Reduced Female Gene Flow in the European Flat Oyster Ostrea edulis

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The geographical structure of 15 natural populations of the flat oyster (Ostrea edulis L.) was assessed by single-strand conformation polymorphism (SSCP) of a 313-base-pair (bp) fragment of the mitochondrial 12S-rRNA gene. Fourteen haplotypes were observed, with one being dominant in the Mediterranean samples and another one in the Atlantic populations. The geographically extreme populations sampled in Norway and the Black Sea appeared differentiated by exhibiting the dominance of a third group of haplotypes. The results were compared to available microsatellite data at five loci. The Atlantic/Mediterranean differentiation pattern was qualitatively the same with both types of markers, confirming an isolation-by-distance pattern. The average mitochondrial haplotypic diversity displayed a high among populations variance, reflecting small effective population size in some locations. Additionally, a 10-fold quantitative difference was observed in Fst between the mitochondrial and the nuclear genomes, which could be due to an unbalanced sex ratio or sex-biased differential reproductive success between males and females (or both).

The European flat oyster, Ostrea edulis, occurs naturally from Norway to Morocco in the northeastern Atlantic and in the whole Mediterranean. It has been a harvested species for at least 6,000 years. The genetic structure of these European populations has probably been influenced both by its intrinsic evolutionary history and past and present human activities, which are not always easy to tell apart (for review, see MacKenzie et al. 1997).

During the last decade, the nuclear genetic diversity and geographical structure of O. edulis populations were investigated, mostly by the use of enzymatic markers (Jaziri 1990; Saavedra et al. 1993; Saavedra et al. 1995). These studies have revealed moderate differentiation between Atlantic and Mediterranean populations (Fst = 0.058 between the two marine basins; Saavedra et al. 1993; Saavedra et al. 1995). Jaziri (1990), basing conclusions on a lower genetic diversity of Atlantic populations, considered that these stocks originated from Mediterranean populations, after the last Quaternary glacialiation, whereas Saavedra et al. (1995) interpreted some clinal and V-shaped patterns of allelic frequencies as the result of interglacial secondary contact of Atlantic and Mediterranean stocks. The question of the genetic discontinuity between both sides of Gibraltar was thus left open, and has recently been reanalyzed.

In a survey based on five microsatellite loci, Launey et al. (2002) revealed very congruent structuring patterns as compared with Saavedra and colleagues’s (1993; 1995) allozyme data, but the V-shaped patterns were not observed. This study also revealed a good correlation between genetic and geographic distances for both types of markers, supporting isolation-by-distance as a model corresponding to the effective dispersal mode. The mitochondrial (mt) genetic structure and diversity of European flat oyster populations remained to be investigated.

Because this maternally inherited genome has generally an increased sensitivity to drift, its study could provide a more precise picture of factors that influence genetic variability. Moreover, the mtDNA molecule being easily accessible to sequence analysis, its variants can be ordered in a phylogenetic framework allowing in certain cases chronological reconstitution. Actually, numerous studies have shown noncongruent patterns between nuclear and mitochondrial markers (e.g., Hansen et al. 1999), patterns which have been interpreted as the result of different factors, such as natural selection (e.g., Pogson et al. 1995), founder effect (e.g., Poteaux et al. 2001), or introgression (e.g., Krafsur 2002). Nevertheless, differentiation patterns are primarily determined by the level of dispersal between populations. Differentiation patterns can be qualitatively but not quantitatively congruent because of either sex-biased or cytoplasmic effective population size differing from the nuclear one. Such effects have been shown to occur in several marine organisms (Arnaud et al., 2003b; Fitzsimmons et al. 1997;
chain reaction (PCR), specific primers were designed on the basis of alignment of several sequences obtained with samples of this species. Sequences of new specific primers are 12SOeduF, 5′-GAGCAGCTGGTAAAACGTCG-3′; and 12SOeduR, 5′-GTTAATTCCTTGCTTCC-3′. Amplification was performed with 2.5 mM MgCl₂, 200 mM of each dNTP, 1 mM of each primer, 0.7 U of Tag polymerase, and 10X PCR reaction buffer. PCR was carried out with an initial denaturation step at 95°C, followed by 30 cycles of amplification (denaturation at 95°C for 1 min, annealing at 54°C for 1 min, and elongation at 72°C for 1 min) and a final elongation step at 72°C for 7 min.

**SSCP Polymorphism and Sequencing**

PCR products were screened for mutational differences by single-strand conformation polymorphism analysis (SSCP; Orita et al. 1989). The amplified fragments were denatured at 95°C for 7 min before being run by electrophoresis in a 10% 37.5:1 acrylamide/0.6X TBE gel, 0.4 cm tick at 30 W (150 mA, 200 V), maintained at a constant temperature (5°C) for 14 h. We observed DNA single-strand bands by staining the gel with an ethidium bromide solution (10 mg/L) during 20 min.

At least two samples of each SSCP class were sequenced, in order to verify that different SSCP variants consistently underlay distinct DNA sequences (Sunnucks et al. 2000). Sequencing reactions (35 cycles of 30 min at 95°C, 30 min at 60°C, and then 1 h at 72°C) were performed forward and reverse with 32P radioactive primers on purified DNA PCR products. Sequence products were separated in a high resolution 6% 29:1 acrylamid/0.5X TBE gel. Sequences were revealed by autoradiography of dried gels.

**Data Analysis**

For each sample, Nei’s unbiased haplotypic diversity $H_{fb}$ (Nei 1987) was estimated as well as $Fst$ (Wright 1951), estimated by 0 (Weir and Cockerham, 1984) with Genetix 4.02 software package (Belkhir et al. 1996–2001). An analog of $Fst$, $\phi_{st}$ (Excoffier et al. 1992), which takes into account divergence between haplotype sequences was estimated between pairs of populations with Arlequin 2.0 software package (Schneider et al. 2000). A hierarchical classification was constructed from Reynolds’ genetic distances $D = -\ln (1 - Fst)$; Reynolds et al. 1983) between samples by using the neighbor-joining method as implemented in the Phylip 3.57 software package. Correlation between genetic [measured as $Fst/(1 - Fst)$] and $\phi_{st}/(1 - \phi_{st})$, following Rousset, 1997] and geographic distance matrices was tested with a Mantel nonparametrical
permutation test (Mantel 1967) as implemented in Genetix 4.02. The geographic distances were measured along the coast and accounted for principal current flow in each area.

Results

12S-rRNA Polymorphism

Analyzing SSCP in the 313-bp 12S-rRNA fragment revealed 14 haplotypes (named with capital letters). The frequencies are detailed in the Appendix. “A” and “B” haplotypes are present in all samples except in the Black Sea (BS) and show a frequency shift between Mediterranean and Atlantic seas: the “A” haplotype was the most common in the Atlantic ocean (except Norway), whereas the “B” haplotype was more frequent within Mediterranean samples. Black Sea (BS) and Norwegian (ANa) samples presented peculiar haplotypic compositions, with their principal haplotype being, respectively, “I” and “C,” two haplotypes rare elsewhere. The sequences of the 14 SSCP haplotypes were registered in GenBank as accession nos. AY157516 to AY157529. The neighbor-joining tree (Figure 2A) identified three different groups of haplotypes: group I + C (including haplotypes I, N, M, C, and E), group B (B and the rare haplotypes F, J, and H), and the less well defined group A, with unclear roots (A and the rare haplotypes L, S, G, and D).

Comparison Between Mitochondrial and Microsatellite Diversity

Mitochondrial and microsatellite gene diversities ($H_{mt}$) for each sample are presented in Figure 3. Average mitochondrial haplotype diversity was almost as low as ($H_{mt} = 0.49 \pm 0.17$) microsatellite genotypic diversity ($H_{gt} = 0.91 \pm 0.02$). As reported by Launey et al. (2002), average microsatellite diversity was lower in the Atlantic than in the Mediterranean + Black Sea samples. The mitochondrial diversity was not correlated ($r^2 = 0.11$, ns) with microsatellite diversity and was much more variable. The lowest levels of variability in this study are registered in the Black Sea (BS: $H_{mt} = 0.30$) and on the southern European Atlantic coast (e.g., ASb: $H_{mt} = 0.17$). These last samples showed a significantly lower $H_{mt}$ than values recorded for Mediterranean samples (e.g., MEb: $H_{mt} = 0.71$) and on some North Atlantic samples (e.g. ANa: $H_{mt} = 0.68$). This was not true for the Portuguese sample, which showed a high diversity, despite its low sample size (ASd, $n = 14$: $H_{mt} = 0.62$). On its side, the nucleotidic diversity II ranged from 0.001 (in the BS sample) to 0.01 in the Oslo sample (Ana), and was significantly correlated with the haplotype diversity ($r^2 = 0.33$).

Population Structure

Differentiation among populations obtained with each marker is presented in Table 1. The global $\Theta_c/\Theta_N$ ratio was 12.8. Although the mitochondrial genetic distance dendrogram between populations showed a global differentiation between Atlantic and Mediterranean populations (Figure 2B), the Mediterranean group of populations was the only one to be not significantly structured in terms of its haplotype diversity. Allele and haplotype frequency variations both fitted a one-dimension isolation-by-distance model (Figure 4). There was a slight but significant regression ($r^2 = 0.34$, $P = 0.001$; slope: 0.08 $Fst$ per 1000 km) between 12S-rDNA genetic distance and geographic distance by pairs.
of populations (one-dimension isolation-by-distance model). This significant regression is maintained even if the most differentiated external populations from Norway and the Black Sea are excluded ($r^2 = 0.22$, $P = 0.009$; slope: $0.07 \text{ Fst per 1000 km}$), even for $\phi_{st}$ ($r^2 = 0.5175$, $P = 0.002$; $0.003 \phi_{st}$ per 1000 km), despite the molecular relatedness of haplotypes I and C, which are the dominant ones in Black Sea (BS) sample and in Norway (Ana) sample, respectively.

**Discussion**

**Intrapopulation Variability**

Microsatellite diversities in flat oyster Mediterranean populations have been shown to be significantly higher than in the Atlantic ones. Allozymic data exhibited the same differences between North Atlantic populations and western Mediterranean ones (Saavedra et al. 1995), and eastern Mediterranean populations showed as low a diversity as Atlantic ones. This was not the case, however, for 12S-rRNA diversity, which appeared much more variable across samples than the diversity observed for microsatellites. Because cytoplasmic markers have an increased sensitivity to drift, due to a generally lower effective population size (Birky et al. 1989), a given population may lose all its mtDNA variability during a bottleneck and still retain a significant fraction of its nuclear variability (Kolman and Bermingham 1997). However, this is often recognized as insufficient to explain the observed discrepancies (Baker et al. 1998; Escorza-Trevino and Dizon 2000).

In the case of the European flat oyster, the populations are not small (compared with these human ones). However, some areas have encountered several human and biological stresses that may have drastically reduced their effective

**Table 1.** Global $\theta$ and associated $P$ values calculated in the present study from haplotypic frequencies ($\theta_N$)

<table>
<thead>
<tr>
<th></th>
<th>$\theta_C$</th>
<th>$P$</th>
<th>$\theta_N$</th>
<th>$P$</th>
<th>$\theta_C\theta_N$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global $\theta$</td>
<td>0.244$^a$</td>
<td>$P \leq .001$</td>
<td>0.019$^{a}$</td>
<td>$P \leq .001$</td>
<td>12.8</td>
</tr>
<tr>
<td>Atlantic $\theta$</td>
<td>0.201$^a$</td>
<td>$P \leq .001$</td>
<td>0.014$^{a}$</td>
<td>$P \leq .001$</td>
<td>14.3</td>
</tr>
<tr>
<td>Atlantic-Norway $\theta$</td>
<td>0.084$^a$</td>
<td>$P \leq .001$</td>
<td>0.010$^{a}$</td>
<td>$P \leq .001$</td>
<td>8.4</td>
</tr>
<tr>
<td>Mediterranean $\theta$</td>
<td>0.013</td>
<td>$P \leq .115$</td>
<td>0.012$^{a}$</td>
<td>$P \leq .001$</td>
<td>1.1</td>
</tr>
<tr>
<td>Mediterranean + Black Sea $\theta$</td>
<td>0.209$^a$</td>
<td>$P \leq .001$</td>
<td>0.016$^{a}$</td>
<td>$P \leq .001$</td>
<td>13.1</td>
</tr>
</tbody>
</table>

Comparison with $\theta_N$ values obtained with microsatellite markers.

$^a$ Significant values.

**Figure 3.** Nei’s diversity $H_{nb}$ calculated in the populations on the basis of the nuclear microsatellites data and the mitochondrial 12r-RNA data.

**Figure 4.** Regression of genetic distance, $Fst/(1 - Fst)$, versus geographic distance in the flat oyster populations for nuclear microsatellite data (filled dots) and mitochondrial 12rRNA data (open dots).
population size. This is particularly true along the French Atlantic coast where populations suffered (1) an over-exploitation of the natural beds, especially during the 18th century, (2) a massive mortality in the 1920s, and (3) two parasitic epizooties (Bonamia ostrae and Marteilia refringens) in the 1960s (Gouletquer and Héral 1997). No such data exist for the Mediterranean Sea, but it appears that the prevalence of B. ostrae is smaller in this sea. The recruitment of natural spat from this species has been strongly reduced, and the French production of flat oyster dropped by a 20× factor since the 1960s, though it is still 2,000 tons per year at present. Such human and disease factors could explain the lower level of diversity observed along the French Atlantic coast as compared to the northern Atlantic countries (like Ireland), where such factors have not been reported. However, marine organisms seem to be quite resilient to such low effective population size effects, even when stocks are apparently demographically depleted.

Interpopulation Differentiation

In the present study, the cytoplasmic/nuclear (θC/θN) ratio was greater than 10. Such observations have already been made in whitefish populations (Hansen et al. 1999) and in the blue marlin (Buonaccorsi et al. 2001), but the discrepancy was never so high. In gonochoric species with equilibrated sex ratios, mitochondrial effective population size (Ne) is classically expected to be fourfold lower than nuclear Ne, which would translate in inversely proportional ratio for the equilibrium Fst values when Ne/w is large. In hermaphroditic species, each individual can theoretically become female and transfer its mitochondrial genome to the next generation. Thus, nuclear genomes are expected to have effective population sizes only twofold greater than mitochondrials ones, so Fst values reached by nuclear markers should be twice as small as those reached with cytoplasmic markers.

Mutation rates have been proposed as influencing the patterns observed (Buonaccorsi et al. 2001; Shaw et al. 1999; Turan et al. 1998). However, recent reevaluation has shown that migration appears to be the prime factor controlling heterogeneity of gene frequencies among populations when it overrides mutation, as is probably often the case (Estoup et al. 2002). Male-biased migration or female philopatry have indeed been suspected in several instances (Baker et al. 1998; Fitzsimmons et al. 1997; Lytholm et al. 1998), but such arguments are not applicable to sessile hermaphrodite species. Thus, what could explain the 12.8-fold ratio observed in European flat oyster? In this species, sex ratio within a reproductive season is male biased, attaining as much as a 3:1 ratio; furthermore, female gonads develop more slowly (Le Dantec and Marteil 1976). These facts probably reflect a heavier energy cost in the development of oocytes and thus a lower probability of becoming female. Moreover, B. ostrae induce high mortalities mainly within adults between 2 and 3 years old (Culloty and Mulcahy 1996). As O. edulis is a protandrous species, the proportion of individuals that achieve reproduction as females in a population may be reduced by the action of this parasite.

The variance in female reproductive success also affects the mitochondrial effective size. This variance is known to be high in bivalves (Hedgcock 1994) and supposedly higher in females than in males (Boudry et al. 2002), thus further reducing mitochondrial relative to nuclear Ne, as has been proposed to occur in a similar case (the hermaphroditic bivalve Pinctada margaritifera) by Arnaud et al. (2003a).

The Case of Populations at the Border of the Geographical Distribution

Genetic differentiation of the Norwegian flat oyster population (Ana) has been observed with all genetic markers analyzed to date: allozymes (Johanesson et al. 1989; Saavedra et al. 1995), microsatellites (Launey et al. 2002) and mtDNA (present work). Actually, the haplotype C is the most frequent one in this population, whereas it is rare in the other samples, with the exception of two samples from the east of the Mediterranean. It has to be noted that this Norwegian sample is located at the boundary of the Atlantic Ocean and the Baltic Sea. Therefore, this pattern may reflect a reduced gene flow with Atlantic stocks, a reduction caused by a harsh selection against immigrants if local populations are adapted to the particular conditions of the Baltic.

It is interesting to point out that the I + C haplotypic group is dominant in the two extremes of the geographic distribution of European flat oysters: in the Norwegian sample, C haplotype is dominant and I haplotype frequency increases, and in the Black Sea sample the I haplotype is dominant with its two related haplotypes, M and N. One explanation could be that the I + C group represents ancestral haplotypes that independently became dominant in both populations (both generated after the last Quaternary glaciation). This hypothesis does not seem to fit well, however, with the haplotypic tree in Figure 4, although the root is unknown. Another one would imply selective considerations, since the Baltic and Black Seas share some common ecological features, such as low salinity. Third, the high frequency of I + C haplotypes in both extremes of the flat oyster distribution may also have been generated by anthropogenic transfer. Although we do not have any precise data about trade in flat oysters between the Black Sea and the Baltic Sea, any accidental or intentional introduction of Black Sea oysters to the Baltic Sea or in the opposite direction cannot be totally excluded.

The Black Sea population studied raises another question because one of the lowest measures of genetic diversity of O. edulis populations has been found in this sample. If gene flow is really occurring all along the species range as indicated by the isolation-by-distance model followed by the populations, it is difficult to understand the presence of the I group only in this sample. In other words, if gene flow is limited between Black Sea and Mediterranean populations, this fact should have been detected by the nuclear markers that estimate the parameter well. Another possibility is that the Black Sea population may derive from a foundation event. As the populations have been drastically reduced during this century by problems derived from eutrophication, as well as by the
introduction in 1946 of a bivalve predator, the Japanese gastropod *Rhabana thomassia* (Kholodov 1994), a recent bottleneck cannot be excluded. If so, it did not lead to the disappearance of the rare haplotypes M and N. The presence of these rare haplotypes, derived from haplotype I, found only in the Black Sea, could also be considered the result of a more ancient foundation. From 22,000 to 9,000 years B.P., the Black Sea depression was occupied by a giant freshwater lake (*Denser* in Ross, 1977). Nine thousand years ago, a Mediterranean water inflow started into this basin, and the I haplotype may have entered then, giving birth to M and N in this lapse of time.

In such an isolation-by-distance model fitted by the *O. edulis* populations in Europe, a relatively homogeneous continuum of haplotypic frequencies is observed. This typical pattern is settled by the gene flow occurring between populations. At first inspection, the high gene flow estimated from the microsatellite-based *Fst* would seem sufficient to prevent the occurrence of the high variance in mitochondrial diversity observed. Biased sex ratio, higher female variance in reproductive success, and epizooties-induced population size crashes probably play a prominent role in reducing female gene flow.

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**References**


Appendix Table A. Frequencies (*100) of the 12S haplotypes

| Population | A | B | C | D | E | F | G | H | I | J | M | N | S | L |
|-----------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ANa       | 36| 8 | 43| 13|   |   |   |   |   |   |   |   |   |   |
| ANb       | 74| 7.5| 7.5| 11|   |   |   |   |   |   |   |   |   |   |
| ANc       | 54| 27| 2| 15| 2 |   |   |   |   |   |   |   |   |   |
| ANd       | 36| 41| 3 |   |   |   |   |   |   |   |   |   |   |   |
| ANe       | 78| 9.5| 6.5| 3 | 3 |   |   |   |   |   |   |   |   |   |
| ASa       | 85| 11| 2 |   |   |   |   |   |   |   |   |   |   |   |
| ASb       | 90| 10|   |   |   |   |   |   |   |   |   |   |   |   |
| ASC       | 86| 8 | 6 |   |   |   |   |   |   |   |   |   |   |   |
| ASD       | 50| 36|   |   |   |   |   |   |   |   |   |   |   |   |
| MWA       | 29| 58| 4.5| 4.5| 2 | 2 |   |   |   |   |   |   |   |   |
| MWB       | 41| 51| 2 |   |   |   |   |   |   |   |   |   |   |   |
| MWC       | 36| 48| 4 | 2 | 2 | 2 | 2 |   |   |   |   |   |   |   |
| MEa       | 9 | 65| 13| 4.3| 4.4| 4.3| 4.3|   |   |   |   |   |   |   |
| MEb       | 34| 40| 14| 2 | 2 | 2 | 4 |   |   |   |   |   |   |   |
| BS        | 83| 8.5| 8.5|   |   |   |   |   |   |   |   |   |   |   |