Aquat. Living Resour. 19, 277–282 (2006)

DOI: 10.1051/alr:2006028
www.edpsciences.org/alr

Aquatic
Living
Resources

The role of genetics in shellfish restoration

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Received 20 October 2005

Abstract – Restoration of shellfish populations is becoming an increasingly common practice worldwide, as natural fisheries succumb to pressures of overharvesting, habitat loss or degradation, and challenges from invasive competitors and pathogens. Primary genetic concerns relevant to shellfish restoration projects are reviewed, using the cupped oysters *Crassostrea gigas* and *Crassostrea virginica* as case studies. Molecular genetic tools can be used to delineate the geographic distribution of germ plasm diversity at the species and intraspecific levels, enabling more informed selection of genetic material for hatchery breeding and production. Maintenance of genetic variability and prevention of excessive inbreeding in hatchery stocks may be facilitated by the use of genetic markers for regular pedigree monitoring. The effect of hatchery supplementation on the effective population size of a recipient wild population (Ryman-Laikre effect) is reviewed in light of the population biology of bivalve molluscs, and appears to be minimal. Deployment of selected enhancement stocks may be a valuable tool for improvement of degraded wild populations, reversing the negative effects of generations of selective harvesting. Molecular genetic tools can be used effectively to monitor restoration programs, when the discriminatory power of the marker(s) is high and high-throughput scoring methods currently available are used.

Key words: Effective population size / Genetic diversity / Restoration / Genetic monitoring / *Crassostrea virginica* / *Crassostrea gigas*

Résumé – Le rôle de la génétique dans la restauration des populations de mollusques. Le repeuplement est une pratique de plus en plus courante chez les mollusques dont les populations souffrent de surexploitation, de perte ou dégradation d'habitat, ou des effets néfastes d'espèces envahissantes ou de pathogènes. Les aspects fondamentaux de la génétique de la restauration des populations de coquillages sont ici passés en revue, se basant sur les huîtres creuses, *Crassostrea gigas* et *Crassostrea virginica*, comme cas d'étude. Les outils de la génétique moléculaire peuvent être utilisés pour étudier la répartition géographique de la diversité des ressources génétiques aux niveaux inter- et intra-spécifiques, permettant davantage de sélection pour les productions aquacoles issues d'écloseries. La conservation de la variabilité génétique et la protection contre la consanguinité excessive, dans les stocks issus d'écloseries, peuvent être facilitées par l'utilisation de marqueurs génétiques dans le suivi régulier des générations. L'impact des écloseries sur la taille efficace des populations sauvages (effet Ryman-Laikre) est montré à la lumière de la biologie des populations de mollusques bivalves, et semble être minime. La mise en culture de souches sélectionnées peut conduire à l'amélioration de populations sauvages dégradées, en inversant les effets négatifs de l'exploitation sélective au fil des générations. Les outils de la génétique moléculaire peuvent être utilisés pour un suivi efficace de programmes de restauration, lorsque le pouvoir discriminant de marqueur(s) est élevé et que des méthodes de discrimination à haut-débit, aujourd'hui disponibles, sont utilisées.

1 Introduction

Restoration of shellfish populations is becoming an increasingly common practice worldwide, as natural fisheries succumb to pressures of overharvesting, habitat loss or degradation, and challenges from invasive competitors and pathogens. Because the typical restoration project involves hatchery propagation of stocks for outplanting, some genetic impact on the recipient (wild) population is inevitable if the planted stocks survive and reproduce. The primary issues of concern are 1) changes in effective population size (N_e) of the wild population, and 2) changes in the genetic composition of the recipient population. These changes may range from benign to harmful, depending on the particular situation.

Populations targeted for restoration programs generally share two features: 1) census numbers that are greatly reduced from historical levels, and 2) altered life history traits as the result of selective overfishing. For example, size-selective harvesting often favors the evolution of reduced somatic growth rate and earlier reproduction, resulting in phenotypes of lower commercial value (Moav et al. 1978; Conover 2000; Stokes

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ICSR'05, 8th Int. Conf. Shellfish Restoration, Brest, France
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and Law 2000). Restoration programs offer the potential of reversing both negative trends, i.e., increasing census numbers and returning life history phenotypes to their earlier state. Initial optimism over the prospects of genetic improvement of degraded wild populations (Moav et al. 1978) has given way to a pessimistic and at times alarmist outlook (Naylor et al. 2000; Heath et al. 2003).

Three primary genetic concerns relevant to shellfish restoration projects will be reviewed here, using the cupped oysters *Crassostrea gigas* and *Crassostrea virginica* as case studies: 1) identification and use of the correct genetic material for producing hatchery lines; 2) maintenance of genetic variability in hatchery stocks; 3) maintaining N_e in the recipient (wild) population. The utility of molecular genetic tools for monitoring restoration programs will also be discussed.

2 Identification of germ plasm diversity

A first step in creating hatchery lines for restoration is the selection of germ plasm. An informed decision takes into account the nature and geographic distribution of germ plasm diversity, which will reflect contemporary (often human-mediated) gene flow superimposed on historical population structure. In the case of some shellfish species, even species-level taxonomy may be inadequate, and genetic tools may be used to clarify the number and geographical distribution of species.

The eastern oyster *C. virginica* ranges from maritime Canada to the Yucatan peninsula. While many reports describe this species as occurring in Venezuela (e.g., Nirchio et al. 2000) and Brazil (Carriker and Gaffney 1996), it appears now that these cases were in fact *Crassostrea gasar*. We found that specimens identified as *C. virginica* from Venezuela (Julio Perez, pers. comm.) had mitochondrial large subunit (16S) rRNA sequences (GenBank ##) virtually identical to those of *C. gasar* from west Africa and Brazil (Lapègue et al. 2002).

Genetic analysis of population structure in this species began with the allozyme work of Buroker (1983), who observed a high degree of genetic similarity in populations from Cape Cod to Texas, with genetically distinct peripheral populations in southern Texas and Nova Scotia (see Gaffney 1996 for review). These findings were interpreted as evidence of widespread gene flow causing genetic homogeneity throughout the majority of the species range, attributed to dispersal during the planktonic larval stage. This picture was substantially altered when Reeb and Avise (1990) showed a dramatic genetic break in mitochondrial DNA between Atlantic populations (New England to Cape Canaveral, Florida) and Gulf Coast populations (Cape Canaveral to Texas), mirroring a phylogeographic pattern seen in diverse other organisms (Avise 1992). Subsequently, Cunningham and Collins (1994) showed that the same phylogeographic split can be observed in the allozyme data, depending on the method of tree construction employed.

Evidence of further population subdivision within the Atlantic and Gulf Coast assemblages was reported by Wakefield and Gaffney (1996), who observed several common haplotypes in a mitochondrial 16S rRNA fragment detectable by denaturing gradient gel electrophoresis (DGGE). Sequence

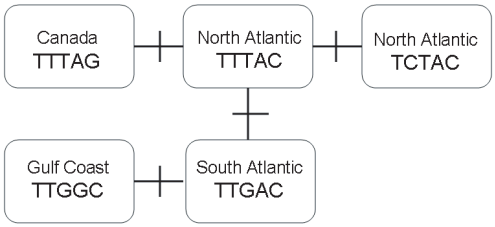

Fig. 1. Parsimony network showing common 16S mitochondrial rRNA haplotypes in *C. virginica*. The five variable nucleotides correspond to nucleotides 1942, 1948, 2096, 2104 and 2124 in GenBank Accession AY905542. All connected haplotypes are separated by a single mutational step.

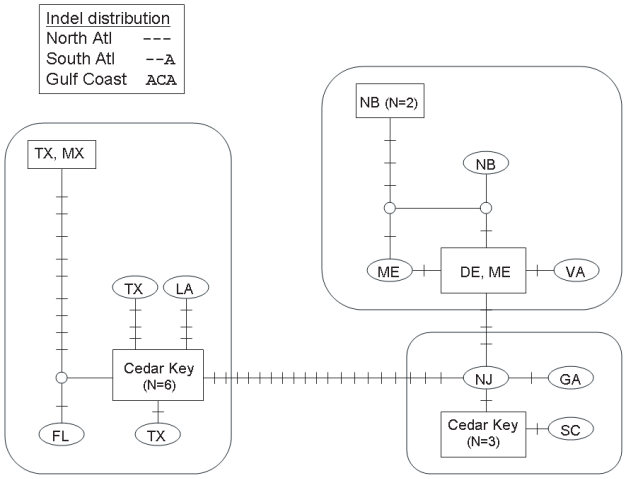

Fig. 2. Parsimony network showing haplotypes of 25 individuals for a 681 bp fragment of the *C. virginica* mitochondrial genome spanning ND2 (partial)-tRNA^{Arg}-tRNA^{His}-ND4 (partial), corresponding to nucleotides 11293 and 11973 in GenBank Accession AY905542. The three major haplogroups (Gulf Coast, North Atlantic and South Atlantic) are distinguished by an insertion/deletion polymorphism occurring in a noncoding region between tRNA^{Arg} and tRNA^{His} at nt 11410-14111 in AY905542. Abbreviations: DE = Delaware, FL = western Florida, GA = Georgia, LA = Louisiana, ME = Maine, MX = Mexico, NB = New Brunswick, NJ = New Jersey, SC = South Carolina, TX = Texas, VA = Virginia. All individuals from Cedar Key, Florida possessed unique haplotypes falling into Gulf and South Atlantic haplogroups, consistent with a hypothetical trans-Floridian exchange occurring via the Suwanee Seaway.

analysis showed a phylogeographic pattern for the haplotypes (Fig. 1), which is confirmed by the pattern of haplotype variation in a more variable mitochondrial region (Fig. 2). The primary geographic subdivisions in *C. virginica* consist of a northern Atlantic haplogroup, ranging from the mid-Atlantic northward, a southern Atlantic haplogroup occurring from the mid-Atlantic southward, and a cluster of Gulf Coast haplotypes, which shows greater sequence diversity than the Atlantic asssemblage. In addition, one western Florida population, Cedar Key, contains two unique haplotypes with different affinities (Fig. 2). This anomaly may represent an ancient vicariant event associated with the Suwanee Strait, which connected the Gulf of Mexico with the Atlantic Ocean across northern Florida, as has been hypothesized to explain

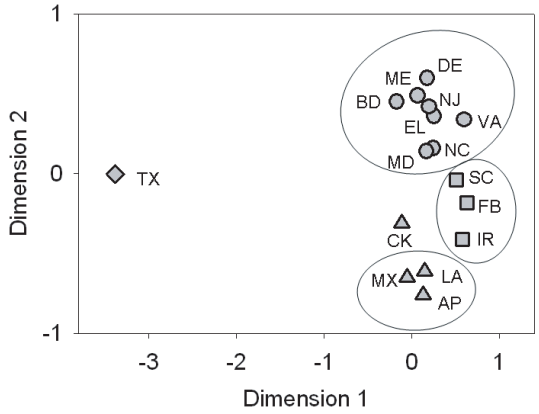

Fig. 3. Nonmetric multidimensional scaling of Nei's (1978) genetic distances among eastern oyster populations, based on PCR-RFLP analysis of four nuclear genes (Hoover and Gaffney 2005). Population clusters are consistent with mitochondrial phylogeography. Additional abbreviations: AP = Appalichicola Bay (northwest Florida), BD = Bras d'Or, Nova Scotia, CK = Cedar Key (northwest Florida), EL = Ellerslie, Prince Edward Island, FB = Fernandina Beach (northeast Florida), IR = Indian River (east central Florida), MD = Maryland, NC = North Carolina.

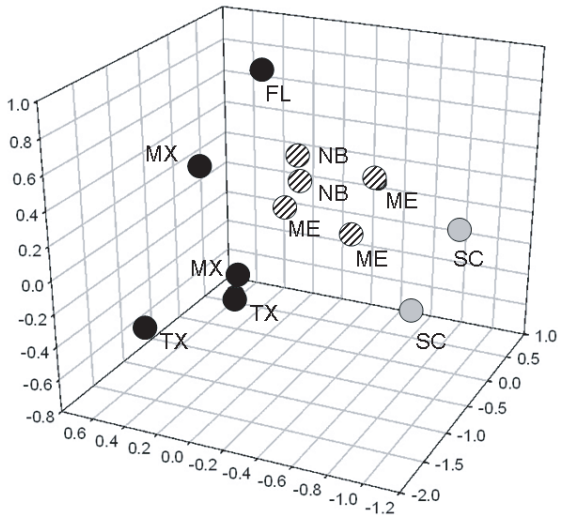

Fig. 4. Nonmetric multidimensional scaling of genotypic similarities (allele-sharing at >350 SNP loci) in *C. virginica*. Amplified fragments (200–700 bp) from nine gene loci were sequenced for 12 individuals representing the geographic distribution of the species. Individual oysters cluster into regional groups (Gulf Coast = black; South Atlantic = gray; North Atlantic = hatched).

disjunct distributions of other marine invertebrates in the region (Bert 1986; Lee and Ó Foighil 2004). A similar result is obtained by RFLP analysis of five PCR-amplified mitochondrial regions (> 5500 bp total) (data not shown). Nuclear DNA markers likewise show subdivision within and between the major assemblages, in studies employing either RFLP analysis (Hoover and Gaffney 2005; Fig. 3) or single nucleotide polymorphisms (SNPs) scored by direct sequencing (Fig. 4).

The distribution of germ plasm diversity in *C. gigas*, the most widely cultivated cupper oyster worldwide, is not well characterized. The origin and relationship of the closely related Portuguese oyster (*C. angulata*) has been clarified in recent years by molecular genetic analyses (Boudry et al. 1998, 2003; Ó Foighil et al. 1998), but less attention has been given to population structure in *C. gigas*, perhaps because cultured rather than wild populations are the primary focus for most researchers. Preliminary analyses of SNPs in *C. gigas* suggest the existence of regional differentiation that should be explored further and utilized in breeding programs (Fig. 5). One additional point should be considered in selecting broodstock for the creation or improvement of hatchery lines: phenotypic values are often poor indicators of genetic potential in breeding programs (Tanksley and McCouch 1997). Given our current rudimentary understanding of the genetic basis of performance traits in shellfish, it would be prudent to explore the use of diverse germ plasm sources, even when logistical or political considerations encourage the use of local material.

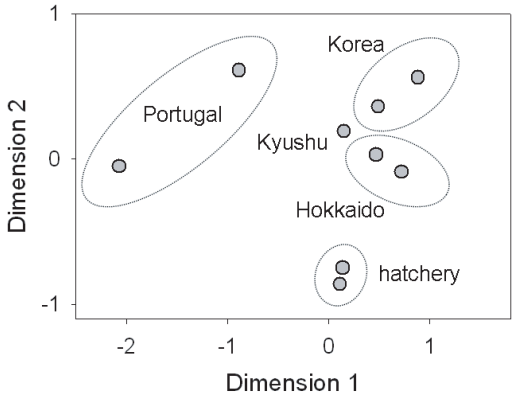

Fig. 5. Nonmetric multidimensional scaling of genotypic similarities (allele-sharing at 164 SNP loci) in *C. gigas*. Amplified fragments from ten gene loci were sequenced for 7 individuals from geographically diverse wild populations, and two individuals from an inbred hatchery line provided by D. Hedgecock. Individual oysters cluster into regional groups and are distinct from the hatchery line.

3 Maintaining genetic variability in hatchery stocks

The importance of maintaining genetic variability in hatchery stocks is well appreciated. The enormous fecundity of most shellfish species makes it possible to produce large numbers of progeny from a small number of parents, allowing the erosion of genetic diversity through random genetic drift as well as inadvertent hatchery selection. Even when adequate numbers of parents are used for mass spawning, their disproportionate gametic contributions can lead to effective population sizes well below the nominal N_e (Gaffney and Scott 1984). This problem can be addressed in a simple manner, by combining multiple small spawns at later stages, to equalize family numbers. Nevertheless, it is common for hatchery stocks of shellfish to exhibit reduced allelic diversity (Gaffney et al. 1992; Hedgecock et al. 1992) and to a lesser extent lowered heterozygosity (Allendorf 1986; Dillon and Manzi 1987). In *C. virginica*, Cordes et al. (submitted) found that five microsatellite loci in a hatchery line showed reductions in absolute numbers

of alleles of 25% to 59%, but little change in overall heterozygosity. In the *C. gigas* shown in Fig. 5, heterozygosity at 149 SNP loci for two hatchery individuals (Nei's mean unbiased gene diversity = 0.104) was lower than in wild individuals, in which mean unbiased gene diversity ranging from 0.140 in Hokkaido to 0.237 in Portugal.

For hatchery stocks used for aquaculture, some loss of allelic diversity is a necessary consequence of selective breeding, and not a cause for alarm. The level of inbreeding in the hatchery is a more serious concern, and can be addressed through proper pedigree monitoring using genetic markers. This will also enable the detection of broodstock contamination, which is difficult to avoid completely in most hatchery settings. However, there is another issue that arises when hatchery stocks are used for enhancement or restoration of wild populations: how does N_e in the hatchery line affect N_e in the recipient population?

4 Maintaining N_e in the wild population

Ryman and Laikre (1991) first called attention to the impact of supportive breeding (enhancement of wild populations with captive-reared animals) on effective population size, noting that deploying captive stocks with small N_e could severely reduce N_e in the recipient population in a simple way that depended on $N_{e(wild)}$, $N_{e(captive)}$ and the relative proportions of wild and captive animals. For example, a 50% reduction in N_e occurs when the hatchery stock makes up ≈30% of the post-enhancement population, if $N_{e(wild)}$ = 200 and $N_{e(captive)}$ = 10. This model, biologically realistic in the case of endangered salmonid populations, highlighted the potentially damaging effects of restoration programs and led to considerable hand-wringing over the use of hatchery lines for shellfish enhancement.

In contrast to salmonids, even decimated shellfish populations typically show large census numbers. For example, the Chesapeake Bay population of *C. virginica*, which has suffered a decline in abundance of two orders of magnitude over the last century, nevertheless is estimated at several billion (http://www.vims.edu/mollusc/cbope/basin.htm). This suggests that even the most ambitious restoration programs in this estuary are unlikely to make a major numerical contribution to the total population size. However, the important parameter is not census number, but N_e, and it appears that N_e is often several orders of magnitude lower than census number for organisms with enormous fecundities. This may result from high variance in progeny number, a phenomenon dubbed the Hedgecock effect (Waples 1998). As an extreme example, Hedgegock (1994) estimated the Pacific oyster population of Dabob Bay, Washington to have $N_e \leq 400$, while N was 10^8, a difference of 5-6 orders of magnitude.

If this low N_e/N ratio is applied to oyster populations in Chesapeake Bay, the predictions of the Ryman-Laikre model are noteworthy. For a population of 10^8, typical of minor basins within the Chesapeake Bay and $N_{e(wild)}$ = 500, outplanting several million seed will cause a net increase in N_e, even when N_e of the hatchery stock is very low (Fig. 6). If the actual $N_{e(wild)}$ is doubled, a net gain in N_e is still predicted, except when the $N_{e(hatchery)}$ is very small and the number of seed planted is large (Fig. 7). Thus, for the range of conditions relevant to oyster restoration programs in Chesapeake Bay, the effect of hatchery enhancement is likely to be an increase in N_e, in contrast to the scenarios envisioned for endangered salmonid populations.

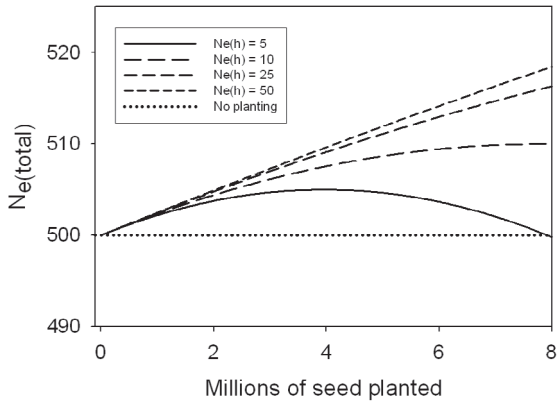

Fig. 6. Ryman-Laikre effect in a wild population with census number = 10^8 and N_e = 500, supplemented with hatchery seed, half of which survive to reproduce. Supplementation increases total N_e, even when hatchery N_e is very small.

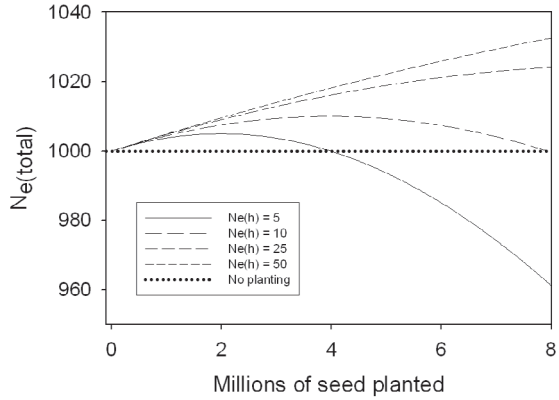

Fig. 7. Ryman-Laikre effect in a wild population with census number = 10^8 and N_e = 1000, supplemented with hatchery seed, half of which survive to reproduce. Effect of supplementation on total N_e is marginal, even for extremely small hatchery N_e.

5 Genetic improvement of degraded wild populations

Resource managers have come to recognize that fisheries practices can significantly alter the genetic composition of wild populations. In the case of *C. virginica*, more than a century of selective overharvesting (Rothschild et al. 1994) combined with the onslaught of two serious pathogens may have reduced the frequency of faster-growing, more disease-tolerant genotypes, accelerating the decline of the commercial fishery (S. Allen, pers. comm.). Following the logic of Moav et al. (1978), this situation could potentially be reversed by seeding the wild population with genetically improved seed. In particular, the outplanting of F_1 hybrids between selected hatchery strains (with enhanced growth rates and improved disease

resistance) and wild animals offers the potential of capturing hybrid vigor in the F_1 generation, as well as providing a conduit for introgressing favorable alleles into the wild population. The $_{F1}$ hybrid strategy has another practical advantage in the case of shellfish restoration: ample supplies of eggs from naturally conditioned wild females may be easily obtained, and fertilized with strip-spawned sperm from selected hatchery lines, avoiding bottlenecks in broodstock size and larval production that might occur if only hatchery lines were used to generate seed for outplanting.

6 Monitoring restoration efforts with genetic markers

A final application of genetic methods and concepts to shellfish restoration is the use of genetic techniques to evaluate the effectiveness of an enhancement program: have the outplanted animals survived and reproduced in reasonable numbers? Because shellfish restoration methods are largely still in a trial-and-error stage, rigorous evaluation of their efficacy is essential to determine which methods are most effective. Although some restoration programs in the past have simply used the total number of animals planted as a measure of success, this is clearly inadequate. The use of genetic tags to track the survival and reproduction of enhancement stocks provides a solution to this problem, when properly implemented.

The basic elements of a genetic monitoring scheme include a genetic signature that allows hatchery seed (and ideally, their offspring) to be distinguished effectively from native animals, and a technical approach that allows an adequate number of candidate specimens to be processed. The latter requirement is particularly important in cases where the census number of wild animals in the restoration area is high. A recent study employing genetic tags to track shellfish restoration efforts illustrates some of the challenges inherent in this enterprise.

In 1997, approximately four million hatchery-produced *C. virginica* (spat on shell) were planted on natural oyster bars in the Choptank River, Chesapeake Bay. Dive surveys indicated annual mortality rates of 40%, 67% and 81% over the next three years. Milbury et al. (2004) employed a mitochondrial DNA marker found only in the hatchery stock, which derived from the Gulf of Mexico, to estimate the reproductive success of the outplanted spat, by sampling newly recruited spat in 1999, 2000 and 2001. Because the mitochondrial marker is inherited maternally, the gametic contributions of hatchery oysters reproducing as males (likely to occur in 1998 and 1999, since *C. virginica* is a protandrous hermaphrodite) would not have been detected; however, all progeny of oysters reproducing as females (increasingly likely from 1999 onwards) would have possessed diagnostic mitochondrial signatures.

Given the abundance of native oysters in the Choptank River at the time of the restoration effort, estimated at 8.9×10^7 adults, Milbury et al. (2004) calculated that the planted seed should have generated approximately 1% of the 1999 year class, and about 0.1% of the 2000 year class, if their reproductive contributions were comparable to native animals. Detecting this modest level of enhancement signal with any statistical confidence requires processing large sample sizes using high-throughput DNA screening techniques. In this case, Milbury et al. (2004) used Pyrosequencing^{TM} to screen >4500 spat, of which three contained Gulf Coast mitochondrial haplotypes and were attributed to the enhancement effort. This level is severalfold lower than the null expectation of 5-15 individuals based on equal reproductive success for the outplanted spat. These results demonstrated the efficacy of high-throughput screening for detecting a faint signal, and suggested that the planting of Gulf Coast oysters in Chesapeake Bay is probably not an effective means of directly increasing census number, although some introgression of favorable alleles may have resulted from the modest contribution of the planted oysters. Recent work by Hare et al. (2006) employing both nuclear and mitochondrial DNA markers to track the success of hatchery seed with improved disease tolerance (DEBY line) planted in the Chesapeake Bay likewise noted a detectable but marginal effect of the enhancement stocking.

Genetic monitoring programs should focus on the two key elements – diagnostic signatures of planted stocks, and high-throughput assays – in order to screen large numbers of individuals effectively, with minimal frequencies of false negatives and false positives. This is particularly challenging in the case where the enhancement stock is derived from local material rather than a genetically distinct exotic population, as in the case described above. One approach currently in development involves identification of mitochondrial haplotypes and/or nuclear alleles that are rare in natural populations but have elevated frequencies in first-generation hatchery-produced seed owing to the population bottleneck inherent in hatchery propagation. Diagnostic SNPs that mark a particular batch of hatchery seed can then be assayed in large population samples using high-throughput methods. The importance of adequate sample sizes is illustrated by the work of Wilbur et al. (2005), who used mitochondrial signatures to monitor the success of a scallop restoration project in Florida. Although they were able to obtain definitive signatures of hatchery scallops by direct sequencing of a mitochondrial fragment (879 bp), the number of candidate individuals that could be assayed by the same method was limited, resulting in the inability to detect a contribution of <10% to the native population.

7 Conclusion

Several genetic considerations should play a central role in shellfish restoration projects. Broodstock selection for the creation of synthetic lines, selective breeding and introgression should be informed by an understanding of the geographic distribution of germ plasm diversity. The control of N_e and inbreeding in hatchery lines destined for use in restoration efforts can be facilitated by regular pedigree monitoring using genetic markers. Gauging the potential impact of restoration on N_e in the wild population will require reliable estimates for both the hatchery and wild populations; large population sizes and low N_e/N ratios in wild shellfish populations suggest that harmful reductions in N_e are unlikely to result from hatchery supplementation. Enhancement of degraded wild populations with genetically improved strains may help to reverse decades of negative fisheries selection, if coupled with appropriate harvesting practices. The efficacy of restoration programs can be

rigorously evaluated with the use of high-throughput molecular markers.

Acknowledgements. I thank Stan Allen, Dennis Hedgecock, Don Meritt, Kim Reece and other participants in the 2002 workshop "Genetic Considerations for Hatchery-Based Restoration of Oyster Reefs" held at the Virginia Institute of Marine Science for thoughtful and lively discussions of the genetic aspects of shellfish restoration.

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