Report of the Kumamoto Brood Stock Workshop

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Oregon State University
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Organized by

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EXECUTIVE SUMMARY

The Kumamoto oyster *Crassostrea sikamea* is a distinct biological species distinguishable from its close relative the Pacific oyster *C. gigas* by a one-way gametic incompatibility (Kumamoto oyster sperm cannot fertilize the Pacific oyster egg but the opposite cross produces viable offspring), by differences in certain genetically encoded enzymes that can be visualized by electrophoresis, and by differences in the nucleotide sequence of a mitochondrial gene encoding the large subunit of ribosomal RNA. The magnitude of molecular genetic differences suggests that the Kumamoto and Pacific oysters diverged from each other at least one million years ago.

Confusion over the specific status of the Kumamoto oyster has unfortunately contributed to contamination of commercial brood stocks on the west coast of the United States. The long term persistence of this species in the west coast oyster industry is threatened not only by hybridization with Pacific oysters, but by the effective propagation of inadequate numbers of individuals, which, if unchecked, would lead ultimately to loss of genetic diversity and inbreeding depression. Both of these problems can be remedied by the infusion of new, pure, Kumamoto oyster stocks. Unfortunately, the Kumamoto oyster has apparently disappeared from its native Japanese grounds, eliminating this most obvious source of new stock.

The west coast oyster industry should take immediate steps to preserve the genetic integrity and diversity of its Kumamoto oyster brood stocks. Employing currently available diagnostic methods, the initial steps for identifying pure Kumamoto brood stock would be: (1) strict selection of 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) sampling of larvae for mitochondrial DNA typing; (5) sampling of progeny at an early juvenile stage for enzyme typing; (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 are capable of steps 1 to 3, and based on these alone, step 6. The highly accurate diagnostic tests based on DNA and enzymes (steps 4 and 5), could be carried out with the help of academic laboratories capable of providing molecular diagnostic services. A combination of government and private funding is needed to carry out all steps of the screening program in a coordinated manner.

Research that would improve the efficiency and reliability of diagnostic methods for the Kumamoto oyster are identified: 1) an understanding of the basis of the gamete incompatibility and whether Kumamoto x Pacific oyster hybrids pass the sperm incompatibility test; 2) tests of whether differences in egg size allow species separation by a simple screening procedure; 3) development of biopsy methods for safe typing of adult brood stock; 4) identification of nuclear DNA markers that would allow rapid discrimination of hybrids from pure Kumamoto oysters.

INTRODUCTION

The distinctive, deeply cupped Kumamoto oyster, which commands a premium price in the half-shell trade, has been widely regarded as a variety of the more common Pacific oyster *Crassostrea gigas*. Unfortunately, over the past several years, oyster producers in California, Oregon, and Washington have reported that Kumamoto hatchery seed has been growing up to resemble the typical and less valuable Pacific oyster, implying that commercial Kumamoto brood stocks may be contaminated by mixture or hybridization of the two oysters. Confusion over the biological and taxonomic status of the Kumamoto oyster may underlie the problem of contaminated seed and may imperil the long-term persistence of this valuable oyster in the west coast oyster industry.

Over the past several years, Dr. Anja Robinson has been collecting, spawning and rearing Kumamoto oysters in the hope of preserving a pure brood stock. Like the commercial oyster breeders, she has had to identify pure Kumamoto brood stock on the basis of their distinctive shell morphology, their small size owing to slow growth, and a late summer or fall peak in gonad maturation and spawning. Dr. Dennis Hedgecock's laboratory, on the other hand, has developed techniques to distinguish genetic differences between the Kumamoto and Pacific oysters and has recently confirmed, through experiments and carefully controlled observations, several lines of evidence supporting the naming of the Kumamoto oyster as a distinct species, *Crassostrea sikamea*. Moreover, preliminary screening of some Kumamoto stocks for diagnostic molecular markers has confirmed mixture and hybridization with Pacific oysters.

In view of these recent events and findings, Drs. Hedgecock and Robinson organized a workshop, which was funded by the Oregon Sea Grant College Program, to achieve a better understanding of the Kumamoto brood stock situation and to devise a strategy for ensuring a reliable source of high quality Kumamoto seed. The workshop, which was held at the Portland Center of Oregon State University on January 24, 1992, was attended by university and government scientists and by oyster producers (Appendix I). This report summarizes the proceedings and conclusions of the workshop.

TAXONOMIC STATUS OF THE KUMAMOTO OYSTER

The taxonomic history of the Kumamoto oyster, together with significant milestones in its distinction from the Pacific oyster, is chronicled in Appendix II. The name, *sikamea*, from the Japanese word for wrinkled, was applied by Amemiya (1928) to the cupped oyster from Ariake Bay, as a distinct variety of the Japanese oyster, *Ostrea gigas* var. *sikamea*. (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.) The Kumamoto Prefecture borders on Ariake Bay.

In the late 1950s and early 1960s, two reports of experimental crosses among Japanese oysters yielded opposite results concerning the specific status of the Kumamoto oyster. Numachi (1958; cited in Numachi,

1978) described two cupped oysters from Ariake Bay: Type A, whose sperm could fertilize neither Type B eggs nor the eggs of oysters from the Miyagi, Hiroshima or Hokkaido Prefectures, and Type B, which was fully interfertile with the other geographical populations (see Fig. 1 for locations). Unfortunately, Numachi's work, which suggested that the name Kumamoto failed to distinguish between two sympatric species of oysters, was ignored by subsequent workers.

Imai and Sakai's (1961) paper concerning a large, long-term inbreeding and crossbreeding study reported that oysters from the 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, was likely responsible for spreading the notion to western biologists that variation among the geographical populations of Japanese oysters was a continuum of racial differences. The apparent morphological similarity of Type A and Type B Kumamoto oysters (see below) 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 proposed that the name *C. laperousi* Schrenk be restored for the Hiroshima oyster and that Amemiya's variety be elevated to full species, *C. sikamea*. Although no evidence has surfaced to support the status of *C. laperousi* Schrenk, Ahmed's judgement regarding *C. sikamea* has been strongly affirmed by more recent studies (reviewed below). Still, Ahmed failed to establish the correspondence of Numachi's Type A and *C. sikamea* and to distinguish between the two sympatric Kumamoto oysters. The use of Kumamoto as a common name should be restricted to *C. sikamea* as defined by the reproductive and biochemical features described below.

Unfortunately, native Kumamoto oysters may be nearly extinct in Japan. Historically, the Japanese did not regard small Kumamoto oysters as commercially valuable (Amemiya 1928), which may account for the demise of their native stocks. Ozaki and Fujio's (1985) survey of protein variation in natural and hatchery-propagated oyster stocks found no evidence for any oyster but *C. gigas*. These authors attributed this result to the widespread distribution of *C. gigas* seed among Prefectures. Likewise, several recent attempts by U.S. oyster producers to locate and import, through Japanese counterparts, original stocks of Kumamoto oysters in Japan have failed (K. Cooper, pers. comm.). Ariake Bay is now devoted almost entirely to nori culture (Chew, pers. comm.). Dr. Chris Langdon of OSU and the Hatfield Marine Science Center, a workshop participant, volunteered to personally search for *C. sikamea* while on sabbatical leave in Japan this year.

HISTORY OF THE KUMAMOTO OYSTER ON THE WEST COAST

The first documented importations of the Kumamoto oyster were made experimentally in 1947 by John Glude and Cedrick Lindsay of the Washington Department of Fisheries (WDF) for the purpose of increasing the

supply of cocktail oysters on the west coast (Woelke, 1955). Between 1947 and 1953, a total of 98 cases of experimental seed were planted on oyster beds in Washington, Oregon, California and Hawaii. Plantings were successful, *i.e.*, seed survived for two years, in a variety of localities in Washington (Gray's Harbor, Oyster Bay, North Bay, Belfair, Hoods Port, Pt. Whitney, Dabob Bay) and Oregon (Coos Bay, Yaguina Bay, and Tillamook Bay). The success of these early plantings induced several oyster growers to purchase small commercial quantities of Kumamoto seed oysters annually, so that the total number of cases of seed imported from 1947 to 1953 was 3181. Interestingly, a WDF experiment in 1952 compared native Kumamoto seed set in May *vs* seed set in August or September; spring-caught seed showed poorer survival (Woelke, 1955). That spring-caught seed may have been Type A (*sikamea*) and fall-caught seed, Type B (*gigas*) is suggested by reports of differences in the reproductive season of these oysters in their native habitat (see next section). Unfortunately, comparative growth data on these two types of seed were not reported by the WDF.

Subsequent importations of Kumamoto seed by the industry are less well documented. Workshop participants recalled commercial seed importations after World War II to several additional west coast localities besides those used in the WDF studies (Willapa Bay, Oakland Bay, Poulsbo, WA; Humboldt Bay, CA). They also recalled that some of this seed grew quite fast and may have been Kumamoto Type B or *gigas* ("Verne Hayes of Eureka Oyster Co. got a bad lot"; "Kumamotos from Eureka and Willapa grew fast"). Putative Kumamoto oysters recently obtained from Tasmania were reported to have grown to 3 inches shell height in 18 months (Chew, pers. comm.). Thus, even the earliest importations of the Kumamoto oyster to the west coast may have contained two species and led to contamination of subsequently developed hatchery stocks.

DISTINGUISHING PACIFIC AND KUMAMOTO OYSTERS

Morphology

Qualitative differences in shell shape and size among the geographical populations of Japanese cupped oysters are summarized in Table 1 (from Numachi, 1978). Oysters from Ariake Bay (Kumamoto and Saga Prefectures) are characterized by slow growth, hence small shell size, a deeply cupped left valve, a high ratio of meat weight to total weight, and a wrinkled, dark violet or brown shell surface. That these characters overlap considerably, however, between the Type A and B Kumamoto oysters of Numachi (1958) and even with those of the Hiroshima oysters is evident from Imai and Sakai's (1961, Table 1) description of the parent oysters used for their breeding experiments. Nevertheless, recent studies of west coast commercial stocks of Kumamoto oysters, in which diagnostic genetic differences have been used for classification (see below), have shown that skilled oysterman can discriminate the Kumamoto and Pacific oysters on shell characters fairly accurately. Of course, absolute accuracy is necessary for brood stock identification.

Reproductive Traits and Gametic Incompatibility

Differences in reproductive traits between *C. sikamea* and *C. gigas* are more clear-cut than differences in shell traits. The spawning season of the Kumamoto oyster is given by Numachi (1978; see Table 1) as very early, with eggs maturing in winter; the other oysters spawn in spring and early summer. Along the west coast of the U.S., the Kumamoto oyster is reproductively mature from late summer through early winter (Robinson, 1992; see CONTROLLED CROSSES), while the Pacific oyster, which is primarily derived from imported Miyagi stocks, spawns in late spring and early summer. Of course, spawning seasons can be manipulated by artificial conditioning, and there is some overlap of spawning seasons in late summer. The mature eggs of the Kumamoto oyster are smaller than those of the Pacific oyster, averaging about 45 μm *vs* 55 μm in diameter, respectively. Whether this difference in egg diameter is large or consistent enough to allow for physical separation of egg mixtures by screening remains to be studied.

A defining difference between the Kumamoto (Type A) and Pacific oysters is the inability of the Kumamoto sperm to fertilize the Pacific egg, which was first described by Numachi (1958). Crosses in the other direction, *i.e.* Pacific sperm with Kumamoto eggs are fully fertile, although Numachi noted a reduced rate of fertilization when Type B sperm was used. Banks et al. (1991; 1992b) recently confirmed this one-way gametic incompatibility in carefully controlled crosses using Kumamoto stocks from Hayes Oyster Co. and Coast Oyster Co. Hundreds of Pacific oyster eggs that were exposed to and were bound by Kumamoto sperm were examined for signs of successful fertilization, but none was observed to undergo maturation or cleavage. This result obtained even when Pacific oyster eggs were stripped from the ovary and exposed directly to high concentrations of Kumamoto sperm, conditions which normally produce polyspermy (Stephano and Gould, 1988). In contrast to these results, greater than 90% of eggs in *sikamea* x *sikamea*, *gigas* x *gigas*, and *gigas* x *sikamea* crosses were successfully fertilized. This one-way gametic incompatibility in artificial fertilizations is evidence of at least partial reproductive isolation and supports the classification of the Kumamoto oyster as a separate species, *C. sikamea*. Most importantly, the inability of Kumamoto oyster sperm to fertilize Pacific oyster eggs provides commercial hatchery operators with a simple means of distinguishing the two species (see CONTROLLED CROSSES).

Biochemical and Molecular Genetic Differences

The first study to quantify genetic variation within and between the Kumamoto and Pacific oysters was by Buroker et al. (1979), who used the techniques of starch gel-electrophoresis and specific enzyme staining to demonstrate variation at 26 enzyme-coding genes. They found that while the Hiroshima and Miyagi populations had an average genetic similarity of 99%, the average similarity of these oysters and a Kumamoto population was only 74%, a value within the range of genetic similarities reported in other studies of morphologically distinct oyster species. Banks et al. (1991; 1992b) recently confirmed these results for west coast commercial stocks, using 17 protein-coding genes and comparing a total of 77 Kumamoto oysters of the 1987 and 1991 year-classes in Humboldt Bay with 78 natural

set Pacific oysters from Dabob Bay, WA. The average genetic similarity in this comparison was 67%, close to Buroker et al.'s 74%.

Some of the enzyme-coding genetic differences found by Buroker et al. and Banks et al. can be used to diagnose whether a juvenile or adult oyster is Kumamoto or Pacific (Table 2). For example, Kumamoto oyster genotypes for one of two isocitrate dehydrogenase isozymes (*Idh-1*) are completely distinct from those occurring in the Hiroshima, Miyagi or Dabob Bay population samples. In both studies, the two species have nearly or completely distinct genotypes at four enzyme-coding genes (symbolized by abbreviations for each enzyme name): *Aat-2*, *Idh-1*, *Idh-2*, and *Mpi-2*. An additional gene, *Mdh-2*, appears to be diagnostic for the two species in some west coast stocks (Table 2). Banks et al. found Kumamoto stocks from Coast Oyster Co. to be homozygous for a different *Mdh-2* allele than the one occurring in all Pacific oyster populations. However, this Kumamoto-specific allele occurred at a frequency of only 5% in Buroker et al.'s earlier sample of native Kumamoto oysters. Kumamoto stocks derived from the Oregon State University hatchery, on the other hand, have an intermediate frequency for this Kumamoto allele of 70%, so that about 30% of alleles are Pacific type and 9% of individuals in this stock are indistinguishable from Pacific oysters for this gene. The fixing of what was originally a rare *Mdh-2* Kumamoto-specific allele in the Coast Oyster Co. stock is likely the result of genetic drift in small populations, as demonstrated previously for hatchery-propagated stocks of the Pacific oyster (Hedgecock and Sly, 1990). Indeed, changes in the frequencies of alleles between the 1987 and 1991 year classes of this Kumamoto stock yield an estimate of only 4 individuals as the effective number of brood stock.

Additional genetic evidence that the Kumamoto oyster is a distinct species from the Pacific oyster comes from a study of maternally inherited mitochondrial DNA (mtDNA; Banks et al. 1991, 1992a), which utilized a new technology for enzymatic amplification of DNA, the polymerase chain reaction or PCR (Saiki et al., 1988). Samples selected for study were Pacific oyster adults from the native population in Dabob Bay, WA, and Kumamoto oysters from commercial stocks cultivated by Coast Oyster Company in Humboldt Bay, CA, and by Hog Island Oyster Company in Tomales Bay, CA. Working with DNA extracts from these different oysters, Banks et al. amplified and sequenced a portion of the mitochondrial gene that codes for the large ribosomal RNA subunit (the 16S rRNA gene), an essential component of the mitochondrion's molecular apparatus for protein synthesis. This mtDNA sequence can now be repeatably amplified from a variety of tissues including individual larvae and spat.

Three of the Tomales Bay Kumamoto oysters were found to have Pacific oyster mtDNA sequences in this initial survey of mtDNA variation; these same individuals were also found to be homozygous for Pacific oyster enzyme markers, indicating that these were simply Pacific oysters mixed in with Kumamoto production stocks. However, among Kumamoto brood stock subsequently sampled (derived from OSU hatchery stock and from Coast Oyster Co. stocks), first generation hybrids were identified. Interestingly, the shells of some hybrids are indistinguishable from pure Kumamoto oysters in size and shape. Thus, purposeful or unwitting hybridization between the species, which is possible through crosses of

Pacific males and Kumamoto females, has resulted in contamination of Kumamoto brood stocks. This contamination is likely responsible for the bad Kumamoto seed that has recently surfaced in the industry.

Differences between the mtDNA types of the two species can presently be rapidly diagnosed by three methods (Fig. 2). First, a PCR primer specific to the Kumamoto sequences can be used, together with the original non-species-specific primers, in the initial PCR reaction of an unknown sample; if the unknown individual is a Kumamoto oyster, the reaction yields two products that differ in length and are easily resolved on agarose gels stained with ethidium bromide (Fig. 2A,B). Second, digestion of the single PCR product obtained from a Pacific oyster with the restriction endonuclease enzyme Dra I, which recognizes and cleaves the sequence TTTAAA, yields two pieces of DNA (Fig. 2A,B). Kumamoto PCR products, having a TTAAAA sequence at this site, are not cut by Dra I. Finally, oligonucleotide probes specific for alternative Pacific and Kumamoto sequences allow for rapid typing of individual PCR products in dot blot hybridization reactions (Fig. 2C).

A SCREENING PROGRAM FOR PURE KUMAMOTO BROOD STOCKS

This is a two-part task. The first step is to determine whether existing Kumamoto brood stocks are pure *C. sikamea* or hybrids. Gametic incompatibility, strict morphological criteria, and diagnostic molecular markers can be used for this purpose. The second phase, the development of genetically diverse Kumamoto brood stocks, is a longer term project; methods for developing and improving oyster brood stocks are being evaluated by a project funded by the USDA's Western Regional Aquaculture Consortium. The workshop considered only the more immediate, concrete objectives of the first task.

Unfortunately, no non-destructive diagnostic method can at present definitively identify whether an adult brood oyster is Kumamoto or Pacific. The maternal lineage of an individual can be identified by PCR and mtDNA typing of eggs or progeny as soon as two days after fertilization; tissue biopsy could undoubtedly be perfected for confirming the maternal lineage of brood oysters. But typing of maternally inherited mtDNA cannot distinguish pure Kumamoto oysters from Pacific male x Kumamoto female hybrids, the most likely contaminants, because both carry the Kumamoto mitochondrial genome. Discrimination of hybrids from pure Kumamotos can only be made by testing for paternally inherited Pacific oyster genes.

Paternal lineages of brood stock can be identified by study of nuclear genes, which are inherited from both father and mother. The genetically encoded enzymes discussed above serve this purpose, but enzymes typically cannot be identified before a young spat stage. Thus, with present technology, definitive diagnosis of pure Kumamoto brood stock requires making crosses non-destructively and sampling progeny after one to two months of culturing for enzyme typing. Improvements in the technology are possible, however. Hedgecock's laboratory is currently working on the development of PCR methods for nuclear DNA markers that would facilitate paternal identification at the larval stage or even in biopsied adult tissue samples.

Employing currently available diagnostic methods, the initial steps for identifying pure Kumamoto brood stock would be: (1) strict selection of 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) sampling of larvae for mitochondrial DNA typing; (5) sampling of progeny at an early juvenile stage for enzyme typing; (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 are capable of steps 1 to 3, and based on these alone, step 6. Steps 4 and 5, the highly accurate diagnostic tests based on DNA and enzyme differences, could be carried out with the help of academic laboratories, such as Hedgecock's, which are capable of providing molecular diagnostic services. A combination of government and private funding is needed to carry out all steps of the screening program in a coordinated manner.

CONTROLLED CROSSES

Methods for non-destructive spawnings and pairwise controlled crosses of valuable Kumamoto brood stock have been developed by Anja Robinson at the Hatfield Marine Science Center, Oregon State University, Newport. Whenever possible, commercial breeders should also employ non-destructive methods in mass spawns.

In Yaquina Bay, OR, Kumamoto oysters have mature gametes in late summer (August-October) and retain them through February (Fig. 3). Kumamoto oysters may be artificially conditioned to spawn earlier in the year, but higher percentages of straight-hinged larvae and spat are obtained from oysters conditioned starting in April than from oysters conditioned in February or March (Robinson, 1992). Conditioned brood oysters must be spawned in individual containers to assure successful isolation of gametes and controlled, one-to-one matings. All spawning oysters should be crossed in all possible combinaions and larvae from each mating reared separately for molecular diagnosis of breed purity. Extreme care must be taken in handling eggs, sperm and larvae to avoid cross-contamination. Unfertilized eggs should be reserved as a control for inadvertant fertilization by contaminating sperm, and all screens should be immersed in hot fresh water to kill any retained eggs or larvae before re-use.

A simple means of distinguishing true Kumamoto oysters, *C. sikamea*, in commercial hatcheries is afforded by the inability of Kumamoto oyster sperm to fertilize Pacific oyster eggs. At the time of Kumamoto spawning, the hatchery operator should have on hand eggs from a Pacific oyster. Then, sperm from each putative Kumamoto male can be tested first for its ability to fertilize the Pacific oyster eggs. If fertilization occurs (*i.e.*, if the male were a Pacific instead of a Kumamoto oyster), polar bodies and first cleavage would be evident within an hour of mixing the gametes. Failure of this simple fertilization test would define the male as a Kumamoto oyster and still allow its sperm to be used in the commercial spawn. The one caveat to this simple test is that the gamete compatibility of hybrids, which have been detected in commercial stocks, is not yet known. Sperm from

pure-bred Kumamoto males may be preserved in liquid nitrogen to assure fertilization of eggs at the time of spawning since the majority of old oysters tend to be females (Lannan, 1971). Pure-bred Kumamoto brood oysters should be labeled and kept for propagation of pure commercial Kumamoto stocks.

COLLECTIONS AND BASELINE POPULATION STUDIES

Two different genetic problems have been identified with respect to commercial Kumamoto brood stocks. First, the genetic integrity of Kumamoto brood stocks may have been contaminated through mixing or hybridization with Pacific oysters. Second, the genetic diversity of at least one major commercial stock has apparently been restricted owing to effective propagation of a very small number of parents. Both of these problems can be addressed by incorporating new, pure brood stock into commercial breeding programs. Because the native Kumamoto oysters may have been lost in Japan, the only sources of new genetic material are the few isolated and presumably pure populations of Kumamoto oysters that exist along the west coast. Some oyster companies have what are believed to be pure Kumamoto stocks. Also, workshop participants agreed to search fallow oyster beds where native Japanese Kumamoto seed was originally planted, in order to save any surviving Kumamoto oysters as potential *sikamea* brood stock. Collections from these stocks should be made, tested by the available diagnostic methods and carefully propagated by controlled crosses in order to establish a pedigreed base population for the west coast industry.

SURVEYING COMMERCIAL STOCKS

Commercial breeders of Kumamoto oysters can participate immediately in a Kumamoto screening program in two ways. First, they can apply strict morphological and growth criteria to their own Kumamoto brood stocks, and they can incorporate the sperm incompatibility test as a part of their protocol for mass spawns. Progeny from mass spawns could be sampled both at the D-hinge and early spat stages for subsequent molecular diagnosis by a cooperating academic laboratory. Second, commercial breeders can send any Kumamoto brood stock not required for commercial hatchery spawns to Dr. Robinson at the Hatfield Marine Science Center for the making of controlled pedigreed crosses, the progeny from which would also be typed by molecular diagnostic methods. Parents and progeny of confirmed pure Kumamoto oysters would be returned to the commercial breeder. This screening program should begin in 1992. **There is no reason to delay and very good reasons to proceed with a screening program. Available diagnostic methods can and should be applied to ensure the persistence of the Kumamoto oyster in the west coast oyster industry.**

RESEARCH NEEDS

Although existing diagnostic methods can be used to identify pure Kumamoto brood stock for commercial hatchery propagation, new research in several areas could improve the efficiency or reliability of such diagnosis.

For example, the basic mechanisms of the gametic incompatibility between the Kumamoto and Pacific oysters should be elucidated. At issue is whether sperm from Pacific x Kumamoto oyster hybrids can fertilize Pacific oyster eggs. If hybrid sperm fertilizes Pacific oyster eggs, then the sperm incompatibility test provides commercial breeders with a very simple and effective means of ensuring the purity of male Kumamoto brood stock, which by definition fail to fertilize Pacific oyster eggs. Whether differences in egg sizes between Kumamoto and Pacific oysters allow for reliable separation by screens also needs to be evaluated for its potential as a simple diagnostic tool for commercial breeders. Biopsy methods that would provide tissue for molecular diagnostic tests without harming potentially valuable brood stock need to be perfected. Finally, the development of methods for typing diagnostic nuclear DNA markers would allow the discrimination of brood stock directly from biopsy samples, without need for progeny testing.

REFERENCES

Ahmed, M. 1975. Speciation in living oysters. Advances in Marine Biology 13:375-397.

Amemiya, I. 1928. Ecological studies of Japanese oysters, with special reference to the salinity of their habitats. J. Coll. Agric. 9(5):333-382 + 5 plates.

Banks, M. A., McGoldrick, D. J. and D. Hedgecock. 1991. Discriminating Kumamoto and Pacific oysters using molecular markers. J. Shellfish. Res. 10(2):511-512.

Banks, M. A., Waters, C., and D. Hedgecock. 1992a. Discrimination of closely related oyster species (*Crassostrea gigas* and *C. sikamea*), based on enzymatic amplification of rRNA-coding mitochondrial DNA. MS in preparation.

Banks, M. A., McGoldrick, D. J., Borgeson, W. and D. Hedgecock. 1992b. Specific status of the Kumamoto oyster (*Crassostrea sikamea*), based on gametic incompatibility and molecular genetic differences from the Pacific oyster *C. gigas*. MS in preparation.

Buroker, N. E., Hershberger, W. K. and K. K. Chew. 1979. Population genetics of the Family Ostreidae. I. Intraspecific studies of *Crassostrea gigas* and *Saccostrea commercialis*. Mar. Biol. 54:157-169.

Galtsoff, P. S. 1964. The American oyster *Crassostrea virginica* Gmelin. United States Department of the Interior Fishery Bulletin of the Fish and Wildlife Service 64:iii+480p.

Hedgecock, D. and N. B. Okazaki. 1984. Genetic diversity within and between populations of American oysters (*Crassostrea*). Malacologia 25(2):535-549.

Hedgecock, D. and F. Sly. 1990. Genetic drift and effective population sizes of hatchery-propagated stocks of the Pacific oyster *Crassostrea gigas*. Aquaculture 88:21-38.

Imai, T. and S. Sakai. 1961. Study of breeding the Japanese oyster, *Crassostrea gigas*. Tohoku J. Agr. Res. 12:125-171.

Lannan, J. E. 1971. Experimental self-fertilization of the Pacific oyster *Crassostrea gigas*, utilizing cryopreserved sperm. Genetics 68:599-601.

Numachi, R. 1978. Japanese species, breed and distribution. In: Aquaculture in Shallow Seas: Progress if Shallow Sea Culture, Imai, T. (Ed.), Part II, Chapter 1. Biological research on the oyster. Section 2.3, pp. 123-126, Balkema, Rotterdam.

Ozaki, H. and Y. Fujio. 1985. Genetic differentiation in geographical populations of the Pacific oyster (*Crassostrea gigas*) around Japan. Tohuku J. Agri. Res. 36:49-61.

Robinson, A. M. 1992. Gonadal cycle of *Crassostrea gigas* kumamoto (Thunberg) in Yaquina Bay, Oregon, and optimal conditions for broodstock conditioning and larval culture. Aquaculture, in press.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487-491.

Stephano, J. L. and M. Gould. 1988. Avoiding polyspermy in the oyster (*Crassostrea gigas*). Aquaculture 73:295-307.

Woelke, C. E. 1955. Introduction of the Kumamoto oyster *Ostrea* (*Crassostrea*) *gigas* to the Pacific coast. Washington Dept. of Fisheries, Fisheries Research Papers 1(3):41-50.

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APPENDIX II. A Brief History of the Kumamoto Oyster *Crassostrea sikamea*.

1793. Thunberg names the first oyster from Japan *Ostrea gigas*.

1861. Schrenk names *Ostrea laperousi* (=Hiroshima).

1928. Amemiya names Kumamoto as a new variety, *O. gigas* var. *sikamea*.

> "This variety is common in the muddy shallow water of Ariake Bay in Saga Prefecture. It is a dwarf or stunted form, and is devoid of commercial value owing to its small size."

1955. ICZN validates *Crassostrea* (Sacco, 1897) for cupped oysters.

1958. Numachi reports two types of oysters in Ariake-Kai. Sperm from Type A can fertilize neither Type B eggs nor the eggs of the other races; eggs from Type A can be fertilized by sperm from any other race. Type B is fully interfertile with the other races.

1961. Imai and Sakai show that the Hokkaido, Miyagi, Hiroshima, and Kumamoto (Type B?) races are interfertile.

1975. Ahmed proposes that *C. gigas* refer to Hokkaido and Miyagi oysters (after Hirase 1930), that the name *C. laperousi* Schrenk be restored for Hiroshima oysters, and that Amemiya's variety be raised to the status of full species, *C. sikamea*.

1979. Buroker, Hershberger and Chew support Ahmed's judgement about Kumamoto with electrophoretic data on enzyme differences.

1985. Ozaki and Fujio find that all oysters sampled throughout Japan are the same (*C. gigas*).

1991. Banks, McGoldrick and Hedgecock confirm Numachi (1958) and Buroker et al. (1979) and provide mtDNA differences as further support for *C. sikamea*.

Table 1. Comparison of Morphological and Reproductive Characteristics of *Crassostrea* from various regions in Japan (after Numachi, 1978).

Characteristic	Geographical Population			
	Hokkaido	Miyagi	Hiroshima	Kumamoto
Growth	Very rapid	Rapid	Slow	Very slow
Size	Maximum	Large	Small	Minimum
Depth of Shell	Shallow	Medium	Deep	Deep
Meat wt:Total wt	Minimum	Low	Maximum	High
Shell Texture	Smooth	Slightly wavy	Wrinkled	Wrinkled
Shell Color	Pale gray	Gray-violet	Dark violet	Drk violet-brown
Mass Mortality	High in south	High in south	High in north	Low
Spawning Period	Early spring	Spring	Early summer	Winter
Egg Diameter	Large (55 μm)	Large (55 μm)	?	Small (45 μm)

Table 2. Diagnostic differences in allozyme genotypes between Kumamoto and Pacific oysters. Data from Banks et al. (1991) and Buroker et al. (1979) are pooled for *C. sikamea* and *C. gigas* except for the *Mdh-2* locus.

	Genotypes for aspartate amino transferase, *Aat-1*						Total No. individuals
	CC	BC	BB	AB	AA	AC	
sikamea	101	1	0	0	0	0	102
gigas	0	12	54	51	19	6	142

	Genotypes for isocitrate dehydrogenase, *Idh-1*					Total No. individuals
	DE	DD	BC	BB	AB	
sikamea	1	107	0	0	0	108
gigas	0	0	1	110	11	122

	Genotypes for mannose phosphate isomerase, *Mpi-1*						Total No. individuals
	CD	CC	BC	BB	AB	AA	
sikamea	10	96	4	0	0	0	110
gigas	0	0	6	112	16	1	135

	Genotypes for malate dehydrogenase, *Mdh-2*			Total No. individuals
	BB	AB	AA	
Buroker et al. (1979)				
sikamea	69	9	0	78
gigas	83	0	0	83
Banks et al. (1991)				
sikamea (Coast Oyster Co.)	0	1	33	34
gigas	25	0	0	25

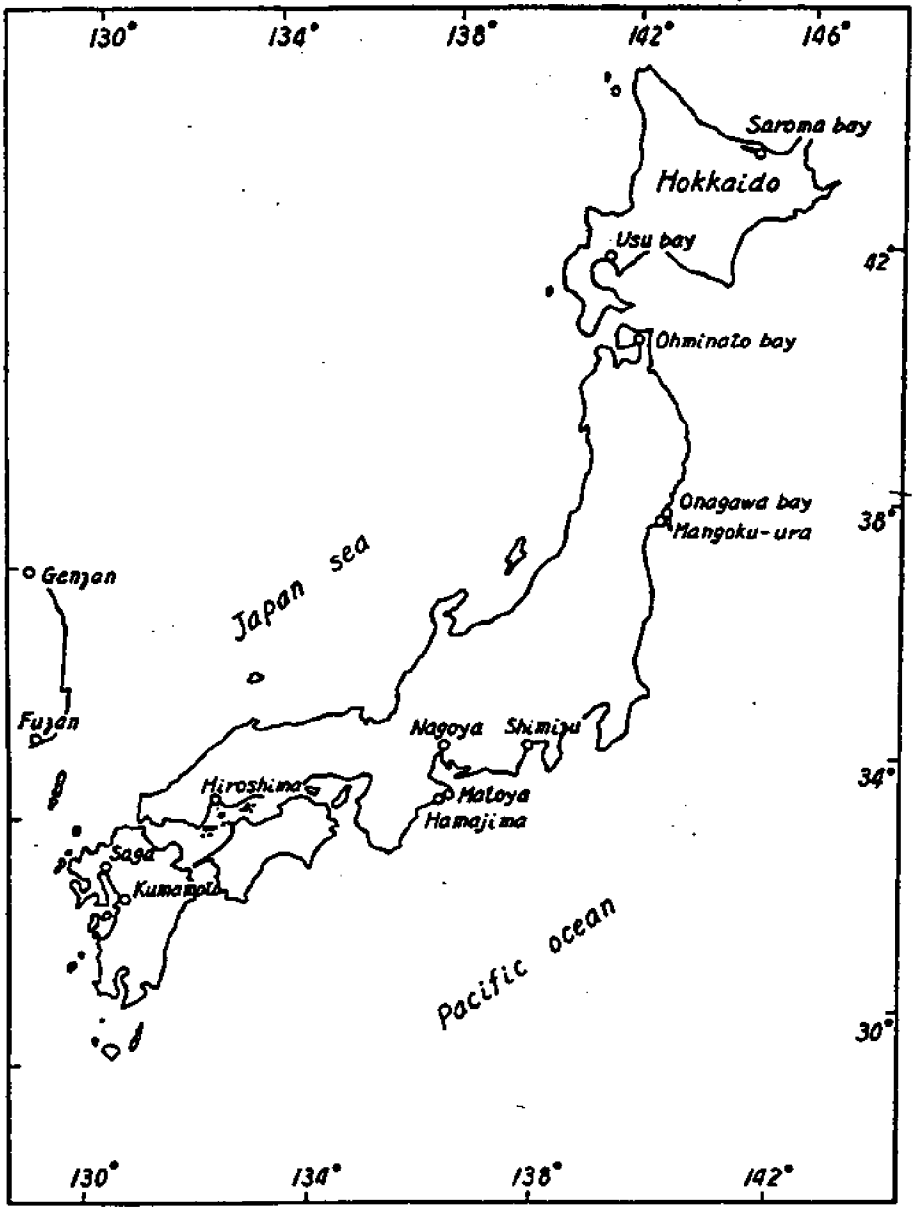
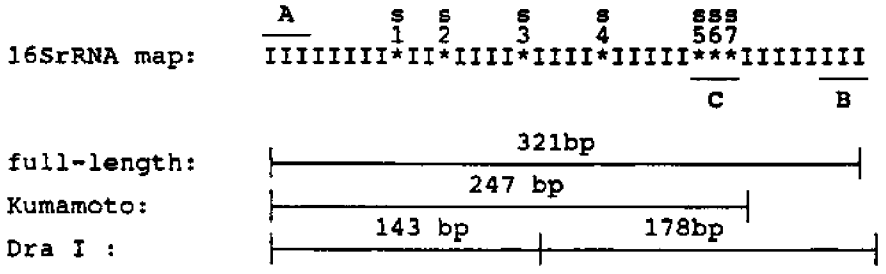


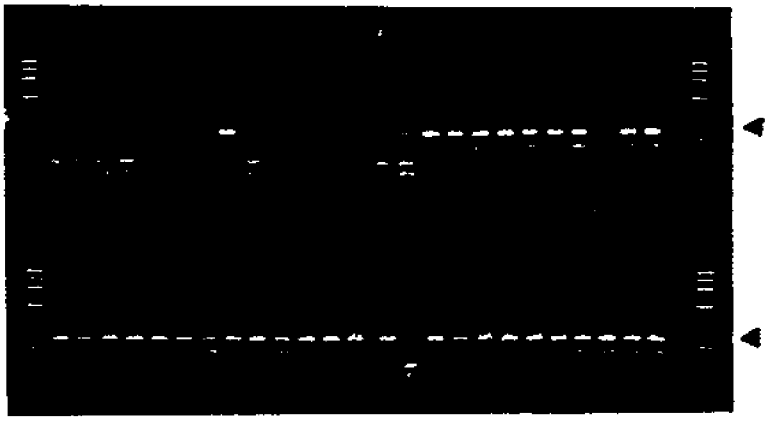
Figure 1. Map of Japan showing the localities of origin of native oysters used for breeding and the beds where oysters were cultured.

Figure 2

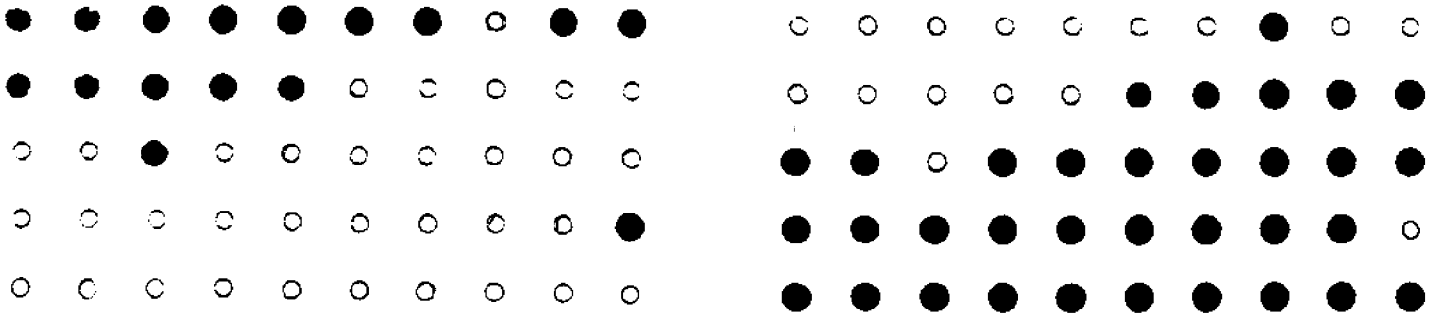
A. Map of a mitochondrial gene amplified by PCR from oysters (line 1). Bars labeled A and B represent universal PCR primers; amplification using these primers results in a 321 nucleotide base-pair product from oysters (line 2). Bar C represents the *C. sikamea*-specific primer; when all three primers are used and sample DNA is Kumamoto, both 321 bp and 247 bp fragments are produced. Asterisks labeled with "s" represent nucleotide differences between *C. gigas* and *C. sikamea*. Site 3 is cut by the Dra I restriction endonuclease, cleaving the *C. gigas* product into fragments of 143 and 178 bp (line 3).



B. Gel separation of PCR products from Kumamoto and Pacific oysters. PCR done with primers A, B, and C; products then treated with Dra I. Lanes 1 and 28 on each of the rows are DNA size standards; lane 27 in each row is a PCR negative control (no template DNA). Lanes 2 to 26 of each row are PCR products from 50 oyster DNA templates. Pacific oysters have three bands, corresponding to fragments on lines 1 and 3 in A; Kumamoto oysters have two bands, corresponding to fragments on lines 1 and 2 in A. Marker at left indicates 321 bp product.



C. Dot blot hybridizations of species-specific probes for site 1 in A. Samples same as in B; left panel has been hybridized to the Pacific oyster probe; right panel has been hybridized to the Kumamoto probe.



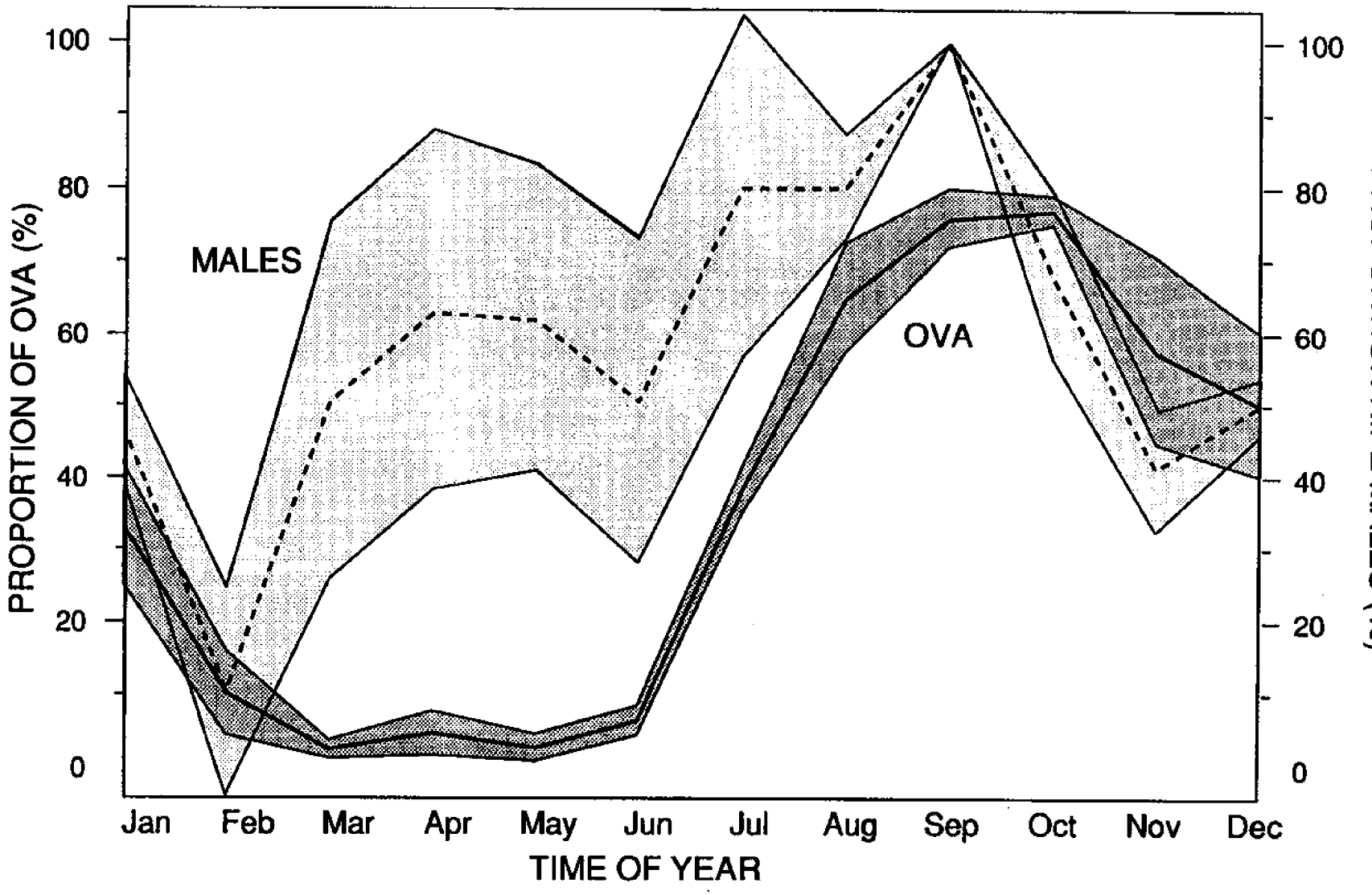


Figure 3. Percent of ova, calculated from oocytes present in monthly tissue samples, and proportion of ripe males, calculated as a percent of total number of sampled males. Shaded areas represent standard deviation from the mean.