Microsatellite Analysis of 6-Hour-Old Embryos Reveals No Preferential Intraspecific Fertilization Between Cupped Oysters Crassostrea gigas and Crassostrea angulata

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Abstract:

Abstract: Experimental examination of reproductive isolation is the first step in understanding hybridization processes. Here, we studied preferential fertilization between 2 cupped oyster taxa, Crassostrea angulata and Crassostrea gigas, as a potential prezygotic reproductive isolation. Early examination of sperm competition is now possible by molecular analysis of oyster embryos. This avoids the confounding effect of differential mortality during the larval stage. Six hundred embryos were sampled from 2 crosses. Three microsatellite loci were enough to determine without ambiguity the taxa of contributing sires of embryos. No evidence of preferential fertilization between gametes from the same taxa was shown. A significantly higher contribution of the C. gigas males was revealed with the C. angulata females, but not with the C. gigas females, which might suggest early heterosis or interaction differences between gametes. In the light of these results, natural hybridization between both taxa can be expected in cases of their geographical coexistence, as in the Southern European populations in which both taxa are in contact as a result of aquaculture development.

Keywords: Crassostrea gigas, Crassostrea angulata, hybridization, in vitro crossings, microsatellites, paternal contributions.

1. Introduction

Identification of mechanisms involved in pre-zygotic reproductive isolation and their characterisation are key questions in evolutionary biology. Despite the fact that hybridisation is a common phenomenon in marine organisms (Gardner, 1997), empirical research has focused mainly on terrestrial species (reviewed by Barton and Hewitt, 1985, 1989; Harrison, 1990; Arnold, 1992). However, the life cycle of sessile marine organisms, often characterised by external fertilization, limits opportunities for pre-zygotic isolation such as behavioural mate choice. Apart from mate preferences and post-zygotic selection, the natural barriers to hybridisation should be classified into 3 other categories (Palumbi, 1994): (i) habitat specialization, (ii) spawning asynchrony and (iii) preferential fertilization, which can be viewed as a kind of assortative mating.

In the present paper, this third mechanism is investigated, studying gamete competition between two cupped oysters of commercial interest, the Pacific oyster *Crassostrea gigas* (Thunberg, 1793) and the Portuguese oyster *Crassostrea angulata* (Lamark, 1819). The main reason for their distinction into two different species, was their separated geographical distribution, *C. angulata* being present in Europe and *C. gigas* in Asia. Based on morphological, physiological, and allozyme data (Mathers *et al*., 1974; Buroker *et al*., 1979; Mattiucci and Villani, 1983), authors concluded there was a single species divided into two sub-species (Menzel, 1974). Furthermore, all attempts to perform inter-taxa crosses were successful (reviewed by Gaffney and Allen, 1993). However, experimental cross-fertilizations were performed, as usual, without gamete competition and consequently did not enable to show differential fertilization success or assortative fertilization, that might occur in natural populations (Arnold,

1993 ; Gregory and Howard, 1994). So the possibility of partial reproductive isolation between *C. angulata* and *C. gigas* remained to be investigated.

Unbiased quantification of sperm competition between taxa was not possible until recently due to the absence of tools which distinguished different taxa and their hybrids during early stages. Furthermore, parental contributions, assigned at juvenile stages, are the product of sperm competition and differential early mortalities (Launey and Hedgecock, 1999), potentially due to hybrid unfitness, or, on the contrary, to hybrid vigour. At the present time, we are able to conduct molecular studies with DNA extractions and PCR on individual embryos or larvae (e.g. Bierne *et al*., 1998) which allow the early examination of gamete competition.

In this paper, we report the study of 2 *in vitro* crosses with paternal taxa assignment of 6-hour-old embryos using microsatellite markers. This allows analysis of sperm competition and testing for the existence of any reproductive isolation between *C. gigas* and *C. angulata*.

2. Material and Methods

Crosses

C. angulata and *C. gigas* oysters were sampled in Spain and France respectively. Their classification was confirmed by PCR-RFLP COI analysis as described by Boudry *et al*. (1998). In order to prevent any environmental effects on the gamete quality, these oysters were used as progenitors in two G0 intra-taxa crosses, and one year-old G1 progenies, reared under common conditions, were then used as progenitors for the experiment. Spermatozoa of 10 males per taxa were collected by stripping the gonad. The sperm was diluted with sea water and the concentration of spermatozoa estimated by replicates using Thoma slides coupled to an image processing system (Alcatel). Spermatozoa of each taxa were then mixed together at a balanced ratio. Ovocytes of 10 females of each taxa were collected using the same procedure, and numbered also by replicates using Mallassez slides. Ovocytes of *C. gigas* (cross 1) and *C. angulata* (cross 2) were distributed in each fertilisation beaker and were fertilised separately and simultaneously with the same 1/1 sperm pool of both taxa (ratio: 500 spermatozoa per ovocyte). G2 embryos were reared in GRP (Glass Reinforced Polyester) tanks filled with sea water (temperature 24°C, salinity 32‰). Six hours after fertilisation, embryos were collected by sieving and preserved in 100% ethanol.

Microsatellite analysis

The parental samples (G1 progenitors) consisted of gill fragments preserved in 100% ethanol. Six hundred embryos (G2) were placed individually into Eppendorf tubes in 20 µl of 100% ethanol. This isolation of individual embryos was performed in a

100% ethanol drop under a microscope using a 2µl pipette. DNA extraction was performed by Chelex-based methods for adults as described by Estoup *et al*. (1996) and for embryos by Bierne *et al*. (1998). Three dinucleotide microsatellite markers (CG44, L10 and L16) were used to assess paternal contribution. Radioactive PCR amplifications and electrophoresis procedures for parental analysis were performed as described by Magoulas *et al*. (1998) (for CG44) and Huvet *et al*. (2000) (for L10 and L16). Protocols were changed in order to improve the amplifications from embryos: 40 PCR cycles were run and the denaturing temperature was decreased to 91°C. An example of autoradiograph is presented in Figure 1.

Determination of contributing paternal taxa

For each locus, the observed parental alleles were ranked into 4 groups according to taxa (*C. angulata* versus *C. gigas*) and sex (female versus male). Then, we determined with certitude (by exclusion of potential sires) to which group of paternal alleles (*i.e. C. angulata* or *C. gigas*) was belonging those of each embryo. A new microsatellite locus was scored until the paternal taxa was unequivocal. This was achieved using 1 to 3 microsatellite markers.

3. Results

The total number of parental microsatellite alleles was 22, 24 and 25 respectively for the CG44, L16 and L10 loci. The mean percentage of alleles shared in common between *C. angulata* and *C. gigas* parents ranged from 18.2% (CG44), 28% (L10) to 33.4% (L16). Over loci, 28.3% of alleles were specific to *C. angulata* and 45.2% to *C. gigas* parents. The 4 groups of the parental alleles (*i.e.* according to taxa and sex) were given for each locus in Table 1 to allow determination of paternal taxa of embryos.

DNA from 373 embryos (62.2%) was amplified. The remaining 37.8% failed to amplify any of the three loci tested. In these cases, PCRs were repeated in order to exclude technical problems at the amplification step. This suggests that non-amplification was due to problems during earlier steps (*i.e.* individual sampling of embryos or DNA extraction) rather than PCR artefacts. Besides, all the genotypes observed in the progenies were compatible with the parental genotypes, excluding any contamination problems.

The determination of contributing paternal taxa of these embryos was realised by establishing to which group of paternal alleles (as defined in Table 1) was belonging those of each embryo. For example, the embryo FA10 from cross 2 (*C. angulata* females) was genotyped 34/40 at the locus CG44 (lane h in Figure 1). Because the allele 40 was not present in the *C. angulata* dams (Table 1), the maternal allele was consequently the allele 34. Among the 3 *C. angulata* females carrying the allele 34, female 5 is one a possible dam of this embryo, its genotype being genotype 25/34 (lane i in Figure 1). We can then deduce that the other allele of the embryo FA10, allele 40,

came from *C. gigas* sirs, because it was not present in *C. angulata* males, and is consequently specific of *C. gigas* males (Table 1). Among the 3 *C. gigas* males carrying the allele 40, male 6 is one a possible sir of this embryo, its genotype being 39/40 (lane w in Figure 1). So we concluded, based solely on the genotype at the locus CG44, that embryo FA10 was a hybrid.

However, for others embryos, more than one microsatellite locus was needed to determine with certitude the contributing paternal taxa. For example, the embryo FA22 from cross 2 (*C. angulata* females), displayed the alleles 31 and 33 at the locus CG44 (lane a in Figure 1). Because females *C. angulata* only showed the allele 31 (Table 1), we had to search for the allele 33 in paternal groups. Both *C. angulata* and *C. gigas* males showed commonly this allele (Table 1), it was consequently not possible to determine the paternal taxa of the embryo FA22 with the first marker. At the second locus (L10), FA22 displayed the alleles 29 and 45. Both were noticed in *C. angulata* females (Table 1) and the allele 29 was noticed in both male taxa. So the analysis with the third microsatellite marker L16 was needed. Alleles of FA22 were 27 and 37 at this locus. The only possible maternal allele was 37 (Table 1). Because *C. angulata* males showed the allele 27 and *C. gigas* males did not, we concluded that the contributed paternal taxa for the embryo FA22 was *C. angulata*.

Table 2 gives the percentage of embryos for which the paternal taxa was determined on the basis of 1, 2 or 3 microsatellite loci. Assignment rate was 70.5% with the microsatellite locus CG44 alone. Adding locus L10, allowed this rate to be increase to 86%. And finally, adding locus L16 allowed all the embryos from which DNA was successfully extracted to be assigned.

The contributions of *C. angulata* and *C. gigas* males when mixed together would

be expected to produce half "pure" individuals and half of hybrids in each cross type. Pure *C. giga*s embryos and hybrid embryos represented respectively 56.1% and 43.9% in cross 1 (*C. gigas* females). In cross 2 (*C. angulata* females), pure *C. angulata* and hybrid embryos were respectively 33.7% and 66.3% (Table 3). *Chi-square* tests were performed in order to compare observed and expected paternal contributions. No significant deviation from the expected 1:1 ratio ($\chi^2 = 2.58$; d.f.1; P>0.1) was observed in cross 1. However, in cross 2, we observed more hybrids than expected under random fertilization, this difference being statistically significant ($\chi^2 = 21.56$; d.f. 1; P<0.01) and significantly higher than in cross 1 ($\chi^2 = 17.69$; d.f. 1; P<0.01).

4. Discussion

This molecular study on individual embryos allows the examination of gamete competition between two sub-species of cupped oysters. Three intricate steps were necessary to address the paternal contributions: the individual isolation of embryos, their DNA extraction and their analysis with microsatellite markers using PCR. The very small size of 6-hour-old embryos (mean size = 60µm) can induce two sources of error: a bad isolation or a bad DNA extraction. The isolation of individual embryos was highly sensitive and difficult to control. So the placing of more than one embryo was possible (as seen with approximately 20 samples, impossible to assign, showing more than 2 alleles), as well as the placing of no embryo at all. Moreover, DNA extractions on such small organisms as oyster embryos cannot be always successful (80% on 160µm larvae in Bierne *et al*., 1998), and a second extraction is not possible. However, this does not introduce bias in assignment because technical artefacts at the embryo isolation step and the DNA extraction step can be considered as randomised. Furthermore, the influence of possible PCR artefacts were also ruled out by multilocus amplifications and by repeated PCR reactions.

More than two thirds of alleles are specific to parents of both taxa. Roques *et al*. (1999) reported similar specificity for redfish taxa (due to specific allele and/or allelic size mode). Consequently, only three microsatellite loci were enough to assign 373 embryos and the first used microsatellite (CG44) was enough to assign close to three fourth of these embryos because of its highest specificity. The relevance of microsatellite markers as molecular tools in parental assignment studies is underlined here because of their high polymorphism. (e.g. Roques *et al*., 1999; Norris *et al*., 2000;

Gerber *et al.*, 2000). The assignment in these studies was probabilistic whereas, in our study, we assigned the paternal taxa with certitude by exclusion of potential sires. For some embryos, several males were possible, but they always belonged to the same taxa.

Our results did not show evidence of preferential fertilization between gametes from the same cupped oyster sub-species. In cross 1 (*C. gigas* ovocytes), contributions were not significantly different. In contrast, in cross 2 (*C. angulata* ovocytes), significantly more hybrids were observed than expected. Better quality of *C. gigas* sperm is not a satisfactory explanation because, were this the case, the same discrepancies would be seen in the two crosses, and they were not ($\chi^2 = 17.69$; d.f.1; P<0.01). Results might be explained either by an early heterosis effect working in a single direction or, by heterogamy or mate choice interactions. Heterosis in F1 hybrids is a well known observation (Edmands, 1999) and it has been demonstrated to occur as early as the larval phase (Beaumont *et al.*, 1993; Hedgecock *et al.*, 1996). It may therefore be suggested that heterosis had occurred in cross 2 (between *C. gigas* males and *C. angulata* females) as early as during the development. Preferential interaction between *C. angulata* eggs and *C. gigas* sperm might be also explained by variable egg-sperm interactions which are conceivable for free-spawning marine organisms (Palumbi, 1999). Indeed, ovocytes could show a non random selection of sperm from different males, that could be based on specific binding proteins (Palumbi, 1999).

Reproductive isolation explains the co-existence of sibling species in sympatry. It has been clearly documented between closely related sea urchins (e.g. Palumbi and Metz, 1991). When reproductive isolation is not achieved, the outcome of secondary contact is either (i) extinction of one of the two taxa, (ii) stable coexistence with hybridisation or (iii) reinforcement of premating isolation and speciation. The

introduction of *C. gigas* into Europe (Grizel and Héral, 1991) and the present expansion of its aquaculture produced the opportunity for such a secondary contact with *C. angulata* (Huvet *et al*., in press). The results of the present study suggest that natural hybridization can be expected in this geographical zone.

Acknowledgements

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Table 1. Parental microsatellite alleles grouped according taxa and sex at each microsatellite locus.

		Alleles observed in the females	Alleles observed in the males
C. angulata	Locus CG44	23, 24, 25, 28, 29, 31, 32, 34	**26**, **27**, **28**, 29, **32**, 33, 34
	Locus L10	17, 21, 28, 29, 30, 34, 35, 37, 40, 45, 47	**9**, 28, 29, **34**, **35**, 40, **43**, **44**, **47**
	Locus L16	9, 26, 32, 35, 37, 38, 41, 42, 43, 46	**9**, **10**, **27**, 35, **36**, **37**, 39, **41**, **46**
C. gigas	Locus CG44	29, 37, 38, 39, 40, 42, 44, 45, 46, 47	29, **30**, **31**, 33, 34, **39**, **40**, **41**, **45**, **46**
	Locus L10	6, 16, 17, 19, 21, 22, 23, 24	**1**, **4**, **19**, **26**, 28, 29, **30**, **32**, **37**, **39**, 40
	Locus L16	19, 28, 29, 31, 32, 34, 38, 39, 41, 42, 46, 47, 48	**1**, **4**, **8**, **21**, **28**, **31**, **32**, **34**, 35, 39, **42**, **43**

In **bold**: specific males alleles.

Table 2. Paternal taxa assignment of embryos using three microsatellite markers.

Number of loci used in the assignment	1 : CG44	2: CG44, L10	3 : CG44, L10, L16
% of assigned embryos for the *C. gigas* females	63.8	81.8	100
% of assigned embryos for the *C. angulata* females	77.2	89.6	100
Total % (on both female taxa)	70.5	85.7	100

Table 3. Taxa of contributing sires analysed on the 6-hour-old embryos.

Cross	Number of assigned embryos	Number of embryos from *C. angulata* father	Number of embryos from *C. gigas* father
1: *C. gigas* females	171	75 (43.9 %)	96 (56.1 %)
2: *C. angulata* females	202	68 (33.7 %)	134 (66.3 %)

Figure 1. Electrophoresis of PCR products of the microsatellite marker CG44 from a set of embryos. The individual embryos or parents are labelled from lane a to z in the following order : FA22, FG11, FG195, FA33, FG206, FG51, FA207, FA10, *C. angulata* female 5, FG184, FG37, FG127, *C. angulata* female 8, FG116 , FG107, FA215, *C. angulata* male 5, FA265, FA270, FA165, FA185, *C. gigas* female 3, *C. gigas* male 6, FG259, FA196 et FA 197 (with FG = embryo from *C. gigas* females (cross 1) and FA = embryo from *C. angulata* females (cross 2)).