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Comparison of genetic variability and parentage in different ploidy classes of the Japanese oyster *Crassostrea gigas*

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Summary

Chemical treatments with cytochalasin B were used to induce triploidy in the progeny of a mass fertilization of 3 male and 7 female *Crassostrea gigas* parents. Triploids were produced either by retention of the first (meiosis I (MI) triploids) or the second (meiosis II (MII) triploids) polar bodies. These animals, together with their diploid siblings, were divided for two experiments. One set was used to compare physiological performance, and the other set deployed to compare growth in two different natural environments. For both experiments, genetic variability in different ploidy classes was estimated using three microsatellite loci and eight allozyme loci. The microsatellite loci were highly polymorphic, allowing independent confirmation of ploidy status and the unambiguous identification of parentage for each oyster. Significant differences in parentage were found between ploidy classes, despite the fact they originated from the same mass fertilization. This indicates that the assumptions of a common genetic background among random samples of animals taken from the same mass fertilization may not be generally valid. Knowledge of parentage also allowed the more accurate scoring of allozyme loci. As expected, triploids were found to be significantly more polymorphic than diploids. However, MI triploids were not significantly more polymorphic than MII triploids. MII triploid genotypes were used to estimate recombination rates between loci and their centromeres. These rates varied between 0·29 and 0·71, indicating only moderate chiasma interference.

1. Introduction

Ploidy manipulation in bivalves has received much attention, mainly because of potential advantages in aquaculture (Beaumont & Fairbrother, 1991). Triploid shellfish can be produced by applying a thermal, pressure or chemical shock, which leads to the retention of the first or the second polar body during the meiosis of the egg, and the production of meiosis I (MI) and meiosis II (MII) triploids, respectively. The chemical cytochalasin B (CB), which inhibits actin polymerization, has traditionally been used for the induction of triploidy.

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Triploidy in oysters were first induced in early 1980s (Stanley *et al*., 1981). Increased body size for triploids has been a common observation in bivalves. Three genetic hypotheses have been put forward to explain the effects of polyploidy on growth (Zouros *et al*., 1996, and references therein). The first, known as the 'allelic variation' hypothesis, considers that this phenomenon is simply a demonstration of heterozygote advantage, as suggested by numerous positive correlations between body size and multi-locus heterozygosity, especially among bivalves. Triploid animals, by having three sets of chromosomes, are expected to be more heterozygous than diploids, because these individuals have a higher probability of carrying two (or even three) different alleles at the same locus. A second hypothesis to explain effects of polyploidy is

known as the 'gene dose' hypothesis, and suggests that triploids grow faster because they have the gene products in triple dose. Under the 'gene dose' hypothesis, MI and MII triploids should not differ in their growth rate. Alternatively, under the 'allelic variation' hypothesis, MI triploids should grow faster, since di-allelic and tri-allelic individuals are expected to be more common in MI triploids. Precisely speaking, this is true only for loci with recombination distance from the centromere of less than 2/3, but this is the highest distance to be expected in the absence of chiasma interference (Thorgaard *et al*., 1983). Higher heterozygosity and faster growth of MI triploids has indeed been demonstrated in European oyster (*Ostrea edulis*). Hawkins *et al*. (1994) showed that in MI triploids, average multi-locus heterozygosity was 50% higher and growth more than 60% faster than in diploid siblings and MII triploids. A third genetic explanation for the improved performance of triploids is based on the observation that chromosome loss in somatic cells is associated with reduced growth rate. This hypothesis proposes that 'progressive haploidization' acts to reduce growth through the unmasking of deleterious recessive genes, while faster triploid growth occurs because the probability of exposing a deleterious allele in a triploid is much smaller (Zouros *et al*., 1996). There are other non-genetic explanations for the relationship between polyploidy and growth, such as energy reallocation from gonad to somatic tissues, and larger cell volume in triploids (Guo & Allen, 1994). However, these explanations alone would not lead to any difference in performance between MI and MII triploids (cf. Hawkins *et al*., 1994).

In *C. gigas*, it is more difficult to induce viable MI triploids than MII triploids (Gérard *et al*., 1999). Nevertheless, this study aimed to compare the level of genetic variability in MI and MII triploid progeny of 3 male and 7 female *C. gigas* parents produced after treatment with CB. In addition to allozymes, we have used highly variable microsatellite DNA markers to assess variation in the non-coding portion of the nuclear genome, to confirm the ploidy status of each oyster, and to identify parentage. This has been a significant novel approach that enabled much higher accuracy in scoring allozyme variation, thereby helping to achieve a more precise estimation of allelic variation in triploids.

We also estimated the gene–centromere recombination rates for each studied microsatellite and allozyme locus. Provided the mother is heterozygous and the father has contributed an allele that makes the recognition of the two maternal alleles possible, the gametic phase in the ova can be determined from the genotype of an offspring. If a crossover between the locus and the centromere did not take place, ova produced by a heterozygous mother after retention of the first polar body, as in the case of MI triploids, will be heterozygous. That is they will contain both maternal alleles. If such a crossover did take place, half the ova will be homozygous and half will be heterozygous. On the contrary, ova produced after retention of the second polar body, as in the case of MII triploids, will be homozygous if no crossover occurred and heterozygous if a crossover did occur. Therefore, the frequency of second division segregation (y) or recombination rate is expected to determine the frequency of heterozygous eggs produced by a mother in both MI and MII triploids. The parameter y constitutes the best estimate of the proportional increase in genetic diversity among triploid MI and MII progeny. If y for a locus is $< 2/3$, then MI triploids are expected to be more variable than MII triploids, whereas the opposite is true if $y > 2/3$. Mean y over loci gives a good estimate of the overall increase in genetic variability in triploids.

In associated papers, using the same offspring as were studied here, Hawkins *et al*. (2000) compare the genetic and physiological basis of growth effects in both MI and MII triploids, whilst Naciri-Graven *et al*. (2000) describe growth of MII triploids within two different natural environments.

2. Materials and methods

(i) *Experimental animals*

Three male and 7 female oysters (*Crassostrea gigas*) originating from the Seudre river on the Atlantic coast of France were artificially mass-crossed during April 1994 at the IFREMER hatchery in La Tremblade, France. Gametes were stripped and filtered on a 45 μm sieve for males and on a 75 μm sieve for females, before being suspended in 3 μm filtered seawater. The oocyte number was adjusted to 15000/ml, and fertilization performed with a ratio of 100 spermatozoa per ovum. The embryos were treated with CB (Stanley *et al*., 1981) 5–15 min after fertilization or 20–30 min after fertilization, to induce MI (retention of the first polar body) or MII (retention of the second polar body) triploid progeny, respectively. Epifluorescence analysis was used to monitor early embryonic development, and ploidy level assessed by image analysis 24 h after fertilization (Gérard *et al*., 1994). Other information concerning fertilization, triploid induction and the rearing of the progeny is presented in Gérard *et al*. (1999), Hawkins *et al*. (2000) and Naciri-Graven *et al*. (2000).

Oysters were reared in cylindrical sieves in 1000 l tanks with recirculating enriched seawater. When the oysters were 5–8 months old, individuals were biopsied to establish four different classes, according to treatment for ploidy induction and the resultant ploidy status: diploids untreated, diploids refractory

Table 1. *Experimental animals that were analysed genetically*

	Code	Description	Number
A. *Parents*			
1	M	Male parents	3
2	F	Female parents	7
B. *Offspring from experiment I*			
1	2N	Diploids	16
2	R-2N	Diploids refractory to CB	21
3	MI-3N	Meiosis I triploids induced by CB	18
4	MII-3N	Meiosis II triploids induced by CB	22
C. *Offspring from experiment II*			
1	MO-2N	Diploids grown in Marennes-Oléron	76
2	MO-3N	Triploids grown in Marennes-Oléron	75
3	TL-2N	Diploids grown in Thau Lagoon	76
4	TL-3N	Triploids grown in Thau Lagoon	75

CB, cytochalasin B.

to treatment with CB, MI triploids and MII triploids. Each class consisted of approximately 20 animals. Biopsy and image analyses were performed according to Gérard *et al*. (1994).

At least 14 days after biopsy, comparisons of physiological performance were undertaken (experiment I) among all ploidy classes (Hawkins *et al*., 2000). These oysters were later dissected on ice and pieces of the digestive gland and the adductor muscle or the gill were air-shipped on dry ice to the IMBC laboratory in Crete, to confirm the ploidy status and identify the parentage of each individual animal, as well as analysing allozyme and microsatellite variation in the different ploidy classes. Parents were also scored for the same allozyme and microsatellite loci.

Other animals from the same mass fertilization were reared in an open seawater circulating system until the end of May 1995. Diploid and MII triploid animals that had not been biopsied to establish triploidy were then deployed within two natural locations: one oligotrophic (Marennes-Oléron, MO) and the other eutrophic (Thau Lagoon, TL) (experiment II). In this way, four classes resulting from the combinations of locality × ploidy status were established: MO-2N, MO-3N, TL-2N and TL-3N. Each of these classes comprised 150 animals. Oysters were left to grow in the field until January 1996, when half the animals from each class were killed and used for measurements of weight and biochemical composition (Naciri-Graven *et al*., 2000).

Soft tissues from oysters that had been deployed at MO and TL were homogenized in La Tremblade using an Ultra-Turax homogenizer. To perform allozyme and microsatellite analyses as described above for oysters of experiment I, a few cubic centimetres of the homogenate was placed in plastic tubes and diluted with equal volumes of 0·1 M dithiothreitol (DTT), to be used for allozyme analysis, while a small piece of gill from each animal was kept for microsatellite analysis. Samples were airfreighted on dry ice to Crete. Table 1 details the coding and numbers of samples of the parents and progeny used in experiments I and II.

(ii) *Allozyme analysis*

Pieces of about 0·2 cm^3 were dissected from the tender part of the digestive gland and either analysed immediately or kept in Eppendorf tubes at −80 °C to be analysed later. At first, these pieces were homogenized in an equal volume of 0·04% solution of β-mercaptoethanol before the electrophoretic run. Later, 0·1 M DDT replaced the more hazardous β-mercaptoethanol for this purpose. Homogenization was performed in 1·5 ml Eppendorf tubes using a glass stick to squash the tissue, followed by centrifugation for 2 min at 14000 rpm (2500 *g*). Supernatant was used for electrophoresis. Tests showed no detectable change in enzymatic activity by using this extract up to three times.

We also performed a preliminary experiment to test whether total-body homogenate could be used for allozyme analysis, to avoid sampling digestive gland, which could compromise analyses of biochemical content within the total tissues of oysters from the field growth experiment. Living oysters were opened and the soft parts homogenized using a glass-Teflon homogenizer, before diluting homogenates by adding an approximately equal volume of 0·1 M DTT, and storing at −80 °C. To compare the efficiency of scoring allozymes using whole-body homogenate with that using digestive gland, a piece of the digestive gland from certain animals was dissected prior to homogenization and also placed at −80 °C. Allozymes were scored three times using both the

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homogenate and the digestive gland, the first time immediately after homogenization, the second time a week after the homogenization, and third time a month after homogenization. Findings indicated that collective homogenization of the total soft tissues and storage at −80 °C did not compromise the efficiency of scoring (unpublished data).

Five systems corresponding to eight allozyme loci were standardized: *leucine amino peptidase* (*Lap*, EC 3.4.11.1, monomeric), *phosphoglucomutase* (*Pgm*, EC 2.7.5.1, monomeric), *aspartate aminotransferase*-1 and -2 (*Aat-1* and *Aat-2*, EC 2.6.1.1, both dimeric), *phosphoglucose isomerase*-1 and -2 (*Pgi-1* and *Pgi-2*, EC 5.3.1.9, dimeric and monomeric respectively), *isocitrate dehydrogenase*-2 (*Idh*, EC 1.1.1.42, dimeric) and *enolase* (*Enol*, EC 4.2.1.11, dimeric). Electrophoresis was performed on horizontal starch gels using the protocols of Saavedra *et al.* (1993). Gels were made of 12% Sigma starch. For *Aat-1*, *Aat-2*, *Pgi-1*, *Pgi-2* and *Idh-2* gel buffer was diluted 1 in 9 from electrode buffer, which was TC pH 7·0 (citric acid 45 mM and Tris 135 mM). For *Lap*, *Pgm* and *Enol*, gel buffer was diluted 1 in 12 from electrode buffer, which was TME pH 7·4 (100 mM maleic acid, 100 mM Tris, 10 mM $MgCl_2$, 1 mM EDTA). All gels were stained according to Pasteur *et al.* (1988), with the exception of those for *Enol*, which were stained as described by Harris (1966). It should be noted that the identification and scoring of parents facilitated the scoring of each offspring, especially for enzymes with many alleles that differed little in mobility (e.g. *Pgm*).

(iii) *Microsatellite DNA analysis*

The progeny of experiments I and II, as well as their parents, were scored for the microsatellite loci *Cg44*, *Cg49* and *Cg108* with PCR amplification and polyacrylamide gel electrophoresis, according to Magoulas *et al.* (1998). Pieces of muscle or gill were dissected from the animals and used for total DNA extraction, following the proteinase k, salt extraction technique (Miller *et al.*, 1988). These DNAs were used as templates in PCR amplifications and the products were resolved in 6% polyacrylamide denaturing gels after radioactive labelling of one of the primers. In general scoring was straightforward, with only minor stuttering. Details of the development of the primers and the conditions of scoring are given in Magoulas *et al.* (1998).

(iv) *Data analysis*

We considered that the triploid state of an animal was confirmed by microsatellite analysis when three alleles were found in at least one of the microsatellite loci. Given the high level of polymorphism of our microsatellite loci, it is very unlikely that a triploid animal will not possess three alleles in at least one of the loci scored. Moreover, similar evidence was sometimes provided by the allozyme analysis, but in this case the tri-allelic animals were rare (i.e. for *Pgm*), so that a difference in band intensity of heterozygote-like animals was the most common indication of triploidy status.

Deviations of diploid classes from Hardy–Weinberg equilibrium (HWE) were estimated by exact tests with the alternative hypotheses of heterozygote excess or heterozygote deficiency (Rousset & Raymond, 1995*a*) using the GENEPOP 3.1a software package (Rousset & Raymond, 1995*b*). Population differentiation was also tested with an exact test for genic differentiation using the same package.

The recombination frequency between the individual loci and the centromere was estimated among the MII triploid progeny of heterozygous female parents from both experiments I and II, plus a set of 18 MII triploid individuals produced after treatment with 6-dimethyloaminopurine (6-DMAP) (Dufresne *et al.*, 1991). The existence of both maternal alleles in the offspring, originating from a heterozygous ovum, was interpreted as evidence for crossing over, while the presence of one of the two alleles (in double dose, originating from a homozygous ovum) was considered as evidence of lack of recombination. Recombination rates were estimated by the ratio between the inferred heterozygous and the total number of ova. MI triploids were not used for the estimation of recombination rates because of their limited availability, and because estimation is rendered more complicated by the lack of any direct relationship between homozygous or heterozygous ova and the presence or absence of crossing over (see Section 1).

Two different indices were used to characterize the genomic diversity of individual oysters. The first was individual heterozygosity (H), which expresses the proportion of studied loci for which that individual was heterozygous (i.e. with two or, in the case of triploids, three alleles). We refer to the second index as the individual's allelic diversity (D), which we used to distinguish between triploids that possessed two alleles and those that possessed three alleles at each locus. To calculate D, each animal was assigned a score of 0, 1 or 2 for each locus, according to whether there were one, two or three alleles, respectively, and the scores of all loci averaged. It is obvious that for a given locus in a diploid animal the two indices coincide, while for a triploid with three different alleles, H will be 1 and D will be 2. The mean H or D for each class of animals was calculated as the average of individual heterozygosities or allelic diversities in that class. Differences between classes in overall heterozygosity and allelic diversity were tested using the non-parametric Wilcoxon matched-pairs test within the software

package Statistica for Windows, based on per locus heterozygosities and allelic diversities (Sokal & Rohlf, 1995). It should be noted that these tests could not be applied for microsatellite heterozygosities and allelic diversities, because the number of paired comparisons (= number of loci) was smaller than 6 (see Sokal & Rohlf, 1995, p. 444), and this is the reason why microsatellite and allozyme H and D values were not treated separately.

The high level of polymorphism of microsatellite loci and the small number of parents allowed the identification of parentage by comparing the genotypes of offspring with those of the parents according to Mendelian inheritance, with no need for specific software.

Differences in parentage between ploidy classes from experiments I and II were tested by chi-square analysis of heterogeneity between the 21 possible families in each class, through a Monte Carlo simulation using the program REAP (McElroy *et al.*, 1991).

3. Results

The 10 parents, the 77 offspring used in experiment I and the 302 offspring used in experiment II were each scored for the three microsatellite and eight allozyme loci. However, the *Pgi-2* locus gave no readable bands among oysters from experiment II. This was probably because we had homogenized and frozen the total soft tissues collectively, although our preliminary test had given positive results for this and all other loci.

A very high degree of polymorphism was revealed for the three microsatellite loci, with the number of alleles ranging between 12 and 15. Allozymes, as expected, revealed a lower level of polymorphism, with the number of alleles per locus varying between 2 and 6. Table 2 gives the number of heterozygotes and the total number of alleles in the parents. It can be seen that for microsatellites, the male parents were each heterozygous for all three loci, with different alleles for each locus, so that the total number of alleles was the maximum possible (3×6). This greatly facilitated the identification of male parentage.

(i) *Parentage and genetic variation*

(a) *Experiment I*

Genotypic scoring confirmed the ploidy status of all but 3 oysters. One individual thought to be diploid (R-2N class) on the basis of prior biopsy and image analysis (see Section 2) was found to be a triploid. Alternatively, two individuals that had been diagnosed as triploids (MII-3N class) were found to be diploid. These oysters were excluded from further analyses of genetic diversity.

The parents of all but three of the experimental animals were identified (Table 3). Parental contribution varied between ploidy classes ($P < 0{\cdot}004$, Monte Carlo simulation). After pooling all classes, it can be seen that all 3 male and 7 female parents participated in reproduction, but with highly unbalanced contributions. The males each produced from 7 (9·0%) to 58 (75·0%) offspring, and the females from 1 (1·3%) to 32 (41·6%) offspring. Of the 21 (3×7) possible families, 14 were produced, varying in size between 1 (M3 × F6) and 32 (M3 × F3).

The great majority of loci in all four diploid classes were found not to deviate significantly from HWE expectations. Four of the 33 tests gave significant results, of which only one (heterozygote deficiency of *Cg44* in R class) remained significant when adjusted by a sequential Bonferroni procedure (Rice, 1989).

Heterozygosities and allelic diversities were calculated separately for each locus within each ploidy class (Table 4). Average individual heterozygosities and allelic diversities were higher for microsatellites, with heterozygosities ranging from 0·333 to 1, and allelic diversities ranging from 0·333 to 2. For allozymes, heterozygosities ranged from 0 to 0·875, and allelic diversities from 0 to 1. Mean heterozygosity (H) and allelic diversity (D) calculated for microsatellites and allozymes from individual heterozygosities for the parents and the six classes of the offspring are illustrated in Fig. 1. As expected, mean values of H and D were higher in the triploid classes than in the diploid ones for both microsatellites and allozymes. Comparing the two classes of triploids, mean H and D

Table 2. *Number of heterozygotes and total number of alleles* (*in parentheses*) *found at microsatellite and allozyme loci in parents*

Parents[a]	*Cg44*	*Cg49*	*Cg108*	*Lap*	*Pgm*	*Aat-1*	*Aat-2*	*Pgi-1*	*Idh-2*	*Enol*
Male [3]	3 (6)	3 (6)	3 (6)	2 (3)	1 (3)	2 (2)	0 (1)	0 (1)	0 (2)	0 (1)
Female [7]	3 (9)	6 (10)	7 (10)	4 (3)	5 (6)	2 (2)	2 (3)	4 (5)	4 (3)	2 (3)
Total [10]	6 (12)	9 (13)	10 (15)	6 (3)	6 (6)	4 (2)	2 (3)	4 (5)	4 (3)	2 (3)

[a] Numbers in square brackets give the number of parents.

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Table 3. *Parentage identification for offspring from experiment I*

Parents[a]	F1	F2	F3	F4	F5	F6	F7	X	Total
2N									
M1									
M2									
M3	2		7		6			1	16
X									
Total	2		7		6			1	16
R-2N									
M1				1			3		4
M2	3				1				4
M3	3		8			1	1		13
X									
Total	6		8	1	1	1	4		21
MI-3N									
M1		4							4
M2	1								1
M3		3	8	1				1	13
X									
Total	1	7	8	1				1	18
MII-3N									
M1		1			1		1		3
M2									2
M3		2	9		4		1		16
X								1	1
Total		3	9		5		4	1	22
Total									
M1		5		1	1		4		11
M2	4				1		2		7
M3	5	5	32	1	13	1	2	2	58
X								1	1
Total	9	10	32	2	10	1	8	3	77

[a] Parents of uncertain identification are denoted by X.

Table 4. *Heterozygosities and allelic diversities for each locus in ploidy classes from experiment I*

	Cg44	*Cg49*	*Cg108*	*Lap*	*Pgm*	*Aat-1*	*Aat-2*	*Pgi-1*	*Pgi-2*	*Idh-2*	*Enol*
Heterozygosity (*H*)											
Parents	0·600	0·900	1·000	0·600	0·600	0·400	0·200	0·400	0·700	0·400	0·200
2N	0·750	1·000	1·000	0·375	1·000	0·375	0·250	0·438	0·133	0·625	0·125
R-2N	0·444	1·000	1·000	0·350	0·737	0·650	0·000	0·450	0·150	0·250	0·250
MI-3N	0·944	1·000	1·000	0·500	1·000	0·889	0·111	0·765	0·333	0·722	0·389
MII-3N	0·950	1·000	1·000	0·650	0·900	0·526	0·300	0·842	0·300	0·778	0·250
Allelic diversity (*D*)											
Parents	0·600	0·900	1·000	0·600	0·600	0·400	0·200	0·400	0·700	0·400	0·200
2N	0·750	1·000	1·000	0·375	1·000	0·375	0·250	0·438	0·133	0·625	0·125
R-2N	0·444	1·000	1·000	0·350	0·737	0·650	0·000	0·450	0·150	0·250	0·250
MI-3N	1·278	1·944	1·588	0·500	1·778	0·889	0·111	0·765	0·333	0·778	0·389
MII-3N	1·105	1·650	1·722	0·632	1·050	0·526	0·300	0·842	0·300	0·813	0·250

values were higher in MI triploids than in MII triploids for both microsatellites and allozymes.

The results of Wilcoxon tests on overall H and D values between the classes of animals are given in Table 5. Only comparisons between a diploid and a triploid class gave statistically significant differences, while differences between the two diploid or the two triploid classes were not significant.

In all classes of animals, mean individual heterozygosity for microsatellite loci was higher or a little lower than 2 times the mean heterozygosity for allozyme loci. The mean allelic diversity for micro-

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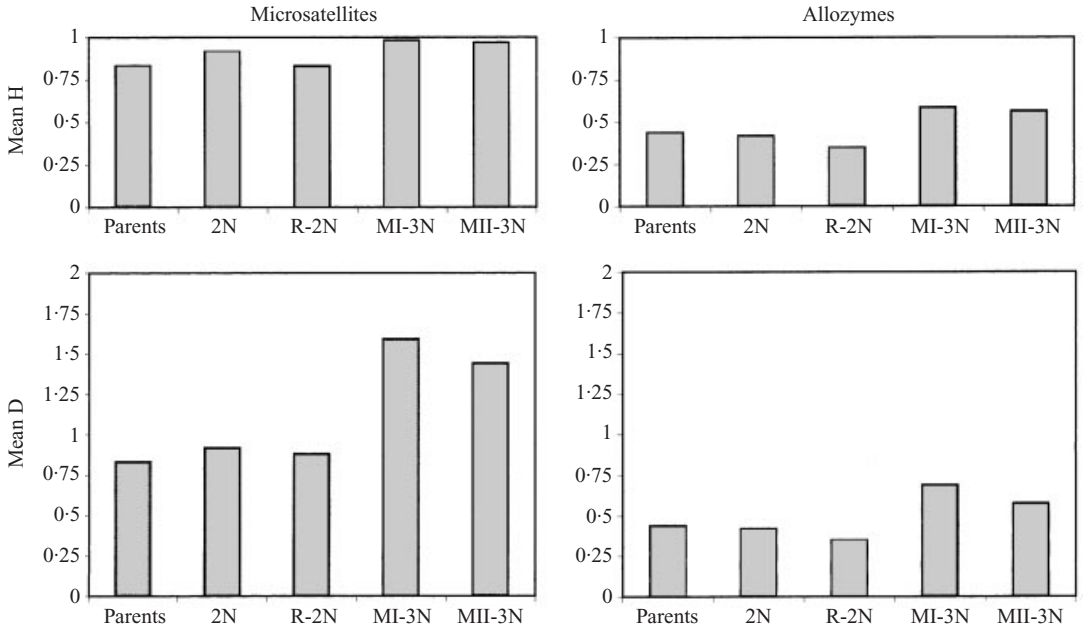

Fig. 1. Comparison of mean heterozygosities and mean allelic diversities between the parents and the six ploidy classes among offspring from experiment I.

Table 5. *Wilcoxon matched pair test for overall heterozygosity and allelic diversity between the ploidy classes from experiment I*

	2N	R-2N	MI-3N
Heterozygosity (*H*)			
2N	—		
R-2N	NS	—	
MI-3N	*	**	—
MII-3N	*	*	NS
Allelic diversity (*D*)			
2N	—		
R-2N	NS	—	
MI-3N	**	**	—
MII-3N	**	**	NS

* $P < 0{\cdot}05$; ** $P < 0{\cdot}01$; NS, not significant, $P > 0{\cdot}05$.

satellites of the two classes of triploids was approximately 2·5 times higher than that for the allozymes.

Pgm was the only allozyme locus for which oysters possessing three alleles were found. Therefore, allozyme D values were greater than H values only for *Pgm*. This is why values of allozyme H and D in the two classes of triploids were quite similar.

(b) *Experiment II*

The triploidy status of 20 oysters from the MO-3N class was not confirmed by genotypic scoring, and the same was true for 15 oysters from the TL-3N class. Compared with experiment I, this higher incidence of error was expected, because no cytological diagnosis of triploidy had been undertaken. In a preliminary ANOVA for dry tissue weight, in which diploid, confirmed triploid and doubtful triploid animals were considered as different categories, the doubtful triploids were found to be very similar to diploids (average weight 1·06 g and 1·07 g respectively), while both these categories had a significantly smaller weight than the confirmed triploids (average 1·49 g). This was consistent with their status as diploids, and they were treated as such in subsequent analyses. Numbers within the four resulting classes were as follows: MO-2N, 96; MO-3N, 55; TL-2N, 91; TL-3N, 60 (total: 302).

Both parents of all but 7 animals were unambiguously identified (Table 6). As for experiment I, parental contribution varied between ploidy classes. Again, all male and female parents participated in reproduction, but with unbalanced contributions. The males each produced from 15 (5%) to 233 (77%) offspring, and the females from 5 (2%) to 151 (50%) offspring. Nineteen families were produced out of the possible 21, ranging in size from 1 (three families) to 141 (M3 × F3) offspring. Monte Carlo simulation showed marginally insignificant heterogeneity (probability 0·054 of exceeding the observed chi-square value by chance alone).

In the two classes of diploids, deviations from HWE were only observed as significant heterozygote excesses in eight of the 20 single-locus tests (MO-2N: *Cg49*, *Cg108*, *Lap*, *Pgm*, *Pgi*-1; TL-2N: *Cg49*, *Cg108*, *Pgm*). After Bonferroni adjustment for multiple tests, five tests continued to show significant heterozygote

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Table 6. *Parentage identification of the offspring from experiment II*

Parents[a]	F1	F2	F3	F4	F5	F6	F7	X	Total
MO-2N									
M1	10	2	4		4	1	1		22
M2	2	1	3		1		1	1	9
M3	14	3	38		6	1	1	1	64
X									
Total	26	6	45		11	2	3	2	95
MO-3N									
M1	2	2			6			1	11
M2					1				1
M3	4	2	31		5		2		44
X									
Total	6	4	31		12		2	1	56
TL-2N									
M1	6			2	1	1			10
M2	3		1		1				5
M3	14		40	1	10	4	4	2	75
X					1				1
Total	23		41	3	13	5	4	2	91
TL-3N									
M1	1		2		7				10
M2									
M3	9	2	32	2	3	1		1	50
X									
Total	10	2	34	2	10	1		1	60
Total									
M1	19	4	6	2	18	2	1	1	53
M2	5	1	4		3		1	1	15
M3	41	7	141	3	24	6	7	4	233
X					1				1
Total	65	12	151	5	46	8	9	6	302

[a] Parents of uncertain identification are denoted by X.

Table 7. *Heterozygosities and allelic diversities for each locus in ploidy classes from experiment II*

	Cg44	*Cg49*	*Cg108*	*Lap*	*Pgm*	*Aat-1*	*Aat-2*	*Pgi-1*	*Idh-2*	*Enol*
Heterozygosity (*H*)										
MO-2N	0·871	1·000	1·000	0·435	0·865	0·540	0·066	0·330	0·505	0·283
TL-2N	0·813	0·988	1·000	0·379	0·902	0·379	0·080	0·224	0·541	0·149
MO-3N	0·946	1·000	1·000	0·714	0·870	0·700	0·179	0·582	0·673	0·464
TL-3N	0·891	1·000	1·000	0·644	0·914	0·792	0·119	0·525	0·695	0·339
Allelic diversity										
MO-2N	0·871	1·000	1·000	0·435	0·865	0·540	0·066	0·330	0·505	0·283
TL-2N	0·813	0·988	1·000	0·379	0·902	0·379	0·080	0·224	0·541	0·149
MO-3N	1·125	1·706	1·755	0·750	1·098	0·700	0·179	0·580	0·722	0·464
TL-3N	1·151	1·754	1·732	0·695	1·345	0·792	0·119	0·525	0·763	0·339

excess (*Cg49* and *Cg108* in both MO-2N and TL-2N and *Pgm* in MO-2N).

Table 7 summarizes the heterozygosities and allelic diversities for each locus within each ploidy class. Individual heterozygosities ranged from 0·250 to 0·900, and allelic diversities from 0·250 to 1·333. As in experiment I, there was no homozygous individual for microsatellite locus *Cg108*. For microsatellites and allozymes, mean individual H and D values are illustrated for all four classes (MO-2N, MO-3N, TL-2N, TL-3N) in Fig. 2. As expected, values of H and D were higher in the triploid populations than in the diploid ones. However, microsatellite H values were only slightly higher in the triploid populations.

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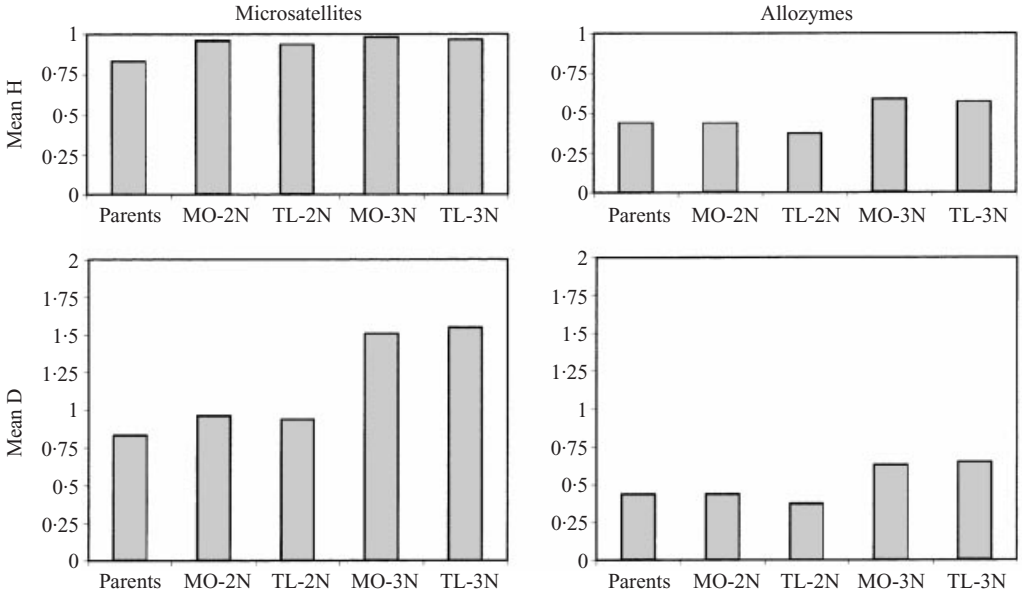

Fig. 2. Comparison of mean heterozygosities and mean allelic diversities between the four ploidy classes of offspring from experiment II. Parents are also depicted here.

Table 8. *Wilcoxon matched pair test for overall heterozygosity and allelic diversity between the ploidy classes from experiment II*

A. Separate classes				B. Pooled classes	
	MO-2N	*TL-2N*	*MO-3N*	*2N*	
Heterozygosity (*H*)					
TL-2N	NS	—		*3N*	**
MO-3N	*	*	—		
TL-3N	*	*	NS		
Allelic diversity (*D*)					
TL-2N	NS	—		*3N*	**
MO-3N	**	**	—		
TL-3N	**	**	NS		

* $P < 0{\cdot}05$; ** $P < 0{\cdot}01$; NS, not significant, $P > 0{\cdot}05$.

No major differences were observed for the two indices between the two diploid or the two triploid populations.

Table 8 summarizes the results of Wilcoxon tests on overall H and D values between the separate classes and the pooled classes (2N, 3N). For all comparisons (H and D, separate or pooled samples), there were no differences between the two diploid classes or between the two triploid classes, but there were significant differences between the diploid and triploid classes. Again, the differences between diploids and triploids were more significant for D than for H. Also, in all four classes, mean heterozygosity for microsatellite loci was higher or a little lower than 2 times the mean heterozygosity for allozyme loci. The mean allelic diversity for microsatellites of the two classes of triploids was approximately 2·5 times higher than that for the allozymes.

Table 9. *Number of heterozygous and homozygous ova and frequency of second division segregation* (*y*) *at three microsatellite and eight allozyme loci*

	Heterozygous ova	Homozygous ova	*y*
Cg44	37	51	0·42
Cg49	101	42	0·71
Cg108	102	45	0·69
Lap	68	45	0·60
Pgm	50	54	0·48
Aat-1	46	34	0·58
Aat-2	21	12	0·64
Pgi-1	71	56	0·56
Pgi-2	5	12	0·29
Idh-2	45	45	0·50
Enol	48	30	0·62
Average	594	426	0·55

See text for details.

Of the 10 loci, tests for genic differentiation (differences in gene frequencies) between the MO-2N and TL-2N classes only showed two significant differences: *Cg108* and *Enol*. Of those, only *Cg108* remained significant after Bonferroni adjustment.

(ii) Recombination rates

Frequencies of second division segregation between individual loci and their centromeres are summarized in Table 9. Estimated recombination rates ranged from 0·42 to 0·71 for microsatellite loci, and from 0·29 to 0·62 for allozyme loci. The average recombination rate over all loci was 0·55. Three loci (*Cg49*, *Cg108*, *Aat-2*) had recombination rates around 0·67, which is

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the maximum value expected if there is no chiasma interference.

4. Discussion

Polymorphisms were scored at 3 microsatellite and 8 allozyme loci in the offspring from 3 male and 7 female oysters. The mean number of alleles per allozyme locus was 3·5 and per microsatellite locus 13·3. Whilst high compared with other animals such variation is not unusual in marine bivalves, either for allozymes (e.g. Koehn *et al*., 1988) or for microsatellites (Naciri *et al*., 1995; McGoldrick & Hedgecock, 1996). Several explanations can be invoked for this observation, including high mutation rate per gamete (Zouros *et al*., 1996), large effective population size (Hedgecock & Sly, 1990) and balancing selection (Karl & Avise, 1992).

Levels of microsatellite polymorphism were very high. Mean H values in the parents were 0·6, 0·9 and 1·0 for the loci *Cg44*, *Cg49* and *Cg108*, respectively. This variation was high enough to allow us: (a) to independently confirm the ploidy status of the oysters, and (b) to determine unambiguously the parents of almost all the experimental offspring. To our knowledge, this is the first time that microsatellites have been used to confirm ploidy status and parentage in experiments of this kind. This approach has also helped to answer questions that could not have been asked before. It was found that the different progeny classes, even though originating from the same mass spawning, can differ significantly in their family structure. This was especially true for experiment I. A possible explanation for this phenomenon would be genotype-dependent differences in rate of embryonic development, since production of either MI or MII triploids depended upon treatment time. Also, larvae were screened for ongrowing on the basis of size, and if a particular genotype resulted in an especially slow development time, then that genotype will have been lost. This finding of different parental contribution leads to the conclusion that different genetic background should be considered as an additional factor when trying to explain physiological or other differences found between different classes of progeny.

Confirmation of ploidy facilitated comparisons of performance between distinct ploidy classes (Hawkins *et al*., 2000; Naciri-Graven *et al*., 2000), which has not been possible in most previous studies. Determination of ploidy from microsatellites also allowed the resolution of ambiguities in scorings of allozymes, especially for allozyme systems with many alleles with small mobility differences, such as *Pgm* in the present work. In addition, because most enzyme loci have few alleles, a triploid animal will most of the time appear to have two bands, with one of the bands having stronger staining density. This difference alone will not allow safe scoring of the animal, because in many instances the intensity of the band may reflect allozyme affinity for the substrate. However, if differential staining is associated with microsatellite scoring and parentage identification, safe scoring of allozyme variation is possible. This means that it was possible to distinguish between the two possible heterozygotes in triploid animals (e.g. between AAB and ABB). For these reasons, we believe that our allozyme scorings yielded more reliable results than in standard allozyme studies.

The identification of both parents of each experimental oyster allowed us to distinguish the influence of genetic variation on phenotypic variance in performance, as presented in the two associated papers (Hawkins *et al*., 2000; Naciri-Graven *et al*., 2000).

In the two diploid classes from experiment I, there was no excess in heterozygosity according to HWE expectations, which is known to occur when there is a limited number of parents (Robertson, 1965). On the contrary, in the two classes of diploids from experiment II, there were significant heterozygosity excesses. This may have been due to the higher viability of heterozygotes, given that the animals of experiment II were older, and had been deployed in the natural environment.

For allozymes, heterozygosities were much higher in triploids than in diploids. But in the case of microsatellites, the difference was not as great. This appeared to result from the high degree of polymorphism at microsatellite loci, so that diploids already had high heterozygosities (0·82–0·94). For both allozymes and microsatellites, allelic diversities were much higher in triploids than in diploids. This means that allelic diversity (D) may be a more appropriate index for measuring genetic diversity.

MI triploids were found to be more polymorphic than MII triploids, but the difference was not statistically significant. Given the estimated mean recombination rate of 0·55, MI triploids are expected to be more variable, since this value is lower than 2/3. It should be noted that aneuploidy may be higher in MI triploids than in MII triploids (Gérard *et al*., 1999), which may lead to a proportionally higher decrease of apparent heterozygosity in MI than in MII triploids.

High recombination rates (y values near 1·0) suggest that a single crossover occurs between the gene and its centromere in virtually all cases (Thorgaard *et al*., 1983). This happens if there is complete chiasma interference, that is if the existence of one crossover between the gene and the centromere inhibits a second crossover. High levels of chiasma interference have been observed in several cases (e.g. Allendorf *et al*., 1986, and references therein). However, results from the present study suggest only moderate levels of

chiasma interference, since there were only two loci (*Cg49* and *Cg108*) with recombination rates slightly higher than 2/3, which is the highest value expected if there is no chiasma interference. This finding is also in accordance with the fact that MI triploids were found to be more variable than MII triploids.

Following ongrowing in Thau Lagoon, diploid oysters had smaller mean heterozygosities and diversities for both microsatellite and allozyme loci than diploids that had been deployed in the bay of Marennes-Oléron. Although not statistically significant, this difference may indicate that under the unfavourable oligotrophic environment of Marennes-Oléron, there was a stronger selection for genetically more diverse individuals. The fact that such a difference was not observed in the case of triploid animals may indicate that the possession of three chromosomal sets provided enough genetic diversity to cope with the unfavourable conditions prevailing in Marennes-Oléron, when there was not sufficient selective pressure to create differential mortality. Alternatively, given the possible negative effect of somatic aneuploidy on fitness (Zouros *et al*., 1996), diploids would suffer more in an unfavourable environment, when any correlation between heterozygosity and survival would be stronger. This argument is supported by observations that correlations between heterozygosity and growth are more likely to appear under stressful conditions (Stiven, 1995; Audo & Diehl, 1995).

Results presented here cannot alone explain either the advantage of more heterozygous/genetically diverse individuals or the better performance of triploids. Mechanisms underlying these advantages are addressed in associated papers, in which genetic variation is examined in combination with various physiological and biochemical characteristics (Hawkins *et al*., 2000; Naciri-Graven *et al*., 2000).

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