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RESEARCH ARTICLE

Characterisation and genetic polymorphism of metallothionein gene *CgMT4* in experimental families of Pacific oyster *Crassostrea gigas* displaying summer mortality

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Abstract
Summer mortality events have been observed in Pacific oyster *Crassostrea gigas* for several decades. This paper examines the selective pressure exerted by summer mortality on the polymorphism of a newly identified oyster metallothionein gene. *CgMT4* cDNA and genomic sequences were obtained. *CgMT4* was studied in two generations of oysters reared in three sites on the French Atlantic coast, using single strand conformation polymorphism analysis. Four alleles were detected. Individuals carrying genotype *MT4*-CD seem to have higher susceptibility to summer risk conditions. The *MT4* gene could be a potential new genetic marker for susceptibility; further validation studies are recommended.

Keywords: Pacific oyster, metallothionein, susceptibility, genotypes, selection

Introduction

Metallothioneins (MTs) are low molecular weight (6–7 kDa) ubiquitous cytoplasmic metal-binding proteins (Kojima & Kagi, 1978). The defining characteristics of metallothioneins are a high cysteine content (~30 %) and the presence of conserved Cys-X_n-Cys motifs, where X can be any amino acid other than cysteine (Jenny et al. 2006). Metallothioneins are involved in the cellular regulation of essential metals (copper, zinc, iron, manganese, etc.) and in the detoxification of non-essential heavy metals (cadmium, mercury, etc.). Metallothionein genes have been identified in all major classes of invertebrates and vertebrates (Kägi 1993) and are known to show increased expression in the presence of heavy metals or causative agents of oxidative stress (Andrews 2000). However, metallothionein genes have been reported to be inducible by many other biotic and abiotic factors (Hamer 1986; Ghoshal & Jacob 2000). In the Pacific oyster *Crassostrea gigas*, three metallothionein genes have been already described (*CgMT1*, *CgMT2* and *CgMT3*) (Tanguy et al. 2001; Tanguy & Moraga, 2001; Marie et al. 2006). Moreover, a partial sequence of a new *Crassostrea gigas* metallothionein gene was obtained in SSH libraries constructed in oysters subjected to hypoxia as opposed to normoxia (David et al. 2005). Metallothionein expression analyses have been conducted on these different isoforms (Tanguy et al. 2001; David et al. 2005; Marie et al. 2006), and a sensitive enzyme linked immunosorbent assay was developed to quantify metallothionein protein expression in *C. gigas* (Boutet et al. 2002). However, to our knowledge, very few studies have been conducted on the polymorphism of metallothionein genes. Tanguy

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et al. (2002) monitored the polymorphism of two exons of *CgMT1* in field populations exposed to various metal concentrations and in experimentally exposed populations. Using single strand conformation polymorphism (SSCP), they identified two haplotypes in exons 2 and 3 of *CgMT1* that were significantly associated with tolerance to metals. Polymorphism was also observed in 5′ and 3′ untranslated regions (UTRs) of oyster metallothionein genes or cDNA (Tanguy et al. 2001). A few studies have been done on the polymorphism of metallothionein genes in humans, mice (Hayashi et al. 2006; Kita et al. 2006; Miura 2009; Giacconi et al. 2009; Gundacker et al. 2010; Chen et al. 2010) and even soil insects (Roelofs et al. 2006; Timmermans et al. 2007; Janssens et al. 2007; Janssens et al. 2008; Janssens et al. 2009) that suggest that it could be affected by environmental pressures.

As classically described in the studies dealing with evolutionary toxicology (Bickham 2011), differences in sensitivity to pollution (hydrocarbons, pesticides, nutrient inputs) and other environmental stressors can cause differential mortality between individuals of a single population. Consequently, the genetic structure of populations can be modified as a result of selection (Gillespie & Guttman 1993; Moraga et al. 2002; Tanguy et al. 2002). Massive summer mortality events, such as those described for oysters (Samain et al. 2004; Samain & McCombie 2008), may also lead to genetic changes in populations. Studies of genetic polymorphism in specific candidate genes might therefore allow us to identify genetic biomarkers of sensitivity or resistance to environmental stressors in marine populations, reflecting genetic variation in the stress response, linked to variations in organism sensitivity (Forbes & Depledge 1996; Depledge 1996). By this method, previous studies allowed us to identify some alleles of *C. gigas* glutamine synthetase and delta-9 desaturase genes that seemed to be associated with susceptibility to summer mortality, depending on environmental factors and genetic background (David et al. 2007).

The aim of this study was (1) to characterize a new candidate gene in the Pacific oyster *C. gigas* and (2) to characterize new genetic markers (alleles or genotypes of this gene) that could be linked to sensitivity during summer mortality events. First, the isolation and characterization of the full-length cDNA and genomic sequence encoding MT4, the new metallothionein of *C. gigas* (*CgMT4*), are described. A phylogenetic analysis was then performed to situate the *CgMT4* sequence among other species and to identify potential evolutionary divergences in the sequence. The polymorphism of this gene was also studied in selected oyster families characterised by a high level of mortality, to find out the possible selective effect of environmental parameters and mortality on *MT4* allelic frequencies in *C. gigas*.

Material and methods

Cloning and sequencing of *CgMT4* cDNA and 5′ and 3′ flanking regions

Total RNA was extracted from the gills, mantle and digestive gland of freshly opened oysters *Crassostrea gigas* using the guanidium isothiocyanate method (Strohman et al. 1977). Gills, mantle and digestive gland were all chosen in order to increase the chances of obtaining samples where *CgMT4* was sufficiently expressed. Gills and mantle are the first tissues in contact with the environment; digestive gland is involved in digestion, accumulation and detoxification. The generation of cDNA of the 5′ and 3′ UTRs for *CgMT4* was achieved by following the commercial protocol for the rapid amplification of 3′/5′ cDNA ends (5′/3′ RACE Kit, Roche, Mannheim, Germany), using specific primers. The primers were designed from the partial sequence of *C. gigas* metallothionein 4 gene (*CgMT4*) previously identified (David et al. 2005) and are shown in Table 1. Briefly, cDNA was synthesized using oligo dT anchor primer 1 and M-MLV reverse transcriptase (Promega, Madison, WI, USA), and was then purified using the Wizard® DNA Clean-Up System (Promega). A poly-A tail was generated at the 5′ end of the reverse transcription product, using terminal deoxynucleotidyl transferase (Promega) and dATP. Amplification of the 5′ and 3′ UTRs was then carried out using oligo dT anchor primer 2 and 5′ (MT4 Rev then MT4 Rev2) or 3′ (MT4 For) specific primers (Table 1). The

Table 1. Sequences of the primers used for the *C. gigas MT4* cDNA and gene cloning and in the *CgMT4* gene polymorphism analysis.

Primer name	Sequence
Oligo dT anchor primer 1	5′-CGCTCTAGAACTAGTGGATCTTTTTTTTTTTTTT-3′
Oligo dT anchor primer 2	5′-GACCACGCGTATCGATGTCGACT(16)V-3′
MT4 For	5′-GCCCAGACGGGAAAATGCGTGTG-3′
MT4 Rev	5′-CAGTTACACGATGCTTTGGCGCA-3′
Anchor primer	5′-GACCACGCGTATCGATGTCGACT-3′
MT4 Rev 2	5′-ACGCATTTTCCCGTCTGGGCGA-3′
MT4 A	5′-ACTACAGGAAAAGATCAGCTCAC-3′
MT4 B	5′-CAGTTACACGATGCTTTGGCGCA-3′
MT4 C	5′-GACAAGTGTAAATGCGCCAAAGCATCGTG-3′
MT4 D	5′-TCAGGTATACAGTATATACTGAAGCG-3′
Forward primer P1	5′-ATCCTACACAAACCTTCTTTTTCAGCGGG-3′
Reverse primer P2	5′-ATGTCCGTTTACTTACTAGTACAGCAGCA-3′

resulting 150- and 450-base pair cDNA fragments generated by these procedures corresponded to the 5′ and 3′ UTRs, respectively; these were inserted into pGEM-T vector (Promega), cloned, and then sequenced using a LiCOR IR^2 (Sciencetech) and Thermo Sequenase Primer Cycle Sequencing Kit (GE Healthcare Europe, Freiburg, Germany). Sequences were subjected to a homology search using the BLASTX program (http://www.ncbi.nlm.nih.gov/BLAST/).

Molecular phylogeny analysis

Phylogenetic analysis was performed on the amino acid sequences from full-length metallothionein cDNA from different oyster species. Amino acid sequences were aligned using ClustalW Software (Thompson et al. 1994). Molecular phylogenetic trees were constructed using the phylogenetic component of the MEGA3.1 software (Kumar et al. 2004). Amino acid differences were corrected for multiple substitutions using a γ correction. In this correction, α, the shape parameter of the γ distribution, was set to 2. With $\alpha = 2$, the distance between any two amino sequences, d_{ij}, is approximately equal to Dayshoff's PAM distance per site. Support for the major nodes within trees was evaluated by bootstrapping the data, with 1000 bootstrap replicates of the whole data set generated.

Cloning and sequencing of the *CgMT4* gene

Total genomic DNA was isolated from oyster gills using phenol/chloroform/isoamyl alcohol (25:24:1). The *CgMT4* gene sequence was amplified using two primer combinations designed from the cDNA sequence (MT4 A and MT4 B; MT4 C and MT4 D; Table 1), with Uptitherm DNA polymerase (Interchim, Montluçon, France). Two hundred nanograms of genomic DNA were used for amplification with 2 mM MgCl2, and 10 pmol of each primer though the following steps: denaturation at 94°C for 5 min, then 56°C or 58°C for 2 min for the different primer combinations, respectively, 72°C for 1 min 30, then 40 cycles at 94°C for 30 s, 56°C or 58°C for 40 s depending on the primer combination, 72°C for 1 min 30, and a final step at 72°C for 10 min. Cloning and sequencing were performed according to the procedures described above.

Family production and field collection

Production of the oyster families used in the *CgMT4* polymorphism analysis is described in Dégremont et al. (2005, 2007, 2010a) and Samain and McCombie (2008). Briefly, as part of the MOREST program (Samain & McCombie, 2008), a first experimental generation (G1) of *C. gigas* was bred in 2001 at the IFREMER hatchery in La Tremblade (France). This G1 consisted of 72 full-sib families produced using 18 males and 72 females as G0 parents (Figure 1). G1 oysters were subsequently placed at three sites along the French coast: Baie des Veys in Normandy (1°06′ W, 49°23′ N), Auray in Brittany (2°57′ W, 47°36′ N) and Ronce-les-Bains in the Marennes-Oléron basin (1°10′ W, 45°48′ N). Further details about family production, sites and mortality records are described in Dégremont et al. (2005, 2007, 2010a). To breed the next generation, 12 G1 families were selected, 6 showing high survival (average = 95%) and 6 showing low survival (average = 41 %), judged by their performances in Auray, the site with the highest mortality (Dégremont et al. 2005). These were used to produce 48 G2 'batches' in 2002. Half of these batches were bred from the G1 families showing high survival and the other half from those showing low survival (Figure 1). Each G2 batch was therefore a form of family obtained by crossing an average of 55 parents overall (proportions of males and females depended on the sex ratio) from two G1 families. In this study, we focused on the susceptible G1 families 4–16, 14–54, 14–55 and 7–25; their (G0) parents were males 4, 7 and 14, and females 16, 25, 54, 55; and their (G2) progeny which were the G2 batches J, L and Z (Figure 2; Table 2). These G2 progenies were chosen on the basis of their G1 parental families having shown the highest mortality rate of their generation.

The G1 oysters were sampled from all three sites in autumn 2001 and the G2 oysters were sampled in autumn 2002. The three sites differ notably in temperature

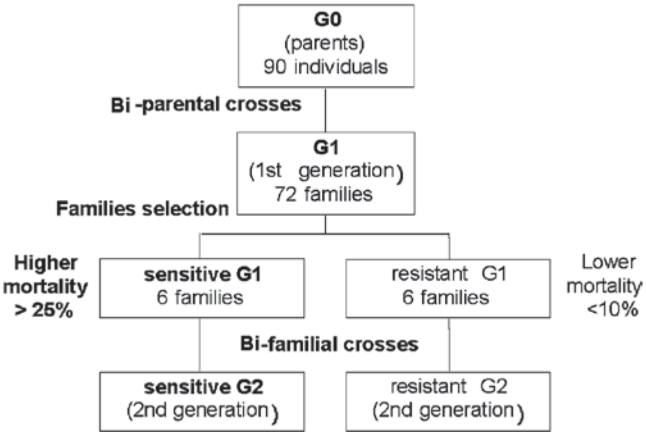

Figure 1. Crosses and resulting generations bred as part of the MOREST program and used in the present polymorphism study.

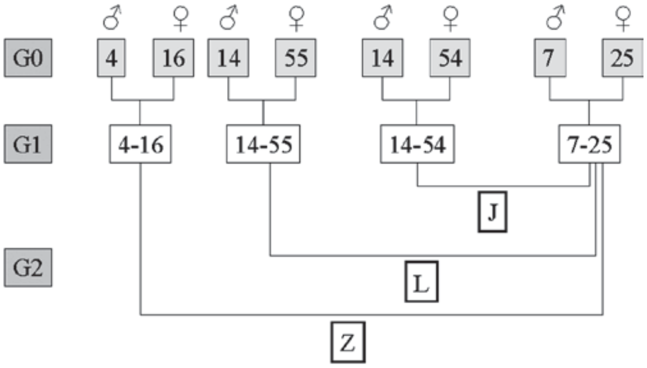

Figure 2. MOREST families used in this study. Parents (G0) are identified by numbers. G1 families are identified by « x-y » where x is the male and y the female used for the cross. G2 batches are identified by letters.

dynamics (north to south gradient) and trophic conditions (food availability is highest in Baie des Veys).

PCR-SSCP analysis

Total genomic DNA was isolated from oyster gills using phenol/chloroform/isoamyl alcohol (25:24:1). Depending on family and site, 13–48 individuals were studied for each of the G1 families, and 42–48 individuals were studied for each of the G2 batches. Primers were designed to amplify exons 2 and 3 (exon 1 being too short). Exon 2 was selected due to its polymorphism level and reproducibility of band patterns. Exon 2 of the *CgMT4* gene was amplified using the forward primer P1 and the reverse primer P2 (Table 1). All PCR amplifications were performed in a volume of 25 µL 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 for 5 min at 94°C, primer hybridisation for 2 min at 60°C and elongation for 1 min 30 at 72°C), 40 amplification cycles were performed as follows: 30 s at 94°C, 40 s at 60°C, 1 min 30 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 at 95°C, and quickly chilled on ice to melt and retain single strand DNA. After loading on a neutral 10% polyacrylamide gel (37.5:1, acrylamide: bisacrylamide), the samples were electrophorised at constant voltage (120V) in a 0.6X TBE buffer, for 16 h at 10°C. After electrophoresis, the gels were stained with ethidium bromide and visualised under UV light. Single strand DNA bands from the PCR products visualized on the gel as different conformation types obtained were gel-purified by diffusion into water by freezing at –20°C and thawing. The same PCR-amplifications were done on this recovered DNA as for the SSCP analysis. PCR products were then purified using a Qiaex II gel extraction kit (Qiagen, Courtaboeuf, France), cloned and sequenced as described above.

Table 2. Mortality of the families from the first (G1) and the second (G2) generations of *C. gigas* placed in Baie des Veys, Auray and Ronce-les Bains.

		Mortality (%)		
Generation	Family/batch	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

1350 individuals were initially placed for each family at each site.

Table 3. G0 parental genotypes for exon 2 of the *CgMT4* gene.

Parents	*MT4* Genotypes
Male 7	CD
Female 25	CD
Male 14	BC
Female 54	AC
Female 55	BC
Male 4	CD
Female 16	CD

Four alleles, named alleles A, B, C and D, were detected in experimental families of *C. gigas*, Three parental genotypes were detected and named genotypes AC, BC and CD.

Statistical and genetic analysis

For G1 families, observed genotypic frequencies were compared to expected Mendelian genotypic frequencies in samples of equivalent size, using χ^2 conformity tests in Statistica software (Statsoft). Yates corrections were made when sample sizes were between two and five individuals. Expected frequencies could not be calculated for G2 offspring because many parents (in variable ratios of female and male) from each G1 family were used to produce the G2. Expected Mendelian frequencies could not be calculated on the progeny from these “bi-familial” crosses, since the exact genotypes of each parental individual used are unknown. Population expectations could not be used either, as population size cannot be considered as infinite or in panmixia in our experimental design. Contingency table analyses were therefore used to compare genotypic frequencies at Auray and Ronce-les-Bains sites with those at Baie des Veys, where mortality rates were much lower. 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 ($\alpha' = \alpha/k$ where $\alpha = 0.05$, k = number of tests carried out).

Results

Molecular characterization of the *CgMT4* gene

The cDNA sequences of *MT4* from *C. gigas* showed a 261-bp coding region corresponding to 87 amino acids with a calculated molecular weight of 9.0 kDa (MWCALC software, http://bioinfo.hku.hk) and an isoelectricpoint of 7.89. The 5′ and 3′ UTR sequences are 166 and 181 bp, respectively. Several ATTTA(G) motifs are present in the UTRs of the *MT4* cDNA sequence: there is one ATTTG motif in the 5′ UTR and two ATTTA motifs in the 3′ UTR. Three untypical Cys-Cys-Cys and one Cys-X-Cys-X-Cys were found in *CgMT4*. The size of the *MT4* gene is 2196 bp from the 5′ UTR to the poly-A site (Figure 3). The sequence contains 3 exons of 9, 42 and 36 amino acids, respectively, separated by 2 introns of 501 and 1084 bp, respectively. The 5′ UTR also contains an intron of 103 bp. All the introns of the *CgMT4* gene start and end with the consensus GT and AG splicing signals.

Molecular phylogeny

A range of 24 oyster metallothionein amino acid sequences was analysed, including that of *CgMT4*, to

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gctgtaaagcaattaatcggatgatcactacaggaaaagatcagctcacacaggtaaatatatatccaatat    72
tgtcaacagatcttaacagcaaaaaatttgttattgttttacggtgcatgtttgtgatgtatgaatttctgt   144
                        M   S   D   H   C   T   C   A   Q                      9
tttttctttcagtcccttcaaa ATG TCC GAT CAT TGT ACC TGC GCC CAG A gtaagtacatt   205
atgatatatatatacgtataaatgcgcaaccttcaactgacgtggttaaataataagaaaaagcatagtgca   277
ttggaaaggagaaagtacttacataattctaattaagtcctttctttataaaggattacatttgatcttatg   349
taacacgaatatggtataatattatcagagagagagagagagagagagagagagagagagagagagagaaga   421
gaagagaagagagagagaattgtgtgcctccctatatacatgtactgcttataacaatctaatttattttat   493
tttagtatcttatgactaaaacattgtatttcgtttttcgatttatccattttgatacacacaaaacgatct   565
attgagagcggatcaaagatctaatgctagttagattgttagtgaattatcaactagtttgacgatgttttg   637
                                                          T   G   K   C     13
ataccatcttcagctaaaagttttcgctctctcatcctacacaaaccttctttttcagCG GGA AAA TGC   706
 V   C   C   D   T   C   P   P   D   G   N   C   S   C   G   D   K   C        31
GTG TGT TGT GAC ACG TGC CCG CCG GAC GGG AAC TGT AGT TGC GGA GAC AAG TGT    760
 K   C   A   K   A   S   C   N   C   T   C   K   G   K   A   K   C   C        49
AAA TGC GCC AAA GCA TCG TGT AAC TGT ACA TGT AAA GGC AAA GCC AAG TGC TGC    814
 C   T                                                                      51
TGT ACT A gtaagtaaacggacattataatagaaatccatatatactgattgtaaagtacgcttaattca   883
tgtgcccaacattcgtactgttaggtaatcagttgatcgatcaatcgattcattgattggctattcgcttat   955
ttggtgcaccgttactaaagaaggaatgtataagtttcataaaagttttccacacaaaagcctaagagctgt  1027
cccgaattcctttatagtattacatttatatggttgaaggtcctataaaaatccacagggtccaaatttcga  1099
cggtgttcttttttttttttttgcttacatctcgttcgttcttcaatgaacatgaatatattttatgcat  1171
atggtactatggtagacaaacaatcaactggatgagtatttattaaaaatgcaggtttaacaagtaagaaat  1243
taattcttgaagttttcctaaagaaaaagagtaaattttgttaacccacctgtactccaaatttaaatcatc  1315
gtactacaaaaataaaaacgctatgacgaaccctatgatgtatcatttgcaaagactggagacgcattaatg  1387
ttaaacaagtaaggttgtgaagataaatttatctgactaggcaatatagttaaatcgccgaatttaggactt  1459
ggtaatggtggcacctgttattttgttgatggatatactctgtgaatggaagtaatgaaatgtgcaaaggtt  1531
aatcagcttatgttaaaaaagcaagctatgatcgatattcatgataaacatatcggatttcaaaagtattgc  1603
tcaatccattcgaaaagaagaccctatagtctaagactgtcgaaatatgggcacacaatgatacttggacac  1675
gggcctttgcaacacgtaatcatgtcattttggatcccttaacttggatggatatgaagcttataaattact  1747
ctccatggaatcagtccatgaattaatttggtttaattcttttaaatgccgaaaattgacacaattggcaac  1819
tttattattatccatttttctacggagctttgaagtttctacataaaatagcttgttataatatatacgca  1891
              K   E   K   C   C   C   G   K   G   C   H   G   P   E   T     66
cattttttcttcagAA GAA AAA TGC TGC TGC GGA AAG GGG TGT CAC GGA CCA GAA ACC  1949
 C   K   C   P   A   D   C   C   C   K   K   E   H   D   A   C   S   K        84
TGT AAA TGT CCG GCC GAC TGC TGC TGT AAG AAG GAG CAT GAC GCA TGC TCA AAG   2003
 A   G   H   *                                                              88
GCG GGG CAC TGA  acggtaactgcgcagaaattatctcttattcgcttcaatatatactgtatacctg  2070
agacatggaaataaaataacttgatatttttgcaagcgtatatttattaaatgctatgtatactaataattt  2142
atgtgtaactgctgtatatcatgtatacttatggaataaactgaacgaaaagct                    2196
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Figure 3. The nucleotide sequence and predicted amino acid sequences of the *C. gigas* metallothionein 4 gene. Bold characters indicate the predicted amino acid sequence. The polyadenylation sequence is double underlined and messenger stability motifs ATTTA and ATTTG are shown in bold characters and double underlined. C-X-C; C-X-C-X-C and C-C-C motifs are highlighted. The intron in the 5′ UTR is underlined.

build a phylogenetic tree. The tree places the *CgMT4* sequence close to the *C. virginica MT-IV* group, but closer to *C. virginica MT-III* than to other *CgMT* genes (Figure 4).

Polymorphism analysis

SSCP analysis performed on exon 2 of the metallothionein 4 gene in the experimental families allowed four different alleles to be characterised; these were named A, B, C and D. G0 parental genotypes are presented in Table 3. The genotypic frequency distributions in the different generations, families/batches and locations are presented in Table 4 (G1) and Table 5 (G2), with their χ^2 values for the tests made for significant deviation from expectations. In the first generation, significant differences were observed between expected and observed frequencies in family F7-25 at the Auray and Baie des Veys sites. In these sites, observed frequencies of CD heterozygotes were lower than expected, contrary to CC homozygotes. A significant decrease of CD heterozygote frequencies was also observed in family F4-16 at Auray, whereas an increase in DD homozygotes was noticed at

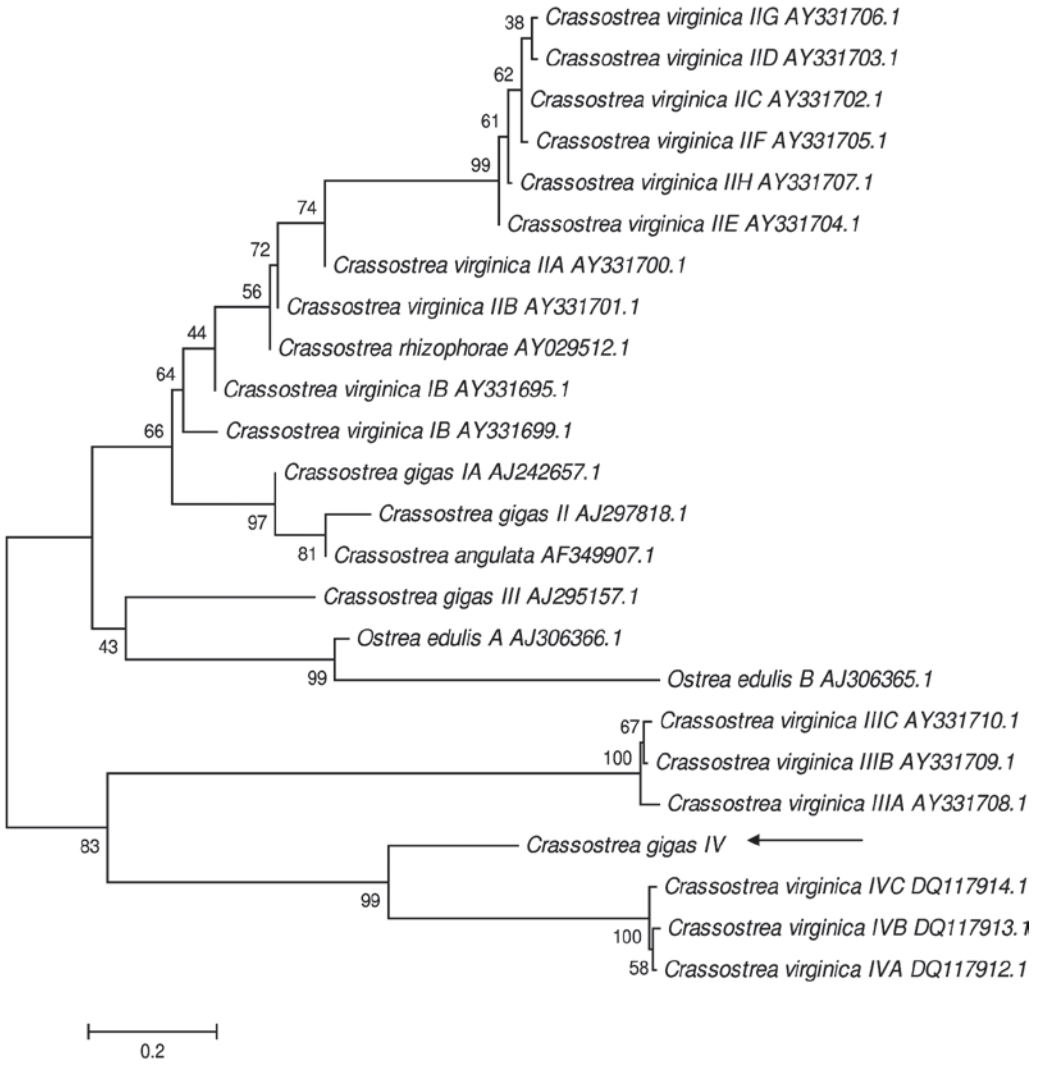

Figure 4. An unrooted phylogeny showing the most likely relationships between representative MT4 amino acid sequences. Branch lengths are proportional to estimates of evolutionary change. The number associated with each internal branch is the local bootstrap probability, which is an indicator of confidence. The sequences are *Crassostrea gigas* MT 1A (AJ242657.1) MT 2 (AJ297818.1), MT 3 (AJ295157.1) and MT4 (AM265551), *Crassostrea virginica* MT IA (AY331695.1), MT IB (AY331699.1), MT IIA (AY331700.1), MT IIB AY331701.1), MT IIC (AY331702.1), MT IID (AY331703.1), MT IIE (AY331704.1), MT IIF (AY331705.1), MT IIG (AY331706.1), MT IIH (AY331707.1), MT IVA (DQ117912.1), MT IVB (DQ117913.1), MT IVC (DQ117914.1), MT IIIA (AY331708.1), MT IIIB (AY331709.1), MT IIIC (AY331710.1), *Ostrea edulis* A (AJ306366.1) and B (AJ306365.1), *Crassostrea angulata* (AF349907.1) and *Crassostrea rhizophorae* (AY029512.1).

Ronce-les-Bains for the same family. In the second generation, no significant difference was observed in any of the batches or families, when comparing observed frequencies at Auray and Ronce-les-Bains with those from Baie des Veys.

Sequencing the different alleles detected for exon 2 of the *MT4* gene revealed that only allele B presented a mutation in the nucleic acid sequence that resulted in an amino acid change: asparagine 24 is changed to lysine. Two silent mutations were common to allele B and allele D (amino acids 26 and 27). Allele A differed from the other alleles by one silent mutation in position 22.

Discussion

This study characterised a new *C. gigas* metallothionein gene, called *CgMT4*. This gene contains three coding exons, the type of organisation classically described for metallothionein genes in *C. gigas*, apart from *CgMT3*, which shows no intronic sequence (Tanguy & Moraga 2001; Tanguy et al. 2001). The amino acid sequence has features in common with other metallothionein amino acid sequences, i.e. a high content of Cysteine residues arranged in typical Cys-X-Cys motifs, and non-aromatic amino acid residues. However, the obtained sequence has relatively low identity to *CgMT1*, *2* and *3* at the nucleotide level. The amino acid sequence shows addition of Cys residues, resulting in the presence of three Cys-Cys-Cys motifs. Similar motifs have been described in a *Crassostrea virginica* metallothionein gene known as *CvMT-IV* (Jenny et al. 2006) and phylogenetic analysis revealed that the *CgMT4* sequence aligns with this *CvMT-IV* group better than with other *CgMT* sequences. Cys-Cys-Cys motifs have been classically observed in

Table 4. Distribution of metallothionein 4 exon 2 genotype frequencies observed in tested *C. gigas* G1 families in the three sites, and comparisons with expected frequencies.

			Baie des Veys			Auray			Ronce-les-Bains		
Family	Genotype	Expected frequencies	Observed frequencies	χ^2 [size]	*p*-value	Observed frequencies	χ^2 [size]	*p*-value	Observed frequencies	χ^2 [size]	*p*-value
14–54	AB	0.25	0.17	6.44 (3)	0.09	0.08	5.33 (3)	0.15	0.17	1.11 (3)	0.77
	AC	0.25	0.33	[18]		0.42	[12]		0.28	[18]	
	BC	0.25	0.06			0.08			0.22		
	CC	0.25	0.44			0.42			0.33		
14–55	BB	0.25	0.26	0.04 (2)	0.98	0.20	0.40 (2)	0.82	0.00	5.71 (2)	0.06
	BC	0.50	0.48	[23]		0.53	[30]		0.65	[17]	
	CC	0.25	0.26			0.27			0.35		
7–25	CC	0.25	0.65	17.20 (2)*	0.00*	0.52	20.17 (2)*	0.00*	0.30	2.22 (2)	0.33
	CD	0.50	0.20	[20]		0.25	[48]		0.35	[23]	
	DD	0.25	0.15			0.23			0.35		
4–16	CC	0.25	0.42	4.68 (2)	0.10	0.46	6.38 (2)▲	0.04▲	0.06	8.06 (2)*	0.02*
	CD	0.50	0.26	[19]		0.15	[13]		0.41	[17]	
	DD	0.25	0.32			0.38			0.53		

Numbers in rounded brackets correspond to degrees of freedom. Numbers in square brackets under χ^2 values indicate sample sizes. *Indicates a significant difference between expected and observed frequencies at the α' significance level. ▲Indicates a significant difference between expected and observed frequencies at the α significance level.

Table 5. Distribution of metallothionein 4 exon 2 genotype frequencies observed in tested *C. gigas* G2 batches from the three sites, and comparison of frequencies observed in Auray and Ronce-les-Bains with frequencies observed in Baie des Veys.

		Baie des Veys		Auray			Ronce-les-Bains		
Batch	Genotype	Observed frequencies	χ^2 [size]	Observed frequencies	χ^2 [size]	*p*-value	Observed frequencies	χ^2 [size]	*p*-value
Z	CC	0.56		0.40	4.64 (2)	0.10	0.54	0.09 (2)	0.96
	CD	0.24	[45]	0.46	[48]		0.27	[48]	
	DD	0.20		0.15			0.19		
L	BC	0.31		0.28	0.71 (3)	>0.90	0.27	1.42 (3)	>0.75
	BD	0.04	[48]	0.06	[47]		0.09	[45]	
	CC	0.56		0.57			0.58		
	CD	0.08		0.09			0.07		
J	AC	0.19		0.10	3.88 (4)	>0.50	0.18	8.65 (4)	>0.10
	AD	0.00	[42]	0.08	[48]		0.11	[44]	
	BC	0.12		0.10			0.02		
	BD	0.05		0.06			0.02		
	CC	0.57		0.58			0.50		
	CD	0.07		0.06			0.16		

Numbers in rounded brackets correspond to degrees of freedom. Numbers in square brackets under χ^2 values indicate sample sizes. No significant differences were observed between observed frequencies in Auray or Ronce-les-Bains and observed frequencies in Baie des Veys at α and α' significance levels.

metallothionein genes of the protozoan *Tetrahymena* (Díaz et al. 2007; Guo et al. 2008) and more infrequently in the metallothionein of other organisms such as the yeast *Yarrowia lipolytica* (Accession number P41928), the annelid *Eisenia fetida* (P81695) (Gruber et al. 2000) and blue crab *Callinectes sapidus* (AAF08966). Furthermore, small, cysteine-rich hypothetical proteins that contain Cys-Cys-Cys clusters and that are potentially good candidates for novel metallothioneins have been described in an increasing number of organisms (Díaz et al. 2007). Metallothionein proteins or genes are known to be involved in responses to various environmental stresses (Isani et al. 2000), such as metal contamination (Boutet et al. 2002; Amiard et al. 2006; Damiens et al. 2006), hypoxia (David et al. 2005), radioactive discharge (Farcy et al. 2007), parasite infection (Desclaux-Marchand et al. 2007), estrogen exposure (Canesi et al. 2007) and, particularly, chemical contaminants that induce oxidative stress (Klaassen et al. 1999). Metallothioneins have also been suggested to respond to temperature changes (Farcy et al. 2009).

Research focused on oyster summer mortality phenomena has shown that a number of different risk factors may be involved. Among these, environmental parameters such as seawater temperature (Soletchnik et al. 2003), biotic stress through pathogen infection (Samain & McCombie, 2008) and environmental stress (mainly chemical contamination and hypoxia) (Samain et al. 2007) seem to be associated with summer mortality risk. Physiological status and ability to resist stress play an important role in the degree to which oysters are sensitive to summer mortality. These observations led us to consider the *MT4* gene as a relevant potential candidate gene for polymorphism analyses in the present study, since *CgMT4* was first identified in response to hypoxia (David et al. 2005).

The SSCP techniques used here demonstrated the existence of polymorphism in the coding sequence of the *MT4* gene in the Pacific oyster *C. gigas*. The *MT4* genotype of oyster families selected for their high level of mortality among the different families produced in the MOREST program (Dégremont et al. 2005; Samain & McCombie 2008; Dégremont et al. 2010a) was examined. Four alleles, named A, B, C and D, were detected. Significant deviations of observed frequencies from expected ones have been shown in some families of the first generation. Four major forces can usually explain deviations in allelic frequencies: mutation, migration, null alleles and selection. The experimental method used to rear the families (oysters placed in the field after settlement were grown in bags whose mesh size was smaller than the juvenile oysters themselves) means that migration can be eliminated. Mutation and null alleles can be eliminated also, because the events are very rare between two successive generations. This leaves only selection (differential selection of phenotypes with different genotypes) as a reliable explanation for our results. Furthermore, segregation distortion due to fitness differences at earlier stages of development can also be dismissed. Polymorphism in family F7-25 was analysed before mortality occurred, and did not show any deviation from Mendelian expectations (data not shown).

In the first generation, SSCP analyses revealed deviations from expected Mendelian genotype frequencies in the families presenting both alleles C and D (families F7-25 and F4-16). CD heterozygotes were less represented than expected, which suggests there had been selection against the corresponding individuals. This heterozygote genotype may therefore be deleterious, making these oysters more sensitive to summer mortality. It has been commonly observed that heterozygosity offers an advantage in fitness related traits (Zouros 1987). Heterozygotes have frequently been reported to use less energy for their metabolism than homozygotes, thus increasing their tolerance to environmental pressures (Holley & Foltz 1987; Hawkins et al. 1989). The kind of selection against heterozygotes observed here has been already reported in various mollusc species. Juvenile mussels obtained from pair-matings revealed heterozygote deficiency on allozyme loci (Mallet et al. 1985), which led authors to hypothesize that genotype-dependent larval mortality occurred. A low level of heterozygosity was also observed at 4 allozyme loci in susceptible stocks of mussels showing a high level of high summer mortality (Tremblay et al. 1998). They suggested a relation between heterozygote deficiency and sensitivity, due to a higher energetic cost of maintenance. Environmental influences, such as chemical contamination, can also exert selection on particular alleles and consequently reduce heterozygosity (De Nicola et al. 1992; Hummel et al. 1995).

Moreover, in the present study, a strong site effect was noticed, since the same family (F4-16) showed different responses in two different sites. CD heterozygotes were counter-selected in Auray and to a lesser extent in Ronce-les-Bains, where a strong decrease of CC homozygotes was seen. Site effect is thought to reflect environmental parameter variations between the three studied sites. Environmental factors (temperature, salinity, nutrient and pollutants inputs, food quantity and quality, etc.) seem therefore to be strongly implicated in summer mortality. Dégremont et al. (2005, 2010a, 2010b) found inter-site variation in survival of the *C. gigas* families used in the MOREST program that are related to environmental conditions and consistent with the differences in genotypic frequencies observed among sites. Such differences confirm the complex interactions between oysters and the environment that lead to summer mortality through genotype × environment interactions. These authors also described a strong family effect on survival and growth, with 46% of variance being explained by family effect in G1 (Dégremont et al. 2005). Differences in genotype frequencies were indeed observed between the families F7-25 and F4-16. Finally, no such variations were observed in the second generation, which suggests that selection effect mostly occurred in the first generation, or that selective pressure was quite equivalent between the three sites in the second generation. However, as some sample sizes were sometimes heavily reduced by the mortality itself, the hypothesis that the differences observed in genotypic frequencies were distorted by lowered sample size cannot be totally ruled out.

Allele C and D are characterised by synonymous mutations. Two hypotheses may help to interpret our results. First, selection that occurs on these two alleles may act through other mutations located elsewhere in the *CgMT4* sequence than in exon 2. The choice of PCR-SSCP was made in order to monitor polymorphism, since this method is sensitive and allows the detection of single base-pair differences (Orita et al. 1989; Hongyo et al. 1993; Sunnucks et al. 2000). However, this method prevented us from analysing the polymorphism of all 3 *CgMT4* exons. Alleles C and D might therefore be characterised by functional polymorphism located elsewhere in the gene sequence (exon 1 or 3). The second hypothesis is that the selection could have resulted from a hitchhiking effect (Maynard Smith & Haigh 1974; Barton 2000). Selection acting on one or more genes generally affects other genes, even if the resulting modifications do not directly affect fitness. The studied locus may have been affected by selective pressure exerted during summer mortality on other loci that are physically close to it. Indeed, the mutations observed in the nucleotide sequence of *MT4* alleles C and D could be linked to mutations in other linked genes.

The results shown here are consistent with those obtained in a previous polymorphism study led on the same *C. gigas* families where the polymorphism of glutamine synthetase and delta-9 desaturase genes was examined (David et al. 2007). Allele C of the glutamine synthetase gene and allele B of the delta-9 desaturase gene were found to be subject to a selective effect caused by summer mortality (depending on environmental factors and genetic background). To our knowledge, DNA polymorphism analyses to assess genetic markers of resistance or sensitivity in oysters are quite scarce. Yu et al. (2011), using 12 SNPs, revealed that a serine protease inhibitor gene polymorphism was associated with improved survival after disease-caused mortalities in the eastern oyster *C. virginica*. SSCP markers developed for *C. gigas* metallothionein genes also showed a correlation between allele frequencies and resistance to heavy metals (Tanguy et al. 2002). In the present study, no relationship was observed between mortality rates and chemical characteristics of the three field sites, as the three sites did not significantly differ in their chemical characteristics but in their temperature dynamics and trophic conditions (data not shown). This observation suggests that environmental pollutants do not directly contribute to summer mortality at these sites or to the differences in allele frequencies among them. If there is some selection against metallothionein genotype CD, it may therefore not be due to sensitivity to pollutants.

The present study has provided a new potential genetic marker of stress in *C. gigas*. Polymorphism analysis revealed that the *CgMT4*-CD genotype, potentially associated with sensitivity, was counter-selected during summer mortality. This study may contribute to providing new genetic tools for the selection of non-susceptible oysters, in order to reduce summer mortality risk in cultured oyster populations. Further investigations and comparison with natural populations are now needed to confirm these conclusions.

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