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Natural hybridization between genetically differentiated populations of *Crassostrea gigas* and *C. angulata* highlighted by sequence variation in flanking regions of a microsatellite locus

A. Huvet[1,2], C. Fabioux[1,2], H. McCombie[1], S. Lapègue[1], P. Boudry[1,*]

[1]**Laboratoire de Génétique et Pathologie, IFREMER, 17390 La Tremblade, France**
[2]***Present address:*** **Laboratoire de Physiologie des Invertébrés, IFREMER, Centre de Brest, BP 70, 29280 Plouzané, France**

ABSTRACT: The marine environment is of special interest for studying hybridization between closely related taxa because of the high dispersal potential of planktonic larvae, such as those of most bivalve species. The oysters *Crassostrea angulata* and *C. gigas* are known to be very close genetically and entirely inter-fertile under controlled conditions. However, hybridization in the wild had not been investigated, mainly due to the lack of nuclear diagnostic markers. In the present paper, we first estimated genetic differentiation between these 2 closely related taxa using 8 microsatellite markers. Interestingly, 5 markers displayed significant differences of allele size between taxa. The subsequent sequencing of alleles of one of these microsatellites showed several mutational events, which suggested null alleles and homoplasy. The presence of 1 insertion/deletion event in its 5′ flanking sequence enabled us to design a new bi-allelic ('C' and 'NC') nuclear PCR-restriction fragment length polymorphism (-RFLP) marker (CG44R). This, together with a mitochondrial DNA marker, was used to analyze populations of *C. angulata* and *C. gigas*. The CG44R allele frequencies were very different between *C. angulata* (f[C] = 0.91) and *C. gigas* (f[NC] = 0.92) populations. This analysis also provided evidence for hybridization between *C. angulata* and *C. gigas* in a wild Portuguese population where the 2 taxa are in contact due to recent transportation of *C. gigas* stocks for aquacultural production. Our results represent the first indication of hybridization between these 2 taxa in the natural environment, and contribute to knowledge of the evolutionary history of the *Crassostrea* genus.

KEY WORDS: *Crassostrea angulata* · *Crassostrea gigas* · Hybridization · Genetic differentiation · Microsatellites · Allele size

INTRODUCTION

Natural hybridization consists of successful mating between individuals from populations which are otherwise distinguishable by 1 or more heritable characters (Arnold 1997). The spatial and temporal overlap of populations or their gametes, allowing hybridization to occur, is commonly known as a 'hybrid zone'. The marine environment is of special interest in the study of such processes because both individuals and gametes are thought to disperse over large distances (Hedgecock 1986, 1994, Palumbi & Wilson 1990, Bohonak 1999, but see Taylor & Hellberg 2003). In marine bivalves, several hybrid zones are well documented in clams (*Mercenaria* spp.: Bert et al. 1993) and mussels (*Mytilus* spp. complex: Koehn 1991, Inoue et al. 1997, Rawson et al. 1999, Bierne et al. 2002). In the Baltic mussel hybrid zone, asymmetric introgression and natural selection were suggested to explain discordance between allozyme and non-allozyme patterns (Riginos et al. 2002). In oysters, despite much experimental evidence of inter-specific crosses in the

*Corresponding author. Email: pboudry@ifremer.fr

genus *Crassostrea* (see for review Gaffney & Allen 1993), natural hybridization between species has never been reported in the wild (Hedgecock et al. 1999).

In the genus *Crassostrea*, *C. gigas* and *C. angulata* were first classified as 2 different species by Thunberg in 1793 and Lamark in 1819, respectively. This classification was chiefly due to their apparently separated geographical distribution. *C. angulata* was described in Europe and *C. gigas* in Asia. However, following morphological comparison (Ranson 1948), experimental hybridization (reviewed by Gaffney & Allen 1993, Huvet et al. 2001, 2002) and allozyme data (Mathers et al. 1974, Buroker et al. 1979, Mattiucci & Villani 1983), the authors concluded that there was only a single species grouping Portuguese and Pacific oysters. However, significant phenotypic differences between the 2 taxa were observed. *C. gigas* shows a superior production yield in the natural environment in France (Bougrier et al. 1986, Héral 1986, Parache 1989, Soletchnick et al. 2002), as its growth is twice that of the Portuguese oyster (Bougrier et al. 1986). Differences were also shown in terms of their ecophysiological characteristics (His 1972, Goulletquer et al. 1999, Haure et al. 2003). Furthermore, genetic differences have now been observed on several levels. Karyotype analyses highlighted the close genetic similarity of these 2 taxa in comparison with other cupped oyster species (Leitão et al. 1999a), although differences between *C. angulata* and *C. gigas* were observed, notably for Chromosome 7 (Leitão et al. 1999b). Two studies on the mitochondrial Cytochrome Oxidase Subunit I (COI) gene (Boudry et al. 1998, O'Foighil et al. 1998) demonstrated a clear genetic difference (nucleotide divergence of 5.26%) between the 2 taxa. Finally, a recent microsatellite analysis of cupped oyster populations of *C. gigas* and *C. angulata* revealed a low but significant genetic differentiation between populations of the different taxa (mean Wright's fixation index; F_{st} = 0.022; Huvet et al. 2000b). This differentiation was twice as large as the genetic differentiation estimated between geographically separated populations of the same taxon (Mean F_{st} = 0.01; Huvet et al. 2000b). These last 3 studies also offered an explanation for the separated geographical distribution of the taxa by supporting the hypothesis of the introduction of *C. angulata* from Asia (and more precisely Taiwan) to the Portuguese coast by merchant ships during the 16th century. For *C. gigas*, voluntary introduction from Japan into Europe was made in the early 1970s to replace *C. angulata* in the shellfish industry (Grizel & Héral 1991) because of an iridoviral infection (Comps 1969) which wiped out *C. angulata* from French coasts between 1967 and 1972. Based on these analyses, there might be 2 putative contact zones, 1 between France and the south of Portugal, where 'naturalized' *C. gigas* and *C. angulata* populations have been described, and a second between Japan and Taiwan. However, despite the absence of reproductive barriers under controlled conditions (Huvet et al. 2001, 2002), natural hybridization in these contact zones remain to be investigated. In the mitochondrial (mt) DNA study (Boudry et al. 1998), a diagnostic mtDNA marker was identified at the population level but is unsuited to the study of hybrids and hybridization processes because of its haploid nature and maternal transmission (Boursot & Bonhomme 1986). A nuclear diagnostic marker that would easily distinguish the taxa and their hybrids was therefore needed to study this phenomenon.

In the present paper, we report the analysis of 8 microsatellite loci on samples of the 2 closely related taxa, *C. gigas* and *C. angulata*, leading to the design of a new nuclear PCR-restriction fragment length polymorphism (-RFLP) marker in the 5′-flanking sequence of one of these microsatellites. This nuclear marker allowed us to study hybridization between *C. angulata* and *C. gigas* in the wild along the Atlantic European coast. The genetic differentiation between wild populations of both taxa, estimated with a mitochondrial marker as opposed to a nuclear marker, is compared and discussed in terms of evolutionary history of the 2 cupped oyster taxa.

MATERIALS AND METHODS

Biological material. Fifteen populations of cupped oysters were collected from a large area along the European Atlantic and Asian Pacific coasts (Table 1). In total, 12 European populations and 3 Asian populations (corresponding to 653 individuals) were sampled, of which 9 (Oga, Seu, Arc, Bou, Mir, Rfa, Cad, Kee and Kao; see Table 1 for abbreviations) had been previously identified as *Crassostrea gigas* (Japan, France) or *C. angulata* (Taiwan, South of Spain and Portugal) using a mitochondrial marker (Boudry et al. 1998, Huvet et al. 2000b). Samples of mantle or gills were preserved in 95% ethanol. DNA extraction was performed by the phenol/chloroform method as described in Moore (1993).

Microsatellite analysis. In total, 8 dinucleotide microsatellite loci (CG44, CG49, CG108, L8, L10, L16, L48 and µsatAMY) were analyzed in 186 individuals (93 per taxa) sampled from 3 *C. gigas* (Oga, Seu, Arc) and 4 *Crassostrea angulata* (Mir, Cad, Kee, Kao) populations (Table 1), as previously determined based on a mtDNA marker (Huvet et al. 2000b). The last 2 previously studied populations (Bou and Rfa) were not considered at this step because they presented haplotypes from both taxa. Three of the markers had previously been used to analyze these samples for genetic differ-

entiation (CG44, CG49, CG108 in Huvet et al. 2000b). Primer sequences were available in Magoulas et al. (1998) for CG44, CG49, CG108, in Huvet et al. (2000a) for L8, L10, L16, L48 and in Sellos et al. (2003) for µsatAMY locus. Radioactive PCR amplifications were carried out in a total volume of 10 µl using 10 ng of oyster genomic DNA. The concentrations of the reaction components were as follows: 1× *Taq* buffer, 0.2 mM dNTPs, 0.14 µM forward primer, 3 mM $MgCl_2$ for CG44, 2 mM for CG49, 1.5 mM for L48 or 1 mM for other loci, and 0.017 U *Taq* polymerase. The reverse primer of each pair (0.23 µM) was end-labelled with 1 µCi γ33P, 1X kinase buffer and 0.6 U kinase for 30 min 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, 55°C for CG108, L10 and µsatAMY, 57°C for L8 and L16 or 60°C for L48, and 1 min extension at 72°C. PCR products were separated on 6% denaturating polyacrylamide gels for 3 to 5 h at 50 W (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 to 48 h. Alleles were numbered according to their size (given in base pairs; bp).

Allele sequencing. After their separation on polyacrylamide gel, 2 different alleles of the CG44 locus were extracted from the gel, and re-amplified by PCR under the same conditions as described above (carried out in a total volume of 50 µl). These single DNA fragments were cloned into the plasmidic vector pCR II (Invitrogen) and then manually sequenced using an oligonucleotide tailing kit (Boehringer-Mannheim) and γ33P radiolabeled dNTPs.

PCR-RFLP analysis. Twelve European and 3 Asian samples of *Crassostrea angulata* and *C. gigas* populations (corresponding to 653 individuals) were analyzed with 2 PCR-RFLP markers: a nuclear DNA marker and the mtDNA COI marker. For this mitochondrial marker, 9 populations had previously been analyzed (Boudry et al. 1998, Huvet et al. 2000b; Table 1).

Nuclear DNA RFLP. The CG44 microsatellite marker was amplified as described above and then digested using the *Bsr*GI endonuclease. The restriction reaction was performed for 2 h at 37°C in a 20 µl volume composed of 10 µl PCR product, 2 µl 10× reaction buffer and 7 U *Bsr*GI enzyme. The digested samples were then separated by electrophoresis on 1% agarose gels in 1× TAE buffer (Tris-acetic acid-EDTA), followed by staining with ethidium bromide. Visualization was carried out under UV light and recorded on a computer linked to a video camera.

mtDNA RFLPs. A 710 bp segment of the COI mitochondrial gene was amplified by PCR as described in Boudry et al. (1998). As previously reported (Boudry et al. 1998, Huvet et al. 2000b), 4 endonucleases (*Taq*I, *Sau*3A, *Hha*I and *Mse*I) were polymorphic and revealed 6 different haplotypes of which 4 appeared to be specific to *Crassostrea angulata* populations (Haplotypes A, B, D and J) and 2 to *C. gigas* (Haplotypes C and E). Separation and visualization were achieved in the same manner as for the nuclear PCR-RFLP. The haplotype network is provided in Boudry et al. (1998), and a phylogenetic tree, based on DNA sequence data of these haplotypes, is presented in Boudry et al. (2003).

Data analysis. All the statistical analyses of size of microsatellite alleles were carried out by analysis of variance using SYSTAT 9.0 (Wilkinson 1990) under the general linear model procedure. Statistical comparison of the number of microsatellite alleles between taxa was made using a chi-square test.

Genetic differentiation was estimated between the *Crassostrea gigas* and *C. angulata* samples, each consisting of 93 individuals. Two estimators of genetic differentiation were obtained. Firstly, *F*-statistics, as described by

Table 1. *Crassostrea gigas* and *C. angulata.* Location and sample size (N) of the 15 populations sampled in Europe and Asia. All samples were analyzed with the 2 PCR-restriction fragment length polymorphism (-RFLP) markers: the CG44R marker and the mitochondrial (mt)DNA Cytochrome Oxidase Subunit I (COI) marker. Number of individuals analyzed with the 8 microsatellite markers is given in parentheses (n)

Origin (country)	Abbreviation N (n)	Previously determined or presumed taxa	N (n[a])
Hiroshima (Japan)	Oga	*C. gigas*[a]	49 (31)
Seudre Estuary (France)	Seu	*C. gigas*[a]	49 (31)
Arcachon Bay (France)	Arc	*C. gigas*[a]	50 (31)
Vieux-Boucau Bay (France)	Bou	*C. gigas*[a]	49
Orio (Spain)	Ori	*C. gigas*	50
Ribadesella (Spain)	Rib	*C. gigas*	50
Vicedo, Rio del Barquero (Spain)	Vic	*C. gigas*	50
Setubal (Portugal)	Set1	*C. angulata*	50
Rio Mira Estuary (Portugal)	Mir	*C. angulata*[a]	30 (24)
Tavira (Portugal)	Tar	*C. angulata*	50
Ria Formosa, Faro (Portugal)	Rfa	*C. angulata*[a]	35
Guadalquivir, Sanlucar de Barrameda (Spain)	Gua	*C. angulata*	50
Cadiz (Spain)	Cad	*C. angulata*[a]	21 (21)
Tungkang (Taiwan)	Kee	*C. angulata*[a]	30 (20)
Kaohsiung (Taiwan)	Kao	*C. angulata*[a]	40 (28)

[a]mtDNA marker allowing taxonomic determination (Boudry et al. 1998, Huvet et al. 2000b)

Wright (1951), were calculated according to Weir & Cockerham (1984) using the package GENETIX Version 4.01 for computations and tests (Belkhir et al. 1996–2001). Secondly, the R_{st} parameter (Slatkin 1995a), which takes into account allele size, was computed and tested using the R_{ST} Calculation package Version 2.1 (Goodman 1997). Unbiased estimates and SDs of heterozygosity at microsatellite loci and the CG44R locus were calculated according to the parameter F_{is} (Wright's inbreeding coefficient). Deviations from Hardy-Weinberg expectations were tested in each sample by generating 50 000 permutations. Sequential Bonferroni adjustment of the p-values (Rice 1989) was used to correct tests of F_{is}.

The nucleo-cytoplasmic linkage disequilibrium was estimated from the Linkdisk algorithm (Black & Krafsur 1985). These computations and tests were done using the package GENETIX Version 4.01.

A test for deviation from selective neutrality was realized for the microsatellite data using Arlequin (Schneider et al. 2000) with the Chakraborty's test of population amalgamation procedure (Chakraborty 1990). This test compares the number of alleles in a sample with the number expected in a stationary population at mutation-drift equilibrium. It is based on the infinite allele mutation model.

RESULTS

Microsatellite genotyping: allele size variation and population differentiation

Individuals were genotyped (n = 186, 93 for each taxon) at 5 microsatellite loci, which enlarged the existing data already produced with 3 other loci (Huvet et al. 2000b). The total number of alleles per locus was very high, ranging from 36 to 52 (mean number = 45, Table 2). The number of alleles present in each taxon was not significantly different for any locus or over all loci (0.1% level). Over all loci, the mean allele size was not significantly different between *Crassostrea gigas* and *C. angulata* either. However, 2 loci, L8 and L48, showed a significantly higher mean allele size (4 bp difference) in *C. gigas* samples than in *C. angulata* samples (Table 2). Conversely, 3 other loci, CG44, CG49 and CG108, showed significantly higher mean allele size in *C. angulata* samples than in *C. gigas* samples (Table 2). The greatest difference in size, 21 bp, was noted at the CG44 locus.

From these 8 microsatellite loci, the genetic differentiation between the taxa was estimated with F_{st} and R_{st} parameters, which gave 0.022 for the F_{st} estimate and 0.15 for the R_{st} estimate (12.5 times higher than F_{st}). Both values were significantly different from 0 (at the 0.1% level). Hardy-Weinberg expected heterozygosity and observed heterozygosity ranged from 0.32 (L8, OGA population) to 1 (CG108, KEE population) and from 0.68 (μsatAMY, Mir population) to 0.98 (L48, Kao population), respectively. Half of the F_{is} values, ranging from −0.05 to 0.67, were found to be significant after Bonferroni correction (27 values out of the 56 tested, Table 3) indicating significant heterozygote deficiencies. One locus, L16, showed significant heterozygote deficiencies in all populations. The F_{st} estimate was re-estimated without this locus due to its consistent heterozygote deficiencies across all populations. The result was 0.024 instead of the 0.022 (described above).

The test for deviation from selective neutrality showed non-significant values ($p > 0.05$), apart from the microsatellite amylase (μsatAMY) result in the Mir population, which appeared significant at the 5% level ($p = 0.0476$).

Characterization of CG44R alleles in the 2 taxa

Because the greatest difference in mean size, 21 bp, was at the CG44 locus (Table 2), we cloned and sequenced 2 CG44 alleles: a large allele (266 bp) from a

Table 2. *Crassostrea gigas* and *C. angulata.* Total allele number and allele number in each taxon, mean size of microsatellite alleles in each taxon (Tg in *C. gigas* and Ta in *C. angulata*) and ANOVA for the 8 microsatellite loci analyzed in 93 oysters of each taxon. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

	CG44	CG49	CG108	L8	L10	L16	L48	μsatAMY
Dinucleotide repeats	CA	CA	CA	AG	AG	AG	GA	TC
Total allele number	46	52	46	41	48	41	51	36
Number of *C. gigas* alleles	32	41	41	35	40	34	42	33
Number of *C. angulata* alleles	36	41	34	36	40	34	44	31
Tg (bp)	256.5 ± 16.25	151.1 ± 11.48	143.4 ± 9.86	229.8 ± 17.5	150.8 ± 17.95	165.6 ± 17.35	130.3 ± 15.61	233.4 ± 13.64
Ta (bp)	277.5 ± 15.78	162.6 ± 9.75	151.3 ± 7.37	225.4 ± 19.7	149.2 ± 21.25	163.6 ± 16.76	126 ± 15.47	236.1 ± 13.76
(Ta – Tg) (bp)	21	11.5	7.9	−4.4	−1.6	−2	−4.3	2.7
p	<0.001***	<0.001***	<0.001***	0.03*	0.45	0.28	0.008**	0.06

Table 3. *Crassostrea gigas* and *C. angulata.* Observed (H_o) and expected (H_e) heterozygosity and F_{is} (Wright's inbreeding coefficient) values for each analyzed population and microsatellite locus. F_{is} (*F*-statistic of Wright 1951) was calculated according to Weir & Cockerham (1984). The significance of the F_{is} values was estimated by generated permutations (50 000) of individual genotypes within each population. *p < 0.05. Significant values after sequential Bonferroni adjustment at the p < 0.05 level are indicated in **bold**. See Table 1 for population abbreviations

		Oga	Seu	Arc	Mir	Cad	Kee	Kao
CG44	H_o	0.43	0.81	0.9	0.79	0.57	0.68	0.93
	H_e	0.96	0.95	0.96	0.92	0.9	0.95	0.96
	F_{is}	**0.56***	0.15*	0.05	0.15*	**0.37***	**0.29***	0.03
CG49	H_o	0.97	0.62	0.81	0.92	0.9	0.63	0.7
	H_e	0.97	0.97	0.95	0.91	0.96	0.95	0.96
	F_{is}	0.004	**0.36***	0.15*	−0.01	0.063	**0.34***	**0.27***
CG108	H_o	0.87	0.74	0.9	0.82	0.76	1	0.89
	H_e	0.96	0.97	0.96	0.96	0.95	0.96	0.96
	F_{is}	0.1*	**0.24***	0.06	0.14*	0.2*	−0.05	0.07*
L8	H_o	0.32	0.43	0.71	0.71	0.6	0.5	0.5
	H_e	0.95	0.95	0.93	0.96	0.86	0.92	0.88
	F_{is}	**0.67***	**0.54***	**0.23***	**0.26***	0.31*	**0.46***	**0.44***
L10	H_o	0.68	0.9	0.97	0.71	0.85	0.89	0.93
	H_e	0.96	0.97	0.96	0.95	0.97	0.97	0.97
	F_{is}	**0.3***	0.07	−0.007	**0.25***	0.13*	0.08*	0.05*
L16	H_o	0.63	0.68	0.63	0.54	0.8	0.7	0.52
	H_e	0.95	0.96	0.96	0.95	0.96	0.94	0.96
	F_{is}	**0.35***	**0.3***	**0.34***	**0.43***	**0.17***	**0.27***	**0.45***
L48	H_o	0.87	0.87	0.84	0.96	0.57	0.85	0.79
	H_e	0.96	0.97	0.97	0.92	0.94	0.96	0.98
	F_{is}	0.1*	0.11*	**0.14***	−0.05	**0.39***	0.11*	**0.2***
µsatAMY	H_o	0.84	0.87	0.71	0.58	0.82	0.95	0.81
	H_e	0.96	0.97	0.94	0.68	0.93	0.95	0.94
	F_{is}	**0.13***	0.1*	**0.25***	0.14*	0.13*	0.003	0.13*

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1     GTGAATTTAACTTCCTGCGTTAAGGAAGCTGGATGATCAACAAATTAACCGTCAGATATAC
      |||||||||||||||| |||||||||||||||||||||||||||| ||||||||||||||
1     GTGAATTTAACTTCCTGTGTTAAGGAAGCTGGATGATCAACAAATCAACCGTCAGATATAC

62    CCCATCATATTTTTGGCTATAAACTGTCATTT.....................ATATACA
      |||||||||||||||||||||||||||||||                     || ||||
62    CCCATCATATTTTTGGCTATAAACTGTCATTTGGTAAAGCCAAATATGTGTACATGTACA

101   TGTATACACACACACACACACACACACACACACA.....................
      |||||||||||||||||||||||||||||||||
122   TGTATACACACACACACACACACACACACACACACACACACACACACATTGTCCTATACC

135   ...............................CTGTCCTTCCATTACTCTATAAACAACGT
                                     || |      ||||||||||||||||| |
182   AGACATCTTTATCAAACGAACTATACAACATCTCTATACAAATTACTCTATAAACAACAT

164   GTCTGTTAGTCAATTATGATAAAAA
      |||||||||||||||||||||||||
242   GTCTGTTAGTCAATTATGATAAAAA
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Fig. 1. *Crassostrea gigas* and *C. angulata.* Alignment of 2 CG44 microsatellite allele sequences (above: 188 bp allele from a *C. gigas* individual; below: 266 bp allele from a *C. angulata* individual). The dinucleotide repeat is framed and the 21 bp insertion is underlined. The restriction site of *Bsr*GI in the first insertion is in **bold**

Crassostrea angulata individual and a small one (188 bp) from a *C. gigas* individual. The sequences of both alleles (GenBank Accession Nos. AF527994 and AF527995) are presented in Fig. 1. Their alignment revealed 11 substitutions, 2 insertions (of 21 and 43 bp), located in both parts of the microsatellite repeat, and a difference of 7 repeated elements (CA). These 2 insertions and the 7 additional repeated elements were present in the large 266 bp *C. angulata* allele, explaining the 78 bp difference between the alleles sequenced. It was not possible to examine the presence/absence of the 43 bp insertion by RFLP because no restriction site was observed, or by specific PCR amplification because of the appreciable redundancy of some interrupted motifs in the 3′-flanking region. However, because the 21 bp insertion contained a *Bsr*GI restriction site, it was possible to seek this insertion by a PCR-RFLP method (PCR amplification with the CG44 primers and

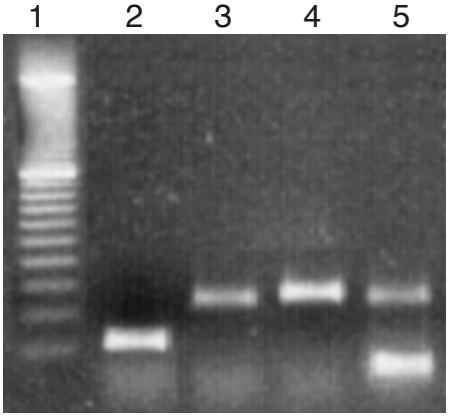

Fig. 2. *Crassostrea gigas* and *C. angulata.* Electrophoresis of digestion products of the CG44R marker from a set of *C. gigas* and *C. angulata*. Lane 1: 1 kb ladder; Lanes 2 to 5: Kao 25 (C/C); Oga 34 (NC/NC); Oga 35 (NC/NC), Tar 50 (C/NC) (see Table 1 for population abbreviations). Genotypes are given between brackets; C and NC correspond to the cut and non-cut alleles, respectively

digestion with the *Bsr*GI endonuclease). The results (obtained on the same 2 sets of 93 oysters per taxon) showed a high frequency of this 21 bp insertion (represented by the cut [C] allele) in the *C. angulata* sample (91%), whereas in the *C. gigas* sample its frequency did not exceed 8% (92% of the non-cut [NC] allele) (Fig. 2). This newly designed marker for analyzing the presence/absence of the 21 bp insertion in the CG44 microsatellite flanking region by restriction with *Bsr*GI was named CG44R. It provided a suitable new tool for the study of hybridization between the 2 taxa.

Hybridization in European populations

Hardy-Weinberg expected heterozygosity at the CG44R locus (Table 4) ranged from 0.032 (Orio) to 0.344 (Tavira). The mean observed heterozygosity, H_o, was equal to 0.149 over all the populations, ranging from 0 (Ribadesella) to 0.304 (Tavira). F_{is} values (departure from Hardy-Weinberg equilibrium) ranged from –0.13 (Kaohsiung) to 1 (Ribadesella) with a mean value over all populations equal to 0.21. Five values of F_{is} out of the 15 tested were found to be significant after Bonferroni correction (Table 4), indicating heterozygote deficiencies in Hiroshima, Seudre, Ribedesella, Ria Formosa and Tungkang populations. The mean F_{st} value between pairs of populations of different taxa was 0.71 for the CG44R locus.

Following the results of Boudry et al. (1998) and Huvet et al. (2000b), who observed haplotypes of the COI marker specific to *Crassostrea gigas* and *C. angulata* populations, the 2 main haplotype clusters are reported with their allele frequencies in Fig. 3 for all

Table 4. *Crassostrea gigas* and *C. angulata.* Non-Cut allele frequency (NC freq) after *Bsr*GI digestion, observed (H_o) and expected (H_e) heterozygosity, and F_{is} values in each population for the CG44R locus. F_{is} (*F*-statistic of Wright 1951) was calculated according to Weir & Cockerham (1984). The significance of the F_{is} values was estimated by generated permutations (50 000) of individual genotypes within each population; *p < 0.05. Significant values after sequential Bonferroni adjustment at the p < 0.05 level are indicated in **bold**. See Table 1 for population abbreviations

Population	N	NC freq	H_o	H_e	F_{is}
Oga	49	0.867	0.133	0.234	**0.432***
Seu	49	0.895	0.105	0.191	**0.452***
Arc	50	0.903	0.194	0.178	–0.091
Bou	49	0.907	0.140	0.171	0.184*
Ori	50	0.984	0.032	0.032	0.000
Rib	50	0.889	0.000	0.199	**1.000***
Vic	50	0.893	0.167	0.194	0.141
Set1	50	0.071	0.143	0.134	–0.067
Mir	30	0.083	0.167	0.155	–0.074
Tar	50	0.217	0.304	0.344	0.116
Rfa	35	0.145	0.132	0.251	**0.479***
Gua	50	0.122	0.204	0.217	0.061
Cad	21	0.111	0.222	0.209	–0.067
Kee	30	0.068	0.046	0.130	**0.656***
Kao	40	0.125	0.250	0.222	–0.130

the populations in the present study. Two geographical groups were distinguished based on the allele frequencies at the COI and CG44R markers. The populations from the French and northern Spanish Atlantic coasts were characterized by high frequencies of *C. gigas* haplotypes. Its frequency was equal to 1, except in the Vieux-Boucau and Ribadesella populations where a few *C. angulata* haplotypes were observed (f = 0.08 and 0.05, respectively). In French and north Spanish populations, the CG44R marker showed high frequencies of the NC allele: from 0.89 (Seudre, Ribadesella and Vicedo) to 0.98 (Orio, Table 4). Similarly, the Japanese *C. gigas* population was fixed for the *C. gigas* haplotypes and showed a high frequency of the NC allele (0.87, Table 4). The second geographic group, composed of southern Spanish and Portuguese populations, was characterized by high frequencies of *C. angulata* haplotypes (f = 1 for all populations, except for Ria Formosa: f = 0.89) and high frequencies of the C allele at CG44R (from 0.86 to 0.93 for Ria Formosa and Setubal populations, respectively; Table 4). The Tavira population displayed the highest mix of specific mtDNA haplotypes (f = 0.18 and 0.82 for *C. gigas* and *C. angulata* haplotypes, respectively) and CG44R alleles (f[NC] = 0.22 and f[C] = 0.78). Finally, the 2 Taiwanese populations revealed only *C. angulata* haplotypes and high frequencies of the C allele at the CG44R locus (f = 0.88 and 0.93 for Kaohsiung and Tungkang, respectively).

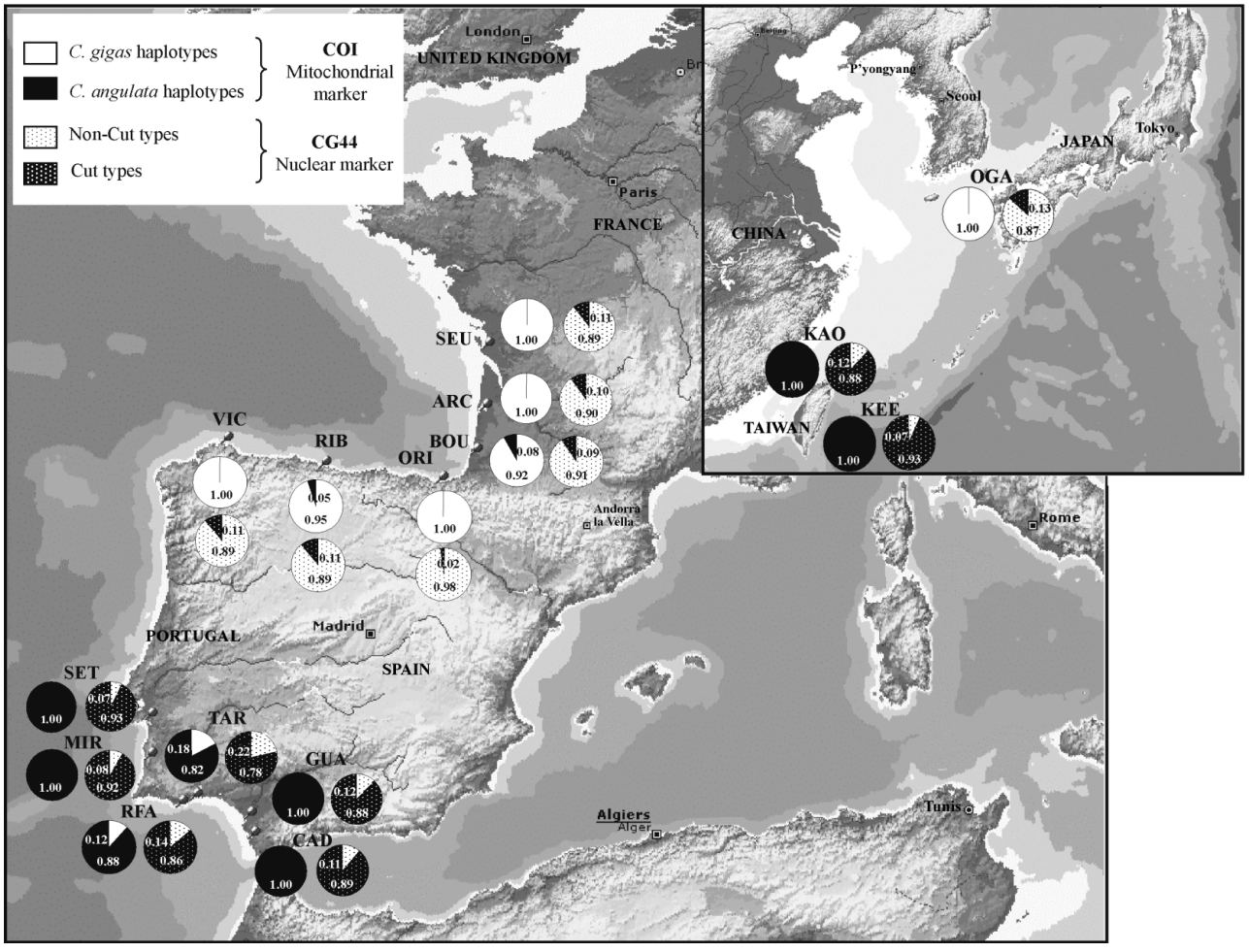

Fig. 3. *Crassostrea gigas* and *C. angulata.* Allelic frequencies per population (see Table 1 for population abbreviations)for the 2 PCR-restriction fragment length polymorphism (-RFLP) markers (mitochondrial Cytochrome Oxidase Subunit I [COI] and nuclear CG44R markers). Left or upper pie charts: COI results with 2 taxa-specific haplotype clusters, *C. gigas* in white and *C. angulata* in black, grouping all observed haplotypes (according to Boudry et al. 1998 and Huvet et al. 2000b); right or lower pie charts: CG44R results with the NC allele associated with *C. gigas* in white with black dots and the C allele associated with *C. angulata* in black with white dots

DISCUSSION

Mutation processes at a microsatellite marker and estimation of genetic differentiation between *Crassostrea angulata* and *C. gigas*

Because of the high variability of microsatellite loci, they are presently one of the main tools in population genetics, paternity analysis and gene mapping. However, many recent inter- or intra-specific studies have demonstrated the complexity of mutational events occurring in these DNA sequences (Angers & Bernatchez 1997, Grimaldi & Crouau-Roy 1997, Steinkellner et al. 1997, Anderson et al. 2000). Inter-specific analyses have often revealed size differences in microsatellite alleles between close species (Deka et al. 1995, Ellegren et al. 1995, Forbes et al. 1995, Rubinsztein et al. 1995a,b, Angers & Bernatchez 1997). Possible explanations may be: (1) difference in mutation rates between close species (Rubinsztein et al. 1995a,b, Cooper et al. 1998), (2) bias in the screening of microsatellites (Ellegren et al. 1995, 1887, Forbes et al. 1995, Pépin et al. 1995, Watanabe et al. 1996, but see also Cooper et al. 1998) and (3) variations in the 5′- and 3′-flanking regions (Angers & Bernatchez 1997, Steinkellner et al. 1997, Anderson et al. 2000). In our results, allele size differences were observed between *Crassostrea gigas* and *C. angulata* at some microsatellite loci but not in a unidirectional manner. Screening bias, detected in some previous studies (see Explanation 2, above), does not appear to be the reason for these size differences, because number and size of our microsatellite alleles was not consistently higher in *C. gigas* (the taxon where these microsatellite loci were

originally screened and isolated) than in *C. angulata*. Consequently, taxa-specific mutational processes or screening biases are unlikely to provide the reason why size differences were observed between *C. angulata* and *C. gigas* at some microsatellite markers. Alternatively, the sequence of 2 microsatellite alleles for the locus CG44 showed that mutation rate was not only very high for the number of dinucleotide repeats, but also in the flanking regions where 2 insertions and many point mutations were characterized. It would not be surprising if such mutations occurred in the primer sequences and consequently imply null alleles. Furthermore, incongruence between size and number of mutation events in microsatellite loci (Angers & Bernatchez 1997) can lead to homoplasy (Grimaldi & Crouau-Roy 1997). Both phenomena were suggested in a previous microsatellite study of *C. angulata* and *C. gigas* populations (Huvet et al. 2000b). Hypotheses of null alleles and homoplasy are therefore supported by the present work, and could explain the significant heterozygote deficiencies observed in *C. angulata* and *C. gigas* populations (48% significant values of F_{is}; Table 3). Among the hypotheses discussed in Huvet et al. (2000b) to explain these heterozygote deficiencies (inbreeding, Walhund effect, selection, null alleles), null alleles appeared to be the best explanation for most of the effect. Null alleles are not detected by PCR because of a mutation in the homologous sequence of the primer (Callen et al. 1993) or an artefact during amplification, such as allelic dropout (heterozygotes for 2 alleles of very different sizes scored as homozygotes for the smaller one due to less efficient amplification of the larger allele). As in our first study (Huvet et al. 2000b), the existence of null alleles is also consistent with the observation of few putative null homozygotes (individuals which showed no amplification at one of the analyzed loci) in the populations showing the strongest heterozygote deficiencies (for example OGA: 3 individuals for CG44 and 2 for L8 and L16 loci). The possibility that natural selection has driven the pattern we observe is unlikely because the results of our test for deviation from selective neutrality indicated that the microsatellites behave in a neutral manner. However, the μsatAMY marker showed a significant p-value, suggesting deviation from selective neutrality. This was consistent with the strong reduction in number of alleles (10) and the very high frequency of 1 allele ($f = 0.54$) observed in the Rio Mira population, compared to those observed in other populations at this marker (mean number of alleles = 20 and $f_{max} = 0.14$). This marker is located in an intron of the amylase Gene A and its neutrality could be affected by linkage disequilibrium of linked polymorphic exons (see Slatkin 1995b, Sellos et al. 2003). Further studies are needed to determine whether the amylase gene could be under direct selective pressure due to its digestive function and whether the μsatAMY marker could be useful for breeding programs.

Such complex mutational processes suggest that allele coalescence cannot be easily inferred from size information, and could consequently induce bias in our estimations of genetic differentiation between populations of closely related oyster taxa, as suggested by Angers & Bernatchez (1997). The genetic differentiation appeared to be 10 times higher between *Crassostrea gigas* and *C. angulata* populations when estimated using the R_{st} parameter compared to F_{st}. Whereas F_{st} estimation is based on allelic identity, R_{st} estimation is based on allelic size, which theoretically depends on number of mutation events in microsatellite motifs. However, in our data, allele sizes were likely to be strongly affected by insertion/deletion events in flanking regions, and R_{st} might consequently be overestimated. The presence of null alleles biases the differentiation pattern observed by F_{st}, but not strongly because its estimation between taxa was no different across all loci, or even without the L16 locus, for which consistent heterozygote deficiencies were observed in all populations.

For the CG44 locus, the 21 bp insertion/deletion fragment was found in most *Crassostrea angulata* individuals and was absent in most *C. gigas*. This, together with the exact correspondence of the size of this fragment (21 bp) with the mean allele size difference between the taxa (21 bp), suggests that this insertion is likely to be the cause of the observed size shift between *C. gigas* and *C. angulata* alleles.

Natural hybridization between *Crassostrea angulata* and *C. gigas* in Europe

The *F*-statistic values reported here were considered with caution in the study hybridization between the 2 taxa. Because F_{st} is a measure of genetic variation among populations relative to the total variation sampled, the within-population variation might therefore be downwardly biased compared to variability between populations. However, in the case of mixed populations without hybridization, F_{is} values can be greatly enhanced, showing strong heterozygote deficiencies (Wahlund effect). A clear difference in allele frequency was observed between *Crassostrea angulata* and *C. gigas* populations with the newly designed CG44R nuclear marker, whose polymorphism reveals the presence or absence of the 21 bp insertion. The CG44R alleles are close to being taxon-specific, with allelic frequencies of 0.1/0.9 (NC/C) for pure *C. angulata* populations and 0.9/0.1 for pure *C. gigas* populations (taxa previously determined with the mitochon-

drial marker). This frequency distinction, plus its co-dominance (i.e. the ability to distinguish heterozygote from homozygote individuals), makes CG44R suitable for searching directly for natural hybridization processes between *C. gigas* and *C. angulata*.

Results from CG44R and mtDNA markers displayed 2 geographical zones along the European Atlantic coasts, corresponding to 2 taxonomic clusters. The geographical distribution of the taxa consequently appeared to be simple in Europe: *Crassostrea gigas* was observed in northern Europe bordered by the headland of northern Spain (La Corogne) in the south, and *C. angulata* was observed in southern Europe. Moreover, we were not able to find any cupped oysters between La Corogne (Spain) and Porto (Portugal), i.e. between the 2 population clusters. Therefore, it can be concluded that no natural contact zone between *C. angulata* and *C. gigas* exists in Europe, in contrast to those observed in other species of marine invertebrates like mussels (Koehn 1991, Inoue et al. 1997, Bierne et al. 2002, 2003) and clams (Bert et al. 1993). No natural gene flow between European *C. angulata* and *C. gigas* populations was shown. Similarly, in the same Spanish region as our study, natural gene flow between algae populations is restricted to within the region due to seawater currents (Alberto et al. 1999). However, natural selective pressure might also play a role, by forcing the allelic repartition we observed here. For example, natural selection between close species of mussels in the Baltic zone was found to explain the discordant patterns of allozyme and non-allozyme loci (Riginos et al. 2002). Selective pressure in the present study could involve the presence or absence of the iridovirus that affected *C. angulata* populations in the late 1960s and/or taxa-specific preferential environmental conditions (Haure et al. 2003). However, this second hypothetical selective factor is unlikely to be major, as *C. angulata* was massively present along the French coasts until the late 1960s, where only *C. gigas* is found today. Concerning the iridovirus, there is limited information about its present distribution in Europe since its last observation in 1984 (Bougrier et al. 1986).

On the basis of the mtDNA marker, a notable mixture of both taxa was observed in 1 population located in the south of Portugal (Tavira). This situation could be the result of the development of oyster farming in this area leading to large-scale importation of *Crassostrea gigas* spat from France (Arcachon and Marennes Oléron bays) into the south of Portugal. Interestingly, the same mixture pattern was observed with the nuclear CG44R marker. Furthermore, whereas heterozygote deficiencies might be expected in the case of only mixed populations of the 2 taxa, no significant heterozygote deficiencies were observed in this population ($p = 0.116$, Table 4) and the observed heterozygosity H_o was clearly higher than those observed in other populations considered as 'pure'. Although linkage disequilibrium has commonly been observed in hybrid zones (Arnold 1997), no significant linkage disequilibrium was observed in the Tavira population. Indeed, the estimation of nucleo-cytoplasmic linkage disequilibrium revealed a non-significant value in the Tavira population ($d = 0.17$, $p = 0.102$) whereas a significant one was observed over all the populations considered together ($d = 0.45$, $p < 0.001$). All these observations lead to the conclusion that natural hybridization occurs in the Tavira population rather than only sympatric distribution of both taxa.

Similarly, Vieux-Boucau and Ribadesella populations were also made up of a mixture of the 2 taxa, though with only a few *Crassostrea angulata* haplotypes observed among a large number of *C. gigas* haplotypes. This mixture might correspond to an ancestral polymorphism related to the past presence of *C. angulata* in these regions (Grizel & Héral 1991). However, unlike the Tavira population, allele frequencies at the CG44 locus in these 2 populations did not reveal any significant shift, probably due to the limited level of mixture. In this context, it would be of great interest to further study *C. gigas* and *C. angulata* populations present in Asia (from Japan to southern China) to determine whether these 2 taxa are sympatric and if they hybridize anywhere.

Evolutionary history and past hybridization of the 2 taxa

The 5.26% genetic divergence between these 2 Asian taxa, *Crassostrea angulata* and *C. gigas*, at the COI gene leads to a rather recent estimate of divergence date at 1 to 2 million yr ago (O'Foighil et al. 1998). Taking into account (1) the large dispersal ability of these taxa; (2) the absence of reproductive barriers between them under experimental conditions (Huvet et al. 2001, 2002); and (3) the low levels of genetic differentiation between populations using allozymes (Mathers et al. 1974, Buroker et al. 1979, Mattiucci & Villani 1983) and microsatellites (mean F_{st} between taxa populations = 0.022 in the present study and in Huvet et al. 2000b), gene flow might exist between these 2 taxa. However, strong genetic differences were observed between the 2 taxa using both the CG44R marker ($F_{st} = 0.71$) and the COI mtDNA marker ($F_{st} = 0.88$; Huvet et al. 2000b). Because of the higher sensitivity of the mitochondrial genome to drift compared with nuclear DNA (due to its haploid character and maternal transmission; Boursot & Bonhomme 1986), and because homoplasy might cause underestimation of genetic differentiation at microsatellite markers (Shaw et al.

1999), one can expect higher estimates of genetic differentiation when using mitochondrial rather than nuclear markers. However, microsatellites are likely to be more resolutive for analysis of the evolutionary history of populations over short temporal and geographical scales (e.g. Angers & Bernatchez 1998, Hauser et al. 2001). We generally observed higher mitochondrial differentiation compared with nuclear DNA differentiation, except at the CG44R locus. A possible scenario is that, following the initial divergence between the 2 taxa, a secondary contact might have occurred before a second spatial and/or temporal separation. This secondary contact may have allowed limited gene flow resulting in the present frequency distribution of the CG44R alleles. However, the effects of such gene flow on the mitochondrial genome may have been lost because of drift, while the mutation rate of nuclear markers is too fast (microsatellites) or too slow (allozymes) to have enabled it to be traced. This hypothetical gene flow event would have occurred before the introduction of *Crassostrea angulata* to Europe (since both alleles of CG44R are present over all the populations and their frequencies are similar in European and Asian populations) but recently enough so that allelic frequencies at 'slow' evolving markers remained unchanged. However, alternative hypotheses involving natural selection cannot be rejected, and further information is needed concerning the potential selective pressures (e.g. presence and impact of the iridovirus that affected *C. angulata* populations). In southern Europe, the recent transplantation of *C. gigas* for aquacultural purposes (Ruano 1997) has created a contact zone where the 2 taxa hybridize, as shown in the present paper, whereas no natural contact zones were observed along the European coasts. If transplantation of *C. gigas* to *C. angulata* areas continues, future studies of this southern European zone would be of great interest to further understand the co-evolution of these taxa.

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