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Reduced Female Gene Flow in the European Flat Oyster Ostrea edulis

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Abstract : 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).

Keywords : Gene, population, flat oyster, polymorphism, Ostrea edulis

Introduction

The European flat oyster, *Ostrea edulis*, occurs naturally from Norway to Morocco in the North-Eastern Atlantic and in the whole Mediterranean. It has been a harvested species for at least 6000 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 using enzymatic markers (Jaziri, 1990; Saavedra *et al.*, 1993, 1995). These studies have revealed moderate differentiation between Atlantic and Mediterranean populations (*Fst* = 0.058 between the two marine basins, Saavedra *et al*., 1993, 1995). Jaziri (1990), based on a lower genetic diversity of Atlantic populations, considered that these stocks originated from Mediterranean populations, after the last quaternary glaciation, while 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 reanalysed. In a survey based on 5 microsatellite loci (Launey *et al.*,2002) revealed very congruent structuring patterns as compared with Saavedra et al'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 which 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 non congruent patterns between nuclear and mitochondrial markers (e.g. Hansen *et al*., 1999) that 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 either sex-biased or cytoplasmic effective population size differing from nuclear one. Such effects have been shown to occur in several marine organisms (Karl *et al*., 1992; Palumbi and Baker, 1994; Fitzsimmons *et al*., 1997; Lyrholm *et al*., 1999; Arnaud *et al*., 2003a). Thus, the comparison of mitochondrial and nuclear markers is expected to provide a complementary insight in the factors shaping the diversity of a given species. To make such a comparison, we report here the polymorphism of a 12S-rDNA mitochondrial gene fragment on the same samples as those of the microsatellite study of Launey *et al.* (2002), avoiding in this manner any bias due to sampling.

Materials and methods

Sampling

Fifteen populations of *Ostrea edulis* L. were sampled (14 to 50 individuals) between 1996 and 1999 along European coasts, from Black Sea to Norway (Figure 1). Sampled individuals' DNAs are those previously studied for microsatellite variation by Launey *et al.* (2002).

12S rRNA amplification

In the present study, the polymorphism of a 313bp fragment from the mitochondrial 12S rRNA gene was analysed. When universal 12S rRNA primers (Kocher *et al.*, 1989) were

used on *O. edulis* genomic DNA, several fragments were amplified. These fragments were sequenced and compared to 12S sequences available in the Genebank database, confirming their identification. In order to optimise 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'-GAGCAGCTGCTTAAAACTCG-3', and 12SOeduR: 5'-GTTAATCTCCCTTTACTCCC-3'. Amplification was performed with 2.5mM $MgCl_2$, 200µM of each dNTP, 1µM of each primer, 0.7 U of TAQ 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 denaturated at 95º during 7 minutes before being run by electrophoresis in a 10% 37.5:1 acrylamide /0.6X TBE gel, 0.4 cm tick at 30W (150mA, 200V), maintained a constant temperature (5ºC) during 14 hours. DNA single strand bands were observed by staining the gel with an ethidium bromide solution (10mg/l) during 20 minutes.

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

Data analysis

For each sample, Nei's unbiased haplotypic diversity $H_e nb$ (Nei, 1987) was estimated as well as Π, the average nucleotide diversity. Sequences alignment was performed using the software Clustalw (Thompson *et al*., 1994). Neighbor joining analysis (Saitou and Nei, 1987) was implemented with Phylip 3.57 (Felsenstein, 1993) using the program NEIGHBOR. Bootstrap analysis was computed using SEQBOOT (500 replicates) and CONSENSE programs from the Phylip package.

Differentiation between populations was assessed using Wright's fixation index *Fst* (Wright, 1951), estimated by θ (Weir and Cockerham, 1984) with Genetix 4.02 software package (Belkhir *et al.*, 1996-2001). An analog of *Fst*, Φst (Excoffier *et al.*, 1992), which takes into account divergence between haplotype sequences was estimated between pairs of populations using 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 non-parametrical permutation test (Mantel, 1967) as implemented in Genetix 4.02. The geographic distances were measured along the coast and accounting for principal current flow in each area.

Results

12S rRNA polymorphism

Analysing single strand conformation polymorphism (SSCP) in the 313bp 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 Genebank as accessions n° AY157516 to AY157529. The neighbor joining tree of haplotypes (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 ($H_e nb$) for each sample are presented in Figure 3. Average mitochondrial haplotypic diversity was almost twice lower ($H_e nb$ = 0.49±0.17) than microsatellite genotypic diversity ($H_e nb$ = 0.91±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, n.s.) with microsatellite diversity and was much more variable. The lowest levels of variability in this study are registered in the Black Sea (BS : $H_e nb$ = 0.30) and on the southern European Atlantic coast (for example ASb: $H_e nb$ = 0.17). These last samples showed a significantly lower $H_e nb$ than values recorded for Mediterranean samples (e.g. MEb : $H_e nb$ = 0.71) and on some North-Atlantic samples (e.g. ANa : $H_e nb$ = 0.68). This is not true for the Portuguese sample, which shows a high diversity, despite its low sample size (ASd, n = 14 : $H_e nb$ = 0.62). On its side, the nucleotidic diversity Π ranged between 0.001 (in the Black Sea sample (BS) and 0.01 in the Oslo sample (Ana), and was significantly correlated to the haplotypic diversity (r^2 = 0.33).

Population structure

Differentiation among populations obtained with each marker is presented in Table 1. The global θ_C / θ_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 as to its haplotypic diversity. Allele and haplotype frequency variations both fit a one-dimension isolation-by-distance model (figure 4). There is 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 (1 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 *Fst* per 1000 km), even for *Φst* (r^2 = 0.5175 ; p≤0.002; 0.003 *Φst* 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

Intra-population 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). as Eastern Mediterranean populations showed as low diversity as Atlantic ones. This was not the case however for 12S-rRDA diversity, which appeared much more variable across samples than the diversity observed for microsatellites. Since cytoplasmic markers

have an increased sensitivity to drift, due to a generally lower effective population size (Birky *et al*., 1989), a given population may loose 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 recognised 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 population size. This is particularly true along the French Atlantic coast where populations suffered : (1) an overexploitation of the natural beds especially during the 18th century, (2) a massive mortality in the 1920's , and (3) two parasitic epizooties (*Bonamia ostreae* and *Marteilia refringens*) in the 1960's (Goulletquer and Héral, 1997). No such data exist for the Mediterranean Sea, but it appears that the prevalence of *Bonamia ostreae* 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 20x factor since the 1960s, but is still 2000 t / year at present. Such human and disease factors could explain the lower level of diversity observed along the French Atlantic coast than in the northern Atlantic countries (like in Ireland) where such factors were not reported. However, marine organisms seem to be quite resilient to such low effective population size effects even when stocks are apparently demographically depleted.

Inter-population differentiation

In the present study, the cytoplasmic / nuclear (θ_C / θ_N) ratio was greater than 10. Such observation have already been made in whitefish populations (Hansen *et al*., 1999) or 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 4 fold lower than nuclear *Ne*, which would translate in inversely proportional ratio for the equilibrium *Fst* values when N_em is large. In hermaphroditic species, each individual can theoretically become female and thus transfer its mitochondrial genome to the next generation. Thus, nuclear genomes are expected to have effective population sizes only two-fold greater than mitochondrial one and thus, *Fst* values reached by nuclear markers should be twice as small as compared with cytoplasmic markers. Mutation rates were proposed to influence the patterns observed (Turan *et al.*, 1998; Shaw *et al.*, 1999; Buonaccorsi *et al.*, 2001). However, recent revaluation has shown that migration appear 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 phylopatry have indeed been suspected in several instances (Fitzsimmons *et al.*, 1997; Baker *et al.*, 1998; Lyrholm *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 slower (Ledantec and Marteil, 1976). These facts are probably reflecting a heavier energetic cost in the development of ovocytes and thus, a lower probability of becoming female. Moreover, *Bonamia ostreae* induce high mortalities mainly within adults between two and three 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 (Hedgecock, 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.* (2003b).

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 analysed 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 that may be 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 (BS), the I haplotype is dominant with it 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 last Quaternary glaciation). This hypothesis however does not seem to fit well with the haplotypic tree in Figure 4, although the root is unknown. Another one would imply selective considerations, since Baltic and Black seas share some common ecological features, as low salinity. Thirdly, the high frequence of I+C haplotypes in both extremes of the flat oyster distribution may also have been generated by anthropogenic transfer. Although we don’t 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 Baltic sea or in the opposite direction cannot be totally excluded.

The Black Sea population studied raises another question as one of the lowest measures of genetic diversity of *Ostrea 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 should have been detected by the nuclear markers that estimate this 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 *Rabana 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, only found in the Black Sea, could also be considered as the result of a more ancient foundation. From 22000 to 9000 years B.P., the Black Sea depression was occupied by a giant freshwater lake (Denser, 1972, 1974 *in* Ross, 1977). 9000 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 *Ostrea 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 to reduce female gene flow.

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Table 1. Global θ and associated p values calculated in the present study from haplotypic frequencies (θ_C). Comparison with θ_N values obtained with microsatellite markers. Significant values are in bold.

	θ_C	P	θ_N	p	θ_C / θ_N
Global θ	**0.244**	p≤0.001	**0.019**	p≤0.001	12.8
Atlantic θ	**0.201**	p≤0.001	**0.014**	p≤0.001	14.3
Atlantic – Norway θ	**0.084**	p≤0.001	**0.010**	p≤0.001	8.4
Mediterranean θ	0.013	p≤0.115	**0.012**	p≤0.001	1.1
Mediterraean +Black Sea θ	**0.209**	p≤0.001	**0.016**	p≤0.001	13.1

Figure 1. Geographical locations of the *Ostrea edulis* populations sampled. Populations are named according to their broad geographical origin. A refers to Atlantic, M to Mediterranean, and N, W, S, E, respectively to North, West, South, and East. ANa: Oslo, Norway; ANb: Grevelingen, Holland, ANc: Lough Foyle, Ireland; ANd: Cork, Ireland; ANe: Port-en-Bessin, France; ASa: Quiberon, France; ASb: La Rochelle, France; ASc: Vigo, Spain; ASd: Ria Formosa, Portugal; MWa: Mar Menor; Spain; MWb: Thau, France; MWc: Port Saint-Louis, France; MEa: Venezia, Italy; MEb: Dubrovnik, Croatia; BS: Sebastopol, Ukraine. Sample sizes are between brackets.

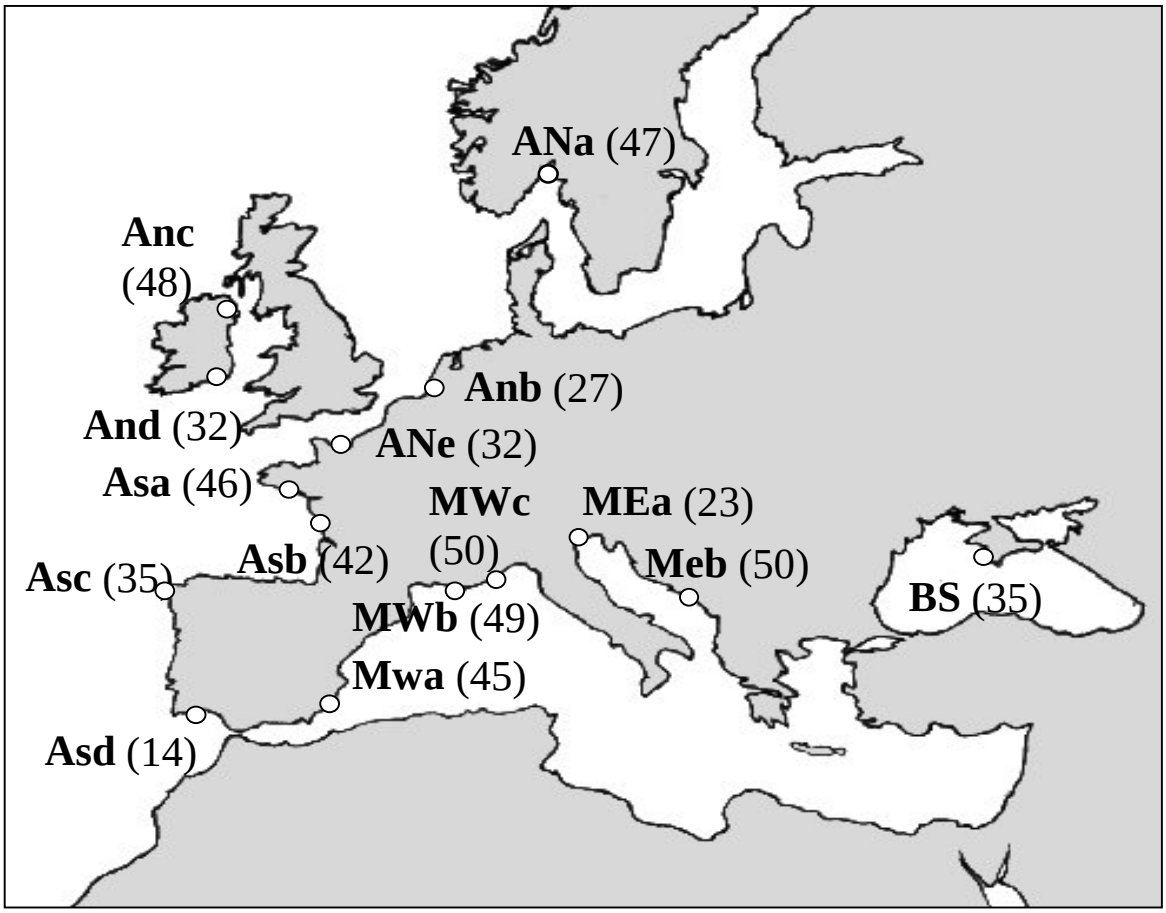


Figure 2. A: Neighbor joining tree based on the mitochondrial 12S rRNA sequences. Bootstrap values (100 replicates) are indicated on the branches. Three groups of haplotypes were separated, named group “A”, “C+I” and “B”. B: Neighbor-joining tree based on Reynold’s mitochondrial genetic distances between flat oyster populations. Location in shaded boxes are from the Mediterranean and Black Seas.

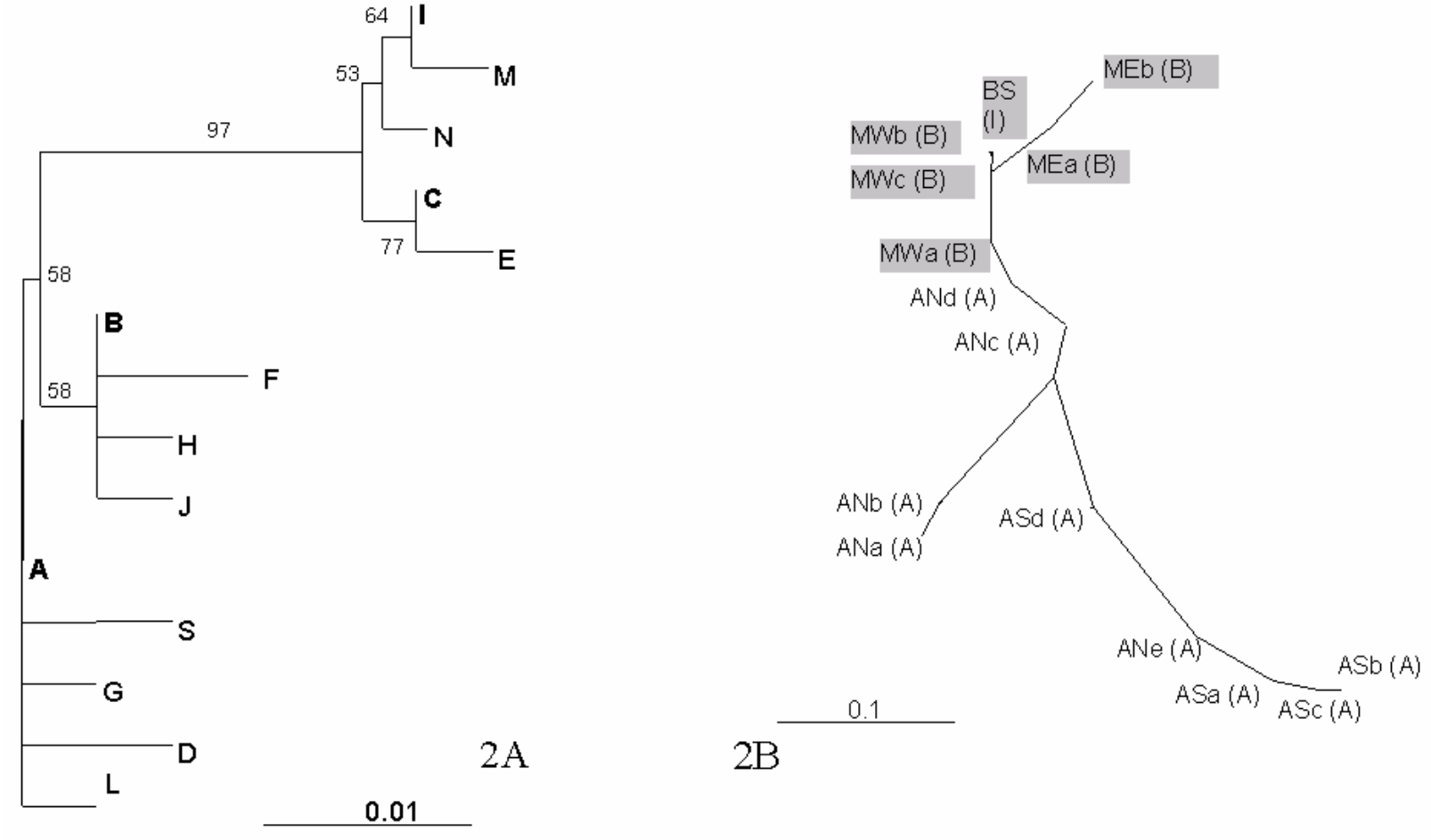


Figure 3. Nei's diversity $H_e nb$ calculated in the populations on the basis of the nuclear microsatellites data and the mitochondrial 12r RNA data.

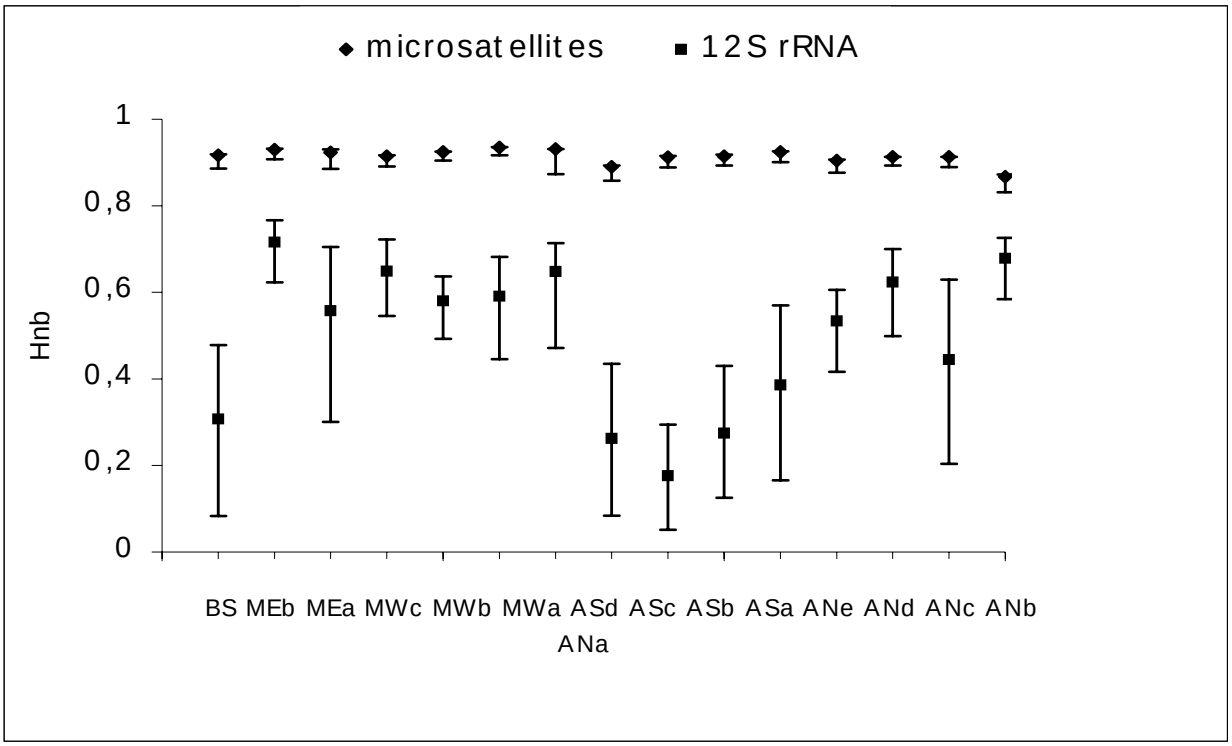
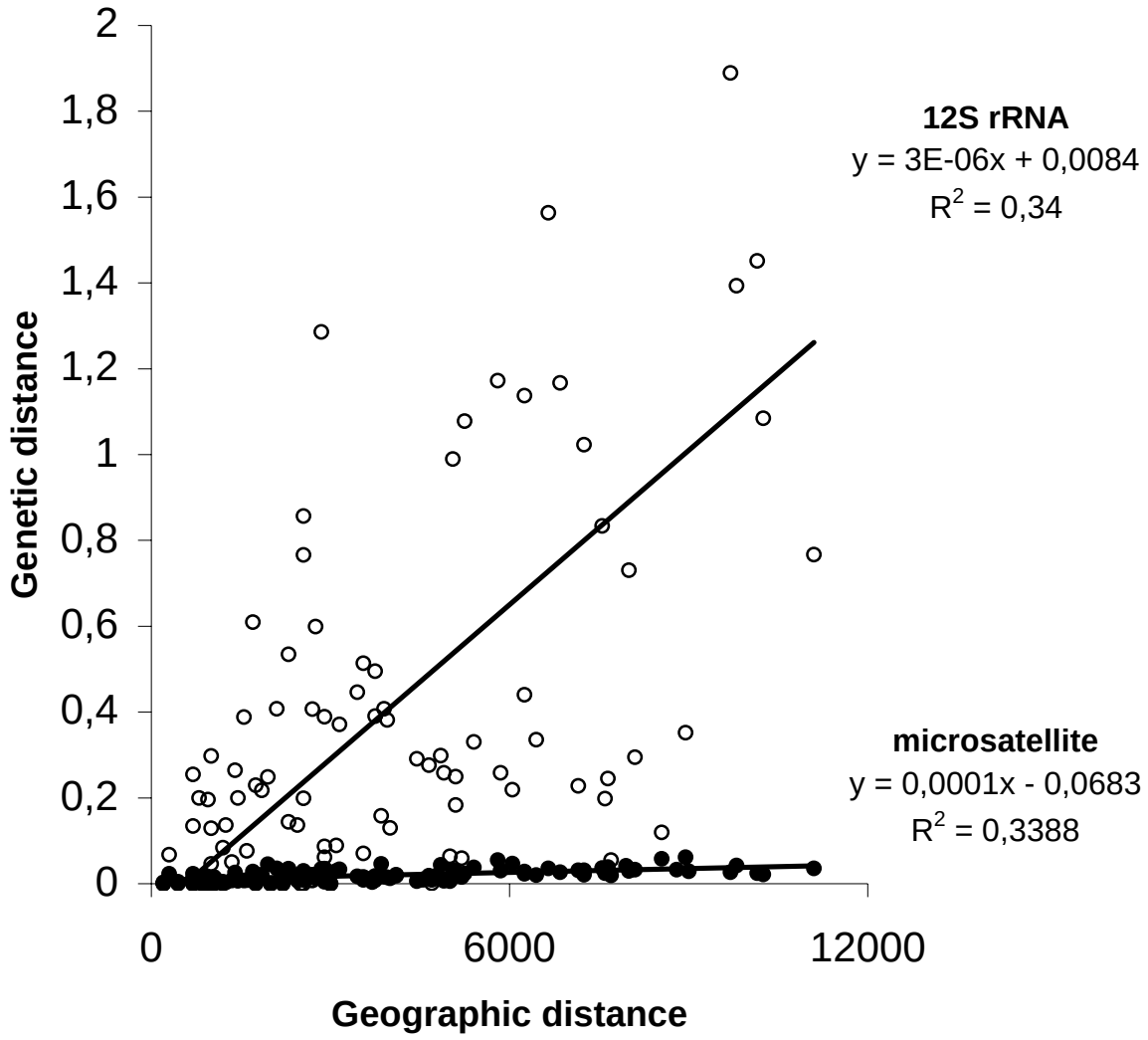


Figure 4. Regression of genetic distance, *Fst/*(1-*Fst*), versus geographic distance in the flat oyster populations for nuclear microsatellite data (filled dots) and mitochondrial 12 rRNA data (open dots).



Appendix. 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	56	41								3				
ANe	78	9.5	6.5	3		3								
ASa	85	11	2						2					
ASb	90	10												
ASc	86	8	6											
ASd	50	36							14					
MWa	29	58	4.5	4.5					2					2
MWb	41	51	2						6					
MWc	36	48	4		2	2		2	2				2	2
MEa	9	65	13		4.3		4.4		4.3					
MEb	34	40	14	2	2				2				2	4
BS									83		8.5	8.5		