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# Mitochondrial and nuclear DNA phylogeography of Crassostrea angulata, the Portuguese oyster endangered in Europe

**Arnaud Huvet[1], S. Lapègue[1], A. Magoulas[2] and P. Boudry[3*]**

[1]Laboratoire de Génétique et Pathologie, IFREMER –, B.P. 133 –, 17390 La Tremblade, France
[2]Genetics Department, Institute of Marine Biology of Crete, P.O. Box 2214, GR 710 03 Iraklio, Greece
[3]Laboratoire de Génétique et Pathologie, IFREMER –, B.P. 133 –, 17390 La Tremblade, France

*: Corresponding author : pboudry@ifremer.fr

**Abstract:** The respective status of the Portuguese oyster, Crassostrea angulata, and the Pacific oyster, Crassostrea gigas, has long been a matter of controversy. Morphological and physiological similarities, homogeneity of allozyme allelic frequencies between populations of the two taxa and the demonstration of hybridization lead most authors to suggest that they should be regrouped within the same species. The risk of introgression and the present expansion of C. gigas aquaculture in Europe raises the question of the need for preservation of C. angulata in Europe, as only a few populations remain. We studied European and Asian populations of C. gigas and C. angulata using microsatellite and mitochondrial DNA markers to estimate their genetic diversity and differentiation. The analysis of genetic distances and the distribution of allelic and haplotype frequencies revealed significant genetic differences between taxa, showing two clusters: (1) C. gigas French and Japanese populations and (2) C. angulata Portuguese and Taiwanese populations. The Asian origin of the Crassostrea angulata taxa is therefore confirmed. Unlike previous studies based on allozymes, significant nuclear genome differences were noted between C. angulata and C. gigas. Despite the presumed history of the introduction of C. angulata into Southern Europe, these populations did not show any significant reduction of variability compared to Taiwanese populations. Any conservation plans for European C. angulata populations should take its non-native origin into account. They represent a valuable genetic resources for European breeding program.

**Keywords:** Crassostrea angulata, Crassostrea gigas - microsatellites - population genetics - phylogeography

## 1. Introduction

The precise status of closely related species is often difficult to define, especially for taxa whose morphology is known to be highly plastic. In oysters, the relative taxonomic status of *Crassostrea gigas*, the Pacific oyster, and *C. angulata*, the Portuguese oyster, which are geographically separated but morphologically similar (Ranson 1948 ; Menzel, 1974), has been questioned for a long time (e.g. Menzel, 1974 ; Buroker *et al*., 1979). Their distinction as two different species, initiated by Thunberg (1793) and Lamark (1819), was chiefly due to their differing geographical distributions, *C. angulata* being present in Europe and *C. gigas* in Asia. However, Menzel (1974) proposed that the Portuguese and Pacific oysters should be classified as the same species based on morphology, physiology and the successful experimental hybridization of the two taxa (reviewed by Gaffney & Allen, 1993).

Several authors (Mathers *et al*., 1974; Buroker *et al*., 1979; Mattiucci & Villani, 1983) examined the genetic differences between the two taxa based on allozyme markers. The observed differentiation between the two taxa was smaller to that reported between reproductively isolated populations within other species (Mathers *et al*., 1974). However, more recently, two studies on the mitochondrial Cytochrome Oxidase subunit I (COI) gene demonstrated a clear genetic difference between the two taxa. O'Foighil *et al*. (1998) compared sequence data from *C. angulata*, three Asian species (*C. gigas*, *C. sikamea* and *C. ariakensis*) and one American oyster, *C. virginica. C. angulata* and *C. gigas* appeared the genetically closest taxa in the Asian complex and were the furthest from the American cupped oyster. This supported the hypothesis that *C. angulata* is Asian in origin. The opposite hypothesis, that Portuguese oysters were introduced to Asia, was therefore rejected because two Asian species (*C. ariakensis* and *C. sikamea*) showed close genetic ties with *C. gigas*, as also revealed by their mitochondrial 16S ribosomal gene sequences (Banks *et al*., 1994). Furthermore, no fossils of cupped oysters have been found in the European Pliocene or Quaternary fossil record (e.g. Ranson, 1960; O'Foighil *et al*., 1998). Boudry *et al.* (1998), using a PCR-RFLP approach, concluded that *C. angulata* was very closely related to a Taiwanese sample, therefore supporting the hypothesis of the introduction of cupped oysters from Taiwan to the Portuguese coast.

Historically, *C. angulata* was introduced from Portugal to the French Atlantic coast in 1868 (Cochard & Dardignac, 1977), where populations expanded and sustained yields of up to 100,000 tons per year by the 1950s (Goulletquer & Héral, 1997). *C. gigas* was introduced from Japan into Europe in the early 1970s, as a substitute for *C. angulata* (Grizel & Héral, 1991) and to counter an iridoviral infection (Comps, 1969) which wiped out *C. angulata* from French coasts between 1967 and 1972. This disease was also observed in the natural populations in Portugal (Ferreira & Dias, 1973). As a result, the extent of remaining *C. angulata* populations is poorly known. Furthermore, the preservation of *C. angulata* in southern Europe is endangered by the present expansion of *C. gigas* aquaculture and by pollution of its natural habitat (Ruano, 1997).

Consequently, the objective of our study is to evaluate the genetic resources and the relative diversity of these two aquaculturally important but non-domesticated taxa. The current picture of their genetic and phylogeographic relationships is unclear due to

differences between allozyme and *mt*DNA results which produce somewhat different patterns. This makes it of great interest to examine the genetic differentiation between these two cupped oyster taxa using another type of nuclear marker: microsatellite markers which are hypervariable and believed, unlike allozymes, to be neutral. In this paper, we report the study of nine geographical samples of *Crassostrea gigas* and *C. angulata* using both microsatellite loci and *mt*DNA PCR-RFLP markers.

## 2. Material and Methods

**Biological material :** Oysters were collected from 9 locations (Table 1), 6 European populations (origins presented in Figure 1) and 3 Asian populations. Five of these populations had been previously identified as *Crassostrea gigas* or *C. angulata* by Boudry *et al*. (1998) using a mitochondrial marker to test about 20 individuals from each population (Table 1). The other 4 populations were provisionally classed according to their geographical origin. In this study, 287 samples were analysed with the mitochondrial marker, to which we added the results from the 95 individuals previously analysed (Boudry *et al*., 1998). The total 382 samples were also analysed with the microsatellite markers. The number of individuals studied per population ranged from 30 to 50. For each population, an abbreviation of 3 capital letters was used (Table 1).
Samples of mantel or gills were preserved in 95% ethanol. DNA extraction was performed by phenol/chloroform method as described by Moore (1993).

***mt*DNA RFLPs:** A 710 bp segment of the COI (Cytochrome Oxidase C subunit I) mitochondrial gene (Folmer *et al.*, 1994) was amplified by PCR as described in Boudry *et al.* (1998). As previously reported (Boudry *et al*., 1998), out of the 9 endonucleases tested, only 4 (*Taq*I, *Sau*3A, *Hha*I and *Mse*I), were polymorphic.

**Microsatellite markers:** Three dinucleotide microsatellite loci (CG44, CG49 and CG108) were used and radioactive PCR amplifications were performed as described by Magoulas *et al*. (1998). These were carried out in a total volume of 15 µl using 2 µl of extract containing oyster genomic DNA. The concentrations of the reaction components were as follows: 1X of *Taq* buffer, 0.2 mM of dNTPs, 0.14 µM of the first primer, 3 mM, 2 mM or 1 mM of $MgCl_2$ for CG44, CG49 and CG108 respectively, 0.017 U of *Taq* polymerase. The second primer of each pair (0.23 µM) was end labelled with 1 mCi of γ33P, 1X of kinase buffer and 0.6 U of kinase for 30 minutes at 37°C. Amplification was performed using 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 53°C for CG44 and CG49 or 55°C for CG108 and 1 min extension at 72°C. PCR products were separated on 6% denaturating polyacrylamide gels for 3-5 hours at 50W (voltage and intensity were set up to give non-limiting conditions, at 1900 V and 150 mA respectively). Gels were run, dried and exposed to film for 6-48 hours. Alleles were numbered according to their size (given in base pairs).

**Data analysis :** *F*-statistics, as described by Wright (1951), were calculated according to Weir & Cockerham (1984), whose method explicitly takes into account differences in sample size such as ours. All the computations and tests were done using the package

GENETIX version 3.3 (Belkhir *et al.,* 1996). Unbiased estimates and standard deviation of heterozygosity were calculated according to the parameter *Fis*. Deviations from Hardy-Weinberg expectations were tested in each sample with 1000 randomly generated permutations, re-sampling individual genotypes within the population. The genetic differentiation for pairs of populations on both types of data was quantified with *Fst* parameters corresponding to the *Theta* of Weir & Cockerham (1984). To test these values, 1000 permutation replicates were generated. Reynolds' genetic distances D (1983) between populations were calculated from the *Theta* values with D = (-ln(1-*Fst*)). Reynolds' genetic distances were used to build two neighbor-joining trees (Saitou & Nei, 1987) with PHYLIP (Felsentein, 1993) and the TREEVIEW package (Page, 1996). A majority-rule consensus topology of the microsatellite tree was derived from 1000 bootstrap replicates with the Seqboot program from the PHYLIP package.

## 3. Results

### Mitochondrial DNA

The use of 4 endonucleases (*Taq*I, *Sau*3A, *Hha*I and *Mse*I) allowed us to distinguish 6 different haplotypes (A-J). These are the same haplotypes as those described by Boudry *et al*. (1998).

Haplotype frequencies per population are presented in Table 2. Two main haplotypes were found: A and C. Most of the populations sampled as *C. gigas* showed a high frequency of haplotype C, ranging from 0.90 to 0.98. Only the French *C. gigas* sample BOU contained a few individuals (4) carrying the haplotype A. The 2 Taiwanese samples showed high frequencies of haplotype A as did the Spanish and Portuguese *C. angulata* samples. Four oysters carrying the haplotype C were found in the Ria Formosa sample (RFA).

Genetic differentiation, given by overall *Fst*, was equal to 0.809. Pairwise *Fst* values are presented in Table 3 and ranged from 0 to 0.99. All the values between taxa (mean *Fst* = 0.88) appeared significantly different from 0. None of the intra-taxa *Fst* values between the populations of *C. gigas* (mean *Fst* = 0.02) or between the populations of *C. angulata* (mean *Fst* = 0.03) were significantly different from 0.

### Microsatellite loci

Over all the populations, the total number of alleles per locus observed was very high, from 52 for the CG108 locus to 53 for the two other loci. The mean number of alleles per locus and per population ranged from 17 to 40 (Table 4).

Observed and expected heterozygosities are shown in Table 4. Gene diversity ranged from 0.89 to 0.96. Fifteen values of *Fis*, from -0.008 to 0.57, out of the 27 tested were found to be significant (Table 4), indicating significant heterozygote deficiencies. Only two French populations SEU and BOU displayed significant heterozygote deficiencies at all three microsatellite markers.

The overall *Fst* value was around 0.017. The level of genetic differentiation between pairs of populations, estimated with pairwise *Fst* values, is presented in Table 3. *Fst* values were found to range from 0.002 to 0.04 (exact mean *Fst* = 0.0173), and most were significant (Table 3). These values were lower in pairs of samples of the same taxon than in pairs with the two different taxa, where all values were significant (mean *Fst* = 0.022; range 0.01 - 0.04). The mean *Fst* value between pairs of populations of *Crassostrea angulata* was 0.018 (range 0.0054 - 0.026) and 0.006 (range 0.0023 – 0.01) between *C. gigas* populations. Moreover, as seen with the mean *Fst*, the genetic differentiation values were significantly higher between *C. angulata* (8 significant *Fst* values out of 10 tested) than between *C. gigas* samples, which were mostly non significant (2 significant *Fst* values out of 6 tested).

### Comparison of mitochondrial and microsatellite data

Two trees of genetic distances (Figures 2 and 3) were built from Reynolds's genetic distances (1983) between all populations for mitochondrial and microsatellite loci. These showed the same general topology. One cluster included the French and Japanese *C. gigas* samples whereas Spanish and Portuguese *C. angulata* samples were grouped with the two Taiwanese samples. The *Fst* values gave the same tendencies for the population structure with both markers even though the mean estimated *Fst* value was 0.5 for the *mt*DNA marker and 0.017 for the microsatellite markers.

## 4. Discussion

Most population genetics studies of marine bivalves to date have been made using allozymes markers (e.g. Volckaert & Zouros, 1989; Liu *et al*., 1995; Saavedra *et al*., 1995; English *et al*., 2000). Between sub-species or closely related populations, genetic differentiation was often absent or only slightly significant, often due to a low level of detectable variation (Buroker *et al*., 1979). Application of DNA markers both from the mitochondrial and nuclear genomes gave valuable new results for the population genetics of *Crassostrea virginica* (Karl and Avise, 1992). Apart from the questionable neutrality of allozymes suggested by these authors, their study of *scn*DNA and *mt*DNA gave consistent but quantitatively different measures of population structure. In the light of these studies, microsatellite loci are expected to be useful markers (Takezaki & Nei, 1996). In the present study, we assayed both the nuclear and mitochondrial genomes in order to have a broad representation of genetic differentiation between *Crassostrea gigas* and *C. angulata* and to make comparisons with previously published allozyme studies.

### Mitochondrial DNA

Our mitochondrial data were fully consistent with initial results obtained by Boudry *et al.* (1998). We confirmed the existence of six haplotypes. No new haplotypes were observed even though a larger geographic zone was studied. Our results support the diagnostic use of these markers to distinguish *Crassostrea gigas* from *C. angulata*.

The *Fst* values showed strong differentiation between populations of the different taxa. In contrast, no significant genetic differentiation appeared between populations of the same taxa. This difference is due to the presence of a low number of taxa-specific haplotypes.

These results confirm that the cupped oysters which occur along the Taiwanese coasts are indeed *Crassostrea angulata*, as Boudry *et al.* (1998) found for one location. This finding was reinforced by our analysis of a second Taiwanese population.

Only two populations showed a limited mixture of the species-specific haplotypes A and C. Contact between the two taxa is therefore restricted, as the rare haplotype does not exceed 11%. The population BOU, located in the South of France, is the only French sample showing any evidence of polymorphism indicating the past presence of *C. angulata*. This persistence might be due to the absence of oyster farming in this area, limiting the recruitment of *C. gigas*. The low frequency of the C haplotype in the sample RFA (Portugal) is likely to be due to some recent introductions of *C. gigas* in this oyster farming area.

**Microsatellite loci**

The observed level of polymorphism, shown by the number of alleles and the gene diversity (*He*), was higher than in most genetic studies using microsatellite markers (e.g. Hansen *et al*., 1999), although no population genetics study with such markers is yet available for marine bivalves. However, we reported, in a primer note, 49 microsatellite alleles at the L10 locus among 468 individuals analysed (Huvet *et al*., 2000) which is comparable to the present study. The high levels of polymorphism we observed are likely to be related to life history traits, which in most marine bivalves include large fecundity, external fertilisation and broad larval dispersal. Cupped oysters also have large effective population sizes.

However, a recent study on Pacific oysters (Li & Hedgecock, 1998) supported the hypothesis of large variance in reproductive success, with only a few adults contributing to the reproductive event, even in a large population. This can lead to genetic drift, reduction of variability and heterozygote deficiency. Heterozygote deficiencies have been seen in many marine bivalve studies based on allozyme markers (e.g. Singh & Green, 1984; Zouros & Foltz, 1984). The most frequently proposed explanations are inbreeding, Walhund effect, selection (Gaffney, 1994) and null alleles (Foltz, 1986). Microsatellite markers are supposedly neutral and do not induce bias in analyses of genetic structure between populations. Inbreeding is unlikely as it would be observed with all markers, which is not the case with our data (e.g. KEE population at locus CG108, Table 4) except with populations SEU and BOU. Wahlund effect would imply differentiated subgroups with limited gene flow within the populations. The estimated *Fis* can therefore be considered as a *Fst* value estimating the divergence between subgroups which are at the Hardy-Weinberg equilibrium. Here, the observed *Fst* values were very much lower than the mean *Fis*, which does not support this hypothesis. Lastly, heterozygote deficiencies might be expected in the case of mixed populations of

the two taxa, but results with the *mt*DNA marker showed a very limited mixture in a few of the populations studied. Even if each of these possible explanations could contribute to a part of the effect, the observed heterozygote deficiencies at microsatellite loci can best be explained by null alleles in our microsatellite data. Null alleles are not detected by PCR, because of a mutation in the homologous sequence of the primer or an artefact during amplification (Callen *et al*., 1993). In our data, a few individuals showed no amplification at one out of the three loci, for which they were probably null homozygous. In these cases, PCRs were repeated 3 times to verify that the results were not due to experimental error. Over all populations, the frequency of null alleles is very similar when estimated by the method described by Chakraborty *et al*. (1992) as by that of Brookfield (1996) which takes into account putative null homozygotes. Vieux-Boucau (BOU) displayed the highest number of presumed null homozygotes (5 individuals: 3 for the locus CG44 and 2 for CG49) and showed strong heterozygote deficiencies (Table 4). Null allele frequencies were 0.25 and 0.24, estimated by Chakraborty's and Brookfield's methods respectively. The agreement of these methods suggests that the number of putative null homozygotes is consistent with the observed heterozygote deficiency and therefore supports the hypothesis of null alleles. The best way to reduce or eliminate these null alleles would be by designing new primers (Hare *et al*., 1996).

The application of microsatellite markers to a population genetics study of cupped oysters has allowed us to examine the genetic differentiation between Portuguese and Pacific oysters. Microsatellite loci are the first nuclear markers revealing significant genetic differentiation, even if low, between populations of *C. angulata* and *C. gigas*. These results should be considered in the light of previous studies based on allozymes (Mathers *et al*., 1974; Buroker *et al*., 1979; Mattiucci & Villani, 1983). These authors concluded that the two taxa were very close genetically, but they did not quantify the genetic differentiation with an *Fst* analysis. In our study, the highest observed *Fst* value was around 4%. Life history traits and large population sizes could explain the genetic homogeneity of the geographically close populations. For more separated populations, human impact must be taken into account. The recent expansion of cultivated stocks of cupped oysters in numerous parts of the world (Chew, 1990) has led to extensive transplantation of oysters. The expected long term consequence would be higher genetic homogeneity of populations of *C. gigas*, which is by far the most important commercial oyster. Indeed, overall *Fst* values appeared significantly higher in *C. angulata* taxa than in *C. gigas* taxa indicating higher homogeneity and gene flow between populations of *C. gigas*.

**Comparison of *mt*DNA and microsatellite data**

Only a few recent articles have compared data based on both mitochondrial and microsatellite markers on the same samples. Some of these showed non-congruent data (e.g. Hansen *et al*., 1999), others very congruent data (e.g. Tessier *et al*., 1997), or data that was qualitatively but not quantitatively congruent. Bérubé *et al*. (1998), working on fin whales, noticed higher differentiation in the mitochondrial data (Mean *Fst* = 0.25) than in the nuclear data (mean *Fst* = 0.175), which could be explained by maternally directed site fidelity.

In our study, the patterns obtained with the *mt*DNA and microsatellite markers were qualitatively similar, as shown by the two trees drawn from the Reynold's distance matrix. One cluster grouped the French and Japanese populations, almost certainly the result of the massive importation of Japanese *C. gigas* into France in 1971 (Grizel & Héral 1991). Secondly, Spanish and Portuguese populations of *Crassostrea angulata* were grouped with the two Taiwanese populations. The results from both *mt*DNA and nuclear DNA therefore support the hypothesis that *C. angulata* was introduced from Taiwan to Portugal. *Mt*DNA markers from both taxa were detected in two populations and consequently these have an intermediate position in the neighbor-joining tree based on population divergences. The same trend is apparent in the neighbor-joining tree based on microsatellite genetic distances. In the Portuguese sample RFA, mainly composed of *C. angulata* haplotypes, two out of the four oysters carrying the *C. gigas* haplotype (C) showed a *C. angulata* specific microsatellite allele at the CG44 locus. This supports the hypothesis that hybridization, already shown with experimental crosses (reviewed by Gaffney and Allen, 1993), also occurs in the wild. Up until now, the contact between the two taxa appears to be restricted, as indicated by the *mt*DNA marker. However, further hybridization is expected in the future, because of the increasing transplantations and production of *C. gigas* in the southern Europe.

Even with very similar qualitative results from both genomes, our mean pairwise *Fst* values were around 0.5 and 0.017 respectively for the PCR-RFLP *mt*DNA and microsatellite markers. Biological reasons can partly explain these quantitative differences between the genomes. Because the mitochondrial genome is haploid and uniparentaly inherited (e.g. Li & Hedgecock, 1998), it is subject to twice as much genetic drift as the nuclear genome. This tends to increase the genetic differentiation between populations when measured with mitochondrial markers rather than nuclear markers. This difference could be emphasised when the sex-ratio is unbalanced in favour of males. As Pacific oysters are protandrous alternative hermaphrodites, observed sex-ratios are known to be highly variable. The proportion of males can be higher depending on age, season and even population size (Buroker, 1983). Behavioural influences are unlikely because of the sessile character of oysters.

Another explanation is that the observed number of microsatellite alleles was very high, due to high mutation rate and corresponding homoplasy. High mutation rate (Slatkin, 1995) and homoplasy lead to apparently low nuclear *Fst* values, as suggested by Shaw *et al*. (1999) for squid. Study of other genetic distance measures, as suggested by Takezaki & Nei (1996), might give a clearer understanding. We built another tree from *Dc* (Cavalli-Sforza & Edwards' (1967) chord distance) based on microsatellite markers but it did not reveal substantial differences from the trees based on Reynold's distances (data not shown). According to Ruzzante (1998), *Fst* is the most appropriate measure to estimate population structure from our samples (unequal sample sizes and mean number of individuals per population less than 50). Given the high allelic diversity (maximum of 40 alleles in 49 OGA samples), the sample size may appear low and analysis of more individuals per population would increase accuracy (Ruzzante, 1998). However, collection of samples was difficult and we could not obtain larger samples from some of the distant locations.

**Genetic impact of the introduction of *C. angulata* into Europe**

A bottleneck or founder effect is to be expected when a population undergoes a severe reduction in size. Few individuals will effectively contribute to reproduction and the next generation, and this will lead to the loss of uncommon alleles and to a decrease in heterozygosity. We would expect that the introduction of *C. angulata* from Taiwan to the coasts of Portugal involved a very small number of oysters. The genetic consequence would be a reduction of variability in the European populations of *C. angulata* compared with the Taiwanese populations. The observed genetic diversity, *He*, was however very close between these two groups of populations. The allele numbers were also very close, except in the Portuguese Rio Mira population (MIR) where the allele number is lower, 23% less at the locus CG108 and 30% less at the loci CG44 and CG49. This population is also monomorphic for the mitochondrial marker. This suggests the action of genetic drift in this population alone. However, the results as a whole suggest that it is more likely that there was a sufficient number of founder individuals imported into Portugal from Taiwan by merchant ships on one or several trips. This movement is presumed to have been unintentional with oysters stuck on the hulls of ships during a period when there was a large amount of commerce and sea traffic between Asia and Portugal (during the $16^{th}$ century). An initial population of a few dozen oysters, introduced into Portugal, could carry as many different alleles as were observed in our data. If these oysters increased in number quickly (due to high fecundity), then the introduced population could retain its original variation. In the same way, no evidence of a bottleneck effect was found in the French populations of *C. gigas* compared to the Japanese one. The same result was produced with allozyme markers (Moraga *et al*., 1989) and was expected because of the massive importation of *C. gigas* from Japan into France (Grizel & Héral, 1991).

## 5. Conclusion

This paper is the first report of a population genetics study on wild cupped oysters using microsatellite markers and demonstrates the usefulness of combining *mt*DNA and microsatellite analyses for addressing phylogeographic questions and concerns about genetic resource preservation.

The state of *C. angulata*, which sustained European oyster industry for more than one century, was poorly known. The recent identification of its Asian origin, based on mitochondrial DNA markers (O'Foighil *et al*. 1998, Boudry *et al*., 1998), meant that we needed to re-evaluate the status of European populations and the importance of their preservation. More information, including results from nuclear markers, was therefore needed to assess their genetic similarity to Asian "parental" populations. The present study shows that, based on microsatellite markers, European and Asian populations of *C. angulata* are genetically close and that any conservation plans for European *C. angulata* populations should take its non-native origin into account. Further studies are needed to document potential adaptive differences between European and Asian *C. angulata* populations. It has been recently shown that populations from different geographical origins have significant differences in oxygen consumption (Goulletquer

*et al*., 1999). Such differences could be of use for selective breeding programs in Europe without the zoosanitary risks associated with the introduction of exotic oysters (Berthe and Boudry, 1999). In addition, the genetic basis of these adaptive physiological traits could reveal stronger nuclear differentiation than the neutral markers examined here.

The increasing production of *C. gigas* in Southern Europe and the hybridization between the two taxa endanger the European populations of *C. angulata*. A fine-scale study with a diagnostic nuclear marker would confirm the natural hybridization and clarify the spatial organisation of these two taxa (e.g. hybrid zone, allopatric distribution) between France and Portugal or between Japan and Taiwan. This could allow us to identify the remaining "pure" *C. angulata* populations in Europe, as the basis for the development of a conservation program.

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**Figure legends**

**Figure 1.** Geographical locations of the 6 European populations.

**Figure 2.** Neighbor-joining tree of the 9 oyster samples based on *mt*DNA genetic distance (Reynolds, 1983) matrix.

**Figure 3.** Neighbor-joining tree of the 9 oyster samples based on microsatellite genetic distance (Reynolds, 1983) matrix. Numbers at nodes are bootstrap values above 50%.

**Table 1.** Locations and sample sizes (N) of the 9 populations of *Crassostrea gigas* and *Crassostrea angulata* sampled in Europe and Asia.

**Table 2.** Haplotype frequencies per population.

**Table 3**. Matrix of pairwise *Fst* values over all loci and statistical tests for microsatellite data (above main diagonal) and for *mt*DNA PCR-RFLP data (below main diagonal).

**Table 4.** Variability parameters for each population and microsatellite locus.

**Figure 1.** Geographical locations of the 6 European populations.

**Figure 2.** Neighbor-joining tree of the 9 oyster samples based on *mt*DNA genetic distance (Reynolds, 1983) matrix.

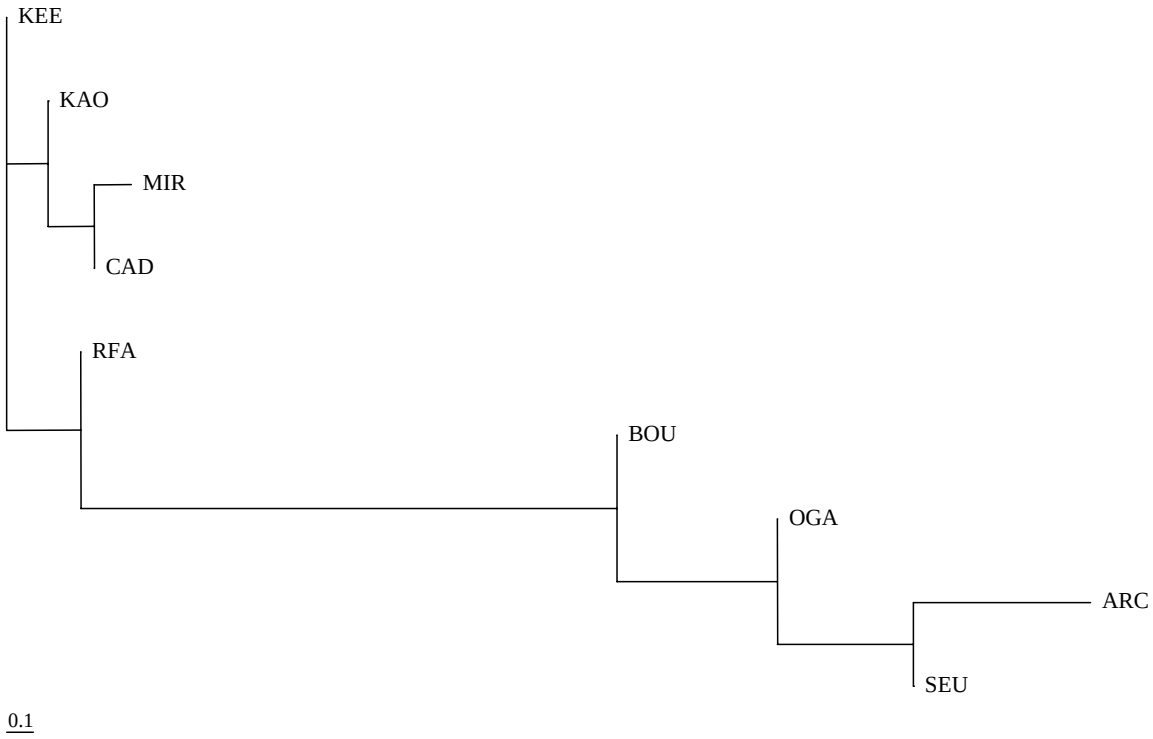


**Figure 3.** Neighbor-joining tree of the 9 oyster samples based on microsatellite genetic distances (Reynolds, 1983) matrix. Numbers at nodes are bootstrap values above 50%.

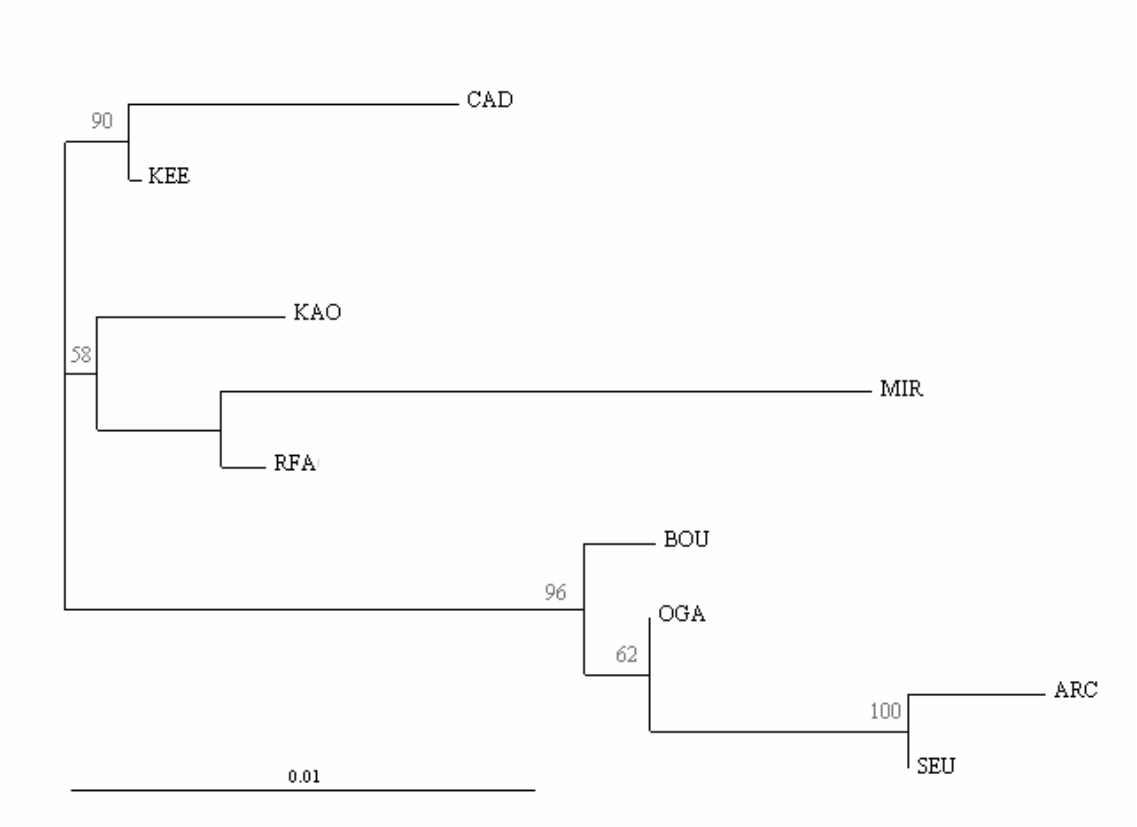

**Table 1.** Locations and sample sizes (N) of the 9 populations of *Crassostrea gigas* and *Crassostrea angulata* sampled in Europe and Asia.

| Origin (country) | Abbreviation | Previously determined (*) or presumed taxa | N (n**) |
|---|---|---|---|
| Seudre Estuary (France) | SEU | *C. gigas** | 49 (20) |
| Arcachon Bay (France) | ARC | *C. gigas** | 50 (15) |
| Vieux-Boucau Bay (France) | BOU | *C. gigas* | 49 |
| Hiroshima (Japon) | OGA | *C. gigas* | 49 |
| Tungkang (Taïwan) | KEE | *C. angulata* * | 30 (23) |
| Kaohsiung (Taïwan) | KAO | *C. angulata* | 40 |
| Ria Formosa (Portugal) | RFA | *C. angulata** | 35 (11) |
| Rio Mira Estuary (Portugal) | MIR | *C. angulata* | 30 |
| Cadiz (Spain) | CAD | *C. angulata** | 50 (26) |

(**) number of samples already analysed in Boudry *et al*. (1998) with the *mt*DNA marker allowing the taxonomic determination (*).

**Table 2.** Haplotype frequencies per population.

| | | | Haplotypes | | | | | |
|---|---|---|---|---|---|---|---|---|
| Country | Population | N | A | B | C | D | E | J |
| France | SEU | 49 | | | 0.98 | | 0.02 | |
| | ARC | 50 | | | 0.98 | | 0.02 | |
| | BOU | 49 | 0.08 | | 0.90 | | 0.02 | |
| Japon | OGA | 49 | | | 0.96 | | 0.04 | |
| Taiwan | KEE | 30 | 0.88 | 0.04 | | | | 0.08 |
| | KAO | 40 | 0.90 | 0.08 | | | | 0.02 |
| Portugal | RFA | 35 | 0.86 | 0.03 | 0.11 | | | |
| | MIR | 30 | 1 | | | | | |
| Spain | CAD | 50 | 0.96 | 0.02 | | 0.02 | | |

Haplotypes are denoted by capitals letters, assigned to each combination of restriction profiles for the four restriction enzymes *Taq*I, *Sau*3A, *Hha*I and *Mse*I (Boudry *et al*., 1998).

**Table 4.** Variability parameters for each population and microsatellite locus.

| | SEU | ARC | BOU | OGA | KEE | KAO | RFA | MIR | CAD |
|---|---|---|---|---|---|---|---|---|---|
| N | 49 | 50 | 49 | 49 | 30 | 40 | 35 | 30 | 50 |
| **CG44** | | | | | | | | | |
| *Nall* | 29 | 26 | 26 | 27 | 26 | 27 | 24 | 18 | 21 |
| *Ho* | 0.775 | 0.88 | 0.532 | 0.5 | 0.766 | 0.9 | 0.857 | 0.8 | 0.54 |
| *He* | 0.947 | 0.937 | 0.927 | 0.944 | 0.941 | 0.948 | 0.943 | 0.909 | 0.906 |
| *Fis* | 0.191 ** | 0.072 | 0.435 ** | 0.479 ** | 0.202 ** | 0.064 | 0.105 | 0.137 | 0.413 ** |
| **CG49** | | | | | | | | | |
| *Nall* | 33 | 32 | 38 | 37 | 26 | 27 | 25 | 17 | 33 |
| *Ho* | 0.659 | 0.837 | 0.574 | 0.917 | 0.666 | 0.763 | 0.412 | 0.9 | 0.96 |
| *He* | 0.949 | 0.936 | 0.964 | 0.956 | 0.942 | 0.95 | 0.939 | 0.893 | 0.957 |
| *Fis* | 0.315 ** | 0.116 | 0.413 ** | 0.051 | 0.308 ** | 0.210 ** | 0.572 ** | 0.009 | 0.007 |
| **CG108** | | | | | | | | | |
| *Nall* | 33 | 34 | 31 | 40 | 26 | 26 | 23 | 20 | 28 |
| *Ho* | 0.775 | 0.82 | 0.82 | 0.837 | 0.966 | 0.875 | 0.823 | 0.828 | 0.8 |
| *He* | 0.955 | 0.956 | 0.949 | 0.965 | 0.943 | 0.942 | 0.936 | 0.934 | 0.948 |
| *Fis* | 0.198 ** | 0.152 ** | 0.146 ** | 0.143 ** | -0.008 | 0.084 | 0.134 | 0.131 | 0.166 ** |

Sample sizes (N), number of alleles (*Nall*), observed heterozygosity (*Ho*), gene diversity (*He*), *Fis* (*F*-statistic of Wright, 1951) calculated according to Weir et Cockerham (1984), which represents an estimate of departure from Hardy-Weinberg proportions. The significance of the *Fis* values were estimated by generated permutations (1000) of individual genotypes within each population. ** Significant at the P<0.01 level.

**Table 3**. Matrix of pairwise *Fst* values over all loci and statistical tests for microsatellite data (above main diagonal) and for *mt*DNA PCR-RFLP data (below main diagonal).

| Population | N | RFA | CAD | MIR | KEE | KAO | BOU | ARC | SEU | OGA |
|---|---|---|---|---|---|---|---|---|---|---|
| RFA | 35 | 0 | 0.01504 ** | 0.0149 ** | 0.0054 | 0.00808 ** | 0.01454 ** | 0.02424 ** | 0.01869 ** | 0.01468** |
| CAD | 50 | 0.0411 | 0 | 0.02615 ** | 0.00735 | 0.01111 ** | 0.02012 ** | 0.03253 ** | 0.02679 ** | 0.02164** |
| MIR | 30 | 0.08194 | 0.00055 | 0 | 0.01727 ** | 0.02017 ** | 0.03107 ** | 0.04007 ** | 0.03733 ** | 0.02915** |
| KEE | 30 | 0.00538 | 0.02002 | 0.07727 | 0 | 0.00804 * | 0.01533 ** | 0.01857 ** | 0.01878 ** | 0.01061** |
| KAO | 40 | 0.013 | 0.00009 | 0.04244 | -0.0209 | 0 | 0.01798 ** | 0.02688 ** | 0.02117 ** | 0.01540** |
| BOU | 50 | 0.73993 ** | 0.84747 ** | 0.87565 ** | 0.78021 ** | 0.80014 ** | 0 | 0.01047 ** | 0.0061 * | 0.00311 |
| ARC | 50 | 0.87721 ** | 0.95743 ** | 0.9998 ** | 0.9206 ** | 0.91561 ** | 0.06306 | 0 | 0.0023 | 0.00577 |
| SEU | 49 | 0.8476 ** | 0.93453 ** | 0.97402 ** | 0.88968 ** | 0.89105 ** | 0.03152 | 0.00086 | 0 | 0.00540 |
| OGA | 49 | 0.82405 ** | 0.91444 ** | 0.95001 ** | 0.86427 ** | 0.87072 ** | 0.01689 | 0.02144 | -0.01428 | 0 |

The statistical significance of the observed values of *Fst* was tested by comparison with 1000 generated permutations of genotypes of populations. * significant at the P<0.05 level, ** significant at the P<0.01 level