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Genetic variation at microsatellite loci in northern populations of the European flat oyster (*Ostrea edulis*)

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Samples of *Ostrea edulis* were collected during 1999 and 2000 from five sites in Scotland, and from one site each in Northern Ireland, Ireland, France, The Netherlands and Norway. Samples were scored at four microsatellite loci. Mean numbers of alleles per locus varied from 12.6 to 16.6 and observed heterozygosity per locus ranged from 0.801 to 0.845. Samples derived originally from hatchery seed showed significantly fewer alleles per locus and significantly reduced expected heterozygosity compared with wild populations. However, observed heterozygosity did not differ significantly between the two groups. Significant deficiencies of heterozygotes were present at one or more loci in four of the ten populations sampled and a significant excess of heterozygotes was present at one locus in a hatchery-sourced Scottish population. Genetic distance indices revealed that the Norway population was the most distinct from all others and that hatchery-sourced populations were also relatively distinct from other wild populations. However, genetic subdivision was generally low implying high historical 'migration' rates for these populations.

INTRODUCTION

The European flat oyster, *Ostrea edulis* L., is a protandrous hermaphroditic brooding bivalve that inhabits highly productive estuarine and shallow coastal marine habitats. It is distributed from the Norwegian Sea, through the North Sea, down to the Iberian Peninsula and the Atlantic coast of Morocco and into the Mediterranean and the Black Sea. In previous centuries it has supported important aquaculture and fisheries activities throughout Europe. Many stocks of *O. edulis* declined massively during the late 19th and throughout the 20th Century due to over-exploitation and disease epidemics. Dwindling stocks were often replenished with oysters from other geographical areas, which has resulted in extensive population admixture all across Europe (Magennis et al., 1983). In addition, although larvae are brooded, they have a planktonic phase lasting more than a week, allowing dispersal through larval transport. Thus, both anthropogenic and natural processes will tend to cause homogeneity across significant geographical distances in populations of *O. edulis*.

Genetic markers have been used in many fish and shellfish species to identify genetic differentiation of populations (Carvalho & Hauser, 1998; Beaumont & Hoare, 2003). Previous genetic studies on oysters have used allozyme markers and have demonstrated that there was little detectable genetic differentiation in European stocks of *O. edulis* (Johannesson et al., 1989; Saavedra et al., 1995). Because of the general low level of allozyme variability—and the belief that this variability may not always be stochastically determined (Karl & Avise 1992)—there is the need to re-examine variability using neutral, highly variable marker loci, with greater resolving power than allozymes. With a high mutation rate, in the range 10^{-2}–10^{-5}, leading to high levels of polymorphism, microsatellite DNA markers have proved highly informative for population genetic studies (Bruford & Wayne 1993; Zane et al., 2002). Isolation of these markers in oysters has opened the possibility of investigating levels of genetic variation in *O. edulis* (Naciri et al., 1995; Morgan et al., 2000; Sobolewska et al., 2001; Launey et al., 2002). Launey et al. (2002), used five microsatellite loci to study Europe-wide genetic variation and reported significant between-region (Atlantic–western Mediterranean–eastern Mediterranean) differentiation, but little variation between Atlantic populations, except at the northern limit of the species distribution (Norway).

Here we report on a small, more localized study, based on variation at four microsatellite loci (Sobolewska et al., 2001) in ten North Atlantic European populations of *O. edulis*. We compared natural populations that have remained relatively undisturbed over the recent past, with populations derived from aquaculture stocks, including a pond-reared Irish population, which is being selected for resistance to the parasite *Bonamia ostreae* (Beaumont et al., 2002). Such comparisons may assist the effective fisheries management and conservation of this important oyster species.

MATERIALS AND METHODS

A total of 351 individuals of *Ostrea edulis* from ten populations was collected from different areas across northern Europe during 1999 and 2000 (Figure 1). Anecdotal evidence suggests that the Scottish, Norwegian, and Irish populations are relatively undisturbed by recent heavy fishing activities, or by the introduction of hatchery produced seed or juveniles. However, the Loch Kishorn (KSN) and Orkney (ORK) populations are known to

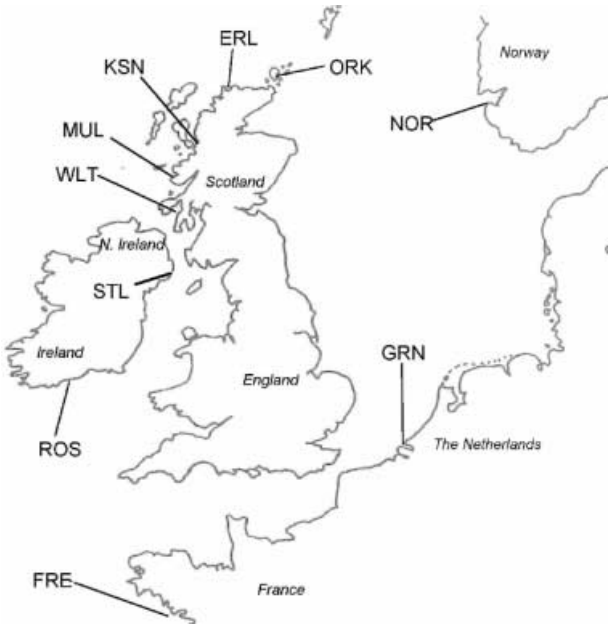

Figure 1. Sample sites of *Ostrea edulis*. NOR, Finnoy Fjord, Norway; ORK, Orkney, Scotland; ERL, Loch Eriboll, Scotland; KSN, Loch Kishorn, Scotland; MUL, Isle of Mull, Scotland; WLT, West Loch Tarbert, Scotland; STL, Strangford Lough, Northern Ireland; ROS, Rossmore Oysters, Cork, Ireland; GRN, Grevelingen, The Netherlands; FRE, Brittany, France.

have originated from seed supplied by Seasalter Hatchery (D. Gowland, personal communication—the KSN are original seed, the ORK oysters are three generations removed from the original seed). The native populations have long been reduced to extremely low numbers and there has been no recent record of natural spatfall at these sites. The Rossmore (ROS) population is derived from a pond production system (David Hugh-Jones, personal communication), where oysters that survived, or were resistant to, infestation by the parasite *B. ostreae* have been selectively bred over several generations to produce a *B. ostreae*-resistant oyster. The oysters from France and The Netherlands (FRE, GRN) were sampled from heavily exploited populations with a long history of fishery management and human interference.

All oysters were sent live to the laboratory, where they were opened and DNA was extracted from the gill tissue using a CTAB (hexadecyltrimethyl ammonium bromide) and phenol chloroform extraction method (Sambrook et al., 1989; Wilding et al., 1997). The four microsatellite loci used in the study were: *Oedu.*HA1, *Oedu.*HA7, *Oedu.*HA21, and *Oedu.*HA11a, with primer sequences and polymerase chain reaction (PCR) conditions as described in Sobolewska et al. (2001). The PCR products were electrophoresed through 6% polyacrylamide gels. Autoradiographs were scanned at a resolution of 300 dpi and imported into GelCompar version 2.0 (Applied Maths BVBA, Kortrijk, Belgium) and the fragments were sized using the M13-G fragments as a size marker.

Diversity was measured as number of alleles per locus (Na), observed heterozygosity (H_o), expected heterozygosity (H_e) under Hardy–Weinberg equilibrium (HWE). Differences between mean N_a, H_e and H_o in hatchery-derived or wild populations were tested for significance using the Kruskal–Wallis test (equal variances) or the Mood median test (unequal variances).

Tests for genotypic linkage disequilibrium (between locus pairs) and deviations from HWE were tested using the exact test of Genepop 3.1c (Raymond & Rousset 1995). Significance levels were determined using the Markov chain method.

Genetic differentiation was estimated using Weir & Cockerham's (1984) estimator of F_{st}, θ in FSTAT (Goudet, 1995). Tests for significant departures from zero were carried out using a permutation test within the program.

Nei's (1978) standard genetic distance G_{st} was calculated using the GENES IN POPULATIONS program (designed by May et al., 1995).

Genetic distance analysis was restricted to genetic indices based on the infinite allele model (IAM) such as F_{st} (Cockerham, 1973; Weir & Cockerham, 1984) and G_{st} (Nei, 1978) rather than indices based on the stepwise mutation model (SMM), such as R_{st} and $\delta\mu^2$ (Goldstein et al., 1995). In recently isolated populations, and when fewer than 20 loci are used, the IAM is believed to be more appropriate (Gaggiotti et al., 1999). Genetic differentiation is more likely the result of allele frequency drift rather than the accumulation of mutational events (Takezaki & Nei, 1996; Perez-Lezaun et al., 1997; MacHugh et al., 1998; Balloux & Goudet, 2002).

Correlation between geographical and genetic distance, a Mantel test was performed between Fst/(1−Fst) and geographical distance in kilometres using Genepop 3.1c (Raymond & Rousset, 1995) with 5000 permutations. Some pairwise distances were difficult to assign due to current flows in the region.

RESULTS

Observed number of alleles (N_a), observed (H_o) and expected (H_e) heterozygosities, and means across populations and loci, are shown in Table 1. Mean numbers of alleles across loci ranged from 7.5 to 18.8, being lowest for KSN and ORK, the populations derived from hatchery production. The median number of alleles for hatchery derived populations (N_a=9.0) was significantly less than the median number of alleles for all other populations (N_a=17.0 H=15.65, $P<0.001$). Excluding the hatchery sourced populations, the GRN and FRE populations exhibited the highest number of alleles while the Norwegian (NOR) and Loch Eriboll (ERL) populations showed the lowest.

No significant linkage disequlibrium was observed ($P>0.05$). Mean observed heterozygosity varied between 0.765 in the FRE and 0.914 in the ERL samples; mean expected heterozygosity ranged from 0.781 in KSN oysters to 0.945 in the West Loch Tarbert (WLT) sample (Table 1).

As expected, with fewer alleles, there were significantly lower expected heterozygosities in hatchery-derived (median H_e= 0.784) populations than in wild populations (median H_e=0.921, Mood test χ^2= 5.53 $P<0.018$). Nevertheless, there was no significant difference between median observed heterozygosities of hatchery-derived (H_o=0.839) and wild populations (H_o=0.829, H=0.18, P=0.67).

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Table 1. *Summary genetic data at four microsatellite loci for ten populations of* Ostrea edulis *from northern Europe. Sample sizes are given in parentheses for each population. For abbreviations of populations see Figure 1.*

Population locus		NOR (20)	GRN (46)	ROS (50)	KSN (39)	STL (29)	ORK (29)	WLT (40)	MUL (34)	FRE (35)	ERL (29)	Mean N_a, H_o, and H_e
HA11	N_a	15	20	14	7	18	15	16	18	20	14	15.7
	H_o	0.900	0.696	0.760	0.921	0.897	0.821	0.769	0.940	0.743	1.000	0.845
	H_e	0.915	0.919	0.890	0.750	0.943	0.932	0.928	0.955	0.937	0.920	0.909
	F_{is}	0.017	**0.245**	0.147	**−0.232**	0.050	0.120	0.173	0.016	**0.210**	−0.088	
HA21	N_a	15	14	11	7	12	9	15	15	15	13	12.6
	H_o	0.800	0.804	0.840	0.868	0.897	0.679	0.897	0.909	0.829	0.828	0.835
	H_e	0.885	0.878	0.843	0.752	0.903	0.716	0.947	0.924	0.883	0.876	0.861
	F_{is}	0.098	0.085	0.004	−0.158	0.007	0.053	0.053	0.017	0.062	0.057	
HA1	N_a	11	19	19	9	20	10	20	21	20	17	16.6
	H_o	0.900	0.739	0.860	0.816	0.759	0.857	1.000	0.818	0.686	0.966	0.840
	H_e	0.868	0.913	0.921	0.850	0.941	0.862	0.974	0.951	0.948	0.927	0.916
	F_{is}	−0.022	0.192	0.067	0.040	**0.196**	0.006	−0.027	0.142	**0.279**	−0.042	
HA7	N_a	13	22	18	7	19	10	18	17	19	15	15.8
	H_o	0.750	0.848	0.800	0.684	0.862	0.857	0.821	0.727	0.800	0.862	0.801
	H_e	0.903	0.933	0.913	0.771	0.933	0.797	0.929	0.892	0.924	0.873	0.887
	F_{is}	0.174	0.093	**0.124**	0.113	0.077	−0.077	0.119	0.187	0.136	0.013	
Mean	N_a,	13.5	18.8	15.5	7.5	17.3	11	17.8	17.3	18.5	14.8	
	H_o	0.836	0.771	0.815	0.822	0.854	0.804	0.872	0.849	0.765	0.914	
	H_e	0.892	0.911	0.892	0.781	0.930	0.827	0.945	0.931	0.923	0.863	
	F_{is}	0.067	0.154	0.086	−0.057	0.083	0.028	0.080	0.090	0.174	−0.016	

N_a, observed number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity at Hardy–Weinberg equilibrium; F_{is}, Wright's (1965) F_{is}values, with those in bold indicating significance ($P<0.001$) after Bonferroni adjustment.

Table 2. *Genetic distance matrix for ten populations of* Ostrea edulis *based on data at four microsatellite loci. Weir & Cockerham's (1984) estimator of F_{st} above the diagonal and Nei's (1978) standard distance below the diagonal. For population abbreviations see Figure 1.*

Population	NOR	MUL	ERL	WLT	KSN	FRE	GRN	ROS	ORK	STL
NOR	****	0.050	0.057	0.054	0.137	0.056	0.052	0.055	0.085	0.038
MUL	0.020	****	0.020	0.025	0.077	0.024	0.016	0.017	0.057	0.015
ERL	0.070	0.046	****	0.034	0.103	0.033	0.030	0.028	0.080	0.022
WLT	0.091	0.030	0.052	****	0.094	0.025	0.028	0.027	0.076	0.017
KSN	0.069	0.071	0.075	0.097	****	0.071	0.079	0.083	0.115	0.068
FRE	0.104	0.072	0.041	0.067	0.101	****	0.016	0.011	0.066	0.016
GRN	0.048	0.062	0.036	0.044	0.095	0.023	****	0.016	0.067	0.016
ROS	0.105	0.144	0.067	0.127	0.166	0.089	0.035	****	0.064	0.017
ORK	0.164	0.162	0.166	0.129	0.231	0.071	0.082	0.121	****	0.054
STL	0.052	0.047	0.034	0.049	0.104	0.015	0.010	0.074	0.098	****

Wright's (1965) F_{is} and F_{it} calculated for all loci in each population (Table 1) showed that significant deficiencies of heterozygotes (significant positive F_{is}values) were present at one or more loci in four of the ten populations sampled. Across populations, significant deficiencies of heterozygotes were recorded at three of the four loci and F_{is} values were positive in 33 cases out of 40. However, against this trend, a significant excess of heterozygotes was present at the HA11 locus in the KSN sample.

Table 2 indicates genetic differentiation measured as Wright's (1965) F_{st} and Nei's (1978) G_{st} genetic distance. Pairwise F_{st} values ranged from 0.011 for FRE and Rossmore to 0.137 for KSN and NOR. The highest mean values for F_{st} were obtained for the two hatchery populations, KSN (0.092) and ORK (0.074); and then for NOR (0.065)—though this is the population with the lowest sample size in the study. Strangford Lough (0.029) and Mull (0.033) were found to be the least genetically differentiated. The overall mean F_{st} was 0.039, and 0.02 if the hatchery populations were excluded (both significantly different from zero $P<0.001$, after strict Bonferroni correction). Overall mean G_{st} was 0.08, and 0.05 when the hatchery populations were excluded. All loci contributed equally to the differentiation. There were highly significant differences between population pairs ($P<0.001$), except for French with Grevelingen, Strangford Lough, and Mull; and for Mull and Strangford Lough.

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The correlation between $\log_{10}$ of the separation distance (km) and $F_{st}/(1-F_{st})$ matrices using a one-sided Mantel test was not significant.

DISCUSSION

The high genetic variability recorded for all our samples from North European populations is in accordance with similar levels of variability described in other studies of *Ostrea edulis* using microsatellite loci (Naciri et al., 1995; Launey et al., 2002). Launey et al. (2002) found that northern Atlantic *O. edulis* populations showed a lower level of genetic variability than Mediterranean populations (mean N_a across five loci=18.5 and 20.6 respectively). Our value of mean N_a=15.2 across four loci in Atlantic populations is lower than that of Launey et al. (2002) but this is mainly due to the inclusion of hatchery-sourced samples. If the KSN and ORK data are excluded, mean N_a rises to 16.7. We also have to consider that sample sizes are quite low, ranging from 20 to 50 individuals. Launey et al. (2002) suggested that for the more northern populations, lower summer temperatures might restrict reproduction and result in spasmodic recruitment derived from a small fraction of the population. Such reductions in effective population size will tend to reduce the number of alleles retained in each generation. Excluding the hatchery populations, our most northerly population, NOR, did show the lowest number of alleles (13.5, Table 1), but it must again be stressed that this population had the smallest sample size. The more southerly French and Grevelingen populations gave the highest number of alleles (18.5 and 18.8, Table 1). Heterozygote deficiencies are commonly observed in allozyme data sets from marine bivalve populations (Zouros & Foltz, 1984; Gaffney, 1994) and Launey et al. (2002) found similar deficiencies in their oyster microsatellite study. We report significant deficiencies of heterozygotes at three of our microsatellite loci in a few populations (Table 1: *Oedu*.HA11—in GRN and FRE; *Oedu*.HA1—in STL and FRE; and *Oedu*.HA7—in ROS). There is no consistent pattern of heterozygote deficiencies at one particular locus, nor in a particular population, which would tend to rule out the cause being due to some systematic feature such as inbreeding. Because allele frequencies are not strongly differentiated between populations, the likelihood of there being a cryptic population sub-structure within samples is remote: this rules out the Wahlund effect as a cause of the heterozygote deficiencies. Although selection against heterozygotes is a potential cause of heterozygote deficiencies at allozyme loci, it is unlikely to be a direct cause at microsatellite loci because of their supposed non-coding neutral nature. Nevertheless, there is some evidence that indirect selection (a microsatellite is presumed to be physically linked to a coding locus) can operate at microsatellite loci in oysters (Boudry et al., 2002). In spite of this evidence, it is also possible that the heterozygote deficiencies recorded in this study could be made by the presence of null alleles. Null heterozygotes are unknowingly scored as homozygotes for the 'active' allele while null homozygotes do not score at all. Null alleles have frequently been unambiguously detected at microsatellite and allozyme loci in hatchery crosses of bivalves (Gaffney, 1994; McGoldrick et al., 2000; Boudry et al., 2002). Although every attempt was made to reduce them—through manipulation of the PCR profile—excessive stutter bands can also cause problems with scoring gels.

The only significant heterozygote excess detected was at the *Oedu*.HA11 locus in the KSN sample. This population was hatchery-derived and had significantly fewer microsatellite alleles per locus than wild populations, a feature observed in allozyme studies of hatchery populations of Pacific oysters (Hedgecock & Sly, 1990). Reduction in the number of alleles is explained by the loss of low frequency alleles through a hatchery broodstock bottleneck and the number of alleles is reduced faster than the level of heterozygosity (Cornuet & Luikart, 1996). Saavedra (1997) showed a reduction in allozyme heterozygosity in hatchery compared with wild populations of *O. edulis*.

Nei et al. (1975) demonstrated that reduction in average heterozygosity depends on not just the size of the bottleneck, but also on the rate of growth of the population. If a population grows rapidly, then reduction in heterozygosity levels will be low, even with few founders. Also, heterozygosity is strongly affected by the frequencies of the alleles rather than simply the number of alleles present at a locus (Beaumont & Hoare, 2003). The significant excess of heterozygotes is probably explained by selective processes taking place during hatchery production of the seed oysters. A similar significant excess of heterozygotes is not seen in the ORK sample, but this may be because they were three generations removed from the original hatchery seed introduction. Nevertheless, overall, the hatchery-derived populations have a significantly reduced expected heterozygosity but no significant reduction in observed heterozygosity compared with the wild populations.

Microsatellite markers are useful tools for investigating levels of inbreeding in populations, and can be used to keep track of such changes in populations, especially for those undergoing selection programmes. The Rossmore population is undergoing a selection programme for disease resistance to the parasite *Bonamia ostreae*, and it would be interesting to examine changes over time in this population. This population showed good survival in challenges with *Bonamia*, but it is not known whether it is truly resistant to the parasite—work is ongoing in this area. It is relevant to note that the Rossmore sample did not show the characteristic reduction in allele numbers at the microsatellite loci found for the hatchery-sourced populations (KSN and ORK) but the sample size was much larger for this population. Considering the various events throughout history that have impinged on the European flat oyster wild populations (disease epidemics, over-fishing and transfers) it would appear that the stocks have retained reasonable levels of genetic diversity at microsatellite loci (high N_a, and H_e).

Launey et al. (2002) reported a mean F_{st} of 0.019 as a measure of genetic differentiation of the Atlantic populations they sampled. Our mean F_{st} was 0.039 for all populations, and 0.020 when the hatchery populations KSN and ORK were excluded. Both studies sampled similar numbers of sites from the Atlantic, but this study concentrated more at the northern end of the distribution of *O. edulis* and included several from Scotland, a region not sampled by Launey et al. (2002). All of our F_{st} values

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were <0.1 (except for the hatchery populations KSN and ORK), which implies high historical migration rates for these northern populations of *O. edulis*. Our data did not support the model of isolation by distance but the geographical range was not large and some distances were difficult to calculate owing to current directions around the British coastline. Little has been published about the history of the populations studied, in terms of any original fishery, or the extent and sources of transfers into the sites (Magennis et al., 1983). Results from this study suggest that several populations (e.g. Strangford Lough, Mull, and Loch Eriboll) are only weakly differentiated from other European populations, implying recent (in historical times) admixture. Such populations are therefore unlikely to represent distinct genetic resources of importance to conservation. The Norwegian sample proved to be the most genetically distinct and further sampling in this region would be valuable to assess the extent to which any residual potential genetic resource might remain in northern waters.

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