Aquaculture
November 2015, Volume 448, Pages 521-530
http://dx.doi.org/10.1016/j.aquaculture.2015.06.019
http://archimer.ifremer.fr/doc/00271/38244/

Can survival of European flat oysters following experimental infection with Bonamia *ostreae* be predicted using QTLs?

Harrang Estelle [1, *], Heurtebise Serge [1], Faury Nicole [1], Robert Maeva [1], Arzul Isabelle [1], Lapègue Sylvie [1]

[1] Ifremer, SG2M-LGPMM, Laboratoire de Génétique et Pathologie des Mollusques Marins, Avenue de Mus de Loup 17390 La Tremblade, France

* Corresponding author : Estelle Harrang, email address : estelle@harrang.fr

Abstract :

The present study identifies quantitative trait loci (QTLs) in response to an experimental infection with the parasite responsible for bonamiosis, *Bonamia ostrea*e, in two segregating families of the European flat oyster, *Ostrea edulis*. We first constructed a genetic-linkage map for each studied family and improved the existing genetic-linkage map for the European flat oyster with a set of SNP markers. This latter map now combines the best accuracy and the best estimate of the genome coverage available for an oyster species. Secondly, by comparing the QTLs detected in this study with those previously published for *O. edulis* in similar experimental conditions, we identified several potential QTLs that were identical between the different families, and also new specific QTLs. We also detected, within the confidence interval of several QTL regions, some previously predicted candidate genes differentially expressed during an infection with *B. ostreae*, providing new candidate genome regions which should now be studied more specifically.

Highlights

► The present study improved the previously published genetic-linkage map for the European flat oyster with a set of SNP markers to give the best genome coverage map for an oyster species. ► Several quantitative trait loci (QTLs) in response to an experimental infection with *Bonamia ostreae*, the parasite responsible for bonamiosis, were identified in two new segregating families of *Ostrea edulis*. ► We found a concordance in the localization of previously identified candidate genes differentially expressed during an infection with *B. ostreae* and the QTLs detected in the two analysed families, providing candidate genome regions which should be studied more specifically for Marker-Assisted Selection (MAS) programs.

Keywords : Ostrea edulis, Single Nucleotide Polymorphism, disease resistance, genetic and QTL mapping, candidate genes

1. INTRODUCTION

The European flat oyster *Ostrea edulis* is native to European coasts. Its geographic distribution area extends from the North Sea to the Atlantic coast of the Iberian Peninsula and includes the Mediterranean Sea and Black Sea (Ranson, 1967; Jaziri, 1990). The species has also been introduced to the United States and Canada (Vercaemer *et al.*, 2006) for aquaculture, although most *O. edulis* populations are located in Europe. Since the 1970s, its European production has drastically decreased due to the emergence of two protozoan diseases: marteiliosis, caused by *Marteilia refringens*, and bonamiosis, caused by *Bonamia ostreae*. Nevertheless, the European flat oyster still remains an important economic species (2300 t, 21.4 million USD; FAO, 2011). Like all other marine bivalves, it has two specificities that limit its means to fight disease: (1) it grows in an open environment that cannot be protected, and (2) it possesses an innate immune system that has no adaptive response, making the use of vaccines impossible. In recent decades, new management practices (*e.g.* oyster culture in deep water) have made it possible to avoid marteiliosis-related mortalities, but do not provide an efficient means to counter bonamiosis. To restore European flat oyster culture, the selection of animals naturally resistant to bonamiosis is therefore a very promising approach.

Selective breeding programs for animals resistant to mortality induced by *B. ostreae* have been developed in France and Ireland (Martin *et al.*, 1992; Baud *et al.*, 1997; Naciri-Graven *et al.*, 1998; Culloty *et al.*, 2001). In France, an experimental breeding program initiated by Ifremer in 1985 demonstrated that it was possible to improve the survival of oysters (Naciri-Graven *et al.*, 1998) and to reduce infestation by the parasite (Martin *et al.*, 1992). However, a decline in *Bonamia* tolerance was demonstrated within a few generations of selection. This phenomenon was attributed to a drastic reduction in genetic diversity, but also to a potential accumulation of deleterious alleles over the generations (Launey *et al.*, 2001). To restore a sufficient level of genetic diversity for continued selective breeding, the subsequent crosses were reoriented to follow an intra-familial selection scheme (Bédier *et al.*, 2001). The newly-produced families were issued from crosses among selected strains and showed enhanced survival and lower prevalence of the parasite (Bédier *et al.*, 2001). These studies identified components of the genetic basis of the resistance to the

infection by *B. ostreae*.

Lines of *O. edulis* resistant to bonamiosis could contribute to the sustainable production of this oyster species. To produce such lines it is essential to enhance genetic improvement programs by direct selection on genes or genomic regions affecting economic traits (Dekkers, 2004); in this case, the resistance against the intrahaemocytic parasite *Bonamia ostreae*. The identification and mapping of such genomic regions is commonly performed by a QTL (Quantitative Trait Loci) method. This approach uses genetic marker association and different statistical methods to study phenotypes to identify and delimit genomic regions where putative genes responsible for traits of interest are located. The genomic regions thus identified may provide valuable information and tools for Marker-Assisted Selection (MAS), so as to accelerate the selective breeding process and remove the need for disease exposure as a selection method.

In order to identify QTL regions linked to a phenotype of interest, it is essential to have a genetic linkage map for the studied species. However, in non-model species like the European flat oyster, for which a whole genome sequencing project has not yet been performed, a reference genetic linkage map is usually not available and the number of molecular markers is relatively limited. An alternative method consists in the construction of a genetic linkage map with the largest density of molecular markers possible, to maximize the genome coverage, for each studied segregating family (Vignal *et al.*, 2002). In marine bivalve species of aquacultural interest, identification and localization of QTLs on these low density genetic maps has been done for the Zhikong scallop *Chlamys farreri* (Zhan *et al.*, 2009), hermaphroditic bay scallop *Argopecten irradians* (Qin *et al.*, 2007; Li *et al.*, 2012), Eastern oyster *Crassostrea virginica* (Yu and Guo, 2006), Pacific oyster *Crassostrea gigas* (*e.g.* Sauvage *et al.*, 2010; Guo *et al.*, 2012) and European flat oyster *O. edulis* (Lallias *et al.*, 2009). For *O. edulis*, 10 linkage groups are expected, to match the haploid chromosome number (Thiriot-Quiévreux and Ayraud, 1982).

A previous study on *O. edulis* identified a total of 5 QTLs for resistance to *B. ostreae*; these were detected in one segregating family after a cohabitation experiment. However, these QTLs could not be directly used in a MAS program because the genomic regions identified contained hundreds of genes and, to optimize the

efficiency of the breeding program, it is necessary to reduce the confidence interval (Spelman and van Arendonk, 1997). Moreover, it was also necessary not only to test other genetic backgrounds to confirm the position of QTL regions previously detected, but also to detect potential new QTLs that did not segregate in the family studied by Lallias *et al.* (2009). Finally markers were also needed that were more easily transferable among populations and laboratories than the AFLPs previously used.

The present study aimed to identify QTLs in response to an infection with the parasite *B. ostreae* in two segregating families of *O. edulis*. Individuals from two flat oyster families were reared in contact with wild oysters experimentally over-infected with *B. ostreae* over one year. This infection method enabled us to mimic the spread of the parasite in the natural environment. QTLs were then identified for two phenotypes: survival/mortality and parasite load. We studied the third generation (F_2) of each pedigree in order to know linkage phase of segregating variants. As the grandparents are from different origins (wild or selected), this segregation made it possible to identify the wild or selected origin of QTL alleles. QTLs identified were then compared between the two segregating families and with QTLs previously obtained under similar experimental conditions (Lallias *et al.*, 2009). The previously published parental maps were mainly constituted of AFLP markers (85.7-87.4% of mapped markers). To facilitate transferability, we tested a new set of SNP markers specifically developed for the European flat oyster (Lapègue *et al.*, 2014). These new molecular markers were used to construct the linkage maps of the new tested families and were also added to the QTL map published by Lallias *et al.* (2009).

2. MATERIAL AND METHODS

2.1. Segregating families

We used two different three-generation families initiated in 2003 and 2004. First, we crossed one wild-type oyster (W31 or W120) with an oyster from one of the selected families from the Ifremer selective breeding program against bonamiosis (703-29) or from an inbred line that had shown good resistance to bonamiosis (L002-55) to produce two F_1 biparental families OE.F_1.04.10 and OE.WL.03.30, respectively. Then, in 2009, two full-sibs from each F_1 family (04.10-7 and 04.10-15 for Family 1; 03.30-1 and 03.30-6 for Family 2) were crossed to produce F_2 segregating families (Family 1: OE.F_2.09.04; Family 2: OE.F_2.09.67, respectively). All crosses were performed as described in Lallias *et al.* (2007). The new F_2 families were named according to the existing system. For example, OE.F_2.09.04 refers to a F_2 family of *O. edulis* produced in 2009 as batch number 04.

The individuals from the two F_2 families (referred to hereafter as the tested oysters) were produced in the same environmental conditions, and were also naive to bonamiosis (no prior contact). These two families will be referred to hereafter as Family 1 and Family 2, respectively.

2.2. Experimentally-induced bonamiosis infection

As there is no known means to cultivate *B. ostreae*, naturally infected wild *O. edulis* collected in the Bay of Quiberon (Southern Brittany, France), an endemic zone for bonamiosis, were used to purify the parasite. Then, four-year-old wild oysters, collected in the same natural area and potentially already infected with *B. ostreae*, were injected with the purified suspension of parasites in order to over-infect them.

The parasite was purified according to Mialhe *et al.* (1988). The amount of purified parasites obtained enabled the injection of a 100 µl suspension of $1x10^6$ parasites into the adductor muscle of 300 wild oysters, to be used as donors in the experiment. This procedure was performed on oysters previously anesthetized with $MgCl_2$, using a 1mL syringe fitted with a 23G needle (0.6 x 25 mm).

The experimental design consisted of 6 150-L tanks, each containing 4 trays; the top and the third trays in each stack contained 25 donors, and the second and the fourth trays contained 50 tested oysters, that can be considered as recipients. Each tank

contained oysters from one family only, so three tanks per family were used. A total of 50 donors and 100 recipients per tank were used, except for the last tank of Family 2 for which only 50 recipients were still available. At the beginning of the experiment, the recipients were 1 year old. As the oysters were very small it was not possible to identify each of them as a member of one or the other family, which is why the families were kept in separate tanks. The difference in size between donors and recipients has enabled to distinguish them in the tanks along the experiment.

The cohabitation experiment was run over 15 months from April 2010 to July 2011. During the experimental period, each tank was supplied with phytoplankton-enriched seawater at 60 $L.h^{-1}$. Mortality was monitored daily as described in Lallias *et al.* (2008, 2009) and dead oysters were removed. To balance the parasitic pressure over all tanks despite these deaths, the number of injected wild oysters was systematically re-adjusted between the tanks. For tested oysters, samples of tissue were collected from each dead individual for further analysis. At the end of the experiment, all remaining oysters from both families were sacrificed and tissue samples collected.

2.3. DNA extraction and parasite load

For each tested family, genomic DNA from dead and sacrificed individuals was extracted from 20 mg of gill tissue using a QIAamp DNA mini-kit (Qiagen) according to the manufacturer's instructions. Quality and concentration were assessed on a 2% agarose gel and a NanoDrop Spectrophotometer ND-2000 (Thermo Scientific). Concentrations were equilibrated to 100 $ng.\mu L^{-1}$ per DNA sample.

For each tested oyster, the level of parasitic infection was assessed by real-time PCR using primers targeting one of the *B. ostreae* actin 1 genes according to Robert *et al.* (2009). All reactions were carried out in triplicate in 25 µL, comprising 5 µL genomic DNA at 5 $ng.\mu L^{-1}$ (sample) or plasmid DNA (positive control) or distilled water (negative control). Data were collected with MxPro software (Stratagene). Replicates of the same sample were validated if the Ct values ranged between [-0.5 Ct; +0.5 Ct]. The number of *B. ostreae* actin gene copies per sample was assessed by comparing the Ct value obtained for each sample (mean of triplicates) with the standard curve

prepared with serial dilution of plasmid DNA.

2.4. Genotyping

Each mapping family consisted of two grandparents (F_0), two parents (F_1) and the progeny (F_2). Given the low volume and low concentration of extracted DNA available for analysis, the genomic DNA of the four F_0 was amplified with an Illustra^{TM} GenomiPhi^{TM} V2 Amplification Kit (GE Healthcare) according to the manufacturer's instructions.

A set of 384 SNP markers, specifically developed for European flat oyster (Lapègue *et al.*, 2014) from direct sequencing (Harrang *et al.* 2013) and *in silico* screening from NGS sequencing (Cahais *et al.* 2012), has already been developed. GoldenGate genotyping with VeraCode Technology (Illumina Inc., San Diego, CA, USA) was performed according to the manufacturer's instructions. Clustering was realized using the GenomeStudio software package (Illumina Inc.). Reliability of SNP detection and coding were the same as described in Lapègue *et al.* (2014).

2.5. Linkage mapping

A genetic linkage map was built independently for each of the F_2 families, using JoinMap® 4 (van Ooijen, 2006). The type of segregation at each locus was determined prior to coding the genotypes according to population type (here "Outbreeder full-sib family"). Then, the first step in the mapping was to test the segregation distortion of each locus against normal Mendelian expectation ratios with a normal classification of genotypes, using the Chi-square test (van Ooijen, 2006). The second step was to calculate linkage between each pair of markers, using the independence LOD (Logarithm of the Odds Ratio, Morton, 1955) score for the recombination frequency. A LOD score higher than 3.0 was considered as significant and enabled the grouping of markers into linkage groups (LG). Next, genetic distances were calculated using the method of Kosambi (1944), and the most likely order of markers was determined using permutations. For each order, the corresponding goodness-of-fit (a G^2 likelihood ratio statistic) was calculated. The expected length of the genome was then estimated using method 4 of Chakravarti *et*

al. (1991). Genome coverage was then estimated by calculating the ratio of the observed genome length (sum of the length of all LG) to the expected length.

Additionally, as extracted DNA samples were still available for the 96 individuals (92 F_2, 2 F_1 and 2 F_0) of the family used for the previous QTL detection experiment (Lallias *et al.*, 2009), the 384 SNPs were also genotyped in those individuals and mapped in this family. An updated genetic map was built for this family with the three different types of genetic marker: AFLPs, microsatellites and SNPs. This is referred to hereafter as the completed genetic linkage map of Family OE.F_2.05.04, named Family 3.

The numbering of the different linkage groups (LG) was the same between the different families. When two groups were part of the same LG but not recognized as such in a family, they were labelled <a> and <b> (*e.g*. LG 4a, LG 4b). An LG identified in only one family was labelled as a <supernumerary> group (S).

2.6. QTL analysis

QTL mapping was performed independently for each of the F_2 families and for each studied phenotypes using the MapQTL® 5 program (van Ooijen, 2004) on the newly constructed genetic linkage maps. Phenotypes were either qualitative for survival (0: died; 1: survived) or quantitative for *B. ostreae* load (number of parasites per mg fresh tissue). For parasite load, contrasting phenotypes were studied to optimize the QTL detection, using the selective genotyping method (Lander and Botstein, 1989). Thus, only the extreme phenotypes were considered: “survived-uninfected” (sacrificed individuals with no parasite detected by PCR), and “died-infected” (dead individuals with at least 1.10^5 parasites detected per mg of fresh tissue).

QTL detection was performed using the interval mapping method developed by Lander and Botstein (1989), scanning for QTLs every 1 cM on the linkage map. The likelihood of the presence of a segregating QTL was determined using a LOD score, when its value exceeded the predefined significance threshold somewhere in a linkage group. The empirical distribution of this significance threshold of the LOD score was obtained from 1,000 permutations (Churchill and Doerge, 1994) over each

linkage group and over all linkage groups (the whole linkage map). Several LOD thresholds were defined on a sliding scale (*P*-values = 0.001; 0.005; 0.01; 0.05 and 0.10) and applied to the result of the interval mapping to estimate the significance of the QTLs. A QTL with a *P*-value < 0.10 at the chromosome-wide level was considered as suggestive, and a QTL with a *P*-value < 0.05 at the chromosome-wide level was considered as significant (Le Bras *et al.*, 2011). For suggestive QTLs, genetic effects were not taken into account due to the non-significance of the test because the values could be over- or under-estimated. The estimated position of a QTL on the map was defined by the maximum value of the LOD score and the 95% confidence interval, using the one LOD drop-off method of Lander and Botstein (1989). The percentage of the variance explained by the QTL was estimated by the MapQTL5 program, using the formula: 100*(H0_var – var)/population_variance, where H0_var is the residual variance under the current null hypothesis and var is the residual variance after fitting the QTL (van Ooijen, 2004). The effect of each significant QTL was then defined as the proportion of the genetic variation observed in the segregating family that is explained by the QTL (Erickson *et al.*, 2004). QTLs were classified into three categories according to their effect: 1-"large-effect": those with an effect greater than 15– 20%; 2-"moderate-effect": those with an effect between 1% and 15– 20%; and 3- "weak-effect": those with an effect less than 1% (Manly & Olson, 1999; Erickson *et al.*, 2004).

3. RESULTS

3.1. Genotyping success

Overall, 80.5% of the SNPs (309 out of 384 markers) were successfully genotyped. Among these, 22, 41 and 24 SNPs were monomorphic for Families 1, 2 and 3, respectively, and were removed from the analysis. A total of 121 (39.2%), 106 (34.3%) and 121 (39.2%) SNP markers in Families 1, 2 and 3, respectively, were informative (*i.e.* polymorphic) and were kept for linkage mapping.

A total of 115, 78 and 92 F_2 oysters were successfully genotyped for Families 1, 2 and 3, respectively.

3.2. Genetic linkage mapping

For Families 1, 2 and 3, 53.7% (65 out of 121), 52.8% (56 out of 106) and 48.8% (59 out of 121) of the SNPs, respectively, were distorted from Mendelian segregation (*P*-value < 0.05), and 78.5%, 72.6% and 69.4%, respectively, after Bonferroni correction. All markers were used for mapping irrespective of their fitting to Mendelian segregation.

The Family 1 genetic linkage map was based on the 121 SNPs that were informative for this family. Among these, 117 markers were successfully positioned in a total of twelve groups, including two groups for LG 4 (LG 4a, LG 4b), two groups for LG 5 (LG 5a, LG 5b) and two groups for LG 9 (LG 9a, LG 9b), established with a LOD score between 4 and 10. Considering the 9 linkage groups (joining groups <a> and <b>), the number of markers per linkage group varied from 5 to 29. The average inter-marker distance ranged from 1.2 cM (LG 1) to 5.8 cM (LG 6), with an average spacing of 3.1 cM. The largest interval varied from 5.9 cM (LG 4b) to 22.3 cM (LG 5a) (**Table 1, Table S1, Fig. S1**). The observed map length was 275.15 cM, corresponding to an estimated genome length of 348.65 cM, and an observed genome coverage of 78.9%. Non-Mendelian markers were not homogeneously distributed on the linkage map ($X^2 = 408.563$, df = 11, $P < 0.001$) but tended to cluster in specific linkage groups. In four linkage groups (LG3, LG 4a, LG 8 and LG 9b) 100% of mapped markers were distorted, and in three linkage groups (LG 1, LG 4b and LG 7) from 62% to 86% of mapped markers were distorted.

The Family 2 genetic linkage map was based on the 106 SNPs that were informative for this family. Among these, 96 markers were successfully positioned in a total of thirteen linkage groups, including two groups for LG 4 (LG 4a and LG 4b), two groups for LG 5 (LG 5a and LG 5b), two groups for LG 8 (LG 8a and LG 8b) and one supernumerary group (S1), established with a LOD score between 4 and 10. Considering the nine linkage groups (joining groups <a> and <b>), the number of markers per linkage group varied from 2 to 21. The average inter-marker distance ranged from 1.6 cM (LG 9) to 8.7 cM (LG 6a), with an average spacing of 3.9 cM. The largest interval varied from 2.3 cM (LG 9) to 27.1 cM (LG 2) (**Table 1, Table S2, Fig. S2**). The genetic linkage map coverage was 253.55 cM, corresponding to an estimated genome length of 355.19 cM, and an observed genome coverage of 71.4%. Non-Mendelian markers were not homogeneously distributed on the linkage

map (X^2 = 301.793, df = 12, P < 0.001) but tended to cluster in specific linkage groups. In seven linkage groups (LG 4a, LG4b, LG5a, LG 6, LG 8b, LG 9 and S1) 100% of mapped markers were distorted, and in two linkage groups (LG 1 and LG 7) from 50% to 67% of mapped markers were distorted.

The completed genetic map for Family 3, which improves on the previously-published map by Lallias *et al.* (2009), was based on a total of 293 successfully positioned genetic markers, consisting of 119 SNPs, 17 microsatellites and 157 AFLPs, in a total of ten linkage groups. The number of markers per linkage group varied from 8 (LG 9) to 54 (LG 1), and the average inter-position distance (informative distance between two closely situated but differing positions on the map, considering as "one" position when many markers are located at exactly the same position) ranged from 1.5 cM (LG 1) to 5.1 (LG 9), with an average informative spacing of 3.0 cM. The largest interval varied from 6.2 cM (LG 6) to 28.7 cM (LG 3) (**Table 1, Table S3, Fig. 1**). The genetic linkage map coverage was 536.40 cM, corresponding to an estimated genome length of 580.42 cM, and an observed genome coverage of 92.4%. Non-Mendelian SNP markers were not homogeneously distributed on the linkage map (X^2 = 250.061, df = 9, P < 0.001) but tended to cluster in specific linkage groups. In two linkage groups (LG 8 and LG 9) 100% of SNPs mapped markers were distorted, and in two other linkage groups (LG 2 and LG 4) from 79% to 82% of SNPs mapped markers were distorted.

Comparing genetic maps between the three segregating families, nine homology groups were identified based on a total of 111 SNPs that were common between at least two of the three families (**Table 1**). Among them, 52 SNPs were shared by both Family 1 and Family 2. As shared SNPs represented less than 50% of the mapped SNPs for each family (respectively, 43% and 49%) and as there was a large difference in the distance estimated between loci and no common order, a consensus map would have been particularly complex to build and interpret and was thus not constructed. In Family 1, Family 2 and the completed map of Family 3, 12 SNPs, 38 SNPs and 192 markers (18 SNPs, 17 microsatellites, 157 AFLPs), respectively, segregated exclusively in these families. No homology group was found in Families 1 and 2 for LG 10, or in Family 1 and the completed map for S1. Based on the linkage of several markers that were common between the different families but that were

successfully mapped in only one (or two) of them, LG 4a and LG 4b were grouped to give LG 4, LG 5a and LG 5b were grouped to give LG 5, LG 8a and LG 8b were grouped to give LG 8, and LG 9a and LG 9b were grouped to give LG 9.

3.3. QTL mapping

No significant tank effects on mortalities of donor oysters were found between the different tanks of each family, either for Family 1 ($X^2 = 0.047$, df = 2, P = 0.977) or Family 2 ($X^2 = 1.570$, df = 2, P = 0.456).

During the cohabitation experiment, 73 and 64 recipient oysters died for Families 1 and 2, respectively, but only 57 and 42 had tissues in sufficiently good condition to allow DNA typing and *B. ostreae* quantification. After DNA extraction, some samples (5 and 8) could not be used for further analysis because of low DNA quality.

Finally, the phenotypes of 115 recipient oysters from Family 1 (63 survived; 7 died-infected; 45 died-uninfected) and 78 recipient oysters from Family 2 (44 survived; 34 died-infected; 0 died-uninfected) were studied to detect QTL for survival after contact with donor oysters. The interval mapping method revealed the existence of four significant ($P < 0.05$) and one suggestive ($P < 0.10$) QTL in Family 1, and one significant and one suggestive QTL in Family 2 (**Table 2, Fig. 2**). The significant QTLs were distributed in three different linkage groups: LG 2 (Family 1, Family 2), LG 3 (Family 1) and LG 8 (2 QTLs in Family 1). On LG 2, there was an overlap between the QTL locations in the two families. For Family 1, the significant QTLs explained 15.2%, 10.6%, 9.7% and 8.8% of the genetic variation for survival after contact with donor oysters. For Family 2, the significant QTL explained 17.2% of the genetic variation for this trait.

For the two families, the suggestive QTL was distributed on the same linkage group: LG 1. Given the narrowness of the confidence interval for the suggestive QTL in Family 2, no common markers were located within the interval and only one marker was simultaneously associated with the suggestive QTL for the two families. Consequently, it is not possible to test the hypothesis of an overlap of the QTL locations between the two tested families.

Secondly, QTL were also detected for *B. ostreae* load. For Family 1, the mean *B.*

ostreae load per mg of fresh tissue was $9.6x10^3$ (ranging from 0 to $2.9x10^5$) for dead individuals. For Family 2, the mean *B. ostreae* load per mg of fresh tissue was $1.4x10^2$ (ranging from 0 to $1.9x10^3$) for individuals that survived and $1.6x10^6$ (ranged from $2.7x10^3$ to $8.3x10^6$) for individuals that died. Because many individuals (96.1 %) from Family 1 that died did not have a *B. ostreae* load above 1.10^5 parasites per mg of fresh tissue, extreme phenotypes of parasite load were not available and parasite load has not been assessed for surviving individuals for this family. So, the detection of QTLs linked to parasite load was only performed in Family 2. For this second family, 15 individuals of phenotype "survived-uninfected" and 17 individuals of phenotype "died-infected" were used to detect QTLs for parasite load. Moreover, as quantitative values of parasite load were not normally distributed, the phenotypic data were transformed into qualitative binary data (Lallias *et al.*, 2009).

The interval mapping method revealed the existence of one significant ($P < 0.05$) and two suggestive ($P < 0.10$) QTLs linked to parasite load (**Table 2, Fig. 2**). The significant QTL, located in LG 2, is the same as that detected for survival after contact with donor oysters, but it explained 22.1% of the genetic variation for this trait. The two suggestive QTLs were distributed in two different linkage groups: LG 1 and LG 6.

Overall, for both families, the QTL alleles linked to the "survived" phenotype originated from the selected grandparents (703-29 or L002-55), and the QTL allele linked to the "died" phenotype originating from the wild grandparents (W31 or W120). One exception was observed for Family 1 in LG 1 (snp_Contig25127_1757, **Fig. 2**). Also, for some loci it was not possible to identify the origin of the QTL alleles because of the heterozygosity of the grandparents.

4. DISCUSSION

4.1. Genetic linkage mapping

In the present study, two new genetic linkage maps were constructed for the European flat oyster *O. edulis* for use in the detection of QTLs for survival after contact with wild oysters injected with *B. ostreae* and in relation to parasite load.

The set of 384 SNPs specifically developed on *O. edulis* by Lapègue *et al.* (2014) was applied for the first time. These molecular markers showed a relatively high level

of segregation distortion in the two mapping families, but this was of a similar order of magnitude to those already reported in similar F_2 families of oyster species (*O. edulis*, Lallias *et al*., 2007, 2009; *C. gigas*, Sauvage *et al.*, 2010). Furthermore, as already observed in some oyster species (*C. gigas*: Li and Guo, 2004, *O. edulis*: Lallias *et al.*, 2007, 2009), distorted markers tended to cluster in specific linkage groups on the new genetic linkage maps. The observation of clusters that differed between the families could reflect the proximity of potential deleterious genes in the LGs.

Segregation distortion is commonly observed in oyster species and bivalves in general (Launey and Hedgecock, 2001; Li and Guo, 2004; Lallias *et al*., 2009), where null alleles may account for a large part of this phenomenon. Furthermore segregation distortion in pair crosses of oyster species can be explained by a high genetic load (*i.e.* a large number of deleterious recessive mutations) in the genome (Bierne *et al.*, 1998; Launey and Hedgecock, 2001). The extreme protein heterozygosity of marine bivalves has been hotly debated in the past, at the heart of the neutralist-selectionist controversy, but the question has never been answered definitively. Recently, a high load of segregating non-neutral amino-acid polymorphisms was suggested to contribute to high protein diversity in wild populations of *O. edulis* (Harrang *et al*., 2013).

Although the average marker spacing appeared smaller than that observed in previous studies on *O. edulis* (above 4 cM, Lallias *et al.*, 2007, 2009), the estimated genome length appeared lower (about 550–575 cM) for an equivalent number of mapped markers. In order to make comparison possible with the two new genetic linkage maps, we added the set of 384 SNPs developed by Lapègue *et al.* (2014) to the map published by Lallias *et al.* (2009) This “completed” map showed a genome length very close to previous estimates for *O. edulis* (see above). Thereby, this study presents both the best estimate of the genome coverage (92.4%) of the European flat oyster, and the largest number of molecular markers mapped on a single genetic linkage map for an oyster species (n = 293). Even so, with the availability of new transcriptomic assemblies for some oyster species and the recently sequenced genome of *C. gigas* (Zhang *et al.*, 2012), this performance should soon be surpassed. For the moment, the completed map becomes the reference genetic linkage map for

the European flat oyster.

Comparing this completed genetic linkage map with the new ones (Family 1 and Family 2, this study), a total of 353 different markers were successfully positioned on the three maps: 179 SNPs, 157 AFLPs and 17 microsatellites. The completed map consisted of 10 linkage groups, corresponding to the haploid number of chromosomes in the species (Thiriot-Quiévreux and Ayraud, 1982). However, only nine linkage groups were identified as homologous between the three different segregating families, and two other linkage groups were observed in only one of the families. In particular, the supernumerary group observed in Family 2 consisted of only two linked markers, which may in fact belong to LG 6 and fuse in other genetic backgrounds for which other SNPs could be mapped and exhibit new associations (E. Harrang, data not published). Within the homologous linkage groups, the order of mapped markers is relatively conserved. The variations observed could illustrate the different recombination rates of markers within each family. The present study thereby confirms the primary interest of comparing genetic linkage maps constructed for different genetic backgrounds.

4.2. Comparative QTL mapping

The present study aimed to estimate the diversity of QTLs where a response is noted to an experimental infection with the parasite responsible for bonamiosis, *B. ostreae*, in two segregating families of *O. edulis*. A total of seven QTLs linked to the phenotypes survived / died were identified in the two families. In the second family, the search for QTL regions linked to parasite load led to the detection of a new one. All these QTLs were located on a total of five different linkage groups, confirming the polygenic feature of the response to bonamiosis previously identified by Lallias *et al.* (2009). Two linkage groups were identical between the two families (LG 1 and LG 2) but, given that the rates of recombination between markers were specific to each reproductive event, and each breeding pair, mapped markers and marker order were not identical between family genetic linkage maps. Consequently, QTL regions were not positioned exactly at the same intervals, although these QTL regions might be identical, particularly for LG 2 where several markers were identified within the confidence interval of the QTL in the two segregating families. Even though only two

families were analysed, with a relatively low number of individuals per family, some interesting features can be highlighted.

Interestingly, Family 1 is related to Family 3. Indeed, the parental individuals (F_1) belong to the same family produced from the same grandparents (F_0: W31 & 703-29). Although the effect of a QTL is supposedly not the same in each family even if they share a common genetic background (Knott, 2005), it is also very interesting to compare the QTLs detected in each of these similar experiments. Indeed, if a QTL exists, it must necessarily segregate in more than one family (Knott, 2005), and therefore we should find common regions in homologous linkage groups. The five QTLs identified by Lallias *et al.* (2009) were located in LG 1, LG 3, LG 5 and LG 8 of the completed genetic linkage map of Family 3. Thus, one linkage group (LG 1) was simultaneously concerned in the detection of QTLs in the two newly tested families, and two linkage groups (LG 3 and LG 8) were simultaneously concerned in the related family (Family 1). Confidence intervals and markers associated with QTLs were not identical between the three families but the detection of QTL region(s) in these homologous linkage groups simultaneously in several families tends to confirm the potential of these suggestive regions.

For LG 3 and LG 8, a lack of common molecular markers between Families 1 and 3 prevented any conclusions from being drawn about whether there were one or more QTLs. It is thus not possible to make a deduction about the potential of one or several different or common QTLs between the families for these linkage groups. However, it appears that all of these QTLs were important in the response to bonamiosis. The reliability of their location still needs to be improved by increasing the number of families and number of animals tested per family.

The detection of the same QTL in several families depends on several other factors, such as the frequency of QTL alleles, proportion of the explained variance and density of the markers used for screening. Therefore, while finding the same QTL in several families is important for consistency, finding it in only one family can be explained by these factors, or a different genetic background, differing segregation over the three generations despite an identical genetic background (grandparents not coming from pure lines, case of related Family 1 and 3), the conditions of the

experimental infection, or it is a false positive (type I error).

Another hypothesis is that QTLs detected in related families were associated with different responses of the oyster to the infection because the development of the infection might not be exactly the same in tested individuals, and they might be sampled at different points in the process of host response. Indeed, it has previously been shown that the expression of some immune related genes is variable over time (Morga *et al.*, 2011b) and different QTLs were identified at different stages of development in cultivated rice (Yan *et al.*, 1998). Thus, it is possible that, at a certain time during the infection, only some QTLs would be favourable and subject to selection and thus detectable due to the difference of allelic frequencies between the two phenotypes examined.

A last hypothesis to explain the differences in QTL detection is based on the low number of oysters analysed due to the experimental conditions, especially for Family 2. Although the power of detection of QTLs linked to parasite load was enhanced by the selective genotyping method (Lander and Botstein, 1989), results might have been subject to the Beavis effect (Xu, 2003). However, the concurrent detection of QTL regions in the same linkage groups in several families strongly supports our results, as it is the case for QTL related to summer mortality in Pacific oyster (Sauvage *et al.*, 2010).

Furthermore, it is important to note that the same QTL with a large-effect was identified for the phenotypes “died” / “survived” and “died-infected” / “survived-uninfected”. Thereby, this study confirms the proliferation of the parasite as the probable cause of mortality, despite the fact that it was not detected in some individuals. In the context of our experiment, a load greater than 1.10^5 parasites per milligram of tissue could be a good indicator of the sensitivity of individuals to bonamiosis. According to Robert *et al.* (2009), this proportion corresponds to a light infection by *B. ostreae*. Despite the fact that this quantitative parameter is difficult to measure by biopsy, because it weakens individuals, it may be a good predictor of future mortalities. However, although molecular tools detect parasite DNA they do not confirm presence of viable pathogens and established infection (Robert *et al.*, 2009).

Overall, apart from a few exceptions, QTL alleles linked to flat oyster sensitivity

to *B. ostreae* (phenotypes “died” and “died-infected”) were preferentially transmitted to the F_2 generation by the grandparent of wild origin. Similarly, QTL alleles linked to the response to bonamiosis (phenotypes “survived” and “survived uninfected”) were preferentially transmitted by the grandparent from the selected line. These results tend to confirm the effectiveness of the selection of European flat oyster lines based on death or survival during experimental challenges to bonamiosis even if the use of markers linked to QTLs cannot yet be used to help selection at this stage.

Finally, the 21 SNPs identified as being significantly linked to QTLs correspond to genes that clustered into 7 main categories according to their putative biological function predicted by NCBI using GO (Gene Ontology) (**Table 3**). It is interesting to note that most of these genes (24%) cluster in the “cell communication, membrane receptor and immune system” category. Furthermore, the confidence interval of some QTLs includes some genes previously shown to be involved in the response of the oyster to an infection with *B. ostreae* (Morga *et al.*, 2011a, 2012). However, these SNPs may in no case be directly used to infer the genes involved in the mechanisms of resistance or sensitivity to bonamiosis. Indeed, the confidence intervals of the identified QTLs are relatively large (0.5 cM to 25 cM), and may contain several hundred genes, of which only a portion are involved in the response mechanisms. Thus, these genes are not necessarily related to immune response genes, but may participate in the regulation cascade of other genes directly involved in these mechanisms. However, they can still be considered as good candidates for transcriptomic analyses.

5. Conclusions

To summarize, we first improved and updated the previously published genetic-linkage map for the European flat oyster using a set of SNP markers. Secondly, we identified and confirmed, using two different oyster families, several regions of the European flat oyster genome that are associated (1) with the survival after contact with wild oysters injected with *Bonamia ostreae* and (2) with parasite load. We also detected, within the confidence intervals of these QTL regions, some previously predicted candidate genes differentially expressed during an infection with *B. ostrea*

(Morga *et al.*, 2011a, 2012). However, to improve the knowledge of the genetic architecture of the survival trait, it could be interesting to identify the genomic regions that are involved in the expression of these different candidate genes, and then to make a comparison between these eQTLs (expression Quantitative Trait Loci) and the QTL regions identified in the present study. Furthermore, in the future the localization of the QTL regions could be improved by the use of high-resolution linkage mapping, especially with RAD-Tag technology, as recently applied in the scallop *Chlamys farreri* (Jiao *et al.*, 2014), another marine bivalve species.

ACKNOWLEDGEMENTS

The authors wish to thank all members of staff of the Ifremer hatchery in La Tremblade for technical assistance with the production and rearing of the oysters, and for maintenance during the cohabitation challenge experiment. The authors thank Bruno Chollet for his help with specialised training in the *B. ostreae* purification method. This research was funded by the European Transnational SEAFARE project (Sustainable and Environmentally Friendly Aquaculture for the Atlantic Region of Europe – n°2009-1/123), funded by the Atlantic Area Transnational Program (2007–2013). The authors also wish to thank Bureau de Traduction de l'Université de Brest (Helen McCombie) for English editing.

REFERENCES

Baud, J.P., Gerard, A., Naciri-Graven, Y., 1997. Comparative growth and mortality of *Bonamia ostreae*-resistant and wild flat oysters, *Ostrea edulis*, in an intensive system. I. First year of experiment. *Marine Biology* **130**: 71-79.

Bédier, E., Baud, J.-P., Cochennec, N., Cornette, F., Gérard, A., Goyard, E., Joly, J.-P., Kopp, J., Langlade, A., Le Coguic, Y., Mazurié, J., Nourry, M., 2001. L'amélioration des performances de l'huître plate européenne *Ostrea edulis* par la sélection génétique. In: *Journées Conchylicoles du Département Ressources Aquacoles*. La Tremblade, Ifremer.

Bierne, N., Launey, S., Naciri-Graven, Y., Bonhomme, F., 1998. Early effect of inbreeding as revealed by microsatellite analyses on *Ostrea edulis* larvae. *Genetics* **148**: 1893-1906.

Cahais, V., Gayral, P., Tsagkogeorga, G., Melo-Ferreira, J., Ballenghien, M., Weinert, L., Chiari, Y., Belkhir, K., Ranwez, V., Galtier, N., 2012. Reference-free transcriptome assembly in non-model animals next-generation sequencing data. *Molecular Ecology Resources* **12**: 834-845.

Chakravarti, A., Lasher, L.K., Reefer, J.E., 1991. A maximum-likelihood method for estimating genome length using genetic-linkage data. *Genetics* **128**: 175-182.

Churchill, G.A., Doerge, R.W., 1994. Empirical threshold values for quantitative trait mapping. *Genetics* **138**: 963-971.

Cui, S., He, X., Fu, S., Meng, Q., Gai, J., Yu, D., 2008. Genetic dissection of the relationship of apparent biological yield and apparent harvest index with seed yield and yield related traits in soybean. *Australian Journal of Agricultural Research* **59**: 86-93.

Culloty, S.C., Cronin, M.A., Mulcahy, M.F., 2001. An investigation into the relative resistance of Irish flat oysters *Ostrea edulis* L. to the parasite *Bonamia ostreae* (Pichot *et al.*, 1980). *Aquaculture* **199**: 229-244.

Dekkers, J.C.M., 2004. Commercial application of marker- and gene-assisted selection in livestock: Strategies and lessons. *Journal of Animal Science* **82**: E313-E328.

Erickson, D.L., Fenster, C.B., Stenoien, H.K., Price, D., 2004. Quantitative trait locus analyses and the study of evolutionary process. *Molecular Ecology* **13**: 2505-2522.

FAO, 2011. Global Aquaculture Production: Quantities 1950-2011. www.fao.org/fishery/statistics/global-aquaculture-production/query.

Guo, X., Li, Q., Wang, Q., Kong, L., 2012. Genetic mapping and QTL analysis of growth-related traits in the Pacific oyster. *Marine Biotechnology* **14**: 218-226.

Harrang, E., Lapègue, S., Morga, B., Bierne, N., 2013. A high load of non-neutral amino-acid polymorphisms explains high protein diversity despite moderate effective population size in a marine bivalve with sweepstakes reproduction. *G3: Genes Genomes Genetics* **3**: 333-341.

Jaziri, H., 1990. Variations génétiques et structuration biogéographique chez un bivalve marin: l'huître plate *Ostrea edulis* L.: Thèse de doctorat, Montpellier.

Jiao, W., Fu, X., Dou, J., Li, H., Su, H., Mao, J., Yu, Q., Zhang, L., Hu, X., Huang, X., Wang, Y., Wang, S., Bao, Z., 2014. High-resolution linkage and Quantitative Trait Locus mapping aided by genome survey sequencing: building up an integrative genomic framework for a bivalve mollusc. *DNA Research* **21**: 85-101.

Knott, S.A., 2005. Regression-based quantitative trait loci mapping: robust, efficient and effective. *Philosophical Transactions of the Royal Society B-Biological Sciences* **360**: 1435-1442.

Kosambi, D.D., 1944. The estimation of map distances from recombination

values. *Annals of Eugenics* **12**: 172-175.

Lallias, D., Beaumont, A.R., Haley, C.S., Boudry, P., Heurtebise, S., Lapègue, S., 2007. A first-generation genetic linkage map of the European flat oyster *Ostrea edulis* (L.) based on AFLP and microsatellite markers. *Animal Genetics* **38**: 560-568.

Lallias, D., Arzul, I., Heurtebise, S., Ferrand, S., Chollet, B., Robert, M., Beaumont, A.R., Boudry, P., Morga, B., Lapègue, S., 2008. *Bonamia ostreae*-induced mortalities in one-year old European flat oysters *Ostrea edulis*: experimental infection by cohabitation challenge. *Aquatic Living Resources* **21**: 423-439.

Lallias, D., Gomez-Raya, L., Haley, C.S., Arzul, I., Heurtebise, S., Beaumont, A.R., Boudry, P., Lapègue, S., 2009. Combining two-stage testing and interval mapping strategies to detect QTL for resistance to bonamiosis in the European flat oyster *Ostrea edulis*. *Marine Biotechnology* **11**: 570-584.

Lander, E.S., Botstein, D., 1989. Mapping mendelian factors underlying Quantitative Traits using RFLP linkage maps. *Genetics* **121**: 185-199.

Lapègue, S., Harrang, E., Heurtebise, S., Flahauw, E., Donnadieu, C., Gayral, P., Ballenghien, M., Genestout, L., Barbotte, L., Mahla, R., Haffray, P., Klopp, C., 2014. Development of SNP genotyping arrays in two shellfish species. *Molecular Ecology Resources*, doi: 10.1111/1755-0998.12230.

Launey, S., Barre, M., Gerard, A., Naciri-Graven, Y., 2001. Population bottleneck and effective size in *Bonamia ostreae*-resistant populations of *Ostrea edulis* as inferred by microsatellite markers. *Genetical Research* **78**: 259-270.

Launey, S., Hedgecock, D., 2001. High genetic load in the Pacific oyster *Crassostrea gigas*. *Genetics* **159**: 255-265.

Le Bras, Y., Dechamp, N., Krieg, F., Filangi, O., Guyomard, R., Boussaha, M., Bovenhuis, H., Pottinger, T.G., Prunet, P., Le Roy, P., Quillet, E., 2011. Detection of QTL with effects on osmoregulation capacities in the rainbow trout (*Oncorhynchus mykiss*). *Bmc Genetics* **12**: 46.

Li, H., Liu, X., Zhang, G., 2012. A consensus microsatellite-based linkage map for the hermaphroditic bay scallop (*Argopecten irradians*) and its application in size-related QTL analysis. *PLoS ONE* **7**(10): e46926.

Li, L., Guo, X., 2004. AFLP-based genetic linkage maps of the Pacific oyster

Crassostrea gigas Thunberg. *Marine Biotechnology* **6**: 26-36.

Manly, K.F., Olson, J.M., 1999. Overview of QTL mapping software and introduction to map manager QT. *Mammalian Genome* **10**: 327-334.

Martin, A.G., Gérard, A., Cochennec, N., Langlade, A., 1992. Selecting flat oysters, *Ostrea edulis*, for survival against the parasite *Bonamia ostreae*: assessment of the resistance of a first selected generation. In: Production, environment and quality, Bordeaux Aquaculture 1992.

Mialhe, E., Bachère, E., Chagot, D., Grizel, H., 1988. Isolation and purification of the protozoan *Bonamia ostreae* (Pichot *et al.*, 1979), a parasite affecting the flat oyster *Ostrea edulis* L. *Aquaculture* **71**: 293-299.

Morga, B., Arzul, I., Faury, N., Segarra, A., Chollet, B., Renault, T., 2011a. Molecular responses of *Ostrea edulis* haemocytes to an in vitro infection with *Bonamia ostreae*. *Developmental and Comparative Immunology* **35**: 323-333.

Morga, B., Renault, T., Faury, N., Chollet, B., Arzul, I., 2011b. Cellular and molecular responses of haemocytes from *Ostrea edulis* during in vitro infection by the parasite *Bonamia ostreae*. *International Journal for Parasitology* **41**: 755-764.

Morga, B., Renault, T., Arzul, I., 2012. New insights in flat oyster *Ostrea edulis* resistance against the parasite *Bonamia ostreae*. *Fish and Shellfish Immunology* **32**: 958-968.

Morton, N.E., 1955. Sequential tests for the detection of linkage. *American journal of human genetics* **7**: 277-318.

Naciri-Graven, Y., Martin, A.G., Baud, J.P., Renault, T., Gerard, A., 1998. Selecting the flat oyster *Ostrea edulis* (L.) for survival when infected with the parasite *Bonamia ostreae*. *Journal of Experimental Marine Biology and Ecology* **224**: 91-107.

Qin, Y., Liu, X., Zhang, H., Zhang, G., Guo, X., 2007. Genetic mapping of size-related quantitative trait loci (QTL) in the bay scallop (*Argopecten irradians*) using AFLP and microsatellite markers. *Aquaculture* **272**: 281-290.

Ranson, G., 1967. Les espèces d'huîtres vivant actuellement dans le monde, définies par leurs coquilles larvaires ou prodissoconques. Etude des collections de quelques-uns des grands musées d'histoire naturelle. *Revue des Travaux de l'Institut des Pêches Maritimes* **31**: 127-274.

Robert, M., Garcia, C., Chollet, B., Lopez-Flores, I., Ferrand, S., Francois,

C., Joly, J.-P., Arzul, I., 2009. Molecular detection and quantification of the protozoan Bonamia ostreae in the flat oyster, *Ostrea edulis*. *Molecular and Cellular Probes* **23**: 264-271.

Sauvage, C., Boudry, P., de Koning, D.-J., Haley, C.S., Heurtebise, S., Lapègue, S., 2010. QTL for resistance to summer mortality and OsHV-1 load in the Pacific oyster (*Crassostrea gigas*). *Animal Genetics* **41**: 390-399.

Spelman, R.J., van Arendonk, J.A.M., 1997. Effect of inaccurate parameter estimates on genetic response to marker-assisted selection in an outbred population. *Journal of Dairy Science* **80**: 3399-3410.

Thiriot-Quiévreux, C., Ayraud, N., 1982. Les caryotypes de quelques espèces de bivalves et de gastéropodes marins. *Marine Biology* **70**: 165-172.

van Ooijen, J.W., 2004. MapQTL 5: Software for the mapping of quantitative trait loci in experimental populations. Wageningen, Netherlands: Plant Research International B.V. and Kyazma B.V.

van Ooijen, J.W., 2006. JoinMap 4: Software for the calculation of genetic linkage maps in experimental populations. Wageningen, Netherlands: Plant Research International B.V. and Kyazma B.V.

Vercaemer, B., Spence, K.R., Herbinger, C.M., Lapegue, S., Kenchington, E.L., 2006. Genetic diversity of the European oyster (*Ostrea edulis* L.) in Nova Scotia: Comparison with other parts of Canada, Maine and Europe and implications for broodstock management. *Journal of Shellfish Research* **25**: 543-551.

Vignal, A., Milan, D., SanCristobal, M., Eggen, A., 2002. A review on SNP and other types of molecular markers and their use in animal genetics. *Genetics Selection Evolution* **34**: 275-305.

Visscher, P.M., Thompson, R., Haley, C.S., 1996. Confidence intervals in QTL mapping by bootstrapping. *Genetics* **143**: 1013-1020.

Xu, S., 2003. Theoretical basis of the Beavis effect. *Genetics* **165**: 2259-2268.

Yan, J., Zhu, J., He, C., Benmoussa, M., Wu, P., 1998. Molecular dissection of developmental behavior of plant height in rice (*Oryza sativa* L.). *Genetics* **150**: 1257–1265.

Yu, Z.N., Guo, X.M., 2006. Identification and mapping of disease resistance

QTLs in the Eastern oyster, *Crassostrea virginica* Gmelin. *Aquaculture* **254**: 160–170.

Zhan, A., Hu, J., Hu, X., Hui, M., Wang, M., Peng, W., Huang, X., Wang, S., Lu, W., Sun, C., Bao, Z., 2009. Construction of microsatellite-based linkage maps and identification of size-related quantitative trait loci for Zhikong scallop (*Chlamys farreri*). *Animal Genetics* **40**: 821-31.

Zhang, G., Fang, X., Guo, X. *et al.*, 2012. The oyster genome reveals stress adaptation and complexity of shell formation. *Nature* **490**: 49-54.

FIGURE LEGENDS

Fig. 1: Genetic linkage map of the European flat oyster *Ostrea edulis*, mapping Family 3 (Lallias *et al.*, 2009) with the addition of 384 SNPs genotyped in 92 individuals. The new genetic linkage map consists of 119 SNPs, 17 microsatellites and 157 AFLPs mapped in ten linkage groups, for a total length of 536.4 cM.

Fig. 2: Comparative mapping of significant and suggestive QTLs linked to resistance or sensitivity to bonamiosis in the European flat oyster *Ostrea edulis*, for Families 1, 2 and 3 (completed map). Only linkage groups (LG) with significant or highly suggestive QTL in Families 1 or 2 are shown. In each LG, markers are indicated on the right, and absolute positions on the left (in Kosambi cM). Interval mapping results are shown for Families 1 and 2: the LOD score is plotted against the position (cM) along the LG. Solid lines represent QTL *F* ratio values for the survival trait, whereas dotted lines represent QTL *F* ratio values for the parasite load trait. Straight dotted lines represent the significance threshold at the chromosome-wide level. The confidence interval of each QTL region is shown by a dark line, and the most probable location of a QTL is indicated with an arrow. Each molecular marker shown in colour bounds the confidence interval or is part of a QTL region, and is common to several homologous linkage groups. For each family, markers linked to the QTL regions and their locations in the LG are indicated with dark blue boxes. The additive effect (R: resistance; S: sensitivity), the significance threshold (**: $p < 0.01$; ***: $p < 0.001$) and the grandparent from which the allele at the QTL originated are presented. **(A):** one LG is compared between the three families. **(B), (C) and (E)**: three LG are compared between two families. **(D)** One linkage group is presented in one family.

Fig. 1:

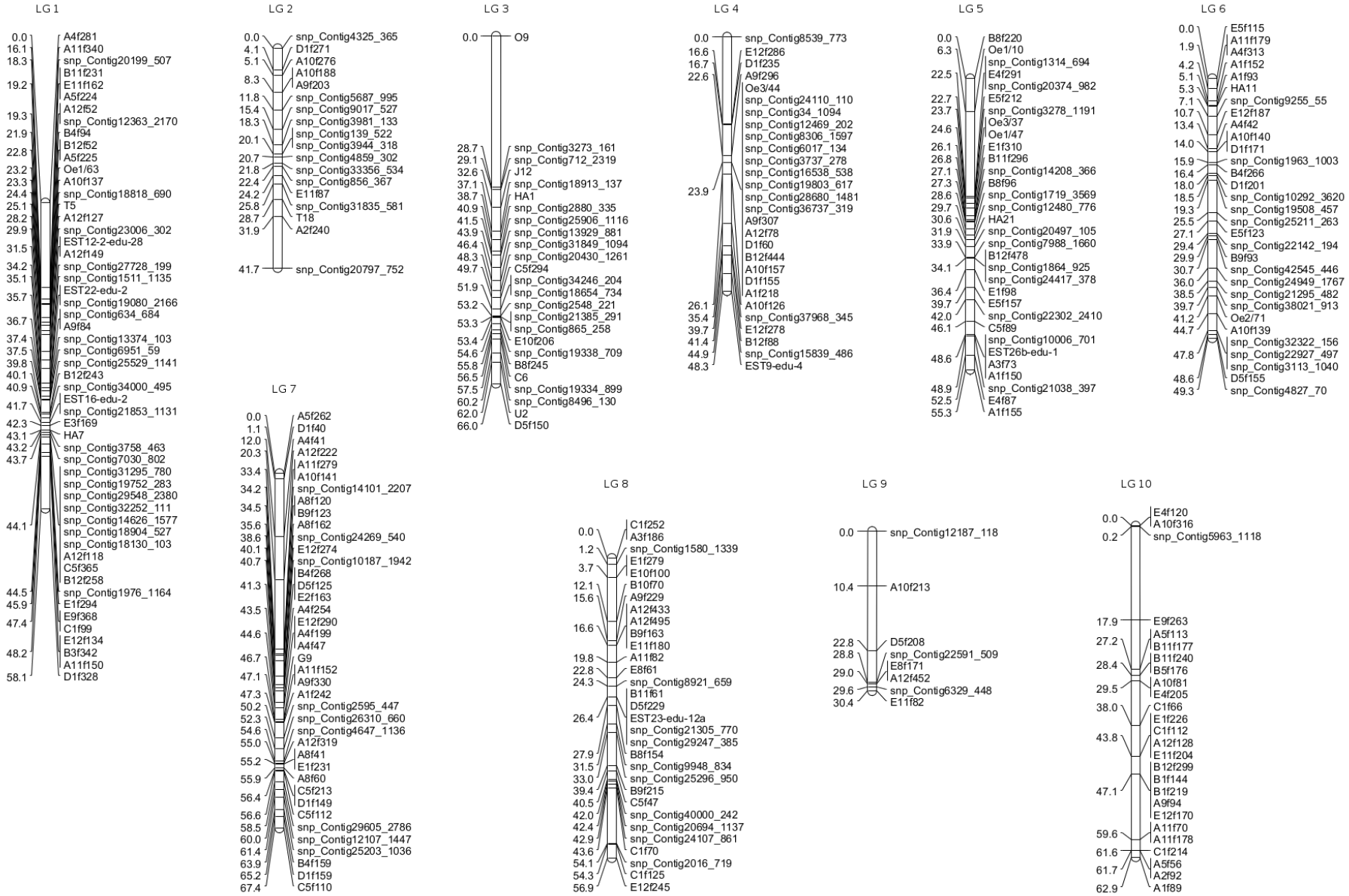
LG 1
0.0 A4f281
16.1 A11f340
18.3 snp_Contig20199_507
B11f231
19.2 E11f162
A5f224
A12f52
19.3 snp_Contig12363_2170
21.9 B4f94
22.8 B12f52
A5f225
23.2 Oe1/63
23.3 A10f137
24.4 snp_Contig18818_690
25.1 T5
28.2 A12f127
29.9 snp_Contig23006_302
EST12-2-edu-28
31.5 A12f149
34.2 snp_Contig27728_199
35.1 snp_Contig1511_1135
35.7 EST22-edu-2
snp_Contig19080_2166
36.7 snp_Contig634_684
A9f84
37.4 snp_Contig13374_103
37.5 snp_Contig6951_59
39.8 snp_Contig25529_1141
40.1 B12f243
40.9 snp_Contig34000_495
41.7 EST16-edu-2
snp_Contig21853_1131
42.3 E3f169
43.1 HA7
43.2 snp_Contig3758_463
43.7 snp_Contig7030_802
snp_Contig31295_780
snp_Contig19752_283
snp_Contig29548_2380
snp_Contig32252_111
44.1 snp_Contig14626_1577
snp_Contig18904_527
snp_Contig18130_103
A12f118
C5f365
B12f258
44.5 snp_Contig1976_1164
45.9 E1f294
47.4 E9f368
C1f99
E12f134
48.2 B3f342
A11f150
58.1 D1f328
LG 2
0.0 snp_Contig4325_365
4.1 D1f271
5.1 A10f276
A10f188
8.3 A9f203
11.8 snp_Contig5687_995
15.4 snp_Contig9017_527
18.3 snp_Contig3981_133
snp_Contig139_522
20.1 snp_Contig3944_318
20.7 snp_Contig4859_302
21.8 snp_Contig33356_534
22.4 snp_Contig856_367
24.2 E11f87
25.8 snp_Contig31835_581
28.7 T18
31.9 A2f240
41.7 snp_Contig20797_752
LG 3
0.0 O9
28.7 snp_Contig3273_161
29.1 snp_Contig712_2319
32.6 J12
37.1 snp_Contig18913_137
38.7 HA1
40.9 snp_Contig2880_335
41.5 snp_Contig25906_1116
43.9 snp_Contig13929_881
46.4 snp_Contig31849_1094
48.3 snp_Contig20430_1261
49.7 C5f294
snp_Contig34246_204
51.9 snp_Contig18654_734
53.2 snp_Contig2548_221
53.3 snp_Contig21385_291
snp_Contig865_258
53.4 E10f206
54.6 snp_Contig19338_709
55.8 B8f245
56.5 C6
57.5 snp_Contig19334_899
60.2 snp_Contig8496_130
62.0 U2
66.0 D5f150
LG 4
0.0 snp_Contig8539_773
16.6 E12f286
16.7 D1f235
22.6 A9f296
Oe3/44
snp_Contig24110_110
snp_Contig34_1094
snp_Contig12469_202
snp_Contig8306_1597
23.9 snp_Contig6017_134
snp_Contig3737_278
snp_Contig16538_538
snp_Contig19803_617
snp_Contig28680_1481
snp_Contig36737_319
A9f307
A12f78
D1f60
B12f444
A10f157
D1f155
A1f218
26.1 A10f126
35.4 snp_Contig37968_345
39.7 E12f278
41.4 B12f88
44.9 snp_Contig15839_486
48.3 EST9-edu-4
LG 5
0.0 B8f220
6.3 Oe1/10
snp_Contig1314_694
22.5 E4f291
snp_Contig20374_982
22.7 E5f212
23.7 snp_Contig3278_1191
Oe3/37
24.6 Oe1/47
26.1 E1f310
26.8 B11f296
27.1 snp_Contig14208_366
27.3 B8f96
28.6 snp_Contig1719_3569
29.7 snp_Contig12480_776
30.6 HA21
31.9 snp_Contig20497_105
33.9 snp_Contig7988_1660
B12f478
34.1 snp_Contig1864_925
snp_Contig24417_378
36.4 E1f98
39.7 E5f157
42.0 snp_Contig22302_2410
46.1 C5f89
snp_Contig10006_701
EST26b-edu-1
48.6 A3f73
A1f150
snp_Contig21038_397
48.9 E4f87
52.5 A1f155
55.3
LG 6
0.0 E5f115
1.9 A11f179
A4f313
4.2 A1f152
5.1 A1f93
5.3 HA11
7.1 snp_Contig9255_55
10.7 E12f187
13.4 A4f42
A10f140
14.0 D1f171
15.9 snp_Contig1963_1003
16.4 B4f266
18.0 D1f201
18.5 snp_Contig10292_3620
19.3 snp_Contig19508_457
25.5 snp_Contig25211_263
27.1 E5f123
29.4 snp_Contig22142_194
29.9 B9f93
30.7 snp_Contig42545_446
36.0 snp_Contig24949_1767
38.5 snp_Contig21295_482
39.7 snp_Contig38021_913
41.2 Oe2/71
44.7 A10f139
snp_Contig32322_156
47.8 snp_Contig22927_497
snp_Contig3113_1040
48.6 D5f155
49.3 snp_Contig4827_70
LG 7
0.0 A5f262
1.1 D1f40
12.0 A4f41
20.3 A12f222
A11f279
33.4 A10f141
34.2 snp_Contig14101_2207
A8f120
34.5 B9f123
35.6 A8f162
38.6 snp_Contig24269_540
40.1 E12f274
40.7 snp_Contig10187_1942
B4f268
41.3 D5f125
E2f163
43.5 A4f254
E12f290
44.6 A4f199
A4f47
46.7 G9
47.1 A11f152
A9f330
47.3 A1f242
50.2 snp_Contig2595_447
52.3 snp_Contig26310_660
54.6 snp_Contig4647_1136
55.0 A12f319
55.2 A8f41
E1f231
55.9 A8f60
56.4 C5f213
D1f149
56.6 C5f112
58.5 snp_Contig29605_2786
60.0 snp_Contig12107_1447
61.4 snp_Contig25203_1036
63.9 B4f159
65.2 D1f159
67.4 C5f110
LG 8
0.0 C1f252
A3f186
1.2 snp_Contig1580_1339
3.7 E1f279
E10f100
12.1 B10f70
15.6 A9f229
A12f433
16.6 A12f495
B9f163
E11f180
19.8 A11f82
22.8 E8f61
24.3 snp_Contig8921_659
B11f61
D5f229
26.4 EST23-edu-12a
snp_Contig21305_770
snp_Contig29247_385
27.9 B8f154
31.5 snp_Contig9948_834
33.0 snp_Contig25296_950
39.4 B9f215
40.5 C5f47
42.0 snp_Contig40000_242
42.4 snp_Contig20694_1137
42.9 snp_Contig24107_861
43.6 C1f70
54.1 snp_Contig2016_719
54.3 C1f125
56.9 E12f245
LG 9
0.0 snp_Contig12187_118
10.4 A10f213
22.8 D5f208
28.8 snp_Contig22591_509
E8f171
29.0 A12f452
29.6 snp_Contig6329_448
30.4 E11f82
LG 10
0.0 E4f120
A10f316
0.2 snp_Contig5963_1118
17.9 E9f263
27.2 A5f113
B11f177
B11f240
28.4 B5f176
29.5 A10f81
E4f205
38.0 C1f66
E1f226
43.8 C1f112
A12f128
E11f204
B12f299
47.1 B1f144
B1f219
A9f94
E12f170
59.6 A11f70
A11f178
61.6 C1f214
61.7 A5f56
A2f92
62.9 A1f89

Fig. 2:

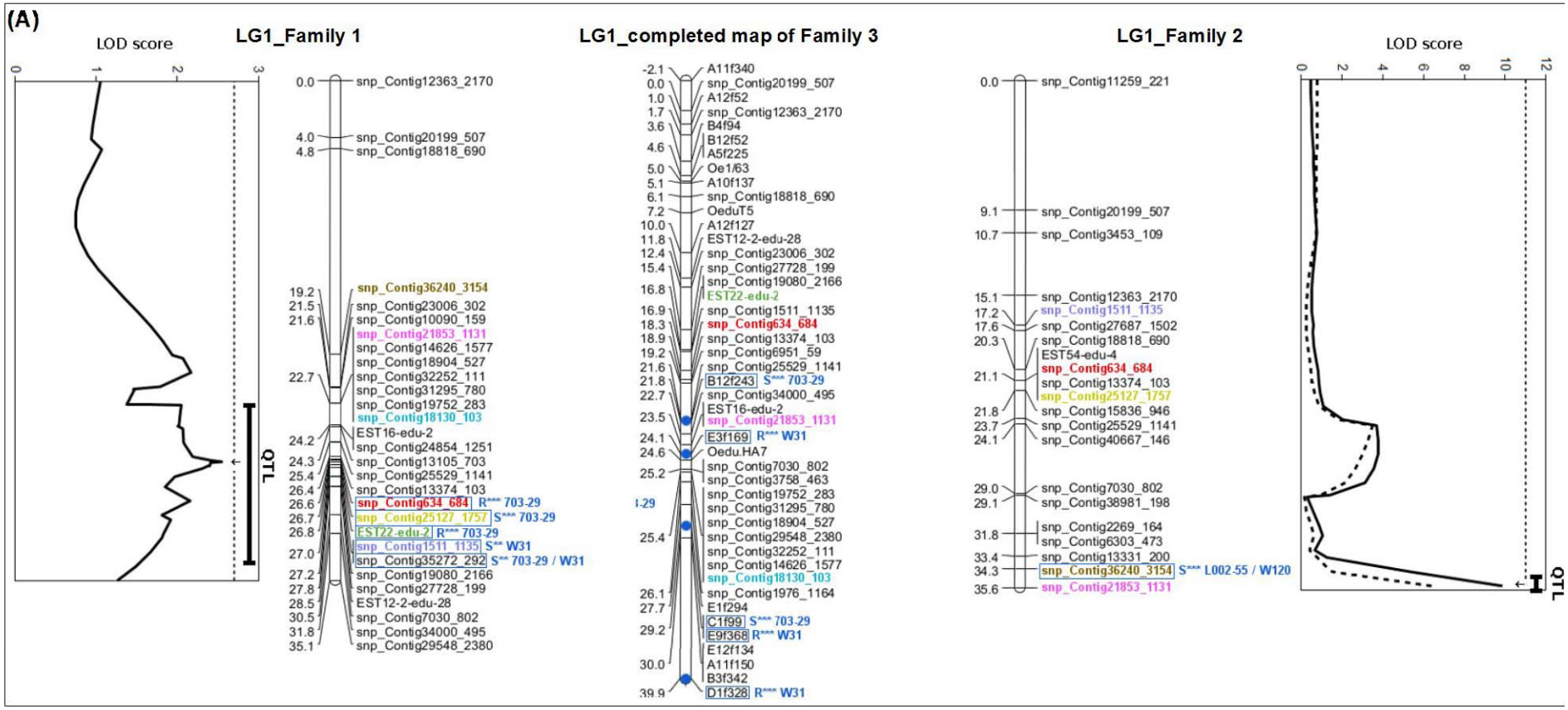
(A)
LOD score
LG1_Family 1
LG1_completed map of Family 3
LG1_Family 2
LOD score
QTL
QTL
0.0 snp_Contig12363_2170
4.0 snp_Contig20199_507
4.8 snp_Contig18818_690
19.2 snp_Contig36240_3154
21.5 snp_Contig23006_302
21.6 snp_Contig10090_159
snp_Contig21853_1131
snp_Contig14626_1577
snp_Contig18904_527
22.7 snp_Contig32252_111
snp_Contig31295_780
snp_Contig19752_283
snp_Contig18130_103
24.2 EST16-edu-2
snp_Contig24854_1251
24.3 snp_Contig13105_703
25.4 snp_Contig25529_1141
26.4 snp_Contig13374_103
26.6 snp_Contig634_684 R*** 703-29
26.7 snp_Contig25127_1757 S*** 703-29
26.8 EST22-edu-2 R*** 703-29
27.0 snp_Contig1511_1135 S** W31
snp_Contig35272_292 S** 703-29 / W31
27.2 snp_Contig19080_2166
27.8 snp_Contig27728_199
28.5 EST12-2-edu-28
30.5 snp_Contig7030_802
31.8 snp_Contig34000_495
35.1 snp_Contig29548_2380
-2.1 A11f340
0.0 snp_Contig20199_507
1.0 A12f52
1.7 snp_Contig12363_2170
3.6 B4f94
4.6 B12f52
A5f225
5.0 Oe1/63
5.1 A10f137
6.1 snp_Contig18818_690
7.2 OeduT5
10.0 A12f127
11.8 EST12-2-edu-28
12.4 snp_Contig23006_302
15.4 snp_Contig27728_199
snp_Contig19080_2166
16.8 EST22-edu-2
16.9 snp_Contig1511_1135
18.3 snp_Contig634_684
18.9 snp_Contig13374_103
19.2 snp_Contig6951_59
21.6 snp_Contig25529_1141
21.8 B12f243 S*** 703-29
22.7 snp_Contig34000_495
EST16-edu-2
23.5 snp_Contig21853_1131
24.1 E3f169 R*** W31
24.6 Oedu.HA7
25.2 snp_Contig7030_802
snp_Contig3758_463
snp_Contig19752_283
-29 snp_Contig31295_780
snp_Contig18904_527
25.4 snp_Contig29548_2380
snp_Contig32252_111
snp_Contig14626_1577
snp_Contig18130_103
26.1 snp_Contig1976_1164
27.7 E1f294
C1f99 S*** 703-29
29.2 E9f368 R*** W31
E12f134
30.0 A11f150
B3f342
39.9 D1f328 R*** W31
0.0 snp_Contig11259_221
9.1 snp_Contig20199_507
10.7 snp_Contig3453_109
15.1 snp_Contig12363_2170
17.2 snp_Contig1511_1135
17.6 snp_Contig27687_1502
20.3 snp_Contig18818_690
EST54-edu-4
21.1 snp_Contig634_684
snp_Contig13374_103
snp_Contig25127_1757
21.8 snp_Contig15836_946
23.7 snp_Contig25529_1141
24.1 snp_Contig40667_146
29.0 snp_Contig7030_802
29.1 snp_Contig38981_198
31.8 snp_Contig2269_164
snp_Contig6303_473
33.4 snp_Contig13331_200
34.3 snp_Contig36240_3154 S*** L002-55 / W120
35.6 snp_Contig21853_1131

Fig. 2 (end):

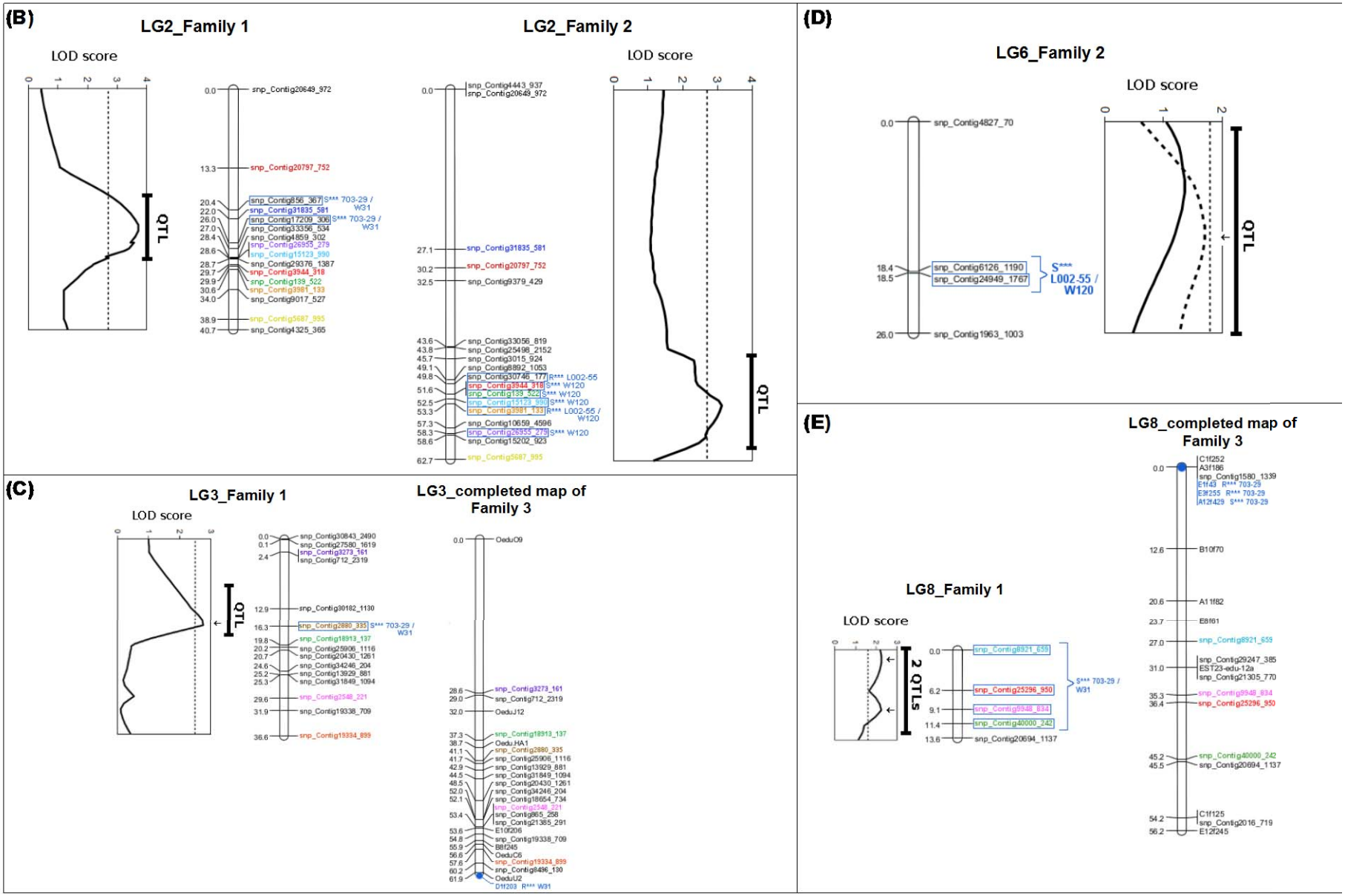
(B)
LG2_Family 1
LOD score
QTL
LG2_Family 2
LOD score
QTL
(D)
LG6_Family 2
LOD score
QTL
(C)
LG3_Family 1
LOD score
QTL
LG3_completed map of Family 3
(E)
LG8_Family 1
LOD score
2 QTLs
LG8_completed map of Family 3

Table 1: Statistics of homology between linkage groups for Families 1, 2 and 3.

		Family 1				Family 2				Improved map of Family 3			
Linkage group (LG)	**Common markers***	**No. of mapped markers**	**Length (cM)**	**Average spacing (cM)**	**Largest interval (cM)**	**No. of mapped markers**	**Length (cM)**	**Average spacing (cM)**	**Largest interval (cM)**	**No. of mapped markers**	**Length (cM)**	**Average spacing (cM)**	**Largest interval (cM)**
LG 1	25(0-0)	29(4)	35.10	1.25	14.40	21(10)	35.60	1.78	9.10	54(31)	58.12	1.10	16.12
LG 2	14(0-0)	16(2)	40.69	2.71	13.32	18(9)	62.70	3.69	27.10	18(7)	41.66	2.45	9.75
LG 3	16(0-0)	15(2)	36.65	2.62	10.43	13(4)	41.20	3.44	18.10	25(10)	65.98	2.75	28.74
LG 4a	15(0-0)	12(0)	0.0	0.0	0.0	13(3)	0	0	0	28(14)	48.30	1.79	16.60
LG 4b		3(0)	9.03	4.52	5.87	2(0)	7.86	7.86	7.86				
LG 5a	15(1-3)	0(0)	44.72	4.97	22.28	7(1)	19.20	3.21	10.10	32(20)	55.35	1.79	16.15
LG 5b		5(1)	19.80	4.95	14.48	2(2)	4.01	4.01	4.01				
LG 6	11(0-0)	11(1)	51.80	5.76	15.70	4(1)	26.00	8.67	18.45	31(20)	49.33	1.64	6.20
LG 7	7(0-1)	7(0)	19.92	3.32	9.35	6(2)	39.80	7.95	11.26	40(33)	67.38	1.73	13.07
LG 8a	6(0-2)	5(0)	13.60	3.40	6.22	3(0)	10.75	5.37	7.45	31(25)	56.88	1.90	10.50
LG 8b						2(1)	3.27	3.27	3.27				
LG 9a	2(2-0)	2(2)	2.04	2.04	2.04	3(3)	3.17	1.58	2.29	8(6)	30.50	4.34	12.40
LG 9b		2(0)	1.79	1.79	1.79								
LG 10	0(0-0)	-	-	-	-	-	-	-	-	26(26)	62.90	2.52	17.65
S1	0(0-0)	-	-	-	-	2(2)	0.0	0.0	0.0	-	-	-	-
Total	111(3-6)	117(12)	275.2	-	-	96(38)	253.5	-	-	293(192)	536.4	-	-
Mean	-	-	-	3.1	9.7	-	-	3.9	9.2	-	-	2.2	14.7

* Common markers are presented as: a(b-c) where a: number of markers common to at least two of the three families; b: number of markers that were successfully positioned in only one family and linked to the same linkage group in another family but not mapped; c: number of markers that were successfully positioned in two families and linked to the same linkage group in the third family but not mapped. For each family, the number of mapped markers segregating specifically in this family is indicated in brackets.

Table 2: Main characteristics of QTLs for survival after contact with wild oysters injected with *Bonamia ostreae* and in relation to parasite load, detected with Interval Mapping in two segregating families of the European flat oyster *Ostrea edulis* tested by experimental infection.

Phenotype	QTL[a]	LG[b]	Interval of markers[c]	Maximum LOD[d]	Location on LG (cM)[e]	Confidence interval (cM)[f]	LG LOD[g] P<0,05	Map LOD[h] P<0,05	Explained variance[i]	Additive effect[j]	Grandparent origin[k]
Survival											
Family 1:	q0904a1-1	1	snp_Contig634_684 –	2.55	26.72	22.7 – 33.6	2.7	3.5		R	703-29
OE.F2.09.04			snp_Contig19080_2166							S	703-29 & W31
	q0904a2-1	2	snp_Contig856_367 –	3.72**	23.48	17.8 – 28.6	2.7	3.5	15.2%	S	703-29 & W31
			snp_Contig17209_306								
	q0904a3-1	3	snp_Contig30182_1130 –	2.77*	16.26	8.3 – 17.5	2.5	3.5	10.6%	S	703-29 & W31
			snp_Contig18913_137								
	q0904a8-1	8	snp_Contig8921_659 –	2.24*	1.50	0.0 – 12.9	1.6	3.5	9.7%	S	703-29 & W31
			snp_Contig25296_950								
	q0904a8-2	8	snp_Contig25296_950 –	2.25*	9.13	0.0 – 12.9	1.6	3.5	8.8%	S	703-29 & W31
			snp_Contig40000_242								
Family 2:	q0967a1-1	1	snp_Contig36240_3154 –	9.87	35.34	35.1 – 35.6	11.0	11.1		R	L002-55 & W120
OE.F2.09.67			snp_Contig21853_1131								
	q0967a2-1	2	snp_Contig30746_177 –	3.13*	53.28	45.2 – 60.5	2.7	11.1	17.2%	S	W120
			snp_Contig26955_279							R	L002-55
Bonamia infection											
Family 2:	q0967b1-1	1	snp_Contig36240_3154 –	6.41	35.34	35.1 – 35.6	10.1	10.2		S	L002-55 & W120
OE.F2.09.67			snp_Contig21853_1131								
	q0967b2-1	2	snp_Contig139_522 –	3.32*	52.47	45.9 – 60.8	2.7	10.2	22.1%	S	W120
			snp_Contig3981_133								
	q0967b6-1	6	snp_Contig24949_1767 –	1.70	13.55	0.6 – 26.0	1.8	10.2		S	L002-55 & W120
			snp_Contig4827_70								

[a] Name of the QTL adapted from Cui et al. (2008): letter <q> followed by an abbreviation of the name of the family (0904 or 0967), followed by an <a> for the phenotype “survived” or <b> for the phenotype “*Bonamia* infected”, then the number of the linkage group, and finally the number of the QTL affecting this phenotype in the linkage group.
[b] Linkage group (LG) in which the QTL is mapped.
[c] Interval of markers between which the QTL is mapped.
[d] Maximum LOD score and level of significance (no indication: $P \approx 0.10$; *: $P < 0.05$; **: $P < 0.01$).
[e] Best estimate of the location of the QTL in each LG.
[f] Confidence interval of the location of the QTL in each LG.
[g] LOD score at $P < 0.05$ obtained after 1000 iterations by the permutation test (Churchill and Doerge, 1994), and corresponding to the significance level to be applied to LG for the detection of QTL.
[h] LOD score at $P < 0.05$ of the global genetic linkage map obtained after 1000 iterations by the permutation test (Churchill and Doerge, 1994).
[i] Percentage of total phenotypic variance explained by the detected QTL. The values are only given for QTL with LOD score exceeding the threshold of significance of the LG.
[j] Estimate of the phenotypic effect of the QTL: R for resistance (phenotype “survived” or “survived-uninfected”); S for sensitivity (phenotype “died” or “died-infected”).
[k] Grandparental origin of the QTL allele.

Table 3: Characteristics of the SNPs located in the confidence interval or linked to QTL for survival after contact with wild oysters injected with *Bonamia ostreae* and in relation to parasite load in two segregating families of the European flat oyster *Ostrea edulis* tested by experimental infection.

Locus	LG	Linked to QTL	Homologous gene	BLAST species	Accession number of the gene	Putative biological function
Located into the confidence interval of the QTL						
EST22-edu-2	1	***	Ribosomal protein L3	*Ostrea edulis*	JN680829	Ribosomal
snp_Contig36240_3154	1	***	Ankyrin repeat domain-containing protein 17	*Crassostrea gigas*	EKC23980	Cells communication, membrane receptor, immune system
snp_Contig25127_1757	1	***	Guanine nucleotide-binding protein G(o) subunit alpha	*Crassostrea gigas*	EKC28055	Cells communication, membrane receptor, immune system
snp_Contig634_684	1	***	Thioredoxin domain-containing protein 12	*Crassostrea gigas*	EKC27271	Detoxyfication, stress protein
snp_Contig1511_1135	1	**	F-box only protein 4	*Crassostrea gigas*	EKC18204	Cell cycle, DNA repair, protein regulation and transcription
snp_Contig35272_292	1	**	Hypothetical protein	N.A.	N.A.	Unknown fonction
EST12-2-edu-28	1	NS	short-chain dehydrogenase	*Ostrea edulis*	JN680824	Respiratory chain
snp_Contig139_522	2	***	FH1/FH2 domain-containing protein 3	*Crassostrea gigas*	EKC35959	Cytoskeleton
snp_Contig26955_279	2	***	Myotubularin-related protein 4	*Crassostrea gigas*	EKC42140	Metabolism
snp_Contig3981_133	2	***	Paired box pox-meso protein	*Crassostrea gigas*	EKC38956	Cell cycle, DNA repair, protein regulation and transcription
snp_Contig17209_306	2	***	Tetratricopeptide repeat protein 17	*Crassostrea gigas*	EKC24151	Unknown fonction
snp_Contig856_367	2	***	Tripartite motif-containing protein	*Crassostrea gigas*	EKC33533	Cells communication, membrane receptor, immune system
snp_Contig30746_177	2	***	Hypothetical protein	*Crassostrea gigas*	EKC41401	Unknown fonction
snp_Contig3944_318	2	***	Hypothetical protein	*Crassostrea gigas*	EKC39636	Unknown fonction
snp_Contig15123_990	2	***	Hypothetical protein	N.A.	N.A.	Unknown fonction
snp_Contig2880_335	3	***	Hypothetical protein	N.A.	N.A.	Unknown fonction

snp_Contig24949_1767	6	***	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase delta-1	*Crassostrea gigas*	EKC40084	Metabolism
snp_Contig6126_1190	6	***	Hypothetical protein	N.A.	N.A.	Unknown fonction
snp_Contig40000_242	8	***	Afadin	*Crassostrea gigas*	EKC42518	Cells communication, membrane receptor, immune system
snp_Contig25296_950	8	***	Toll-like receptor 1	*Crassostrea gigas*	EKC32484	Cells communication, membrane receptor, immune system
snp_Contig8921_659	8	***	Trithorax group protein osa	*Crassostrea gigas*	EKC20059	Cell cycle, DNA repair, protein regulation and transcription
snp_Contig9948_834	8	***	Hypothetical protein	N.A.	N.A.	Unknown fonction
Potentially located in the confidence interval of the QTL						
EST54-edu-4	1	NS	cAMP-responsive element binding protein	*Ostrea edulis*	JN680843	Cells communication, membrane receptor, immune system
EST23-edu-12a	8	NS	Tyrosyl-tRNA synthetase	*Ostrea edulis*	JN680830	Cell cycle, DNA repair, protein regulation and transcription

NS: not significant.
**: $P < 0.005$.
***: $P < 0.001$.
N.A.: not available.