each other and from the native population sampled by Buroker et al. (1979), may have resulted from random genetic drift in both hatchery and natural populations (Hedgecock et al. 1992, Hedgecock 1993). The frequency of the "fast" allele at this locus (*104* in Buroker et al. 1979; *107* in Banks et al. 1993b) was 0.058 in the native Kumamoto population, 0.7 in the Taylor stock and 0.98 in the Coast stock (Table 1). This allele has been detected only once in *C. gigas* samples (N = 262), a frequency of 0.002; thus, *Mdh-2* is a diagnostic locus (*sensu* Ayala and Powell 1972) for the Coast Oyster Kumamoto oyster stock, but not for the Taylor United Kumamoto oyster stock.

We believe that three steps should be taken to conserve the Kumamoto oyster in North America. First, *Crassostrea sikamea* (Amemiya 1928) should be adopted by North American malacologists and the U.S. oyster industry as the scientific name for the Kumamoto oyster. The specific status of this animal is well supported by several concordant lines of evidence indicating reproductive isolation and evolutionary genetic divergence. Use of the correct scientific name should promote the recognition and conservation of this unique species, which, as far as is now known, survives only in the U.S. oyster industry.

Second, in view of their importance to the overall conservation of the species, the species integrity of commercial Kumamoto oyster stocks should be safeguarded (Hedgecock and Robinson 1992). With currently available diagnostic methods, pure Kumamoto brood stock can be identified by a progeny testing scheme involving: (1) strict selection of brood stock candidates with Kumamoto morphology and growth history; (2) non-destructive (thermal) induction of spawning; (3) testing of sperm for inability to fertilize Pacific oyster eggs; (4) testing of larvae from controlled pairwise crosses for mitochondrial DNA haplotype; (5) testing of these same progeny at an early juvenile stage for allozyme genotype; (6) conservation of brood stock whose progeny are diagnosed as pure Kumamoto and culling of those individuals whose progeny carry Pacific oyster genes. Commercial hatcheries should at least practice steps 1 to 3, and on this basis, step 6.

Development of species-diagnostic nuclear DNA markers would improve the efficiency of brood stock testing. The maternal lineage of an individual can be identified by PCR and mtDNA typing of eggs or progeny as soon as one day post-fertilization or of tissue biopsy samples from the brood stock oysters themselves. Yet, mtDNA typing cannot distinguish pure Kumamoto oysters from Pacific male X Kumamoto female hybrids, the most likely contaminants in brood stocks, because both carry the Kumamoto mitochondrial genome. Discrimination of hybrids from pure Kumamoto oysters can only be made by testing for paternally inherited Pacific oyster genes. Allozymes provide such markers, but they cannot be reliably identified in individuals before a young spat stage and they require destructive tissue sampling. There is need, therefore, to develop PCR methods for nuclear DNA markers, which would facilitate identification of paternal species lineage at the larval stage or in biopsy tissue samples from individual adults.

Finally, genetic diversity within commercial brood stocks of the Kumamoto oyster needs to be monitored and conserved through improved brood-stock and hatchery-management practices. Genetic drift and inbreeding in aquatic hatchery populations is made possible by the very high fecundities and very large variances in family sizes of aquatic animals (Hedgecock and Sly 1990, Hedgecock et al. 1992). Breeders should take steps to increase the number of individuals that contribute offspring to a brood stock population and to equalize the reproductive contributions of those individuals in order to reverse the tendency towards small effective population numbers.

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