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Genetic polymorphism of glutamine synthetase and delta-9 desaturase in families of Pacific oyster *Crassostrea gigas* and susceptibility to summer mortality

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Abstract:

Large-scale mortality events have been observed in Pacific oyster Crassostrea gigas on the west coast of France since the early 1980s, particularly during summer. In order to understand the causes of this mortality, two generations of oysters from single-pair matings were studied in three sites on the French Atlantic coast (Baie-des-Veys, Auray and Ronce-les-Bains). The present paper examines the role of two candidate genes in the susceptibility of oysters to summer mortality, and the selective pressure exerted by such mortality on their polymorphism. Glutamine synthetase (amino-acid metabolism) and delta-9 desaturase (lipid metabolism) genes were studied in the successive generations, using polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP). Observed and expected genotypic frequencies were compared. Three different alleles were detected for the glutamine synthetase gene and two for delta-9 desaturase. Allele C of glutamine synthetase seemed to be counter-selected in some second generation families. Allele B of delta-9 desaturase gene was potentially counter-selected at Auray in the families showing higher mortality, and strong selection against BB homozygotes was observed. These observations led us to conclude that any selective effect of summer mortality on allele C of glutamine synthetase gene or allele B of delta-9 desaturase gene could be mediated either directly or via linkage to other loci involved in physiological pathways affecting susceptibility.

Keywords: *Crassostrea gigas*; Delta-9 desaturase; Glutamine synthetase; Mortality; SSCP

Introduction

Coastal ecosystems have been subjected to increased anthropogenic pollution (hydrocarbons, pesticides, nutrient inputs) and other environmental stressors (hypoxia, temperature variations, salinity, etc) in recent decades. Some of these stressors are known to induce modifications in the genetic structure of populations through differential mortality of specific genotypes (Gillespie and Guttman, 1993; Moraga et al., 2002; Tanguy et al., 2002). In fact, it was recently observed that certain individuals within a population may be more vulnerable than others to environmental stressors due to their specific genotypes (Ma et al., 2000). Overall levels of genetic variability can therefore become altered as allele frequencies change (Bickham et al., 2000), which has been observed for enzymatic polymorphisms, extensively studied in marine organisms exposed to contaminants (Moraga and Tanguy, 2000; Moraga et al., 2002; Marchand et al., 2003; Yap et al., 2004). By studying genetic polymorphism in specific candidate genes, genetic biomarkers for sensitivity or resistance to environmental stressors could be characterised in marine populations. Such biomarkers would need to reflect genetic variation in the stress response associated with sensitivity to environmental stress (Depledge, 1996; Forbes and Depledge, 1996). Identification of genetic markers for resistance and/or sensitivity could also be useful as a diagnostic tool to evaluate the consequences of environmental factors for population genetic structure.

The Pacific oyster, Crassostrea gigas, was introduced to French coasts at the end of the 1960s in order to offset high mortalities in the Portuguese oyster Crassostrea angulata (Grizel and Héral, 1991). However, since the 1960s and 1970s in Japan and west coast of the U.S.A. (Beattie et al., 1980), and the early 1980s on the west coast of France (Maurer et al., 1986), significant mortality events have been observed in C. gigas in their first or second year depending on location (Soletchnik et al., 2007). Some mortality events are clearly linked to

pathogen infection or drastic climatic changes (Soletchnik et al., 1999). In order to better understand the ecological mechanisms leading to these mass mortalities, a multidisciplinary program, called MOREST ("MORtalités ESTivales"), was initiated by IFREMER in 2001. Initial results showed that summer mortality is a multi-factorial phenomenon, resulting from interactions between oysters, their environment and opportunistic pathogens (Samain et al., 2004; Dégremont et al., 2005). Moreover, Dégremont et al. (2007) showed that susceptibility or resistance to summer mortality in juvenile C. gigas has a strong genetic basis. In this study, we focus on the genetic impact of summer mortality by following allelic frequencies of two candidate genes through two successive generations of C. gigas experiencing high levels of summer mortality. Different farming sites were compared to evaluate the potential impact of environmental factors on the relation between mortality and genotypes.

Glutamine synthetase is involved in nitrogen metabolism and glutamine synthesis. It converts glutamate to glutamine which is the most abundant non-essential amino-acid in tissues (Tate et al., 1972). The genes encoding glutamine synthetase have been isolated from several vertebrate species (Hayward et al., 1986; Fahrner et al., 1993) and from invertebrate such as Drosophila melanogaster (Caizzi et al., 1990), and recently from C. gigas (Tanguy et al., 2005a). Glutamine synthetase mRNA is up-regulated in oysters exposed to hydrocarbons, hypoxia or pesticides, and potentially plays an important role in resistance to these stressors (Boutet et al., 2004; Tanguy et al., 2005a,b; David et al., 2005).

Delta-9 desaturase is involved in lipid metabolism. This enzyme catalyses the formation of monounsaturated fatty acids and requires acyl-CoA, NADH, NADH-reductase, cytochrome b5, phospholipid and oxygen as co-factors (Ozols, 1997). Delta-9 desaturase has been extensively studied in mammals, chicken, fishes and insects (Tocher et al., 1998). The gene encoding delta-9 desaturase in C. gigas has not been entirely sequenced yet. However, its transcription appears to be down-regulated under experimental hydrocarbon exposure

101 (Boutet et al., 2004) and up-regulated under experimental hypoxia (David et al., 2005).
102 Fragments of coding sequences were obtained during these exposure experiments. To our
103 knowledge very few studies have been published on glutamine synthetase gene
104 polymorphism. Those that have mostly concern plants (Pratta et al., 2004; Nogueira et al.,
105 2005) or, more rarely, human beings (Zhang et al., 2005). To our knowledge, no studies have
106 yet been made on delta-9 desaturase gene polymorphism.

107 The aim of this study was to characterize new genetic markers in the Pacific oyster C.
108 gigas, that could be linked with sensitivity to stress, and to investigate whether some
109 genotypes were associated with better survival. We report a potential relationship between the
110 frequency distributions of a specific glutamine synthetase allele and a specific delta-9
111 desaturase allele with summer mortality in oysters. These relationships were investigated in
112 different families and field sites to obtain a clearer picture of the respective roles of genotype
113 and environment.

114

115 **Material and Methods**

116

117 *Family production and field collection*

118 As part of the MOREST program, a first experimental generation (G1) of C. gigas was
119 bred in 2001 at the IFREMER hatchery in La Tremblade (France). This G1 consisted of 72
120 full-sib families nested within 18 half-sib families produced using 18 males and 72 females as
121 G0 parents (Fig. 1). G1 oysters were subsequently placed in three sites along the French coast:
122 Baie-des-Veys, Auray and Ronce-les-Bains (Fig. 2). Further details about family production,
123 sites and mortality records are described in Dégremont et al. (2005). We selected 6 families
124 showing high survival (average = 95%) and 6 families showing low survival (average = 41%)

125 judged by their performances in Auray, which had the highest mortality out of the three sites
126 (Dégremont et al., 2005), and produced 48 G2 'batches' in 2002.
127 Half of these batches were bred from the G1 families showing high survival and the other half
128 from those showing low survival (Fig. 1). Each G2 batch was therefore a form of family
129 obtained by crossing an average of 55 parents overall (proportions of males and females
130 depended on the sex ratio) from two G1 families. We focused on the susceptible G1 families
131 4-16, 14-54, 14-55 and 7-25; their (G0) parents which were males 4, 7 and 14, and females
132 16, 25, 54, 55; and their (G2) progeny which were the G2 batches J, L and Z (Fig. 3; Table 1).
133 These were chosen on the basis of the families showing the highest mortality rate in G1.
134 The G1 oysters were sampled from all 3 sites in autumn 2001 and the G2 oysters were
135 sampled in autumn 2002. These sites differ notably in temperature dynamics (north to south
136 gradient) and trophic conditions (food availability was the highest in Baie-des-Veys).
137
138 *DNA extraction*
139 Genomic DNA was extracted from gill tissue. About 100 mg of gill was placed in
140 extraction buffer (0.3 M Tris, pH 8, 0.02 M ethylene diamine tetra-acetic acid [EDTA], 0.1M
141 NaCl) with sodium dodecyl sulphate (SDS) and proteinase K at final concentrations of 0.6%
142 and 0.1 $mg.ml^{-1}$, respectively. The mixture was then incubated at 55°C until the tissue was
143 completely dissolved. NaCl was then added to a final concentration of 1.3 M. After
144 homogeneisation, the samples were centrifuged at 3000g at 20°C for 10 min. The supernatant
145 was taken and two phenol/chloroform/isoamyl alcohol (25:24:1) extractions were performed.
146 DNA was precipitated with absolute ethanol, recovered by centrifugation 30 min at 12,000g at
147 4°C, rinsed with 70% ethanol, dried and dissolved in 1mL of TE buffer (10 mM Tris, pH8, 1
148 mM EDTA).
149

PCR-SSCP analysis

Exon 6 of the glutamine synthetase gene (GenBank accession no. **CB617403**) was amplified using the forward primer P1 (5'-CTTGCTCTTTTTTTCAGACACATAGA-3') and the reverse primer P2 (5'-GCATTCATCCAGACAGGTAGTCCTTATGAG-3'). A fragment of the delta-9 desaturase gene (GenBank accession no. **CX069227**) was amplified using the forward primer P3 (5'-TACTGTCTTCTGCTAAACGCCAC-3') and the reverse primer P4 (5'-GTCGTGATATTGAGGTGCCAGCC-3'). All PCR amplification was performed in 25 µL reactions containing 1X Taq polymerase buffer, 2 mM $MgCl_2$, 40 µM deoxynucleotides (dNTPs), 10 pmol of each primer, 0.5 units of Taq Uptitherm DNA polymerase (Interchim, Montluçon, France) and about 100 ng of total genomic DNA. After an initial pre-cycle (denaturation 5 min at 94°C, primer hybridisation 2 min at 55°C, elongation 1 min 30s at 72°C), 35 amplification cycles were performed as follows: 20s at 94°C, 40s at 55°C, 1 min 30s at 72°C, with a final elongation 10 min at 72°C. The PCR products were then combined with 20 µL of loading buffer (bromophenol blue, xylen cyanol, saccharose), heated for 5 min to 94°C, then rapidly chilled on ice to melt and retain single strand DNA. After loading on a neutral 8% polyacrylamide gel (37.5:1, acrylamide: bisacrylamide), the samples were electrophoresed at constant voltage (120V) in 0.6X TBE buffer, for 15h at 10°C. After electrophoresis, the gels were stained with ethidium bromide and visualised under ultraviolet light. Single strand DNA bands from the PCR products on the gel corresponding to different conformation types were gel-purified by diffusion into water frozen at -20°C. The same PCR-amplification conditions used for SSCP analysis were also used to recover double strand DNA. PCR products were then purified using a Qiaex II gel extraction kit (Qiagen, Courtaboeuf, France) and cloned into pGEM-T vectors (Promega, Madison, WI, USA). White colonies were grown in LB medium with 100 mg/l ampicillin and the vector was extracted using an alkaline lysis plasmid minipreparation. The inserts were sequenced using a Li-COR

IR² (Sciencetech Inc., London, Ontario, Canada) and Thermo Sequenase Primer Cycle Sequencing Kit (Amersham Bioscience, Uppsala, Sweden).

Statistical analysis

For G1 families observed genotypic frequencies were compared to expected Mendelian genotypic frequencies using χ^2 conformity tests implemented with Statistica software (Statsoft). Yates' corrections were made when expected sample sizes ranged from 2 to 5 individuals. Because expected genotypic frequencies could not be estimated for the multi-parental G2 batches, we compared genotypic frequencies at Auray and Ronce-les-Bains with those at Baie-des-Veys, where mortality rates were much lower, using contingency table analysis. The standard Bonferroni technique (Lessios, 1992) was used to adjust the significance levels of multiple tests: the predetermined significance level, α, was divided by the number of tests, k, to obtain α' the corrected significance level (α' = α/k where α =0.05, k=number of tests carried out). A multiple correspondence analysis was made on the whole data set using the ADE-4 software package (Chessel et al., 1995), in order to explore possible relationships between glutamine synthetase and delta-9 desaturase genotypes. This kind of factorial analysis allows a graphical representation of several qualitative variables using two-by-two associations.

Results

Glutamine synthetase gene polymorphism

PCR-SSCP, performed on exon 6 of the glutamine synthetase gene, allowed us to characterize 3 different alleles which we named A, B and C. The genotypes of the G0 parents are represented in Table 2. Homozygotes BB and CC, and heterozygotes AB, AC and BC

could be clearly scored (Fig. 4). The genotype frequency distributions in the different generations, families/batches and locations are presented in Tables 3 (G1) and 4 (G2) with their respective χ^2 values for tests of significant deviations from expectations. G1 families did not show significant differences between observed and expected genotype frequencies based on Mendelian segregation (Table 3, Fig. 5a,b). In the second generation, we compared observed genotypic frequencies at Auray and Ronce-les-Bains sites, where higher mortality rates were observed, with frequencies at Baie-des-Veys, where mortality was lower as the expected values (Table 4, Fig. 5c,d,e). We observed significant differences in batch Z but not in the other batches. In batch Z, the frequency of allele C was lower at Auray and Ronce-les-Bains compared to Baie-des-Veys, and even reduced to zero at Ronce-les-Bains.

We also pooled the different G2 batches together and compared the allele frequencies of the three sites to see if there was any overall "site effect". This analysis revealed significant differences between Auray and Baie-des-Veys genotypic frequencies (χ^2=17.59; p=0.0005; α'=0.013; 3 df) and between Ronce-les-Bains and Baie-des-Veys genotypic frequencies (χ^2=18.12; p=0.0004; α'=0.013; 3 df).

The sequences of the different alleles of glutamine synthetase exon 6, characterized by SSCP, revealed that the sequence of allele A differs from the sequences of the other 2 alleles by 2 nucleotide sequence mutations (amino acids 343 and 345), and one of these results in mutation of the corresponding amino acid. The amino acid cystein (amino acid 343) is replaced by arginin. The nucleotide sequence of allele C revealed 2 mutations compared with the other 2 alleles, but these modifications are synonymous (amino acids 338 and 339).

Delta-9 desaturase gene polymorphism

PCR-SSCP performed on a coding fragment of the delta-9 desaturase gene allowed us to characterize 2 different alleles which we named A and B (Fig. 6). Distribution of observed

225 genotypes of the G0 parents is represented in Table 2. The genotypic frequency distributions
226 in the different generations, families, batches and locations are presented in Tables 5 (G1) and
227 6 (G2) with their χ^2 values. Families of the first generation (G1) did not show significant
228 differences between observed and expected Mendelian genotypic frequencies, except for
229 family F7-25 at the Auray site where heterozygote AB was less well represented than
230 expected (Table 5; Fig. 7a,b). We had previously analysed polymorphism of the delta-9
231 desaturase gene in family F7-25 before field placement in the 3 sites and found no deviation
232 of observed genotypic frequencies from expectations (χ^2= 0.02; p=0.89; α'=0.025; 1 df, data
233 not shown).

234 In the second generation, we compared observed genotypic frequencies at Auray and
235 Ronce-les-Bains with those at Baie-des-Veys as the expected. We observed significant
236 differences between Auray and Baie-des-Veys in both batch Z and batch J (Table 6; Fig.
237 7c,d,e). No significant differences were observed for the other batches or sites. Bonferroni
238 adjustement removed the significant result for batch Z. We considered it with caution but we
239 did not reject it as the p-value was close to the significance level (p=0.02; α=0.05; α'=0.013).

240 We also observed a significant decrease in the frequency of allele B at the Auray site
241 in family F7-25 (G1, Table 5, Fig. 7b) and batch Z (G2, Table 6, Fig. 7c). Moreover, no BB
242 homozygotes were observed in batch Z at any site (Table 6). However, in batch J at the Auray
243 site, we observed a greater number of individuals carrying allele B compared to Baie-des-
244 Veys (Table 6, Fig. 7e).

245 Pooling the different batches together to compare the 3 sites, we found no deviation
246 between observed genotypic frequencies at Baie-des-Veys and the other 2 sites (χ^2=0.02;
247 p=0.89; α'=0.025; 1 df; and χ^2=0.05; p=0.82; α'=0.025; 1 df for Auray and Ronce-les-Bains
248 respectively).

249

The sequences of the different alleles characterized by SSCP revealed 2 differences in nucleotide sequences between alleles A and B (nucleotide 151, amino acid number 50; and nucleotide 157, amino acid number 52 of the amplified fragment). However, these mutations do not change the corresponding amino acids.

Multiple correspondence analysis between genotypes at the two loci

The multiple correspondence analysis performed on the whole dataset did not reveal any significant relationship between individuals carrying glutamine synthetase allele C and individuals carrying delta-9 desaturase allele B (Fig. 8). This means that individuals carrying glutamine synthetase allele C are not likely to carry delta-9 desaturase allele B. However, individuals that were homozygous for the B allele of glutamine synthetase were more frequently homozygous at the delta-9 desaturase locus also.

Discussion

The aim of this study was to investigate whether survival of C. gigas was associated with specific genetic markers, by comparing allelic frequencies in (1) families through three generations and (2) three environmentally different culture sites.

Among methods available to detect polymorphism in DNA sequences, we chose the SSCP approach to detect polymorphism in the coding sequences of the two genes: glutamine synthetase and delta-9 desaturase. We observed deviations from expected Mendelian frequencies after mortality, and differences in genotypic frequencies between sites characterized by high or low summer mortality in some G1 families and/or G2 batches. Several hypotheses could explain these results. Deviations in allele frequencies may be explained by a number of factors, particularly migration, mutation, null alleles, or selection.

Our experimental design eliminated migration. Mutation and null alleles can be eliminated also, because these events are very rare between two successive generations. This leaves only selection as a viable explanation for our results. We analysed polymorphism in family F7-25 before mortality and did not observe any deviation from Mendelian expectations. This observation led us to dismiss segregation distortion due to fitness differences at earlier stages. Expected Mendelian frequencies could not be calculated for G2 offspring because many parents (in variable ratios of female and male) were used from each G1 family to produce the G2 and the exact genotypes of each parental individual and their relative contributions to the batch are unknown. For the G2 batches, comparing high mortality sites with a low mortality site allowed us to see how well observed differences in allele frequencies corresponded to differences in mortality assuming only that the allele frequencies were identical at all sites when the batches were planted.

In this work, we chose two genes involved in essential physiological pathways and known to be regulated by a number of environmental parameters. The metabolic functions in which they are involved (nitrogen metabolism, glutamine synthesis and lipid metabolism) are relevant here because of the role of physiological status in susceptibility to summer mortality. With SSCP profiles, we detected three different alleles in exon 6 of the glutamine synthetase gene and analysis of allele segregation in the G1 before field placement showed that observed frequencies did not differ from Mendelian expectations. However, in G2, we observed a strong decrease in the frequency of allele C in the Z batch at Auray and Ronce-les-Bains. The frequencies of AC heterozygotes in both sites and of BC in Ronce-les-Bains fell to 0 suggesting strong selection against allele C in the heterozygous condition. Pooling the different batches together and testing for site effects in the second generation, we observed significant differences between observed genotypic frequencies of the glutamine synthetase

gene at Baie-des-Veys and the 2 other sites. However, the fact that the differences in G2 were only observed for the Z batch and not for the two other batches also indicates an important "family" effect. The differences in counter-selection against heterozygotes among families may be related to the effects of other genes linked to the locus we studied. In any case, it appears that there may be a kind of indirect selection acting on the C allele possibly involving selection on other linked genes, as suggested by the absence of selection against the C allele in G1 and by the fact that allele C sequencing revealed only synonymous mutations. Alternatively, the variation in genotypic frequencies may not be associated with selection exerted by summer mortality but could be the consequence of a family effect.

The SSCP method also detected 2 different alleles of the coding fragment of delta-9 desaturase. Genotype frequencies followed Mendelian expectations in the G1 generation except in family F7-25 at Auray, where AB heterozygotes were less frequent than expected. A similar but non-significant trend was observed in Ronce-les-Bains for the same family. In the G2 generation, differences were found in observed frequencies between Auray and Baie-des-Veys. In the Z batch, AB heterozygotes were significantly less represented in Auray. A complete absence of homozygote BB in the Z batch at any site strongly suggests selection against this genotype. One possible explanation is that the B allele is deleterious, particularly in the homozygous state. When comparing AB heterozygote frequency and associated mortality in the three sites for G1 families F7-25 and F4-16, we observed a clear relationship between decreasing heterozygosity and increasing mortality rate in both families, with a stronger effect in family F7-25. In G2 batches J, Z and L, we also observed that AB frequency decreased where mortality rates were above 50% (batches Z and L in Auray). This tendency seems to suggest that heterozygotes carrying allele B were selected against in the most susceptible families. Furthermore, batch J showed significantly more AB heterozygotes in

Auray than in Baie-des-Veys, which could be explained by the fact that mortality was higher in Baie-des-Veys than in Auray for this batch. In the case of delta-9 desaturase, family effect seems to be more important than site effect, as no significant differences of observed frequencies were obtained among sites considering all the batches of the second generation together.

For both genes, heterozygotes seem to be selected against in families showing high mortality. This result is at odds with the common observation of heterozygote advantage in fitness-related traits (Zouros, 1987). Heterozygotes have been frequently reported to use less energy for their metabolism than homozygotes, thus increasing their tolerance to environmental stressors (Hawkins et al., 1989; Holley and Foltz, 1987). In bivalves, David and Jarne (1997) found that heterozygosity at 9 allozyme loci was associated with higher viability. Heterozygote advantage has also been described in relation to enhanced growth rates in clams (Gentili and Beaumont, 1988; Scott and Koehn, 1990). Volckaert and Zouros (1989) also developed a model based on allozyme studies assuming that energy savings attributed to heterozygosity are used to maximize fitness. Nevertheless, it has been shown in many marine species that environmental conditions can select for certain alleles in particular and also decrease heterozygosity at some allozyme loci in populations exposed to pollutants, *e.g.* in Idotea baltica (De Nicola et al., 1992).

The multiple correspondence analysis performed on the entire data set revealed no association between glutamine synthetase genotypes containing allele C and delta-9 desaturase genotypes containing allele B. Individuals carrying glutamine synthetase allele C do not systematically carry delta-9 desaturase allele B. This suggests that there is no linkage between these two genes. However, each gene considered separately could be linked to another nearby gene or locus. Sequence analysis of the 3 glutamine synthetase alleles and the

2 delta-9 desaturase alleles revealed synonymous mutations in allele C and allele B of these genes respectively, compared with the other alleles. The mutations observed in the nucleotide sequence of these alleles could be linked to mutations elsewhere in the coding sequence of these genes or in other linked genes through a hitch-hiking effect (Maynard Smith and Haigh, 1974; Kim and Stephan 2000; Barton, 2000). We analysed the polymorphism of exon 6 of the glutamine synthetase gene because this exon that showed the highest rate of polymorphism in preliminary analyses. The same arguments may also apply to the delta-9 desaturase gene as we have only studied a fragment of the gene. However, a completely different hypothesis which may explain our results is that no direct selection occurred on the glutamine synthetase and delta-9 desaturase loci, but that changes in genotypic frequencies result from a family effect acting during summer mortality. The absence of large differences in the first generation supports this hypothesis and Dégremont et al. (2005) showed that close to 50% of variation in juvenile oyster survival could be explained by family differences. This family effect on allelic frequencies may be explained at biological level by potential links between the loci we studied and other genes located elsewhere in the oysters genome. Epistatic interactions could be a hypothesis. Family effect we observed on genotypic frequencies could then be a consequence of interactions between glutamine synthetase or delta-9 desaturase alleles and genetic background exerting influence on them.

More generally, in the two genes studied here, changes in genotypic frequencies within a family or batch varied among sites. These family and site effects on genotypic frequencies are consistent with observations by Dégremont et al. (2005) who described a strong family effect on survival and growth of the C. gigas families used in the MOREST program. Dégremont et al. (2005) also showed variation in survival among sites, due to

374 environmental conditions, which are consistent with the differences in genotypic frequencies
375 that we observed among sites.
376
377 To our knowledge, few studies have dealt with genetic or allozyme polymorphism of
378 glutamine synthetase (Pratta et al., 2004; Nogueira et al., 2005; Zhang et al., 2005) and none
379 with that of delta-9 desaturase. However, allozyme polymorphism at different loci has been
380 examines to test for a possible association with survival in molluscs exposed to various
381 contaminants. In C. gigas, 6 alleles and 5 genotypes at 3 loci (Ak, Pgi and Pgm*)* were
382 identified as markers of resistance or sensitivity to herbicide stress and differential survival
383 (Moraga and Tanguy, 2000). Three allozyme loci (Aat-2, Ak and Pgm) were also shown
384 differ in frequency between C. gigas populations that were resistant or susceptible to TBT
385 (Tanguy et al., 1999), and SSCP markers developed for C. gigas metallothionein genes also
386 showed a correlation between allele frequencies and resistance to heavy metals (Tanguy et al.,
387 2002). In the present study, no relationship was observed between mortality rates and
388 chemical characteristics of three field sites. This observation suggests that environmental
389 pollutants do not directly contribute to summer mortality at these sites or to the differences
390 among them in allele frequencies. If there is some selection against glutamine synthetase
391 allele C and delta-9 desaturase allele B, it may therefore not be due to sensitivity to pollutants.
392 Focusing on the causes of mass mortality, Tremblay et al. (1998) studied 4 enzyme alleles in
393 mussels undergoing mass summer mortality, but did not observe any differences in allele
394 frequencies between susceptible and resistant stocks or between one and two-year-old
395 individuals, despite differences in heterozygosity. Xiao et al. (2005) used RAPD markers to
396 investigate the role of genetic factors on scallop mortality in China. They observed reduced
397 genetic diversity in cultured stocks that could result in increased susceptibility to stress
398 leading to increased mortality.

399

400 To conclude, this study has provided new genetic markers, which are in some cases
401 associated with susceptibility to summer mortality in Pacific oyster Crassostrea gigas,
402 depending on environmental factors and genetic background. Indeed, allele C of glutamine
403 synthetase exon 6 and allele B of a coding fragment of delta-9 desaturase gene were less
404 frequent in families and sites showing the highest mortality rates, with a stronger family effect
405 than site effect. If there is selection, this seems to occur on other genes or loci to which the
406 studied genes may be linked. Following the genotypic frequencies of the same genes in a third
407 generation could allow us to verify these results. Frequencies of the 2 counter-selected alleles
408 will also be studied in natural populations of C. gigas submitted to various environmental
409 stresses in the field in order to obtain complementary results. Finally, other candidate genes
410 involved in other physiological functions are currently being examined for genetic
411 polymorphism in the same families, so as to add depth to the initial exploration described
412 here.

413

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555 **Table 1:** Mortality of the families used in this study in the 3 field sites.

Generation	Family / batch	Mortality (%)		
		Baie-des-Veys	**Auray**	**Ronce-les-Bains**
G1	**4-16**	8.9	57.8	41.6
G1	**14-54**	44.4	85.2	75.3
G1	**14-55**	24.7	82.7	66.6
G1	**7-25**	20.0	56.5	58.8
G2	**Z**	6.3	64.2	40.2
G2	**L**	15.2	53.7	30.4
G2	**J**	39.1	33.3	41.4

556 **Table 2:** G0 parental genotypes for exon 6 of the glutamine synthetase gene and the delta-9
557 desaturase coding fragment.

Parent	Glutamine synthetase genotype	Delta-9 desaturase genotype
male 7	BB	AA
female 25	AB	AB
male 14	BB	AA
female 54	BB	AA
female 55	BC	AA
male 4	CC	AB
female 16	BB	AA

558 **Table 3:** Distribution of glutamine synthetase exon 6 genotype frequencies in tested C. gigas G1 families, and comparisons with expected
559 frequencies. Numbers in rounded brackets correspond to degrees of freedom. Numbers in square brackets under χ^2 values indicate sample sizes.
560 No significant differences were observed at the α' significance level.

Family	**Genotype**	**Expected frequency**	**Baie-des-Veys**			**Auray**			**Ronce-les-Bains**		
			Observed frequency	χ^2 **(df)** [sample size]	***p*-value**	**Observed frequency**	χ^2 **(df)** [sample size]	***p*-value**	**Observed frequency**	χ^2 **(df)** [sample size]	***p*-value**
14-55	BB	0.5	0.48	0.04 (1)	0.99	0.38	1.69 (1)	0.64	0.61	0.89 (1)	0.83
	BC	0.5	0.52	[23]		0.62	[29]		0.39	[18]	
7-25	BB	0.5	0.53	0.05 (1)	0.97	0.50	0 (1)	1	0.52	0.05 (1)	0.98
	AB	0.5	0.47	[19]		0.50	[48]		0.48	[21]	

561

562 **Table 4:** Distribution of glutamine synthetase exon 6 genotype frequencies in tested C. gigas G2 batches and comparison of frequencies observed
563 in Auray and Ronce-les-Bains with frequencies observed in Baie-des-Veys. Numbers in rounded brackets correspond to degrees of freedom.
564 Numbers in square brackets indicate sample sizes, * indicates a significant difference between observed frequencies in Auray or Ronce-les-Bains
565 and observed frequencies in Baie-des-Veys, at the α' significance level.

Batch	**Genotype**	**Baie-des-Veys**		**Auray**			**Ronce-les-Bains**		
		Observed frequency	[sample size]	**Observed frequency**	χ^2 **(df)** [sample size]	***p*-value**	**Observed frequency**	χ^2 **(df)** [sample size]	***p*-value**
Z	AB	0.06		**0.40**	**34.13 (3) ***	**0.00 ***	**0.50**	**42.03 (3) ***	**0.00 ***
	AC	0.23	[47]	**0.00**	[43]		**0.00**	[42]	
	BB	0.34		**0.56**			**0.50**		
	BC	0.36		**0.05**			**0.00**		
L	AB	0.11		0.23	3.30 (3)	0.35	0.26	4.35 (3)	0.23
	AC	0.22	[46]	0.13	[48]		0.12	[42]	
	BB	0.50		0.50			0.43		
	BC	0.17		0.15			0.19		
J	AB	0.29		0.30	0.00 (1)	1	0.26	0.03 (1)	0.87
	BB	0.71	[48]	0.70	[44]		0.74	[47]	

566 **Table 5:** Distribution of delta-9 desaturase coding fragment genotype frequencies in tested C. gigas G1 families, and comparisons with expected
567 frequencies. Numbers in rounded brackets correspond to degrees of freedom. Numbers in square brackets under χ^2 values indicate sample sizes. *
568 indicates a significant difference between expected and observed frequencies, at the α' significance level.

Family	**Genotype**	**Expected frequency**	**Baie-des-Veys**			**Auray**			**Ronce-les-Bains**		
			Observed frequency	**χ^2 (df)** [sample size]	***p*-value**	**Observed frequency**	**χ^2 (df)** [sample size]	***p*-value**	**Observed frequency**	**χ^2 (df)** [sample size]	***p*-value**
4-16	AA	0.5	0.29	2.57 (1)	0.10	0.55	0.09 (1)	0.76	0.44	0.25 (1)	0.62
	AB	0.5	0.71	[14]		0.45	[11]		0.56	[16]	
7-25	AA	0.5	0.45	0.20 (1)	0.65	**0.67**	**5.33 (1) ***	**0.02 ***	0.68	2.91 (1)	0.09
	AB	0.5	0.55	[20]		**0.33**	[48]		0.32	[22]	

569

570 **Table 6:** Distribution of delta-9 desaturase coding fragment genotype frequencies in tested C. gigas G2 batches and comparison of frequencies
571 observed in Auray and Ronce-les-Bains with frequencies observed in Baie-des-Veys. Numbers in rounded brackets correspond to degrees of
572 freedom. Numbers in square brackets under χ^2 values indicate sample sizes. ▲ indicates a significant difference between observed frequencies in
573 Auray or Ronce-les-Bains and observed frequencies in Baie-des-Veys, at the α significance level * indicates a significant difference between
574 observed frequencies in Auray or Ronce-les-Bains and observed frequencies in Baie-des-Veys, at the α' significance level.

Batch	**Genotype**	**Baie-des-Veys**		**Auray**			**Ronce-les-Bains**		
		Observed frequency	[sample size]	**Observed frequency**	**χ^2 (df)** [sample size]	***p*-value**	**Observed frequency**	**χ^2 (df)** [sample size]	***p*-value**
Z	AA	0.54		**0.82**	**6.45 (1) ***	**0.02 ▲**	0.60	0.34 (1)	0.71
	AB	0.46	[46]	**0.18**	**[33]**		0.40	[43]	
	BB	0		**0**			0		
L	AA	0.75		0.83	1.08 (1)	0.30	0.75	0.00 (1)	1
	AB	0.25	[48]	0.17	[37]		0.25	[40]	
J	AA	0.79		**0.51**	**8.10 (1) ***	**0.01***	0.68	1.50 (1)	0.71
	AB	0.21	[48]	**0.49**	[45]		0.32	[47]	

Figure 1: Crosses and resulting generations bred as part of the MOREST program and used in the present polymorphism study.

Figure 2: Location of the 3 field sites in oyster farming areas along the French Channel and Atlantic coasts**.**

Figure 3: MOREST families used in this study. Parents (G0) are identified by numbers. G1 families are identified by « x-y » where x is the male and y the female used for the cross. G2 batches are identified by letters.

Figure 4: Diagram of PCR-SSCP profiles obtained for exon 6 of glutamine synthetase in C. gigas families. The genotypes are indicated underneath in capitals and the alleles corresponding to each band are given on the left. Each allele is represented by two bands. One band is at the same level for allele A and allele C.

Figure 5: Glutamine synthetase genotype frequencies in C. gigas G1 families (A: F14-55; B: F7-25) and G2 batches (C: batch Z; D: batch L; E: batch J). Significant differences between expected and observed frequencies (G1) or between Baie-des-Veys and other sites (G2) are marked *.

Figure 6: Diagram of PCR-SSCP profiles obtained for delta-9 desaturase coding fragment in C. gigas families. The genotypes are indicated underneath in capitals and the alleles corresponding to each band are given on the left. Each allele is represented by two bands.

599 **Figure 7:** Glutamine synthetase genotype frequencies in C. gigas G1 families (A: F4-16; B:
600 F7-25) and G2 batches (C: batch Z; D: batch L; E: batch J). Significant differences between
601 expected and observed frequencies (G1) or between Baie-des-Veys and other sites (G2) are
602 marked *.
603
604 **Figure 8:** Multiple correspondence analysis for Crassostrea gigas from all families and all
605 sites, linking glutamine synthetase genotypes with delta-9 desaturase genotypes (GS:
606 glutamine synthetase; D9: delta-9 desaturase). Axis 1: eigen value = 0.536; 26.8% inertia and
607 axis 2: eigen value = 0.500; 25.0% inertia. As no scale appears on the graph, the ovoid box
608 gives the scale of the axes.
609
610

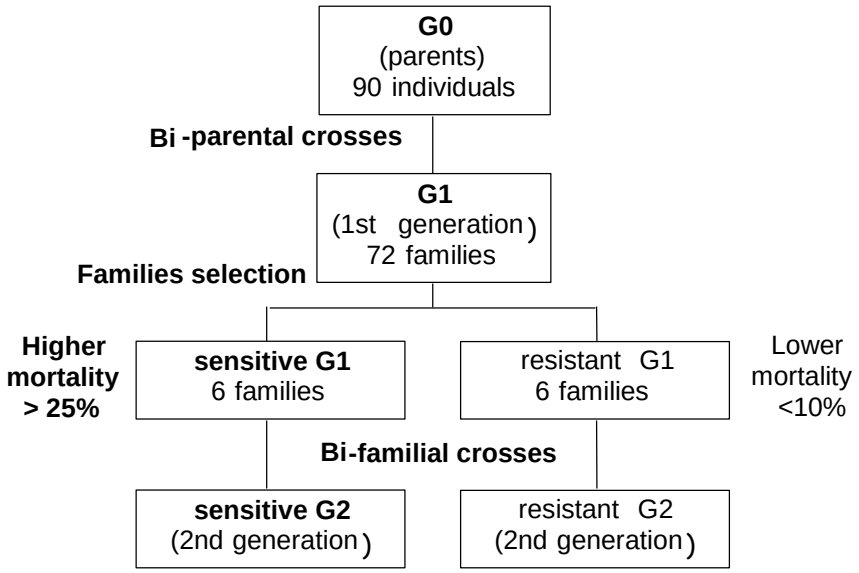

Figure 1

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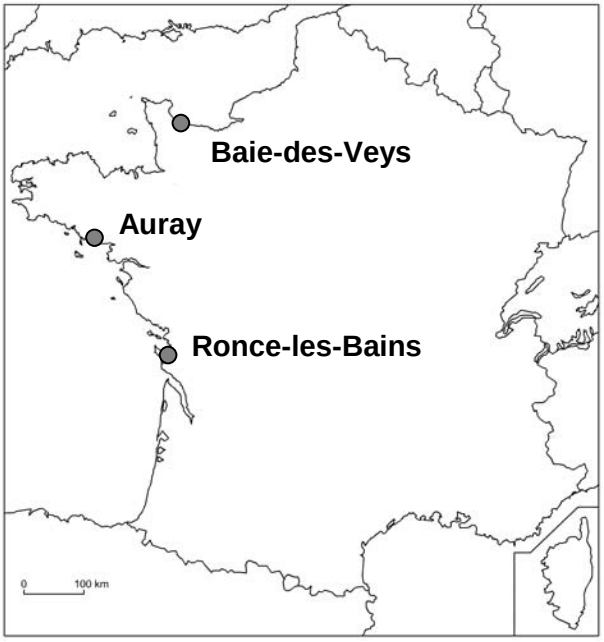

Figure 2

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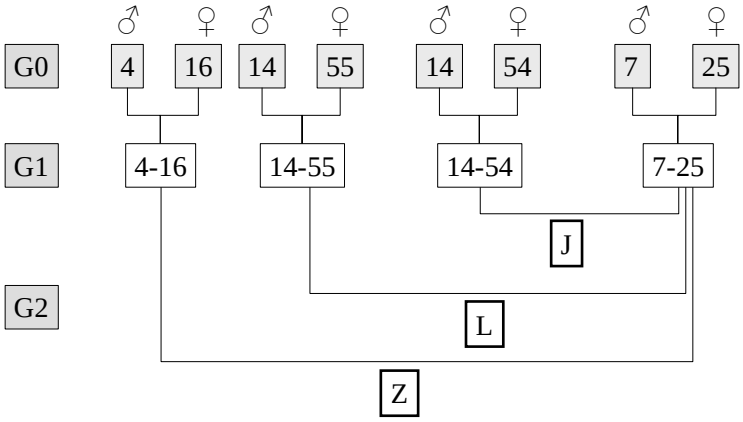

Figure 3

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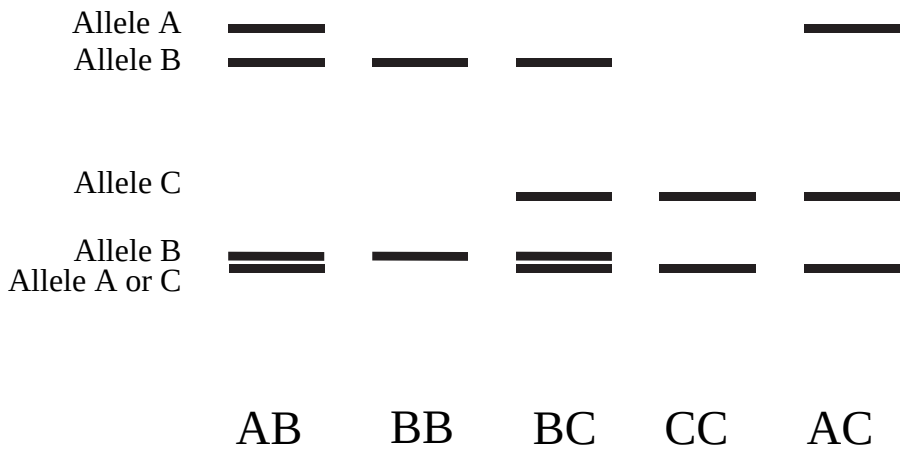

Figure 4

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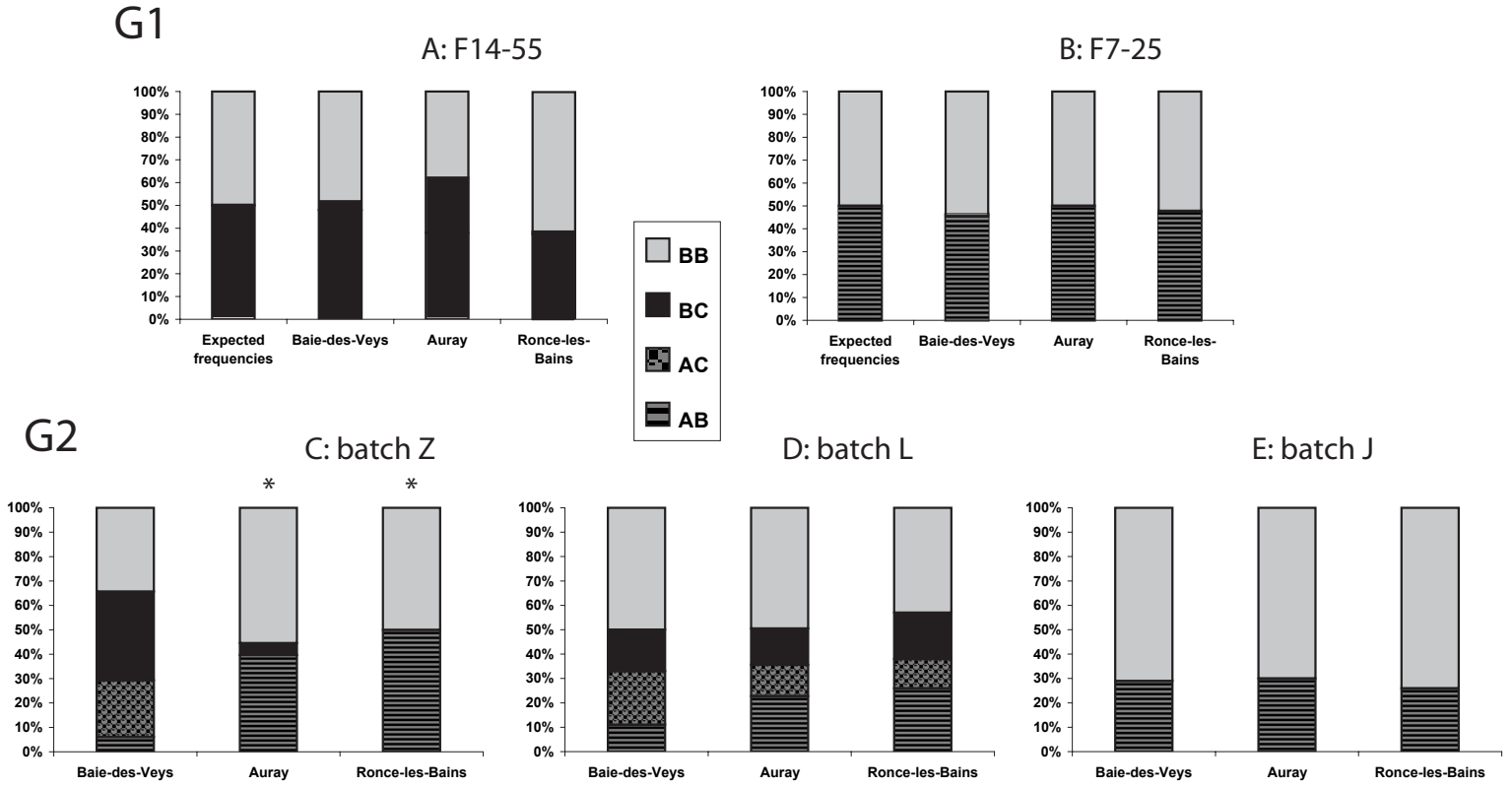

Figure 5

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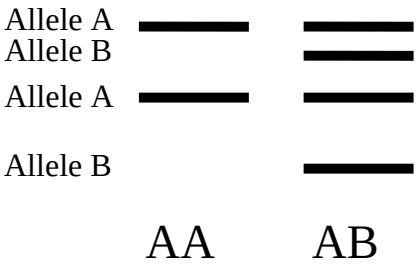

Figure 6

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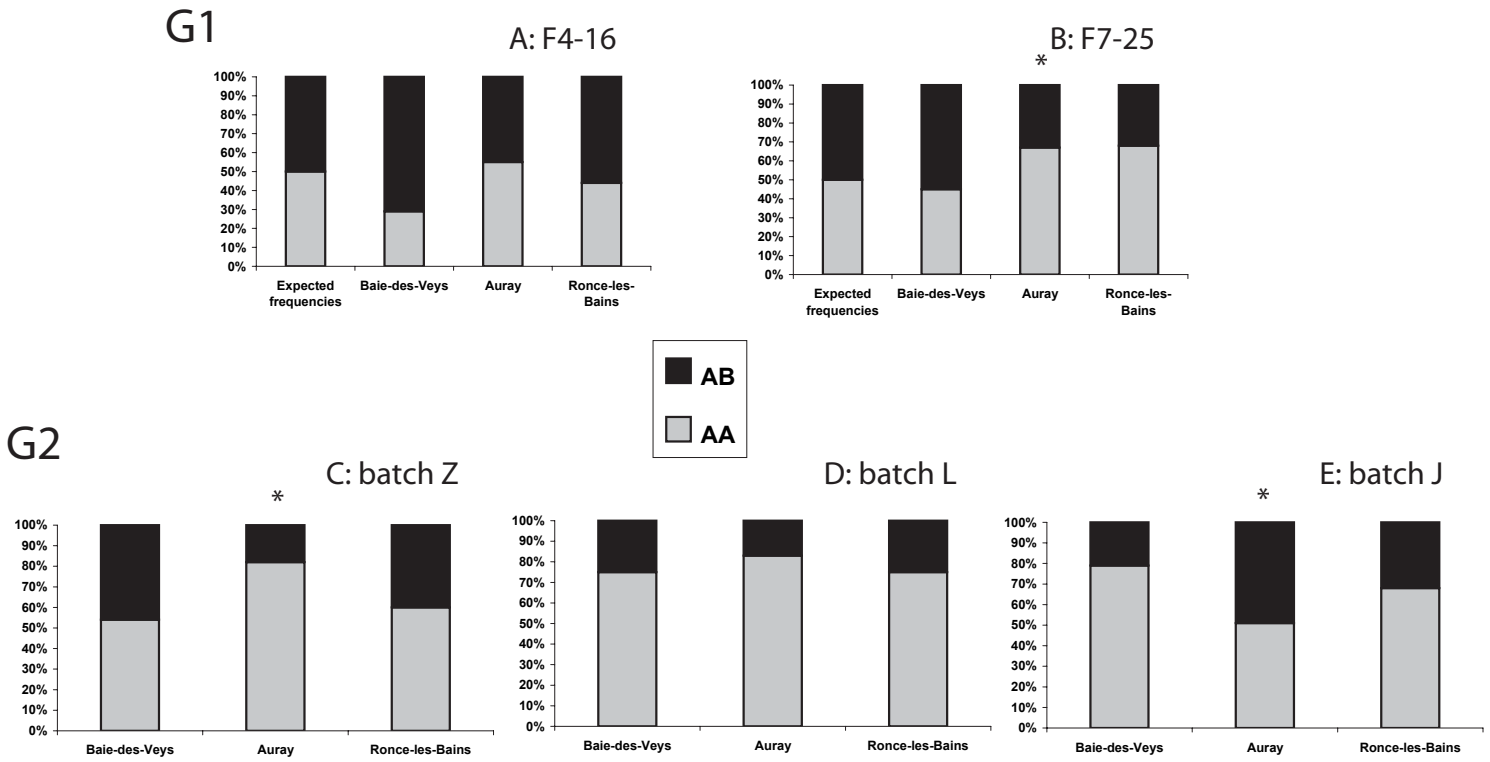

Figure 7

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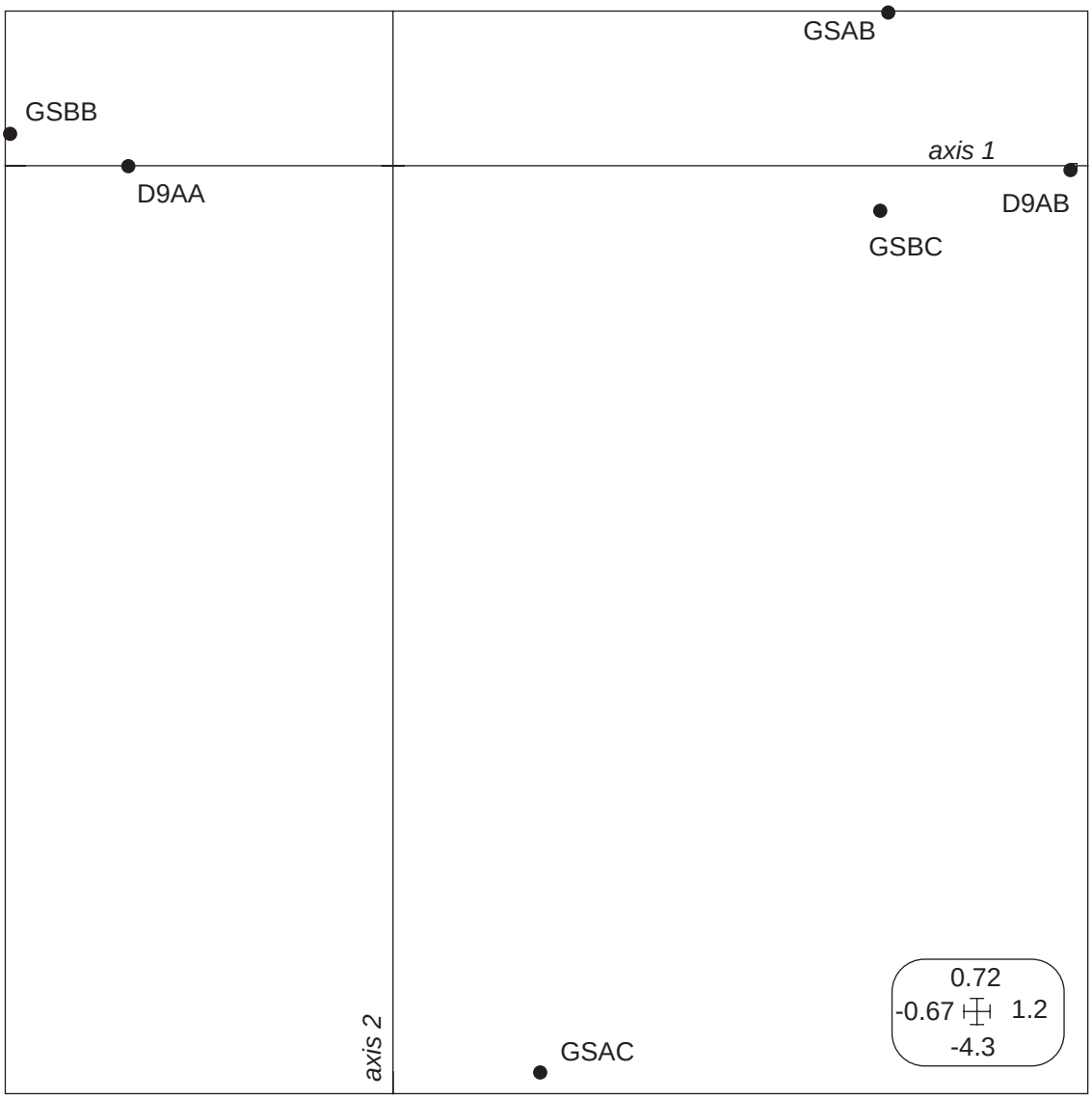

Figure 8

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