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Assessment of genetic diversity of the European oyster (*Ostrea edulis*) in Nova Scotia using microsatellite markers

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ABSTRACT

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The European oyster (*Ostrea edulis*) was introduced to the Nova Scotia aquaculture industry 30 years ago using stocks imported from naturalized populations in Maine whose ancestors originated from the Netherlands. In past years, Nova Scotian hatcheries had successfully produced *Ostrea edulis* spat, but in 2001 and 2002 the two remaining hatcheries in the province suffered 100% larval mortality. One of the factors that may have contributed to the collapse is a suspected loss of genetic diversity due to the limited number of individuals used to establish the Maritimes stocks, and the inevitable subsequent inbreeding during propagation of these populations. This study used microsatellites, neutral molecular markers, to assess the level of genetic diversity in several hatchery stocks and naturalized populations from Nova Scotia, New Brunswick, Maine, and British Columbia. We found that some genetic erosion has occurred in the Maritimes populations, with the largest loss of alleles being found in the hatchery stocks. In spite of this loss, genetic diversity and heterozygosity in the Maritimes populations is still relatively high. The data from this study was also used to look at the relationships between the populations, and an unrooted tree illustrating these relationships was constructed using genetic distances. The results were consistent with our knowledge of the historical transfers of oysters between different locations. Preliminary recommendations for broodstock management of *Ostrea edulis* and the direction of future studies of this species are summarised at the end of the report.

oyster culture
introduced species
mortality causes
genetic diversity
spat

RÉSUMÉ

Vercaemer, B., K. Spence, E. Kenchington, A. Mallet and J. Harding. 2003. Assessment of genetic diversity of the European oyster (*Ostrea edulis*) in Nova Scotia using microsatellite markers. Can. Tech. Rep. Fish. Aquat. Sci. 2453: v + 30 p.

L'huître européenne (*Ostrea edulis*) a été introduite pour l'industrie aquacole de Nouvelle-Écosse il y a 30 ans à partir de stocks importés de populations naturelles du Maine dont les ancestres procvenaient des Pays-Bas. Les écloseries néo-écossaises ont dans le passé produit du naissain d'Ostrea edulis avec succès, mais en 2001 et 2002, les deux écloseries existantes dans la province ont connu 100% de mortalités larvaires. Un des facteurs qui ont pu contribuer à ces échecs est la suspicion d'une perte de variabilité génétique due au nombre limité d'individus utilisés pour l'établissement des stocks des Maritimes et l'augmentation de la consanguinité inévitable lors de la propagation de ces populations. Cette étude utilise des microsatellites, marqueurs moléculaires neutres, pour évaluer le niveau de diversité génétique de plusieurs stocks d'écloseries et de populations renaturalisées de Nouvelle-Écosse, du Nouveau Brunswick, du Maine et de Colombie-Britannique. Nous avons trouvé qu'il y a eu de l'érosion génétique dans les populations des Maritimes, avec la plus grande perte d'allèles dans les stocks d'écloseries. Malgrès cette érosion, la diversité génétique et l'hétérozygosité des populations des Maritimes sont encore relativement élevées. Les données de cette étude ont également permis d'établir les relations entre les populations et un arbre déraciné illustrant ces relations a été construit à partir des distances génétiques. Les résultats sont consistants avec notre connaissance des transfers historiques d'huîtres entre les différents locations. Des recommandations préliminaires pour la gestion des géniteurs d'*Ostrea edulis* ainsi que des avenues de recherche sont résumées à la fin de ce rapport.

INTRODUCTION

European oysters in Nova Scotia

Importations

European oysters (*Ostrea edulis*) were introduced 30 years ago to Nova Scotia principally from Maine populations whose ancestors originated in the Netherlands (Welsh, 1964). This origin has been confirmed by mtDNA analysis (Ellen Kenchington, pers. comm.). They were brought to Nova Scotia to develop oyster aquaculture in the cool, high salinity areas on the Atlantic coast of the province where the native American oyster (*Crassostrea virginica*) populations were marginal. Growing conditions for *Ostrea edulis* are good in the waters off the Atlantic coast of NS but spawning conditions are only marginal in most sites and summer too short to allow good spat growth for subsequent winter survival. Commercial spat supply relies on hatchery production for transfer to grow-out sites at the end of spring/beginning of summer.

Medcof brought 16,000 oysters from Conway, North Wales, UK to St. Andrews, NB and Ellersie, PEI in 1957, 1958 and 1959 but these oysters died over the next few months to years (Medcof, 1961). In the winter of 1959/60, a comparison between oysters from the 1959 Conway import and from Maine, a naturalized stock whose ancestors were originally imported from the Netherlands via Milford, CT in 1949, showed that the Netherlands strain was a more promising stock for introduction in terms of winter survival. In 1969, Drinnan imported 50 oysters from Milford to Ellersie under quarantine. Growth trials were very encouraging (Drinnan, 1970) and in 1970, the Nova Scotia Department of Fisheries (NSDF) received 5,000 oysters from Ellersie and maintained them at the Point Pleasant Station, NS but only 1500 survived after one year. From 1972 onward, seed production was achieved with limited success and inbreeding or a lack of adaptation to the cooler Atlantic water was suspected. In 1978 and 1979, several stocks from Maine were imported under quarantine and were used for extensive genetic studies and pilot seed production at Dalhousie University, Halifax, NS (funded by NSDF) and subsequently at NRC Sandy Cove Station near Sambro, NS. A limited number of broodstock were also obtained from North Wales (Newkirk *et al.*, 1995; Enright, 1995).

European oyster hatcheries in Nova Scotia

The new stock from Maine showed superior growth in the field (Newkirk, 1988) and has since been propagated in hatcheries to supply spat for the aquaculture industry. In 1988, a private hatchery based in Blandford, NS began producing seed but closed in 1994. Two other hatcheries (Port Medway and Lockport Harbour) began producing seed in 1994, and were joined the next year by another hatchery in Lunenburg. In 1999, the Lockport hatchery closed. The two remaining hatcheries (Port Medway and Lunenburg) produced between 700,000 to 1.5 million spat every year each until 2001 when larvae and spat experienced 100% mortality in the two hatcheries. Water quality, such as variation in temperature or organic / bacterial load, along with genetic erosion, were suspected.

From a genetic point of view there are now separate groups of oysters in Nova Scotia that may be differentiated to various degrees by human and environmental influences (e.g. severe winter mortalities in 1990/91 and in 1991/92 or bottlenecks in hatcheries). In addition, there are a few small naturalized populations in sheltered bays that need to be

identified and further characterized (*e.g.* Sambro, Blind Bay, Ship Harbour). The only large naturalized population occurs in New Brunswick in Lake Lockhard where ~2000 juvenile oysters from the Lunenburg hatchery and ~500 mature oysters from Pubnico, NS (originating from a grower who obtained seed from the Port Medway hatchery) were over wintered in 1996/97. This population has now been established for 2 generations. There is also a stock of European oysters on the Pacific Coast of Canada of mixed and poorly documented origins. Transfers have occurred in the last 20 years with individuals originating from California, Scotland and Maine probably via Nova Scotia.

The preservation and utilization of genetic variability in any broodstock program is a critical and complex issue. Numerous studies have shown that genetic variations can be lost at a rapid rate in hatcheries leading to inbreeding and subsequent loss of fitness (*e.g.* Beattie *et al.*, 1987; Naciri-Graven *et al.*, 2000). The effect of loss of variability is addressed in the following population genetics overview. In order to develop a coherent breeding program for the European oyster in Nova Scotia, this project evaluates the existing genetic variability of different groups of naturalized populations and hatchery stocks of oysters using five microsatellite DNA markers.

Population genetics overview

The Hardy-Weinberg equilibrium

English mathematician G.H. Hardy and German physician W. Weinberg expressed the fundamental law of population genetics in 1908. They showed that in large populations undergoing random mating, and without migration, mutation or selection, allele frequencies will remain unchanged over time (the population is in equilibrium). If, in a population, there is a gene with two alleles A and a (biallelic locus), the frequencies of allele A and allele a are equal to p and q such that p+q=1. If a population is in equilibrium, the allele frequencies will not change from generation to generation, and the frequencies of the three possible genotypes (AA, Aa and aa) will be equal to p^2, 2pq and q^2. Individuals with the genotypes AA or aa are homozygotes and individuals with the genotype Aa are heterozygotes.

If the observed frequencies in a population do not show significant differences from the expected frequencies p^2, 2pq, and q^2, then the population is said to be in Hardy-Weinberg equilibrium (HWE). The HWE principle is equally applicable to multiallelic loci, only the mathematical formula is more complicated.

The HWE principle rarely holds true in nature as the basic assumptions on which it is based are often violated. Therefore allele frequencies are constantly changing over time. HWE can, however, be used to assess the effects of factors such as mutation, migration, selection, and genetic drift on allele frequencies in a population. If a population is found to be in equilibrium, it does not necessarily mean that all of the assumptions (no selection, mutation or migration) are valid, as small deviations are hard to detect and there may also be some counterbalancing forces at work. Conversely, a significant deviation from HWE may be due to sampling errors or decrease of polymorphism, for example, through the presence of null alleles (alleles that do not amplify) at a locus.

Microsatellite markers

Microsatellites, also known as Short Tandem Repeats (STRs) or Simple Sequence Repeats (SSRs) are short, highly variable, repetitive sequences of DNA (Figure 1) found

interspersed throughout the genome. They are generally believed to be neutral genetic markers, which means they are not under natural selection and therefore are prime tools for population genetics studies (O'Reilly and Wright, 1995). Microsatellites consist of regions containing numerous repeats of 2-5 nucleotide base pairs, for example $(GT)_n$ or $(ATCT)_n$, where n represent the number of repeats present at a locus. The high level of variability in microsatellite loci, along with their ease of amplification by Polymerase Chain Reaction (PCR) and visualization on acrylamide gels, make them ideal markers for studies of genetic diversity.

Figure 1. Example of microsatellite allele length variation in 2 individuals

	*Oedu*J12		Allele Size*
Ind. A	Forward Primer ----GTGTGTGTGTGTGTGTGT---- Reverse primer	$(GT)_9$	245
	Forward Primer ----GTGTGTGTGTGTGTGTGTGTGT---- Reverse primer	$(GT)_{12}$	251
Heterozygote			
Ind. B	Forward Primer ----GTGTGTGTGTGTGTGT---- Reverse primer	$(GT)_8$	243
	Forward Primer ----GTGTGTGTGTGTGTGT---- Reverse primer	$(GT)_8$	243
Homozygote			

* Allele size is equal to the number of bases between the forward primer to the reverse primer used to amplify the fragment. Differences between allele sizes reflect difference in the number of repeats.

Measurement of genetic diversity within and between populations

Diversity

The observed number of alleles provides an estimate of the variability within a population relative to other populations. However, this measure is highly influenced by sample size, as larger sample sizes will increase the probability of detecting rare alleles. Another simple measure of within population diversity is expected heterozygosity also called gene diversity.

The comparison of expected and observed heterozygosity can give some indication of the genetic structure within a population. One of the most commonly used methods to describe this was developed by Wright (1931, 1951 and 1965). This method is known as F-statistics or fixation indices. The index F_{is} provides a measure of the reduction in heterozygosity in a non-randomly mating population relative to a panmictic population with the same allele frequencies. It is also known as the inbreeding coefficient (f), as it provides an estimate of the loss of heterozygosity that is due to mating between relatives. If a population is in HWE, $F_{is} = 0$ (mating is random). A negative value indicates an excess of heterozygosity (avoidance of mating between relatives or outbreeding) and a positive value indicates a deficit of heterozygosity (an indicator of possible inbreeding), compared with expectations under HWE.

Population structure

F_{ST} measures the effect of population subdivision, which is the reduction in heterozygosity of a subpopulation due to genetic drift. It is also called the co-ancestry coefficient. It is always positive, and varies from 0 (no subdivision) to 1 (extreme subdivision). F_{ST} reflects the level of relatedness of individuals within a population, and allows the determination of the differentiation between populations (if a pairwise comparison of populations has a high F_{ST} value, the individuals within each population are more similar than individuals between the two populations).

Genetic distance

Genetic distance is a measure of the genetic relatedness between populations. The estimate is based on the number of allelic substitutions that have occurred during the evolution of each population. Nei's standard genetic distances (Nei, 1972 and 1978) are still successfully used to characterize diverged populations (Nagamine and Higuchi, 2001). Reynold's distance, based on the co-ancestry coefficient (F_{ST}), is also used to compare populations with small differentiation (Reynold's *et al.*, 1983). Using different algorithms, distances can be processed to produce a phylogenetic tree, which clusters populations based on the genetic distances between them.

Effects of loss of variability

The effective population size (N_e) is the estimated size of an idealized population of breeders that meets all the Hardy-Weinberg assumptions and that will experience the same rate of inbreeding increase as the observed population. The N_e is directly related to the rate of loss of genetic diversity and the rate of increase in inbreeding within a population (Wright, 1969). N_e is a critical parameter for broodstock management as it determines the rate of increase in inbreeding (or loss of genetic variability in a population).

High levels of inbreeding can lead to an overall decline in fitness known as inbreeding depression (Backus *et al.*, 1995). The possible manifestations of inbreeding depression include reduced survival and growth rate, loss of reproductive performance, and increased susceptibility to epidemics due to loss of genetic diversity. As homozygosity levels increase there is a greater chance of deleterious recessive alleles being expressed, which also contributes to the overall loss of fitness.

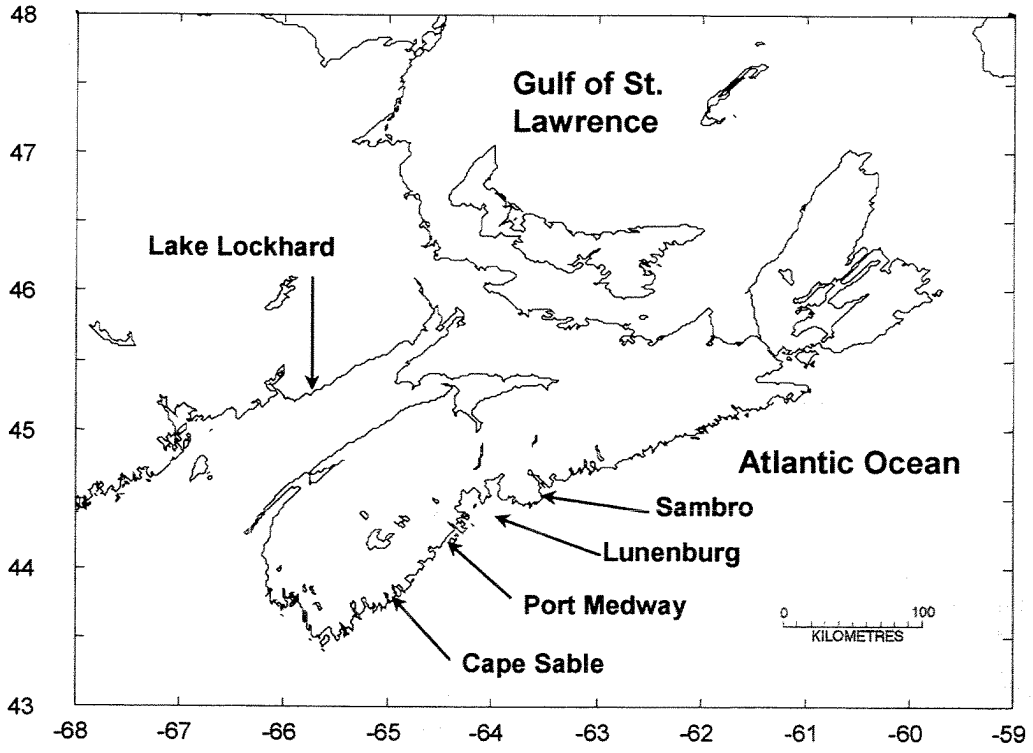
MATERIALS AND METHODS

Collection of samples

Gill or mantle tissue was collected from adult oysters (> 3 years old) using non-lethal sampling techniques (biopsy), and was preserved in 95% ethanol or by freezing at –20°C until DNA was extracted. Samples were taken from nine sites, which included Nova Scotian and British Columbian hatcheries as well as naturalized populations in Maine and New Brunswick (Table 1, Figure 2. A and B).

Figure 2. Maps showing sampling locations for *Ostrea edulis*.

A. Maritime collection sites



B. Maine collection sites

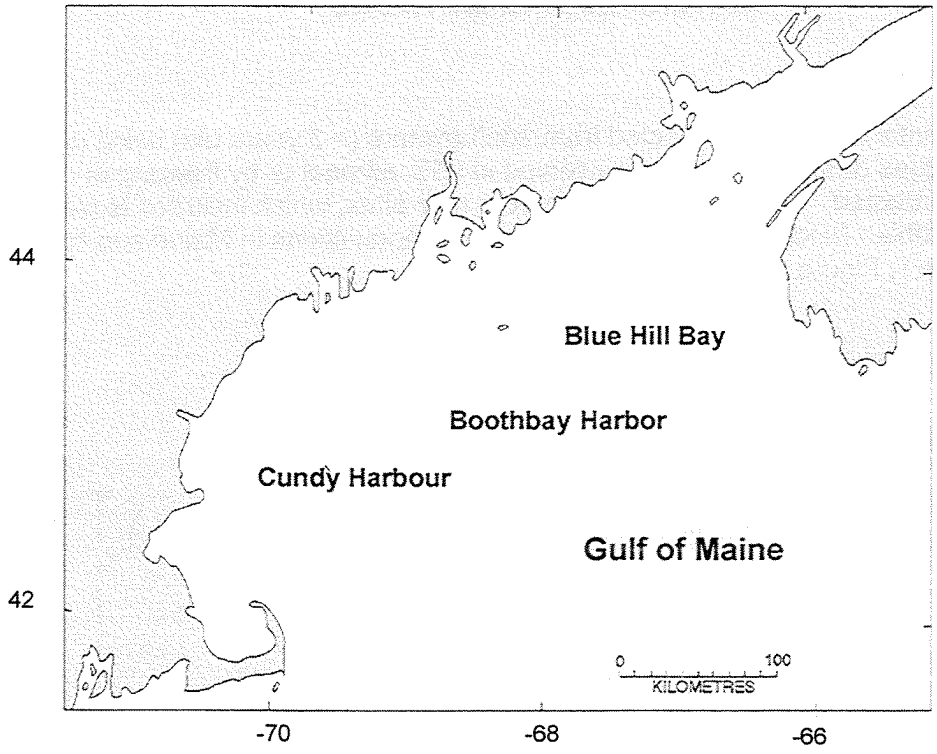


Table 1. Locations and sample sizes for *Ostrea edulis* collected for genetic analysis.

Location	Sample size
Cultured populations:	
British Columbia	
Okeover Inlet	**30**
Nova Scotia	
Lunenburg	**68**
Cape Sable	**126**
Port Medway	**39**
Naturalized populations:	
New Brunswick	
Lake Lockhard	**150**
Maine	
Cundy Harbor	101
Blue Hill Bay	66
Boothbay Harbor	90
Total Maine	**257**
Nova Scotia	
Sambro	**38**
Total number of oysters sampled	**708**

Amplification and visualization of microsatellites

To prepare the samples for DNA extraction, frozen tissue was thawed at room temperature. Both frozen and ethanol-preserved samples were rinsed in distilled water to remove residual salt from the tissue. DNA was extracted using DNeasy Tissue Kits, following the manufacturer's instructions (Qiagen cat #69506).

Polymerase Chain Reaction (PCR) was used to amplify five variable tandem repeat loci (microsatellites). These microsatellites are made up of di- and tetra-nucleotide repeat sequences and, along with the primers (oligos) used to amplify these sequences, were developed by IFREMER (Launey, 1998). However PCR conditions were optimized for use with the platforms available at the MARBL (Maritime Aquatic Resources and Biotechnology Laboratory) at the Bedford Institute of Oceanography (BIO). Primers were labeled at the 5' end with a fluorescent dye for visualization. The characteristics of the loci and the primers are listed in Table 2.

Reaction components were added to 2 μl of DNA from the extraction. The primer concentration in the reaction had to be diluted by a factor of 1/3 - 1/10 depending on the locus and the fluorescent label used (HEX, TET, or FAM) due to the sensitivity of the platform used to visualize DNA fragments. Amplification was performed using a MJ Research Dyad thermocycler (model PTC-220), using a touchdown PCR to increase the specificity of the reaction. PCR components and cycling conditions are given in Table 3.

PCR products were run on a 0.075 mm 5% acrylamide gel with an internal size standard (Megabase ET-400, Amersham-Pharmacia cat #25-0205-01) added to each lane. Electrophoresis and visualization of alleles were performed using a MJ Research BaseStation Fragment Analyzer (Figure 3). Alleles were assigned scores (size in base pairs) by comparing them to the internal size standard using Cartographer software. Allele size was standardized between the MARBL BIO Lab and the IFREMER Laboratoire Génétique et Pathologie of La Tremblade, France by genotyping 10 common samples.

Data analysis

Observed and unbiased expected heterozygosities, F-statistics, and genetic distances were calculated with the programs GENEPOP (Raymond and Rousset, 1995), GDA (Lewis and Zaykin, 2000), GenAlEx (Peakall and Smouse, 2001), and GENETIX (Belkir *et al.*, 1996). PHYLYP (Felsenstein, 1993) was used for drawing phylogenetic trees and CONTRIB (Petit, 1989) was used to calculate allelic richness corrected for sample size. Diversity was compared with that of European collections performed during Sophie Launey's thesis work at IFREMER (Launey, 1998).

Table 2. Characteristics of microsatellite loci in *Ostrea edulis*. Size range is in base pairs and T_a is the annealing temperature. Reprinted from IFREMER (Launey, 1998). Primers sequences obtained from IFREMER.

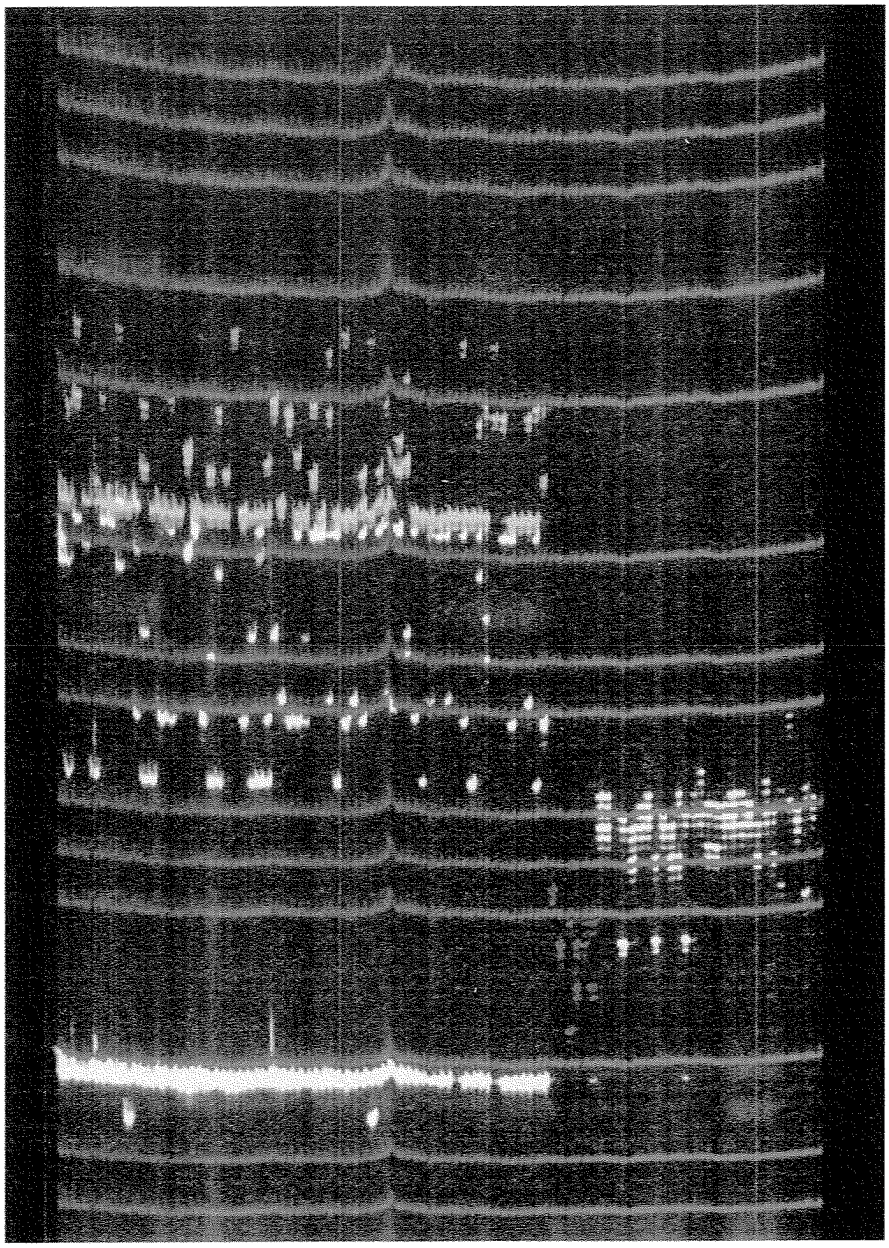
Locus	Repeat	Size range	T_a (°C)
*Oedu*T5	(CA)	106-174	56
*Oedu*H15	(ATCT)	175-227	50
*Oedu*J12	(GT)	217-265	50
*Oedu*O9	(GA)	145-183	50
*Oedu*U2	(AC)(AG)	158-214	60

Table 3. Polymerase chain reaction (PCR) conditions for the microsatellite loci. T_a is the annealing temperature.

PCR cocktail		Cycling conditions
DNA	2.00 µl	1. 94°C x 2 minutes
$MgCl_2$ (15 mM)	1.25 µl	2. 94°C x 1 minute
10x buffer	1.00 µl	3. T_a + 5°C x 1 minute
dNTP mix	1.00 µl	Decrease T_a by 1°C/cycle
Primer F (10 µM)	0.50 µl	4. 72°C x 1 minute
Primer R (10 µM)	0.50 µl	Return to step 2 x 4 cycles
ddH_2O	3.75 µl	5. 94°C x 1 minute
Taq Polymerase	0.08 µl	6. T_a x 1 minute
		7. 72°C x 1 minute
		Return to step 5 x 25 cycles
		8. 72°C x 10 minutes

Figure 3. Screen image from the MJ BaseStation Fragment Analyzer used to genotype the oyster samples.

Four microsatellite loci in three colour channels are represented. The samples on the left are oysters from Cape Sable at *Oedu*H15 (a tetranucleotide locus labeled in HEX - yellow) and *Oedu*J12 (a dinucleotide locus labeled in TET- green). The remaining samples are oysters from various locations at loci *Oedu*T5 (a dinucleotide labeled in FAM - blue) and *Oedu*O9 (a dinucleotide labeled in HEX - yellow). The red lines across the image form a ROX-labeled internal size standard used to measure the size of each allele in base-pairs.



RESULTS AND DISCUSSION

Allelic variability

The five microsatellite loci used in this study were all found to be highly polymorphic. Allele frequencies per locus and per population are given in Appendix A.

Allelic richness is highly dependent on effective population size (Nei *et al.*, 1975) and should be a good indicator of past demographic changes (Petit *et al.*, 1998). The number of alleles found at each locus ranged from 14 (*Oedu*H15) to 29 (*Oedu*U2), with a mean of 22.8.

Table 4. Number of samples genotyped and the number of observed alleles for the combined Canadian and Maine populations. Data for the Northern European populations of *Ostrea edulis* was obtained from Launey, 1998.

	Canadian populations		**Maine populations**		**North European populations (Launey, 1998)**	
	Number of samples	Observed nb. of alleles	Number of samples	Observed nb. of alleles	Number of samples	Observed nb. of alleles
Locus U2	356	26	139	28	254	33
Locus T5	427	21	239	27	254	26
Locus H15	403	14	236	14	254	17
Locus O9	426	12	246	17	254	21
Locus J12	425	23	251	22	254	27
Average		**19.2**		**21.6**		**24.8**

Table 4 compares the observed number of alleles in the Canadian, Maine, and Northern European populations. The observed number of alleles was lower in the Maine populations compared to the Northern European populations (data from Launey, 1998) at four of the five loci used in this study (Table 5). The loss ranged from three alleles (*Oedu*H15) to five alleles (*Oedu*U2). The Canadian populations showed an even greater loss of allelic diversity, ranging from three alleles (*Oedu*H15) to nine alleles (*Oedu*O9), when compared to the Northern European populations. This was true despite the fact that the sample size for the Canadian population was much larger than both the Maine and European populations.

When the Canadian population is compared to the Maine population, there is a loss of alleles at 3 loci: 2 alleles at *Oedu*U2, 6 at *Oedu*T5, and 5 at *Oedu*O9. There was no loss of alleles at *Oedu*H15, and a "gain" of an allele at *Oedu*J12. This "gain" is likely due to the lower number of samples from Maine.

The number of alleles found per locus for each stock/population is given in Table 5. Although variation in sample size makes it difficult to compare these numbers directly, it is apparent from this data that the actual number of alleles at each locus is still relatively high in the Nova Scotia hatchery stocks. However, the naturalized populations in Maine and in the Maritimes (Sambro and Lake Lockhard) both show higher numbers of alleles found at each locus. The low number of alleles found at *Oedu*U2 for Sambro is likely due to the large number of missing data: only 17 of 38 individuals from the Sambro collection amplified at this locus.

Table 5. Sample size (n) and total number of alleles per locus for each population. NS hatchery, Maritimes naturalized, and Maine naturalized combined for comparison.

Population	n	Number of alleles					
		*Oedu*U2	*Oedu*T5	*Oedu*H15	*Oedu*O9	*Oedu*J12	Average
PAC	30	16	12	8	9	11	11.2
LUN	68	14	12	9	9	13	11.4
MED	39	15	13	8	9	9	10.8
CSA	126	18	17	7	10	16	13.6
Total NS hatcheries	**233**	**18**	**17**	**9**	**11**	**20**	**13.6**
SAM	38	12	16	9	11	14	12.4
LLO	150	24	18	11	11	18	15.2
Total Maritimes naturalized	**188**	**25**	**19**	**12**	**12**	**20**	**17.6**
CUH	101	22	23	14	16	19	18.8
BOH	90	22	25	11	12	21	18.2
BHB	66	21	16	9	12	15	14.6
Total Maine naturalized	**257**	**28**	**27**	**14**	**17**	**22**	**21.6**

PAC: Pacific Coast, LUN: Lunenburg, MED: Port Medway, CAS: Cape Sable, SAM: Sambro, LLO: Lake Lockhard, CUH: Cundy Harbor, BOH: Boothbay Harbor, BHB: Blue Hill Bay.

At each locus, some genetic erosion can be seen with smaller number of alleles observed in the Canadian collections even though sample numbers are higher. This is particularly true for locus *Oedu*O9. Nonetheless, all 5 loci still reveal fairly high levels of allelic richness. It would appear that, as of 2002, there **is still a reasonable level of genetic diversity in the Canadian collections**, despite the fact that these populations have been isolated from both their ancestral European and Maine populations, and have been propagated in hatcheries for several generations. As a comparison, in *Ostrea edulis* French populations selected for *Bonamia* resistance, the mean number of alleles was reduced from 19.8 (natural population) to 5.4 to 12.8 (selected populations) in two generations (Launey *et al.*, 2001). The Canadian populations have lost fewer alleles in 6 to 7 generations since their introduction from Maine, indicating that genetic erosion is not occurring as rapidly as it could have under hatchery conditions.

Genetic erosion is a common concern for hatchery stocks. An estimate of the number of breeders contributing to *Crassostrea virginica* MSX resistant lines produced in hatchery varied from 4 to 16 (*in* Hedgecock *et al.*, 1992). Hatchery-propagated Pacific oysters have also lost alleles in three generations and one hatchery stock showed a per-generation effective population size of 9 oysters (Hedgecock and Sly, 1990). Boudry *et al.* (2002) showed that effective population size is strongly reduced in *Crassostrea gigas* because of unbalanced parental contribution. For *Ostrea edulis*, Launey *et al.* (2001) estimated that the effective size of oyster populations selected for *Bonamia* resistance was very low, between 3 and 20 at the most. In the present study, effective population size N_e

was not determined but is suspected to be higher than the populations studied by Launey et *al.* (2001). Alvarez *et al.* (1989) and Saavedra and Guerra (1996) previously reported the number of effective *Ostrea edulis* broodstock to be 6-10 and 3-4 from mass spawning tanks containing 60 and 120 animals respectively. This evidence shows that *Ostrea edulis* is a species susceptible to founder and bottleneck effects in the hatchery environment and that genetic erosion has to be carefully monitored.

Distribution of allelic frequencies

The allelic profiles for each locus are given in Appendix B. All 9 populations have been either plotted together or split into 3 groups: "Total hatchery", "Total naturalized" (Maritime populations) and "Total Maine". All loci showed multimodal distributions as they do in Europe (Launey, 1998). Only the distribution for *Oedu*O9 was close to a normal distribution in a European sample of 507 individuals (Launey, 1998). There are no major discontinuities in the distributions, and increments in allele size correspond to the repeat length for a given locus (dinucleotide for all loci except *Oedu*H15, which is a tetranucleotide locus). The three groups showed differences in the abundance of rare alleles (alleles with frequency < 10%). A high level of rare alleles (87% average over 5 loci) characterizes the Maine population, while the Maritime naturalized populations and hatchery stocks show lower levels of 73% and 69% respectively. The hatchery populations lost 5, 2, 7, 8 and 2 rare alleles at loci *Oedu*U2, H15, T5, O9 and J12 respectively compared to the naturalized populations. These results are usually found when a population experiences a bottleneck event but may also reflect genetic erosion that occurred after an introduction.

Allelic richness corrected for sample size

The large range in sample sizes (30 to 150) makes direct comparisons of allelic richness between populations difficult, as larger samples will have a greater chance of including rare alleles. The program CONTRIB was used to correct for differences in sample size by using a rarefaction number (equal to the lowest number of individuals from a population that amplified at a given locus). Table 6 illustrates the amount of genetic diversity present in each of the sampled populations by showing the actual and corrected numbers of alleles at each locus. The previous trend of hatchery populations showing less allelic diversity than naturalized populations is also seen using this analysis. The NS hatchery stocks have a mean corrected number of alleles ranging from 8.21 (Port Medway) to 9.04 (Lunenburg). The British Columbia hatchery stock is next at 9.72, with the Maritime naturalized populations scoring 10.85 (Sambro) and 11.15 (Lake Lockhard) alleles. The Maine populations range from 10.76 (Blue Hill Bay) to 13.56 (Boothbay Harbor). The large difference between actual and corrected number of alleles seen for most populations at locus *Oedu*U2 is likely due to the fact that allele number was corrected to a smaller sample size because only 17 individuals amplified in the Sambro population at this locus.

Table 6. The actual and corrected (corr.) number of alleles at each locus for all populations. Mean number of alleles across loci (Am) and mean corrected number of alleles (Am corr.) across loci.

Pop	U2	U2 corr.	T5	T5 corr.	H15	H15 corr.	O9	O9 corr.	J12	J12 corr.	Am	Am corr.
PAC	16	12.60	12	11.0	8	7.00	9	8.00	11	10.00	**11.2**	**9.72**
LUN	14	10.16	12	10.44	9	6.86	9	7.16	13	10.59	**11.4**	**9.04**
MED	15	10.07	13	10.60	8	6.89	9	7.26	9	7.02	**10.8**	**8.21**
CSA	18	11.28	17	10.97	7	5.87	10	7.37	16	7.92	**13.2**	**8.56**
SAM	12	11.00	16	13.99	9	7.82	11	9.74	14	11.70	**12.4**	**10.85**
LLO	24	14.04	18	13.73	11	8.24	11	8.05	18	11.70	**16.4**	**11.15**
CUH	22	14.75	23	15.61	14	10.88	16	11.10	19	14.02	**18.8**	**13.27**
BOH	22	14.95	25	17.82	11	9.14	12	9.45	21	16.44	**18.2**	**13.56**
BHB	21	12.95	16	12.99	9	7.71	12	8.98	15	11.16	**14.6**	**10.76**
MNE	*28*	*14.91*	*27*	*16.46*	*14*	*10.21*	*17*	*10.29*	*22*	*15.40*	***21.6***	***13.45***

PAC: Pacific Coast, LUN: Lunenburg, MED: Port Medway, CSA: Cape Sable, SAM: Sambro, LLO: Lake Lockhard, CUH: Cundy Harbor, BOH: Boothbay Harbor, BHB: Blue Hill Bay, MNE: Maine (all 3 locations from Maine combined).

Table 7 shows the corrected number of alleles and mean number of alleles for the combined Nova Scotian hatchery oysters (Lunenburg, Port Medway and Cape Sable) compared to the Maritime naturalized populations (Sambro and Lake Lockhard) and the Maine naturalized populations (Cundy Harbor, Boothbay Harbor and Blue Hill Bay). The hatchery stocks show the least number of alleles at all loci, with a mean of 13.32 alleles for all loci. The Maritime naturalized populations show a mean of 16.6 alleles and the Maine populations a mean of 20.05 alleles.

Table 7. Mean number of alleles across all loci (corrected for differences in sample size) for the hatchery populations, the naturalized Maritime populations, and the naturalized Maine populations.

	Corrected number of alleles per locus					Mean number of alleles per population
	*Oedu*U2	*Oedu*T5	*Oedu*H15	*Oedu*O9	*Oedu*J12	
BC hatchery (PAC)	12.60	11.00	7.00	8.00	10.00	9.72
NS hatcheries (MED, LUN, CSA)	16.26	15.42	7.95	9.75	17.2	13.32
NS naturalized (LLO, SAM)	24.00	18.00	11.00	11.00	19.00	16.60
Maine naturalized (CUH, BOH, BHB)	26.83	25.50	12.67	15.34	19.91	20.05

Allelic richness corrected for unequal sample size clearly indicates that the Lake Lockhard population is the most diverse in the Canadian collections. This is probably due to the fact that it is a relatively large naturalized population. The 5 remaining Canadian populations appear similar, with possibly a slightly higher diversity in the Pacific Coast population. It would thus appear that **there is some degree of on-going genetic erosion in the artificially propagated populations.**

Heterozygosity

Values for expected heterozygosity (H_e), based on allele frequencies under Hardy-Weinburg equilibirum, range from 0.753 (*Oedu*J12) to 0.938 (*Oedu*U2) for all populations combined (Table 8). The observed number of heterozygotes is less than expected at four of the five loci. For three of the loci (*Oedu*U2, *Oedu*T5, and *Oedu*O9) the observed heterozygosity is high and the loci show no deviation from HWE. The remaining two loci (*OeduH15*, *Oedu*J12) show significant heterozygote deficits. Tests for linkage disequilibrium were done and show the existence of linkage disequilibrium for some of the NS hatchery stocks at those two loci.

Table 8. Statistics for microsatellite loci: number of samples per locus (n), number of alleles per locus (A), expected heterozygosity H_e (Nei, 1978), observed heterozygosity H_o, and Hardy-Weinberg Equilibrium deviations estimated by F_{is} or fixation index (f).

Locus	n	A	H_e	H_o	F_{is}
*Oedu*U2	503	29	0.938	0.867	0.076
*Oedu*T5	670	28	0.914	0.889	0.027
*Oedu*H15	645	14	0.849	0.460	0.458**
*Oedu*O9	681	19	0.843	0.847	-0.005
*Oedu*J12	687	24	0.753	0.562	0.254**

** P<0.01

Table 9. Statistics per population/stock: number of samples per locus (n), non biased expected heterozygosity H_e (Nei, 1978), observed heterozygosity H_o, and Hardy-Weinberg Equilibrium deviations estimated by F_{is} or fixation index (f).

Population	n	He	Ho	F_{is}					
				All loci	U2	T5	H15	O9	J12
British Columbia	30	0.860	0.827	0.040	0.134	-0.012	0.044	-0.057	0.079**
Lunenburg	68	0.810	0.670	0.175***	0.003	0.007	0.459***	0.130*	0.335**
Port Medway	39	0.786	0.736	0.064	0.002	-0.094	0.199	-0.166	0.454***
Lake Lockhard	148	0.821	0.707	0.139***	0.076	-0.024	0.547***	-0.060	0.190***
Sambro	38	0.855	0.706	0.176***	-0.043	0.048**	0.478***	0.120	0.317***
Cape Sable	118	0.795	0.695	0.123***	0.071*	0.022**	0.382***	-0.083*	0.290**
Cundy Harbour	100	0.876	0.720	0.179***	0.051	0.056	0.585***	0.049	0.159**
Boothbay Harbour	90	0.889	0.793	0.110***	-0.031	0.017	0.447***	-0.019	0.155***
Blue Hill Bay	65	0.854	0.753	0.119***	0.081	-0.007	0.420***	-0.079*	0.202**
Combined Maine	*257*	*0.884*	*0.753*	*0.149****	*0.055*	*0.029**	*0.501****	*-0.007*	*0.178****

*P<0.05, ** P<0.01, *** P<0.001

When looking at the hatchery stocks (Table 9), levels of heterozygosity were not as dramatically reduced as allelic richness was. The average observed heterozygosity (H_o) for each population ranged from 0.670 (Lunenburg) to 0.827 (British Columbia). It does not seem that there is an association between the observed heterozygosity and the origin of the samples (hatchery *versus* naturalized). This result is similar to earlier studies on *Crassostrea gigas* where the number of alleles was significantly reduced but heterozygosity was retained in hatchery stocks compared with naturalized populations (Hedgecock and Sly, 1990).

The H_o was lower than the expected heterozygosity (H_e) for all populations, but this is typical in bivalves (Hedgecock *et al.*, 1992, Hedgecock and Okasaki, 1994). The difference between H_e and H_o was smallest in the British Columbia and Port Medway samples. This may be a result of a breeding program for certain traits that has led to the incidental selection of heterozygotes, or possibly an artifact of the sampling procedure, as the sample sizes for these populations were both relatively small.

The values calculated for F_{is} or f (an estimate of the level of inbreeding if the heterozygote deficiency is entirely due to mating among relatives) are shown in Table 8 and Table 9. The values for the five loci, all populations combined, range from -0.005 (*Oedu*O9) to +0.458 (*Oedu*H15), which represent a large range of values. The estimated F_{is} values vary much less among samples (from +0.040 to +0.179) than among loci. This result indicates that inbreeding is not the sole explanation for the heterozygote deficiencies.

All populations show positive F_{is} values revealing deficits in heterozygosity, particularly for loci *Oedu*H15 and *Oedu*J12 (Table 9). This result is commonly observed in bivalves and the same pattern was observed in the European populations (Zouros and Foltz, 1984; Launey, 1998). This is likely due to the presence of null alleles (alleles that are not amplifying, possibly due to a mutation in the primer site) which has been hypothesized for *Oedu*H15 by Launey (1998).

Ostrea edulis natural populations have high fecundity and potentially large dispersal of gametes and larvae, so should follow the HWE assumptions for panmixia. However, in the context of the hatchery, this deficit in heterozygosity may possibly reflect blind selection effects, along with small reproductive population size and inbreeding (Hedgecock, 1994).

Genetic differentiation between European oyster populations

The genetic differentiation between populations is revealed by the pairwise F_{ST} values, which are displayed in Table 10. Most of the F_{ST} values under 0.027 are not significant and indicate negligible genetic differentiation. The F_{ST} values are used to calculate the genetic distance between populations, which can be illustrated graphically. For this study, an unrooted tree was constructed using Nei's standard distances (Nei, 1978) and the co-ancestry distances (Reynold *et al.*, 1983). Both distances display the same phylogenic tree (Figure 4). The Maine populations cluster together with the Pacific Coast population and are quite divergent from the Port Medway, Lunenburg, Lake Lockhard and Cape Sable populations, which cluster together. The intermediate position of the Sambro oysters reflects the fact that they are a re-naturalized population consisting of Maine oysters that were maintained at Dalhousie University in Halifax for several years before being released. The results also reflect the founding events that led to the establishment of the

Pacific Coast population through the transfer of oysters from California, Scotland and Maine, probably via Nova Scotia, over the last 20 years.

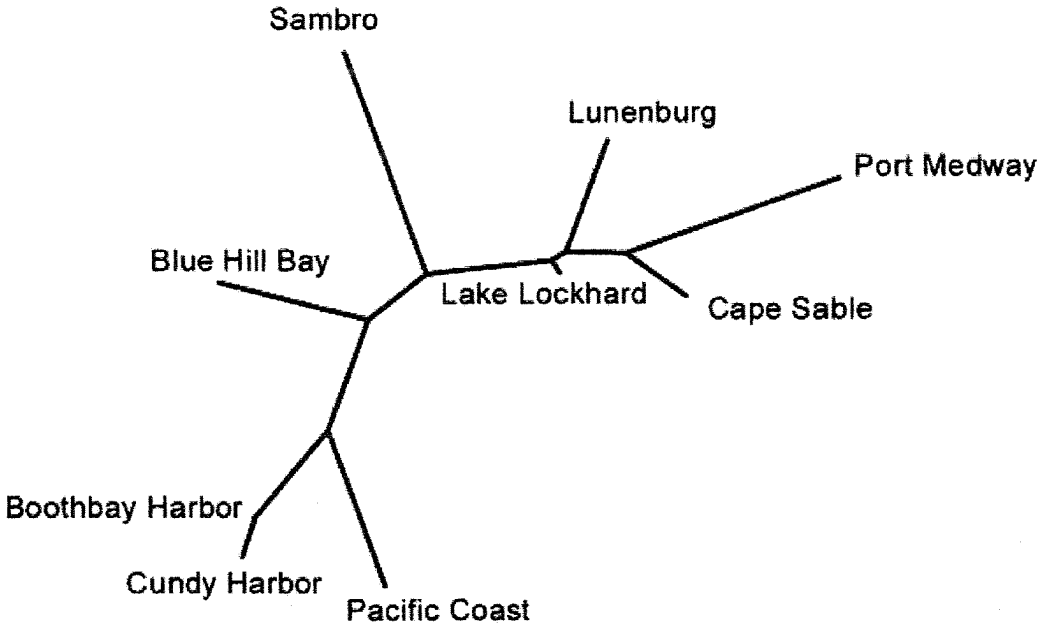
The close clustering of the Nova Scotian populations is explained by **their recent common ancestry and the exchanges of individuals** that have taken place between these populations. For example, the fact that the Lake Lockhard and Lunenburg populations cluster closely together reflects the fact that the Lake Lockhard population was established using oysters mainly from Lunenburg stocks.

Table 10. F_{ST} values for population pairs according to Weir and Cockerham (1984).

Population (F_{ST})	PAC	LUN	MED	LLO	SAM	CUH	BOH	BHB
LUN	0.039							
MED	0.049	$0.027^{n.s.}$						
LLO	0.033	$0.011^{n.s.}$	$0.022^{n.s.}$					
SAM	0.044	0.033	0.054	0.026				
CUH	0.025	0.046	0.054	0.036	0.039			
BOH	$0.019^{n.s.}$	0.043	0.051	0.035	0.037	$0.003^{n.s.}$		
BHB	0.038	0.037	0.040	0.026	0.032	0.024	$0.019^{n.s.}$	
CSA	0.048	0.019	$0.021^{n.s.}$	0.011	0.031	0.046	0.045	0.039

n.s.: P>0.05

Figure 4. Unrooted Neighbor-Joining tree (Saitou and Nei, 1987) obtained from co-ancestry genetic distances.



CONCLUSION AND FUTURE DIRECTIONS

This study provides the first analyses of the genetic structure of *Ostrea edulis* populations/stocks in Nova Scotia. It is based on variation at microsatellite loci, and demonstrates their utility for discerning population structure within and between European oyster stocks across the continent. These markers are also important as a tool to create pedigrees and breeding programs to limit inbreeding and maintain high levels of genetic diversity.

The results of this project show that there is still a relatively high level of genetic diversity in the Canadian populations, but evidence of genetic erosion can be seen in the hatchery-propagated stocks. Some level of inbreeding occurs in hatchery populations of *O. edulis* around the world, and there is evidence to suggest that excessive inbreeding can result in a loss of fitness. For instance, Mallet and Haley (1983) and Naciri-Graven *et al.* (2000) have observed that growth performance of the offspring is negatively correlated with relatedness of their parents. Monitoring the level of inbreeding should be done using those loci showing the lowest frequencies of null alleles.

Increasing the number of loci available for use in *Ostrea edulis* would be useful for continuing and future studies of genetic diversity and population structure in this species. Increasing sample size will also improve the researcher's ability to detect population structure more effectively.

Hatchery production for aquaculture allows the development of genetically improved strains but, simultaneously, the loss of genetic diversity and inbreeding depression can have adverse effects. To manage inbreeding, it is important to utilize pedigree information when producing the next generation. Due to the reduced number of effective broodstock in mass spawning, where only a limited number of individuals contribute most of the offspring to the next generation, it may be wise to increase genetic variability in hatchery populations by introducing individuals from naturalized populations at regular intervals. Another recommended strategy for shellfish hatcheries (Gaffney *et al.*, 1992), already used in the two Nova Scotia European oyster hatcheries, is to pool offspring from multiple spawning groups (*i.e.* multiple lots).

More specifically, we have the following recommendations for broodstock genetic management, both from a R&D perspective and from a practical perspective:

1. There are some weaknesses in our understanding of the reproduction of *O. edulis*. Sex determination, spawning induction, parental contribution and fecundity need to be addressed in future studies.

2. Genetic and environment interactions (GxE) need to be addressed, as this is critical in the development of an effective breeding program. GxE interactions refer to the fact that different genotypes may perform differently in distinct environmental conditions. If GxE interactions are small, the same superior genotypes will perform better in all geographic areas. If, on the other hand, GxE interactions are important, then growers would have to select the stocks most appropriate to their grow-out sites' environnement.

3. The number of *O. edulis* broodstock used to produce larvae in hatcheries should be maximized (at least 50 per lot, preferably 100) in order to maintain genetic diversity and prevent further erosion of genetic diversity in NS hatchery stocks. In addition, the impact of grading of larvae should be evaluated. This is similar to selecting for fast growing larvae in the hatchery who are not necessarily fast growing adults in grow-out sites. Discarding small larvae may not only genetically impoverish the next genration of breeders but may also hinder selection for growth.

4. Outbreeding of *O. edulis* from NS should be carried out within Maritime populations as opposed to more distant populations to avoid the introgression of non-adapted strains into the actual stocks, as well as to reduce the possibility of disease transfer. In particular, history shows that previous transfers survived in Nova Scotia's marginal winter conditions only after a 30-year adaptation to Maine's cold environment. The results of this study can be used in future studies to determine possible outcrosses between most distantly related Maritime populations.

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APPENDIX A

Allele frequency tables for all populations of *Ostrea edulis*

***Oedu*U2**

	PAC	LUN	MED	CSA	LLO	SAM	MNE
150							0.36
154					0.43		1.79
156	1.67				0.43		1.43
158					1.29		2.14
160	3.33	11.76	1.39	2.58	9.48	2.94	6.79
162	15.00	18.38	2.78	16.49	7.33	5.88	5.00
164	1.67	0.74	1.39	0.52	5.17	11.76	5.71
166	8.33		1.39	1.55	0.86		17.14
168	3.33	7.35	1.39	7.22	3.88	8.82	2.86
170		10.29	6.94	1.55	12.07	2.94	1.43
172	8.33	8.09	26.39	8.76	6.47	5.88	8.21
174				0.52	0.86		2.86
176	13.33					2.94	2.14
178		2.94	5.56	10.31	7.33	23.53	3.57
180		1.47		8.76	8.19		11.07
182	5.00	3.68	15.28	4.12	1.29	11.76	3.21
184	13.33	13.97	2.78	12.89	11.21	11.76	6.07
186	5.00				3.02		1.79
188	10.00	3.68	5.56	2.58	1.72		2.14
190				0.52	2.59		1.43
192	1.67	9.56	6.94	7.22	7.33	8.82	6.43
194	3.33						1.43
196	3.33				2.16		0.36
198		6.62	19.44	10.31	2.16		2.14
200		1.47	1.39	2.58	2.16		0.71
202	3.33				1.29	2.94	1.07
204							0.36
206			1.39	1.55	1.29		
214							0.36

OeduH15

	PAC	LUN	MED	CSA	LLO	SAM	MNE
175	20.00	4.62	2.56	21.88	10.53	13.89	18.91
179	6.67				0.38		2.17
183						4.17	0.22
187	11.67	17.69	26.92	25.45	21.05	18.06	4.78
191		1.54	7.69	3.57	4.14	4.17	9.13
195		0.77	2.56		1.13	4.17	3.70
199	1.67	1.54					0.43
203	20.00	20.00	28.21	16.52	21.05	1.39	12.39
207					1.88		1.96
211	3.33						3.04
215	3.33	11.54	5.13	5.80	7.14	12.50	15.65
219					3.01		3.70
223	33.33	34.62	21.79	22.32	25.56	26.39	21.96
227		7.69	5.13	4.46	4.14	15.28	1.96

OeduT5

	PAC	LUN	MED	CSA	LLO	SAM	MNE
106	8.33	6.62	1.28	3.91	6.29	10.81	9.83
114		5.88	1.28	1.30	3.85		3.97
116							0.21
118							0.42
120	16.67	3.68	1.28	3.48	7.34	1.35	11.51
122		5.88	3.85	11.30	4.55	8.11	0.84
126	6.67	15.44	24.36	20.87	9.79	2.70	17.99
128							2.30
130	1.67	8.09		0.43	5.94	5.41	6.07
132				0.43			1.67
134				0.43	1.75	8.11	1.46
136	6.67	5.88	5.13	7.83	4.90	8.11	2.93
138		1.47	12.82	3.04	3.15	2.70	10.67
140	3.33					5.41	1.67
142	10.00	2.21	1.28	5.65	11.89	20.27	5.86
144	21.67	25.74	23.08	16.52	14.34	12.16	9.00
146	13.33	7.35	6.41	1.74	8.04	1.35	2.93
148	5.00	11.76	16.67	20.87	12.94	9.46	1.05
150	1.67			0.43	1.75	1.35	1.26
154			1.28	0.87	1.40	1.35	3.56
156			1.28	0.87	1.40	1.35	0.84
158					0.35		0.84
160					0.35		0.42
162							1.05
164							0.42
166							0.84
168	5.00						
174							0.42

OeduO9

	PAC	LUN	MED	CSA	LLO	SAM	MNE
145	6.67	2.21	1.28	1.67	3.82	2.94	
147							0.41
149							0.41
153							0.41
155							1.83
157			1.28		1.39	1.47	1.63
159							8.13
161	6.67	9.56	1.28	3.75	6.94	23.53	8.74
163	3.33		2.56	3.75	1.39	2.94	8.13
165	10.00	3.68	5.13	1.25	1.74	7.35	7.32
167	31.67	27.21	37.18	22.92	28.47	16.18	24.39
169	25.00	13.24	24.36	24.17	26.39	11.76	20.73
171	6.67	9.56	11.54	14.58	10.07	11.76	9.35
173	3.33	31.62	15.38	19.58	17.01	16.18	5.49
175		1.47		0.42	1.39	4.41	0.41
177	6.67	1.47		7.92	1.39		2.03
179							0.41
181						1.47	
183							0.20

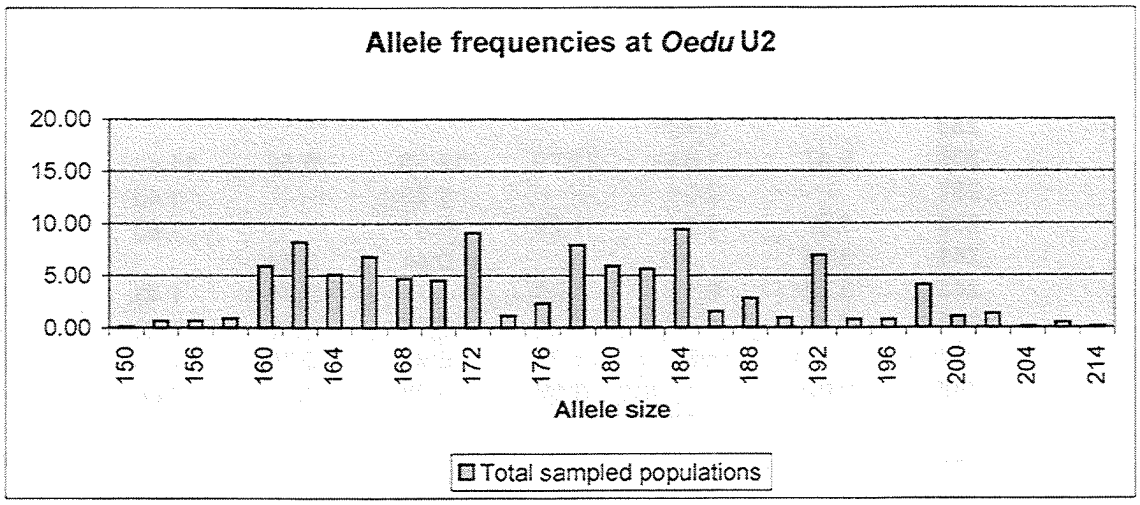
OeduJ12

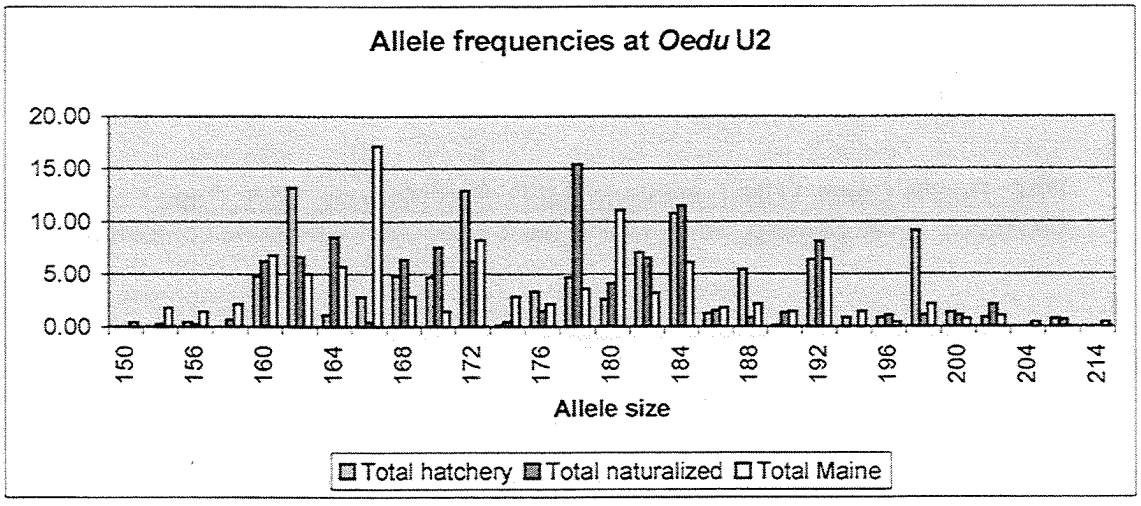
	PAC	LUN	MED	CSA	LLO	SAM	MNE
219	15.00	4.62	5.13	1.23	3.10		6.57
225		2.31					0.40
227	23.33	53.85	53.85	63.11	60.69	42.86	33.67
229	11.67	6.15	11.54	3.28	3.45	1.43	1.39
231	1.67			0.82	1.72	1.43	3.39
233		8.46					1.20
235	6.67	1.54	21.79	12.30	5.52	11.43	6.18
237		2.31		1.23		1.43	0.60
239	1.67	1.54	1.28	0.41		1.43	4.58
241	3.33			0.41	0.34		2.19
243	3.33	6.15	1.28		2.76	1.43	3.78
245	6.67	5.38	1.28	0.41	3.10	1.43	6.18
247		2.31	1.28	9.43	2.76	24.29	6.57
249	20.00		2.56	3.28	3.10	2.86	2.79
251		3.85					6.57
253					2.41	1.43	2.79
255				0.41	1.72	5.71	1.99
257	6.67				3.10	1.43	3.39
259				0.41	1.03		3.39
261		1.54		2.46	3.79	1.43	1.39
263				0.41	0.34		0.20
265							0.80
267				0.41	0.69		
269					0.34		

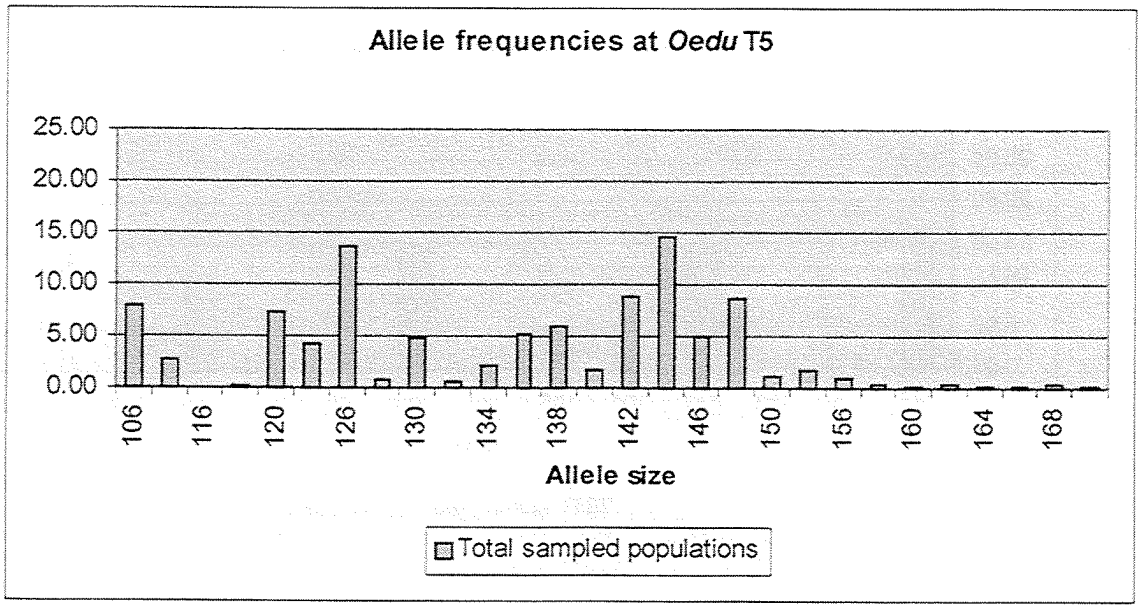
PAC: Pacific Coast, LUN: Lunenburg, MED: Port Medway, CSA: Cape Sable, LLO: Lake Lockhard, SAM: Sambro, MNE: Maine (all 3 locations from Maine combined).

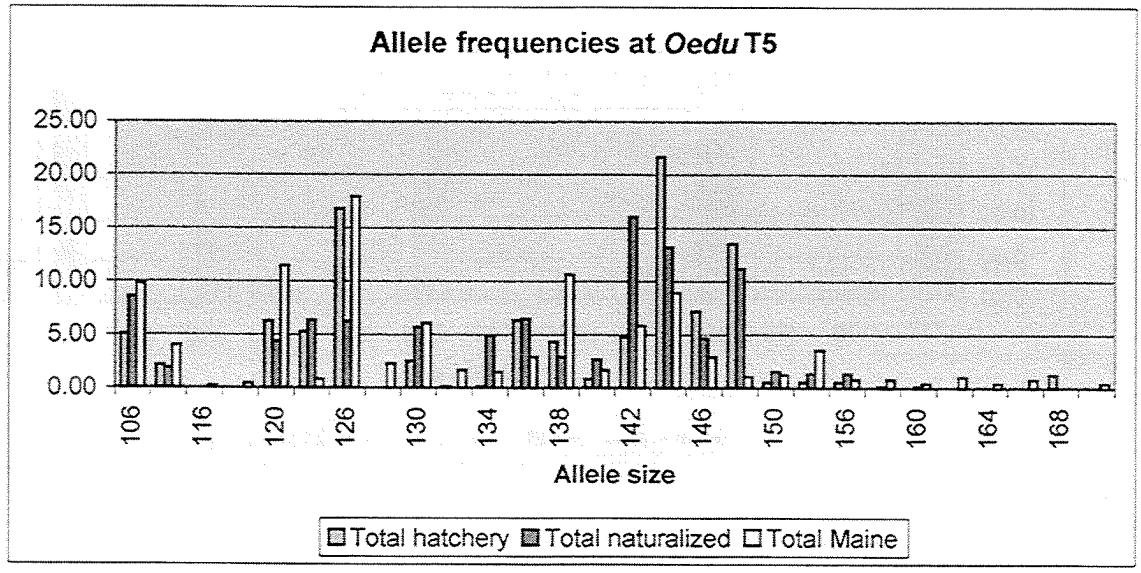
APPENDIX B

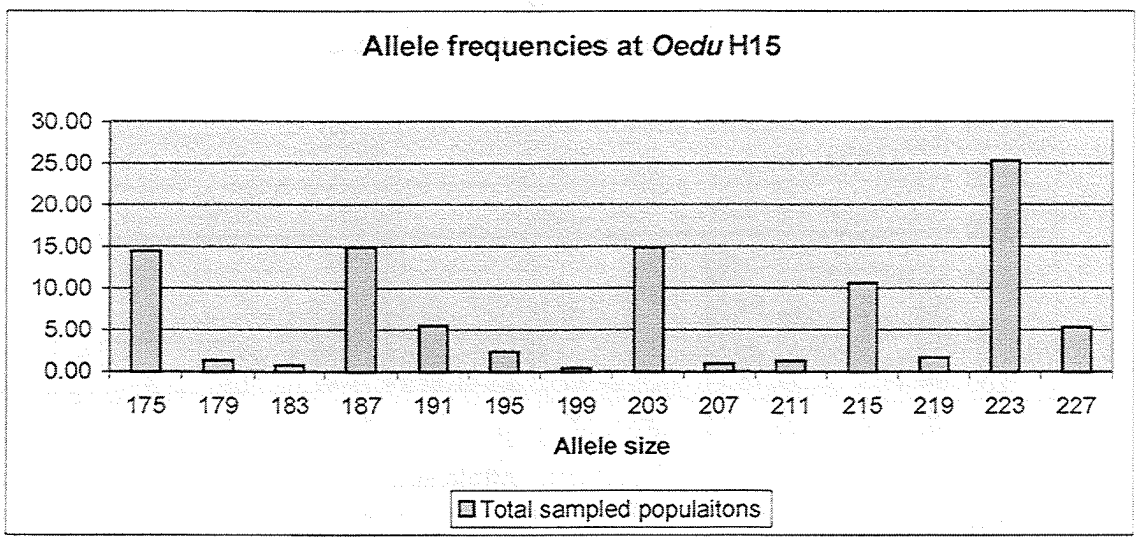
Allele profiles for populations of *Ostrea edulis*

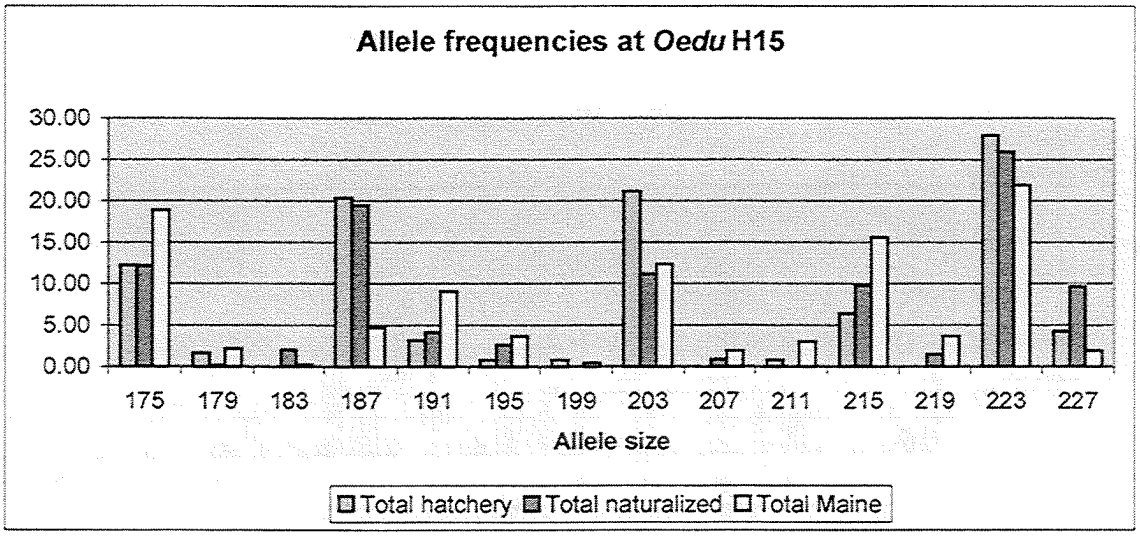


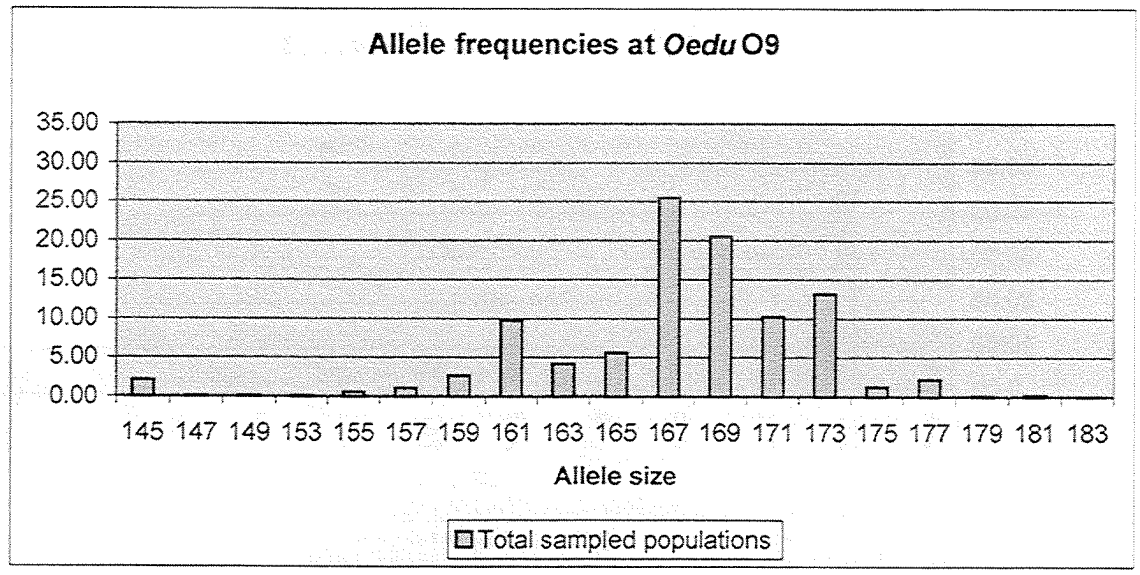


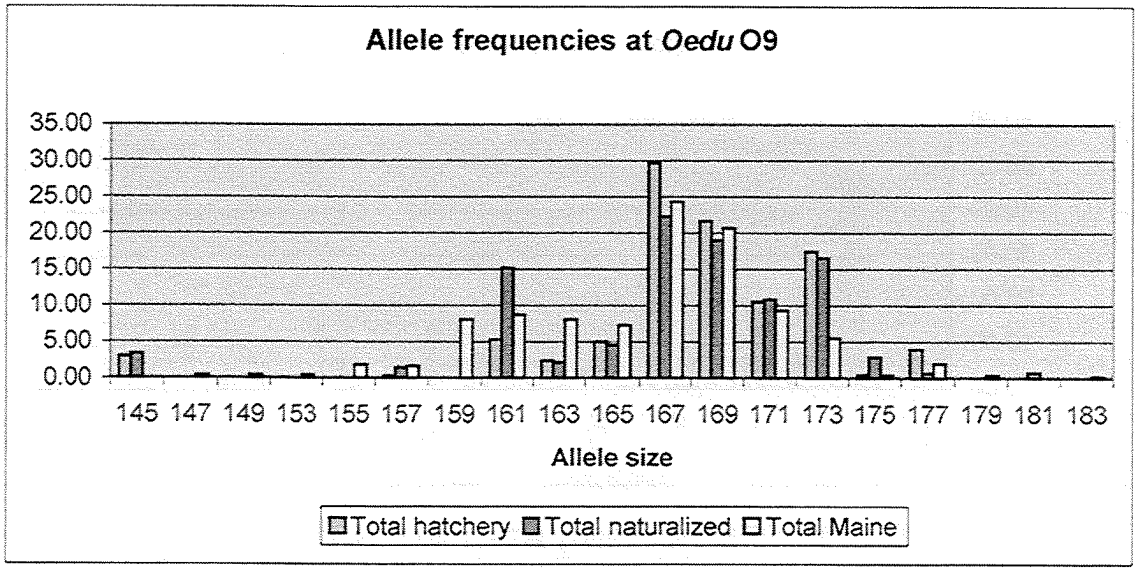

Allele frequencies at *Oedu* T5
25.00
20.00
15.00
10.00
5.00
0.00
106
116
120
126
130
134
138
142
146
150
156
160
164
168
Allele size
Total sampled populations

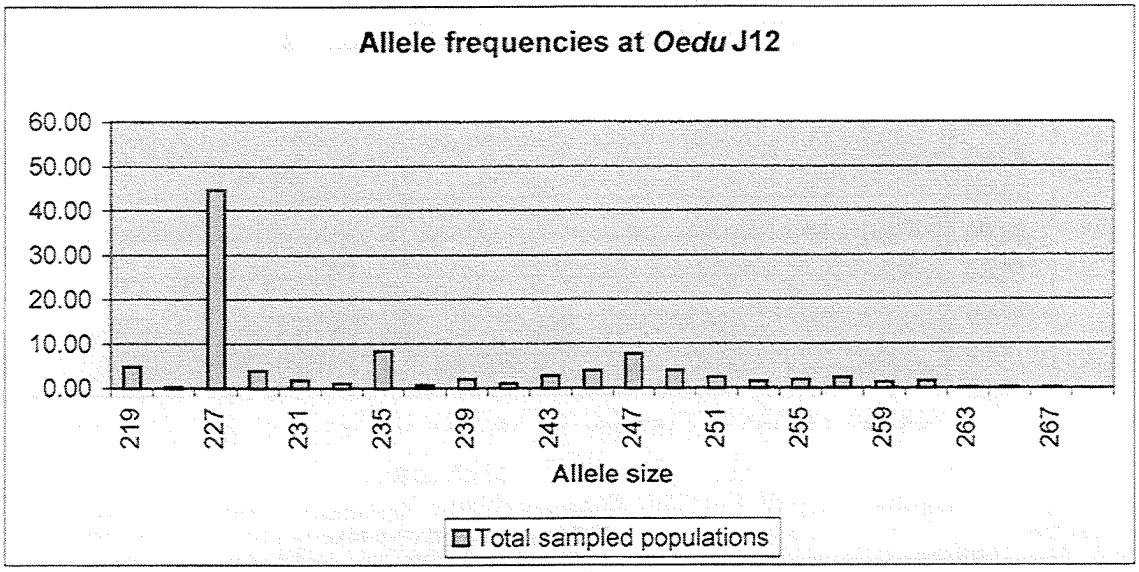

Allele frequencies at *Oedu* T5
25.00
20.00
15.00
10.00
5.00
0.00
106
116
120
126
130
134
138
142
146
150
156
160
164
168
Allele size
Total hatchery
Total naturalized
Total Maine

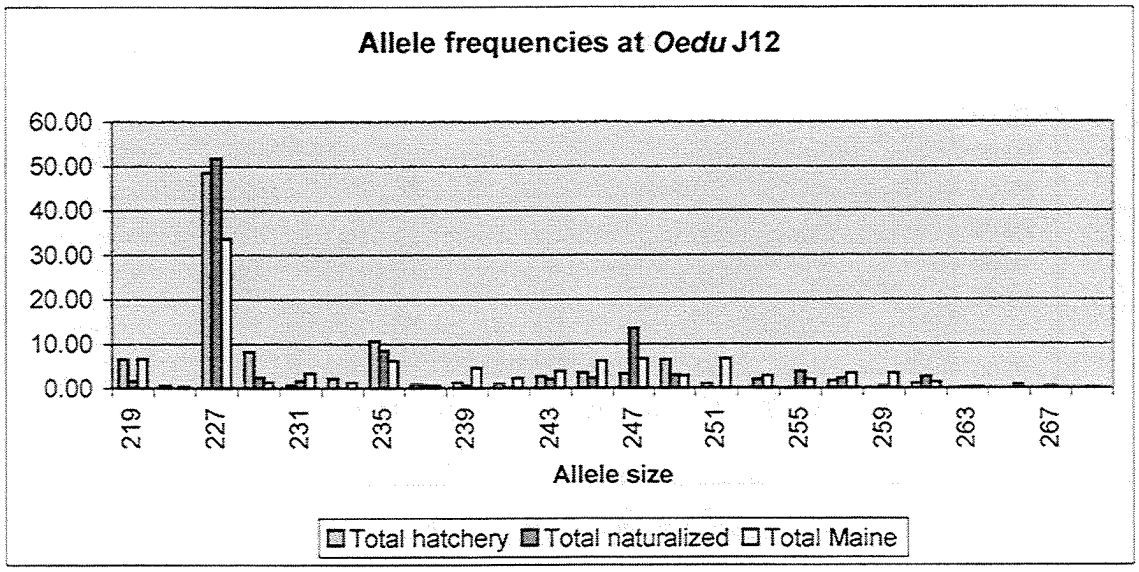

Allele frequencies at *Oedu* H15
30.00
25.00
20.00
15.00
10.00
5.00
0.00
175 179 183 187 191 195 199 203 207 211 215 219 223 227
Allele size
Total sampled populaitons


Allele frequencies at *Oedu* H15
30.00
25.00
20.00
15.00
10.00
5.00
0.00
175 179 183 187 191 195 199 203 207 211 215 219 223 227
Allele size
Total hatchery
Total naturalized
Total Maine


Allele frequencies at *Oedu* O9
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
145 147 149 153 155 157 159 161 163 165 167 169 171 173 175 177 179 181 183
Allele size
Total sampled populations


Allele frequencies at *Oedu* O9
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
145 147 149 153 155 157 159 161 163 165 167 169 171 173 175 177 179 181 183
Allele size
Total hatchery
Total naturalized
Total Maine


Allele frequencies at *Oedu* J12
60.00
50.00
40.00
30.00
20.00
10.00
0.00
219
227
231
235
239
243
247
251
255
259
263
267
Allele size
Total sampled populations


Allele frequencies at *Oedu* J12
60.00
50.00
40.00
30.00
20.00
10.00
0.00
219
227
231
235
239
243
247
251
255
259
263
267
Allele size
Total hatchery
Total naturalized
Total Maine