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Bilan des productions expérimentales d'huîtres plates de 2005 à 2010 au Laboratoire de Génétique et Pathologie de La Tremblade

Sommaire

L'objectif de ce rapport est de détailler les productions d'huîtres plates européennes, *Ostrea edulis*, dans le cadre des projets de recherche développés autour de l'amélioration génétique de cette espèce. Nous avons choisi de synthétiser les actions de recherche dans un premier temps puis la production des huîtres et les expérimentations dans un ordre chronologique.

1. Les activités de recherche en amélioration génétique

1.1. Le programme de sélection pour la résistance à la bonamiose

Plusieurs programmes de sélection de l'huître plate *Ostrea edulis* pour une meilleure résistance à *Bonamia ostreae* ont été démarrés en Europe, en particulier en Irlande et en France. L'existence au niveau individuel, chez les huîtres plates élevées en zones d'endémie, d'une résistance naturelle a été en effet montrée depuis longtemps et suggère la possibilité d'une amélioration génétique. Cette résistance ne semble pas induire pour autant une amélioration de la survie à long terme au sein des populations naturelles. Le fait qu'*Ostrea edulis* se reproduise dès l'âge de 1 an alors que le parasite ne complète son cycle dans l'hôte qu'au bout de 2 à 3 ans peut expliquer en partie cet état de fait. Dans ces conditions, la valeur sélective du caractère resterait faible en conditions naturelles. Un projet CRAFT (regroupant le Royaume Uni, l'Irlande, la Norvège, les Pays-Bas et certaines entreprises privées) a fonctionné de 1998 à 2000 de manière à vérifier le développement de la résistance chez certaines souches irlandaises. Des mortalités et des taux de prévalence moindres ont été observés chez les huîtres sélectionnées par rapport aux témoins, mais ceux-ci provenaient de gisements indemnes de bonamiose ce qui est à même de biaiser les résultats obtenus.

En France, Ifremer a initié dès 1985 des travaux concernant l'amélioration génétique de la résistance à *Bonamia ostreae*, parallèlement aux études menées sur le parasite. La purification de ce dernier, obtenue en 1988 a permis de recourir à des infections expérimentales, et de soumettre les huîtres à une pression de sélection mieux contrôlée que celle obtenue en conditions naturelles. Le programme a évolué d'une sélection massale à une sélection intrafamiliale. La gestion de la diversité génétique des lignées utilisées dans ce programme, et les suivis terrain d'une durée de 3 ans expliquent la longueur de ce programme dont on ne retiendra que les principales étapes :

- En 1995, la comparaison des performances d'huîtres de 3ème génération montrait un gain significatif par rapport au contrôle sauvage (59% de survie contre 13%), alors que les croisements entre ces animaux et le contrôle sauvage suggéraient un déterminisme génétique susceptible d'être sélectionné. Le protocole de croisement massal a été parallèlement modifié en croisements biparentaux de manière à diminuer les effets potentiels de consanguinité.
- De 1997 à 2000, des animaux sélectionnés issus de ce nouveau protocole ont montré des gains significatifs de performances par rapport aux deux cohortes de

naissain naturel captées dans les deux années encadrant leur production en écloserie (61% de survie contre 46 et 13%).

- De 1998 à 2000, le même gain de performances a été montré entre des animaux sélectionnés produits de manière familiale et les contrôles sauvages (83% de survie contre 8% en deuxième année) (Figure 1).

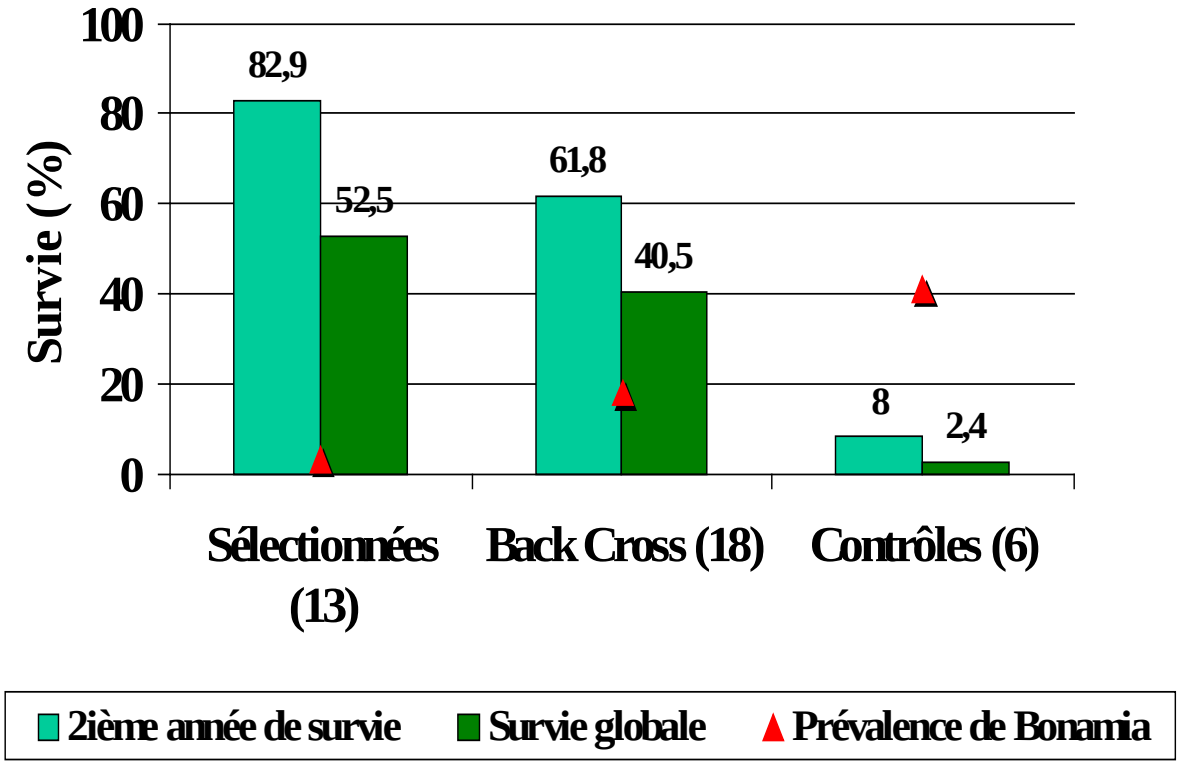

Figure 1. Lien entre la prévalence *à Bonamia ostreae* et la survie enregistrée respectivement pour les huîtres issues des différents types de croisements

Dans tous les cas, les taux de survie apparaissent corrélés aux taux de prévalence en *Bonamia ostreae*. Les résultats expérimentaux disponibles à l'heure actuelle sont donc prometteurs, et laissent penser à une résistance accrue et transmissible des souches développées par Ifremer. Cependant les expériences ont été jusqu'à présent menées, pour des raisons pratiques d'accès aux animaux et d'acquisition des données, en conditions expérimentales (lots de taille limitée, élevage en poches ostréicoles), non transposables au niveau de la production.

En parallèle à ce programme de sélection, des lignées consanguines (frère-sœur) ont été constituées depuis 6 générations (Figure 2). Les derniers croisements remontant à 2000 ou 2003, il devenait nécessaire de refaire une génération supplémentaire. Début 2003, seules les familles en vert avaient des représentants vivants. La famille en jaune avait présenté des survies particulièrement intéressantes ce qui nous a conduit à l'utiliser dans certains croisements ultérieures (voir paragraphe 1.2)

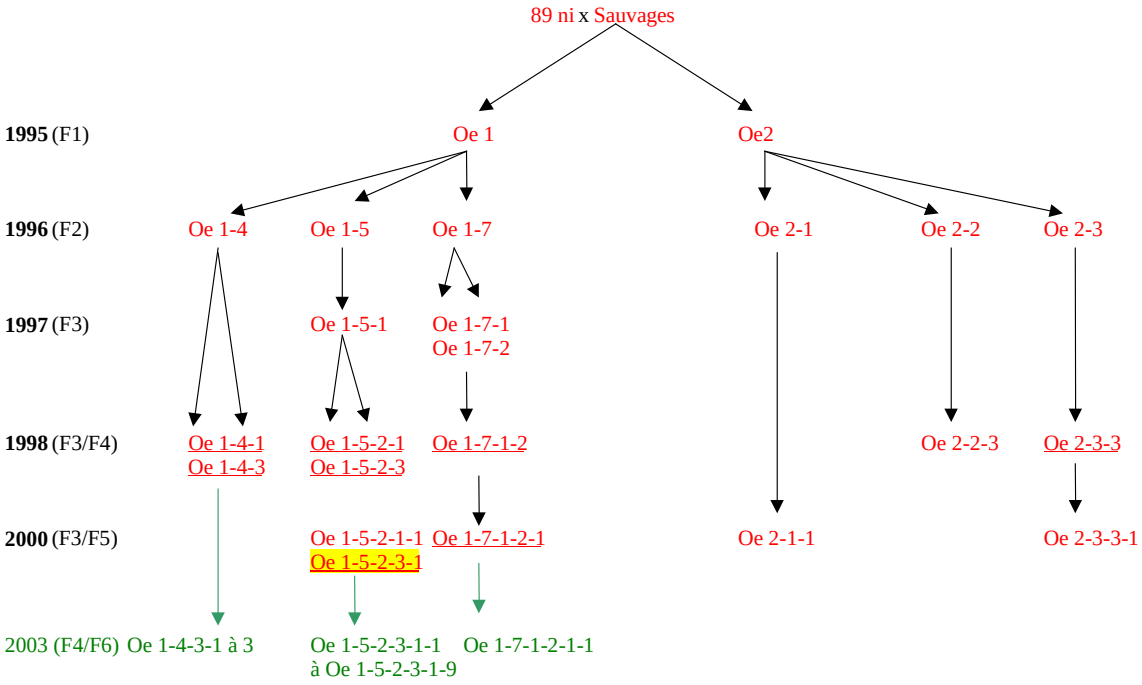

Figure 2. Schéma de l'obtention des lignées d'huîtres plates consanguines en 2003

1.2. INTERREG IIIB AAAG

L'objectif de ce projet AAAG (Groupe Aquacole de l'Arc Atlantique : 2004-2006) était de développer une aquaculture durable dans des pays de l'arc atlantique (Espgne, France, Irlande, Grande-Bretagne). Les thèmes de recherche étaient développés autour de la diversification, la réduction des rejets aquacoles et l'amélioration génétique. Les espèces concernées étaient plus particulièrement l'omble chevalier, la moule, l'huître plate, l'oursin, l'ormeau et la sole. Au sein de ce projet, l'équipe génétique du laboratoire "Génétique et Pathologie" de La Tremblade a collaboré avec l'Université de Bangor pour :

- le développement de nouveaux marqueurs moléculaires chez l'huître plate et la moule,
- l'établissement d'une carte génétique chez l'huître plate, et la moule,
- la détection de zones du génome impliquées dans la résistance à la bonamiose (QTLs) chez l'huître plate (Voir exemple en Figure 3).

Ce travail a correspondu à la thèse de Delphine Lallias, inscrite en thèse à l'Université de Bangor et dont les responsables de thèse étaient A. Beaumont (U. Bangor), P. Boudry et S. Lapègue (Ifremer), et qui a soutenu sa thèse le 28 juin 2007. L'ensemble de ce travail a donné lieu à 6 publications de 2007 à 2009, dont celles directement en lien avec les productions d'huîtres plates sont reprises en annexe.

Pendant cette période, des familles dites « ségrégeantes », c'est-à-dire permettant d'observer une variabilité pour un caractère donné, ont été produites. Cela se traduit par la production successive de deux générations de familles à partir de croisements biparentaux de frères-sœurs. Ces familles sont appelées F1 et F2 pour les deux générations successives. Deux types de familles ont été produits en fonction des géniteurs utilisés au départ :

- les familles F1S puis F2S à partir d'un individu sauvage et d'un individu appartenant à une famille résistante du programme de sélection,
- les familles F1L puis F2L à partir d'un individu sauvage et d'un individu appartenant à la lignée consanguine (Oe 1.5.2.3.1) ayant montré une survie supérieure aux autres lignées.

Il est important de noter que ce projet puis le suivant (AAAG2) ont permis l'acquisition au LGP un séquenceur à 4 capillaires (Applied Biosystems) puis de l'équiper en 16 capillaires.

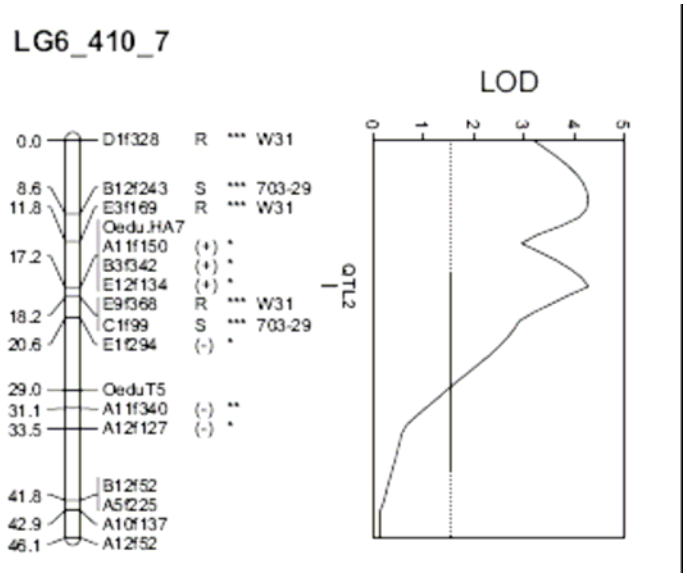

Figure 3. Exemple de localisation d'une zone QTL (notée QTL2) dans le groupe de liaison 6 (LG6) du parent 410_7.

1.3. INTERREG IVB SEAFARE

Le projet SEAFARE est le troisième volet du groupe AAAG qui existe depuis 2004 et a obtenu 2 contrats successifs aux appels d'offres INTERREG IIIB. Pour la réponse à cet appel d'offres, le groupe propose la poursuite de certains travaux engagés mais également de nouveaux sous-projets. L'Ifremer sera en particulier impliqué dans le sous-projet "Aquaculture pour maintenir la biodiversité" en poursuivant les travaux sur la résistance de l'huître plate à la bonamiose (thèse d'Estelle Harrang commençant en 2008 au LGP), mais aussi le sous-projet " espèce invasive" avec l'étude de l'hypothèse adaptative de l'huître creuse, *Crassostrea gigas*, avec des marqueurs AFLP et une approche écophysiologique.

Le travail de thèse qui a débuté en 2008, a pour but de (1) développer de nouveaux outils (marqueurs moléculaires, PCR quantitative de gènes candidats, microarrays) permettant de mieux caractériser la résistance de l'huître plate contre la bonamiose, et (2) de tester l'intérêt des informations fournies par les marqueurs moléculaires (QTLs) pour améliorer et mieux comprendre cette résistance, afin d'envisager une sélection assistée par marqueurs et de contribuer à une production durable de l'huître plate. Trois tâches spécifiques seront ainsi réalisées : (i) L'amélioration de la résolution de la carte génétique en cartographiant des SNPs (Single Nucleotide Polymorphism) détectés dans des fragments de gènes identifiés comme des candidats impliqués dans la résistance à la

bonamiose et en intégrant l'ensemble des marqueurs (ceux-ci et ceux déjà publiés) sur une même carte génétique, et ainsi une meilleure détection des QTLs...; (ii) Une meilleure compréhension de la résistance à la bonamiose par l'utilisation d'outils de PCR quantitative pour des gènes candidats disponibles et dont certains pourraient être co-localisés avec des QTLs, mais aussi l'étude de paramètres hémocytaires.

La réalisation de ces tâches implique notamment auparavant l'obtention de familles F2 à partir des F1 produites en 2004 qui ont participé au projet INTERREG AAAG. Les productions de ces familles ont donc été réalisées en 2009 pour répondre aux besoins des différentes expérimentations.

2. Les productions de 2005

2.1. Production de matériel ségrégeant dans le cadre de AAAG: Familles F2-S

Au cours de l'année 2004, des familles F1-S (S=Sélectionnées) avaient été produites à partir du croisement d'un individu sauvage et d'un individu issu de familles sélectionnées produites en 1998 et obtenues après deux générations de sélection massale pour la résistance à la bonamiose.

En 2005, les 7 familles F1-S restantes conservées pendant l'automne et l'hiver 2004 à La Tremblade pour assurer leur grossissement à savoir : OE.F1.04.01, OE.F1.04.010, OE.F1.04.15, OE.F1.04.25, OE.F1.04.29, OE.F1.04.52 et OE.F1.04.59 ont été utilisées pour la production d'une seconde génération appelée F2-S, obtenue en réalisant des croisements par paire d'individus pleins-frères de la même famille (F1-S x F1-S).

Ainsi, 40 paires d'huîtres plates ont été placées dans des aquariums de 3 litres alimentés individuellement le 27 mars 2005, après 2 mois d'acclimatation puis de maturation progressive au sein de l'écloserie (Figures 4 et 5).

Figure 4. Système pour la reproduction biparentale

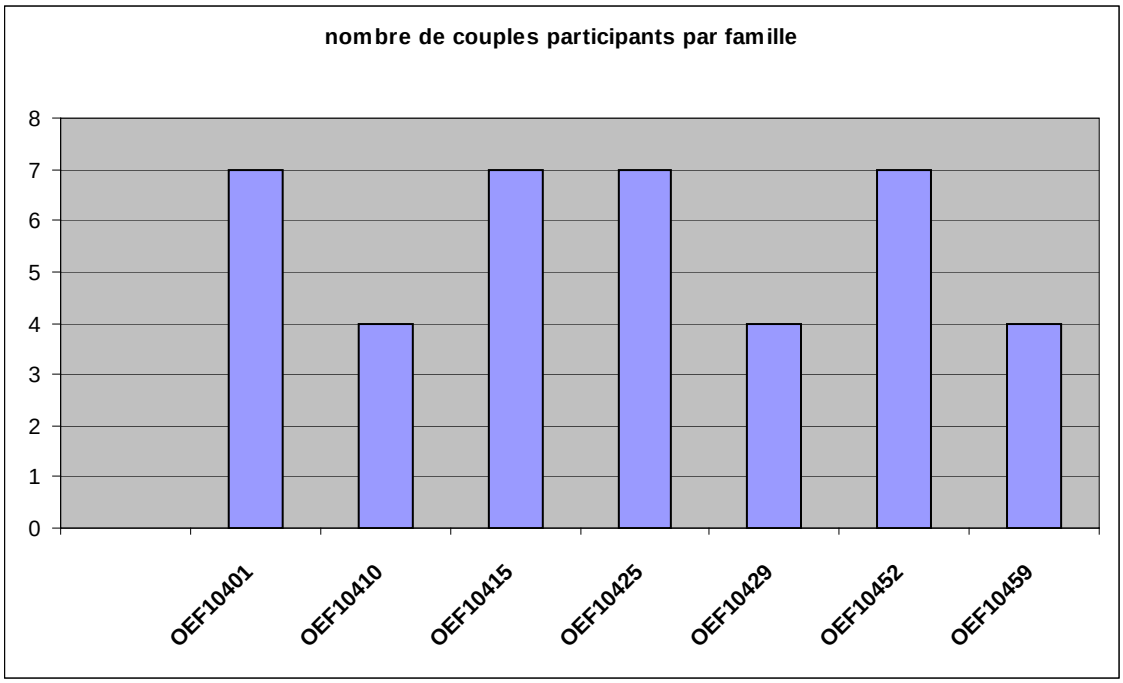

Figure 5. Détail du nombre de couples par famille de 1ère génération

Les premières pontes ont été récupérées à partir du 26 avril 2005, soit près d'un mois après leur conditionnement en aquarium, les autres se sont échelonnées régulièrement jusqu'au 27 juin 2005, date à laquelle nous avons mis fin aux reproductions compte tenu des difficultés rencontrées habituellement en période estivale pour mener à bien la phase d'élevage larvaire qui succède aux pontes (Figure 6).

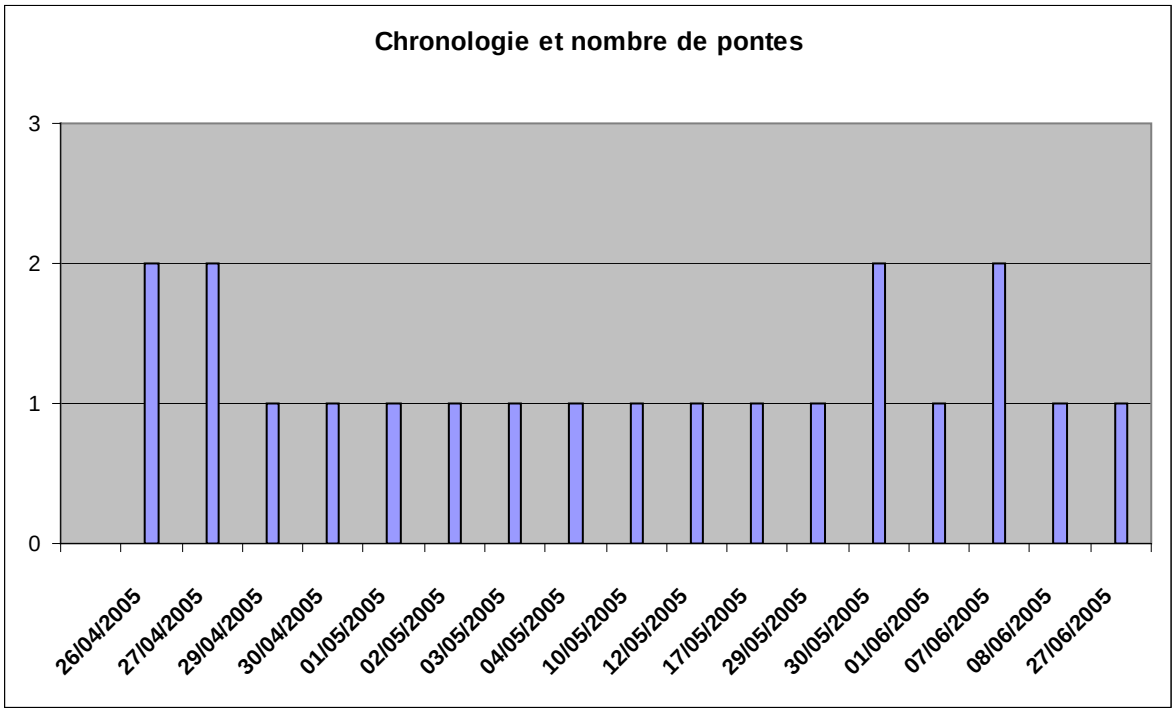

Figure 6. Succession des pontes en aquarium

Au total, 18 pontes ont été récupérées pour 40 couples de reproducteurs, il est à noter la grande disparité de reproduction entre les familles, les 7 couples de la famille OEF10401 donnant 8 fécondations. La famille OEF10425, bien que représentée elle aussi par 7 couples d'animaux, n'a donné aucun descendant au cours de la même période (Figure 7).

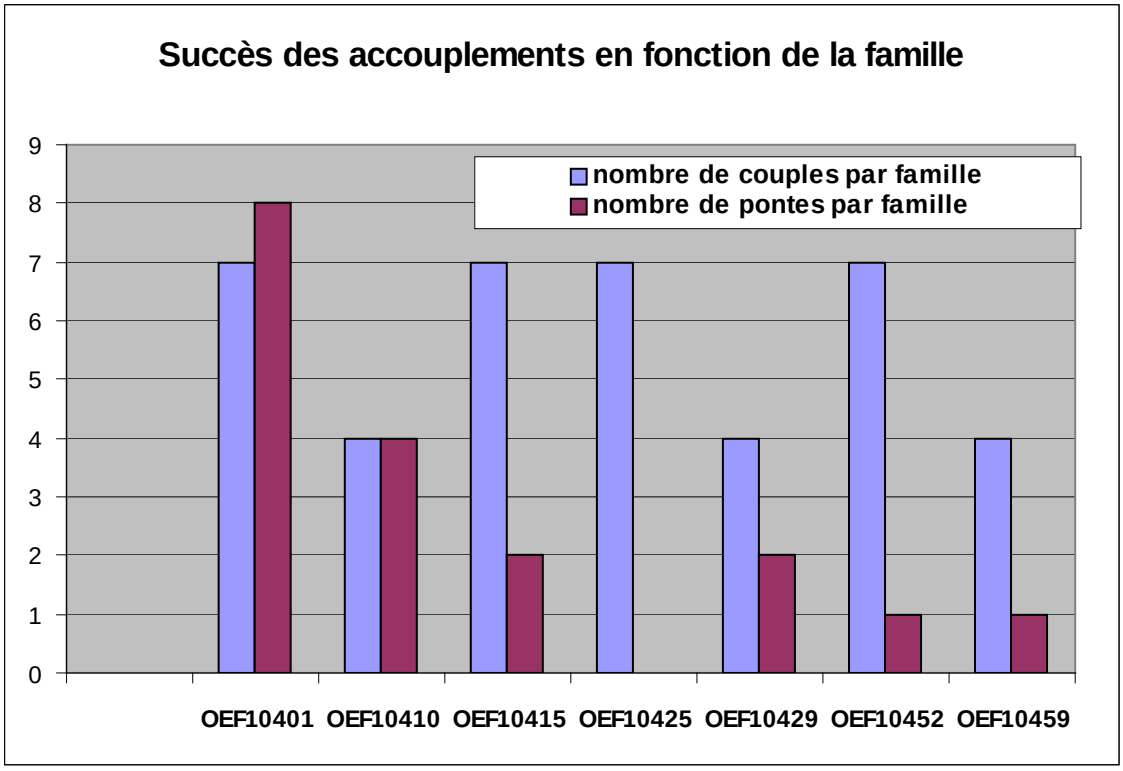

Figure 7. Détail du nombre de pontes obtenues par famille

Les pontes récoltées à l'occasion de ces croisements, ont permis de produire les 17 familles F2-S suivantes à partir de 12 couples différents:
OE.F2.05.01, OE.F2.05.02, OE.F2.05.04, OE.F2.05.05, OE.F2.05.06, OE.F2.05.07, OE.F2.05.08, OE.F2.05.09, OE.F2.05.10, OE.F2.05.11, OE.F2.05.12, OE.F2.05.13, OE.F2.05.14, OE.F2.05.15, OE.F2.05.16, OE.F2.05.17 et OE.F2.05.20. La généalogie de ces familles est précisée sur la figure 8. La notation des familles est comme suit : OE = *Ostrea edulis*; suivi de F1 ou F2 = Famille bi-parentale de 1ère ou 2ième génération; suivi de XX : année de production 05 = 2005; et de YY: numéro de l'élevage.

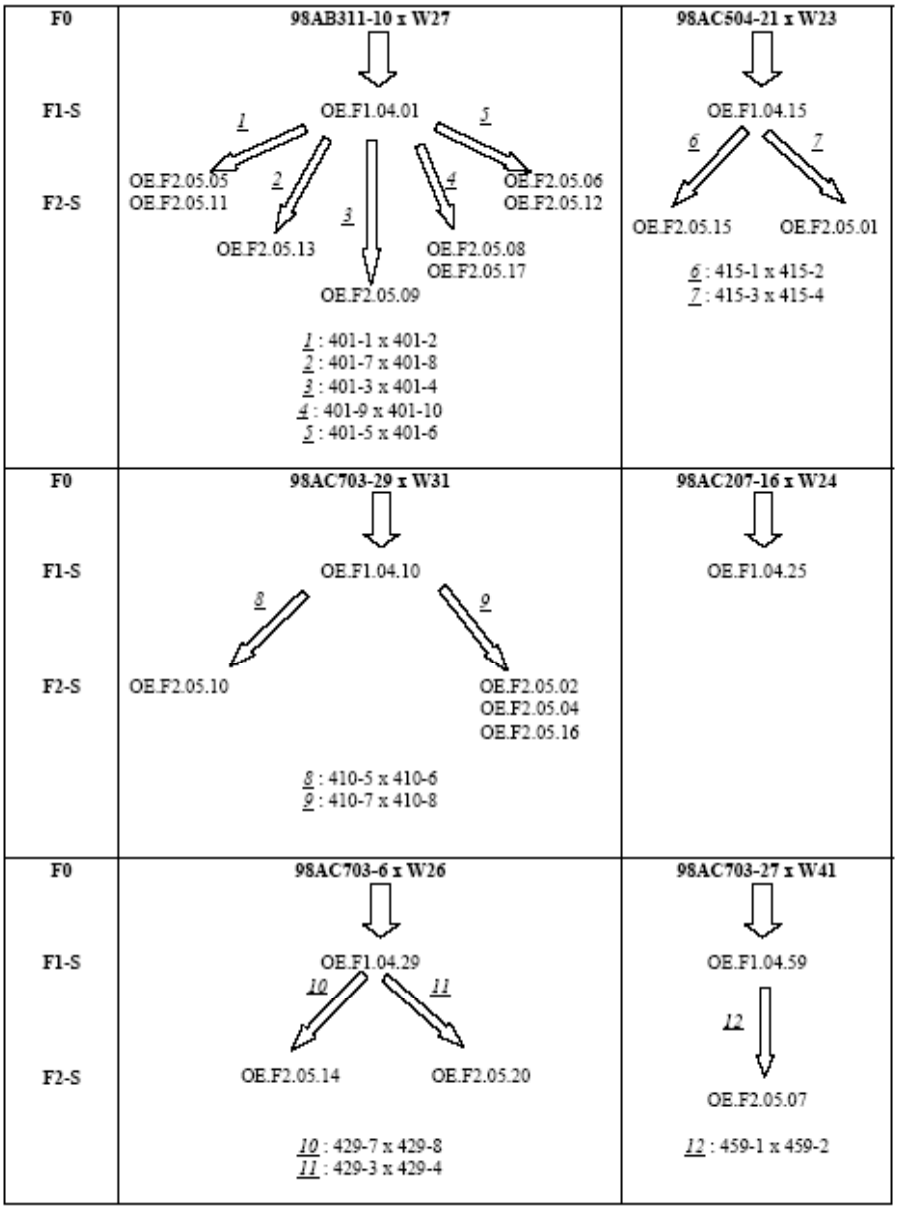

Figure 8. Schéma de production des familles F2-S d'*Ostrea edulis* issues des croisements du programme de sélection à la bonamiose et d'individus sauvages.

Parmi ces différentes familles, la famille OE.F2.05.04 a été retenue dès 2005 sur la base du polymorphisme des marqueurs microsatellites observé au niveau des grands-parents F0 et des parents F1 pour la recherche de QTLs dans le cadre du programme INTERREG AAAG de cartographie génétique de l'huître plate, pour participer à un test d'épreuve expérimental de résistance à la bonamiose. La famille OE.F2.04.45 produite en 2004 et issue d'une autofécondation étant la seconde famille choisie pour des raisons similaires pour ce test d'épreuve.

3. Les productions et expérimentations en 2006

3.1. Reproduction des lignées consanguines

En 2006, les productions d'huîtres plates avaient pour objectif la conservation des lignées consanguines issues du programme de sélection qui sont issues initialement d'une famille consanguine (famille89ni) sélectionnée après inoculation du parasite *Bonamia ostreae* en 1989.

Les dernières lignées consanguines avaient été produites en 2003 et conservées en eau profonde en baie de Quiberon depuis l'hiver 2004.
En avril 2006, elles ont été rapatriées à l'écloserie de La Tremblade, et conditionnées pour leur reproduction jusqu'au 23 mai 2006, date de leur placement en aquarium pour des croisements de type bi-parentaux.

Les familles retenues pour cette production étaient les suivantes :
OELL0301, OELL0309 issues de la lignée OE.1-4-3 (4ème génération de consanguines), OELL0303, OELL0322, OELL0311 issues de la lignée OE.1-5-2-3-1 (6ème génération de consanguines) et OELL0329 issue de la lignée OE.1-7-1-2-1 (6ème génération de consanguines).

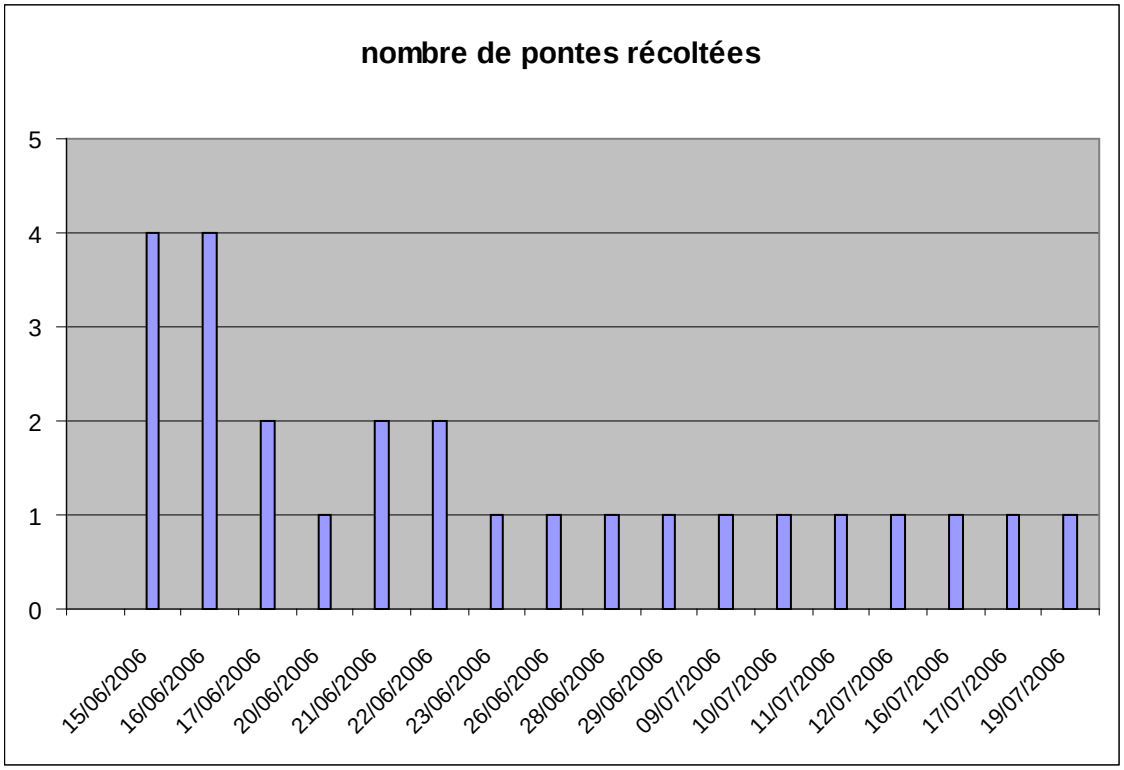

Figure 9. Chronologie des pontes au cours de la période de reproduction

Les pontes ont eu lieu régulièrement sur un temps relativement court entre le 15 juin et le 19 juillet 2006 et ont donc débuté 3 semaines seulement après la mise en aquarium des géniteurs (Figure 9).

Le nombre de larves récupérées à l'occasion des pontes a varié de 300000 à 1 million, quantités suffisantes pour assurer la fixation de plusieurs dizaines de milliers d'animaux en fin d'élevage larvaire.

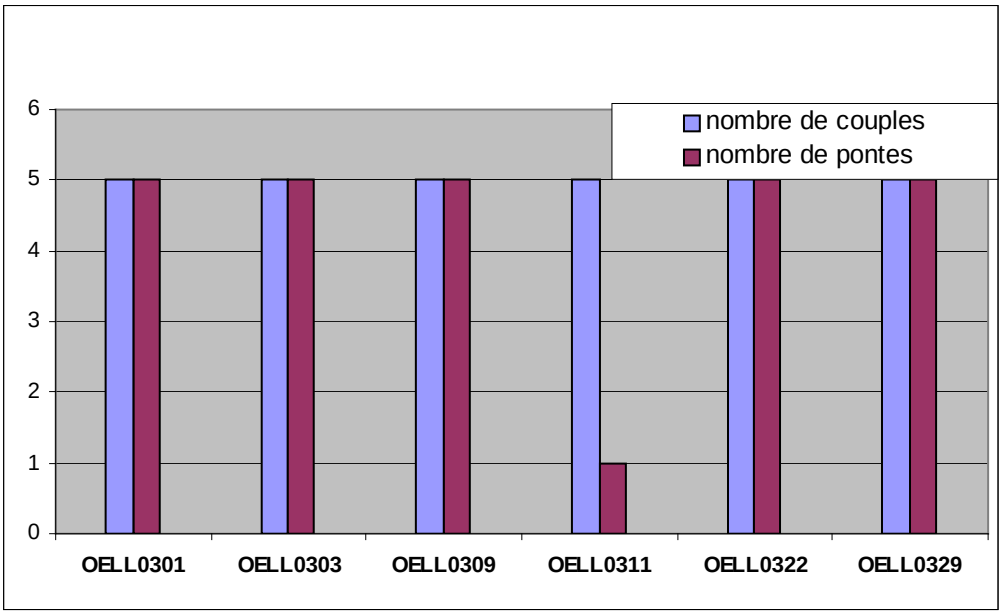

Figure 10. Succès des accouplements en fonction des familles

Un total de 26 lots de lignées consanguines ont ainsi été produits en 2006, chaque famille de géniteurs participant à part presqu'égale à la nouvelle génération, chaque couple de géniteur donnant une ponte, hormis la famille OELL0311 (Figure 10). Il est à noter cependant que des mortalités importantes ont touché certains lots au cours de la phase de nursage au mois d'août 2006, en raison notamment de variations importantes de la qualité de l'eau et du phytoplancton au sein de l'écloserie.

Contrairement aux autres années de production, l'ensemble des lots d'huîtres plates ont été maintenus à partir de 2006 dans les structures d'élevage de la Tremblade, afin d'éviter les mortalités liées au transfert vers la nurserie de Bouin, puis lors du passage et séjour en eau profonde en baie de Quiberon.

3.2. Le test d'épreuve expérimental à la bonamiose

Cette expérimentation s'inscrit dans le cadre du travail sur l'amélioration des espèces cultivées qui consiste notamment en la construction de carte génétique première étape nécessaire à la recherche de QTLs qui sont des marqueurs liés à des caractères quantitatifs d'intérêt tel que la survie à un épisode de mortalité chez l'huître plate.

Un dispositif de 12 raceways a été mis en place pour la réalisation de cette expérimentation qui a eu lieu de janvier à août 2006 et consistait à la cohabitation d'huîtres plates sauvages surinfectées avec des huîtres de deux familles (OE.F2.04.45 et

OE.F2.05.04) issues du programme de sélection pour la résistance à la bonamiose (Figure 11).

Figure 11. Dispositif de cohabitation des huîtres plates

Un suivi des mortalités et du niveau d'infection était réalisé chaque jour et l'ensemble des animaux ont été sacrifiés à des fins d'analyses par marqueurs moléculaires début août 2006.
Les résultats de cette étude sont développés dans la thèse de Delphine Lallias soutenue en juin 2007 (http://archimer.ifremer.fr/doc/2007/these-2603.pdf) et dans les publications citées en Annexes 1, 2, et 3.

4. Etat des stocks en 2008

Un bilan des lots d'animaux maintenus dans les structures de La Tremblade a été effectué en fin d'été 2008 (Tableau 1), afin de faire le choix des géniteurs potentiellement utilisables pour les croisements.

Parmi les lots les plus anciens (2003), un très faible nombre d'animaux était disponible, par rapport aux familles produites de 2004 à 2005.

La notation des familles est généralement comme suit : OE = *Ostrea edulis*; suivi de F1 ou F2 = Famille biparentale de 1ère ou 2ième génération, ou de CS = consanguine, ou de WL = croisement entre un animal sauvage (W) et un animal issu d'une lignée (L); suivi de XX : année de production; et de YY: numéro de l'élevage. Les familles 98AC504 et 98AC703 sont deux familles produites en 1998.

intitulé de lot	nombre d'animaux triés
98AC504	n°1
98AC703	N°2 et 27
OELL0301	35
OELL0303	6
OELL0307	13
OELL0308	8
OELL0309	2
OELL0311	7
OELL0320	6
OELL0322	17
OELL0325	6
OELL0326	12
OELL0329	10
OEWL0321	2
OEWL0323	8
OEWL0327	2
OEWL0330	6
OEF10410	39
OEF10415	13
OEF10425	7
OEF10429	23
OEF10459	29
OEF20502	50
OEF20506	5
OEF20510	30
OEF20512	8
OECS0601	30
OECS0605	30
OECS0606	30
OECS0608	20
OECS0610	40
OECS0611	20
OECS0613	50
OECS0616	40
OECS0620	30
OECS0622	40
OECS0625	65

Tableau 1. Etat des stocks de géniteurs en septembre 2008.

5. Les productions de 2009

Au cours de l'année 2009, trois types de reproductions ont été réalisées pour assurer la conservation des lignées consanguines obtenues en 2006, produire de nouvelles familles F2 pour les besoins d'un nouveau test d'épreuve expérimental de résistance à la bonamiose dans le cadre de SEAFARE et produire des descendants d'animaux survivants d'une expérimentation de sélection en mer réalisée en 2003-2004 à Quiberon.

Le 19 décembre 2008, les lots destinés aux différents croisements ont été rentrés en salle de quarantaine dans des raceways de 150 litres pour une acclimatation progressive aux conditions trophiques de l'écloserie avant l'étape de maturation. L'hiver particulièrement froid notamment en début d'année 2009 a été un frein à la réalisation d'une montée en température régulière des animaux, la température de 15°C n'ayant pu être atteinte qu'au début du mois de février, entraînant un retard important dans la phase de maturation des animaux et compromettant leur reproduction espérée avant la période estivale qui est moins propice à l'optimisation des élevages larvaires de cette espèce.

La température de 19°C souhaitée pour assurer la maturation des géniteurs a été obtenue à la mi-mars et maintenue ensuite jusqu'à la mise en place le 12 mars 2009 des animaux pour la reproduction en masse du lot d'huîtres sélectionnées de Quiberon et le 23 mars 2009 des aquariums pour les couples des différents croisements bi-parentaux.

Tous les lots produits en 2009 ont été maintenus à l'écloserie de La Tremblade pour leur pré-grossissement puis grossissement au cours de l'automne et l'hiver 2009/2010. Les lignées sélectionnées et les lignées consanguines seront conservées à La Tremblade pour de nouvelles reproductions.

5.1. Reproduction des lignées consanguines et des F2 sélectionnées

Les reproductions d'huîtres plates réalisées en 2009 avaient pour objectifs de :

- maintenir d'une part les lignées consanguines obtenues en 2006 dont l'origine a déjà été décrite précédemment,
- produire de nouveau des familles F2 à partir des F1 réalisées en 2004 ayant participé au projet INTERREG AAAG. Ces familles F2 devaient servir de matériel biologique pour un nouveau test d'épreuve à la bonamiose, afin d'améliorer la connaissance de la résistance des huîtres à ce parasite, en prenant en compte les informations moléculaires apportées par l'approche QTLs (lien entre un allèle particulier à un locus donné et le caractère de résistance).

Figure 12. Structure dite « HLM » pour les reproductions des couples en aquarium

Au total, 95 couples ont été mis en place dans la structure « HLM » (Figure 12) :
- 36 à partir de 7 familles (en bleu et jaune, figure 13) pour les familles F2.
- 59 à partir de 11 familles (en rouge, figure 13) pour les lignées consanguines.

Au total, 62 pontes ont été récupérées entre le 23 avril et le 27 juillet 2009 (Figures 14 et 15), date à laquelle les reproductions ont été arrêtées en raison de la faible qualité et du pourcentage de mortalité important de larves émises.

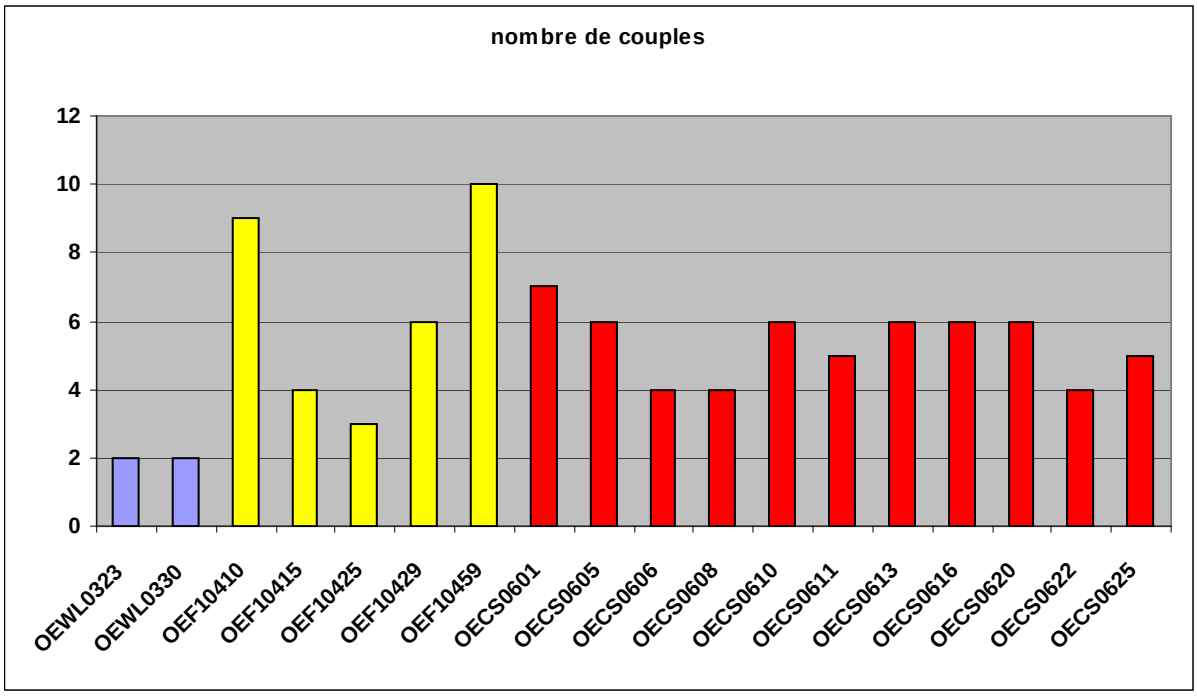

Figure 13. Détail du nombre de couples d'animaux par type de croisement

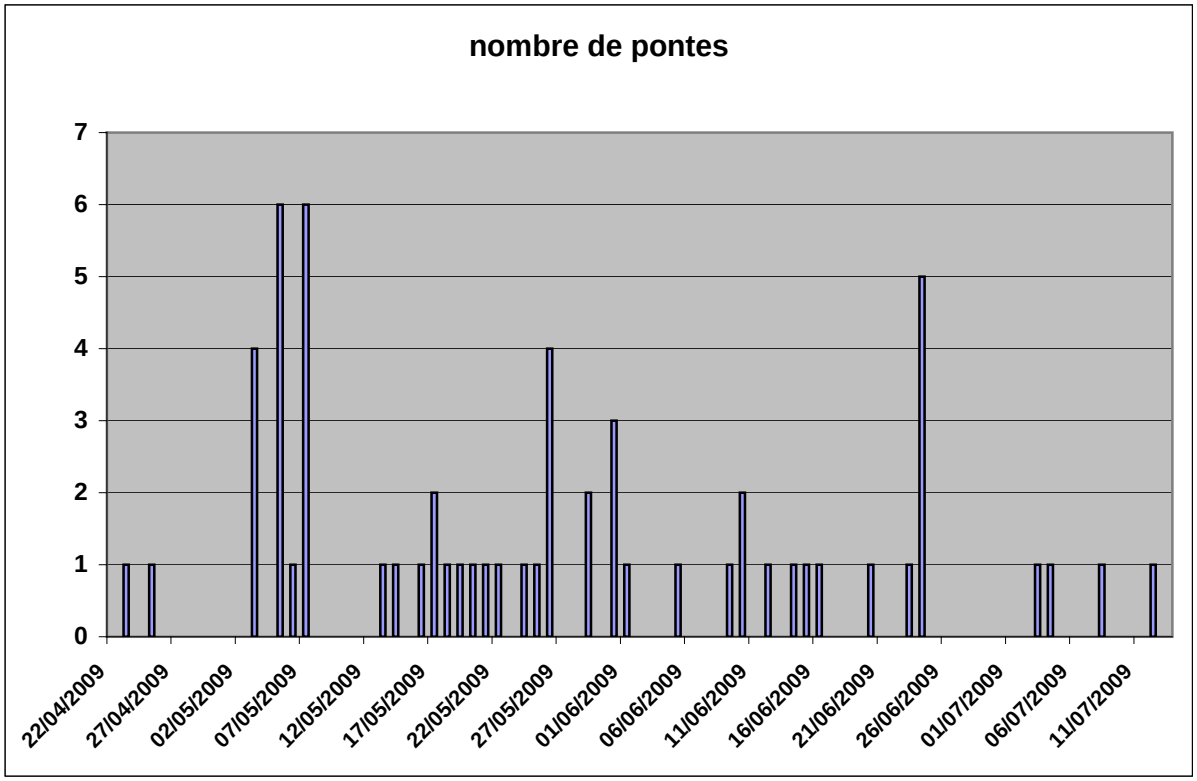

Figure 14. Chronologie et nombre de pontes

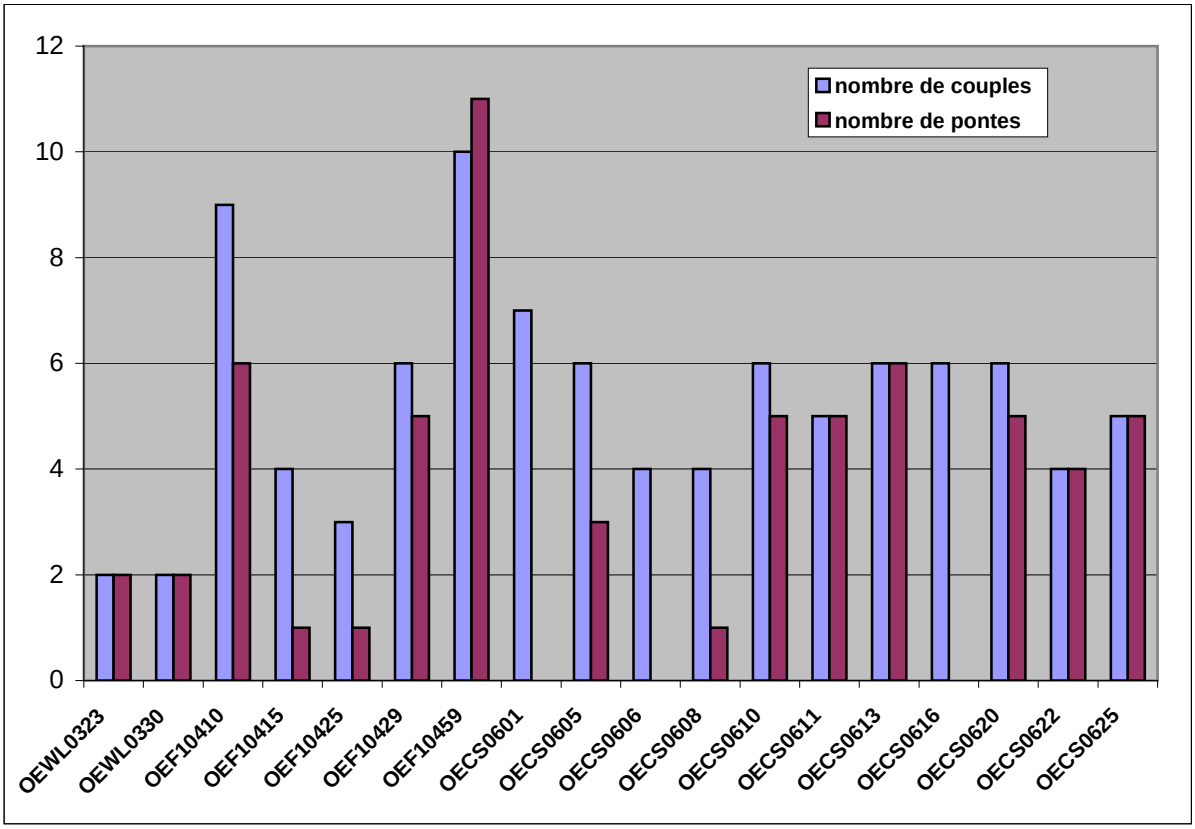

Figure 15. Détail du nombre de pontes obtenues par type de croisement bi-parentaux

Finalement, 26 lots ont été conservés à l'écloserie de La Tremblade sur les 62 pontes récoltées : 10 lots F2 en prévision du test d'épreuve à la bonamiose et 16 lots de lignées consanguines qui constituent les générations F6 et F8 de ces lignées (Figure 16). Ces résultats s'expliquent par les difficultés rencontrées en écloserie en période de reproduction tardive (mois de juin et juillet) contrairement à la période printanière plus propice à la réussite des élevages. Ainsi, 36 lots ont subi des mortalités massives en élevages larvaires et/ou au moment de leur fixation en micronurserie, mais aussi pendant leur nursage, ce qui explique que seulement 40% environ d'entre eux aient pu être conservés.

Le bilan des pontes en comparaison du nombre de couples d'animaux indique que 50 d'entre eux sur 95 ont été à l'origine d'une ponte, le chiffre de 62 pontes s'expliquant par le fait que certains couples ont été à l'origine de plusieurs pontes, la ponte subissant le moins d'aléas de croissance et de mortalité étant préférentiellement gardée. Par ailleurs, Il ne faut pas exclure la possibilité d'autofécondation de certains géniteurs qui peut être vérifiée à posteriori par l'utilisation de marqueurs génétiques.

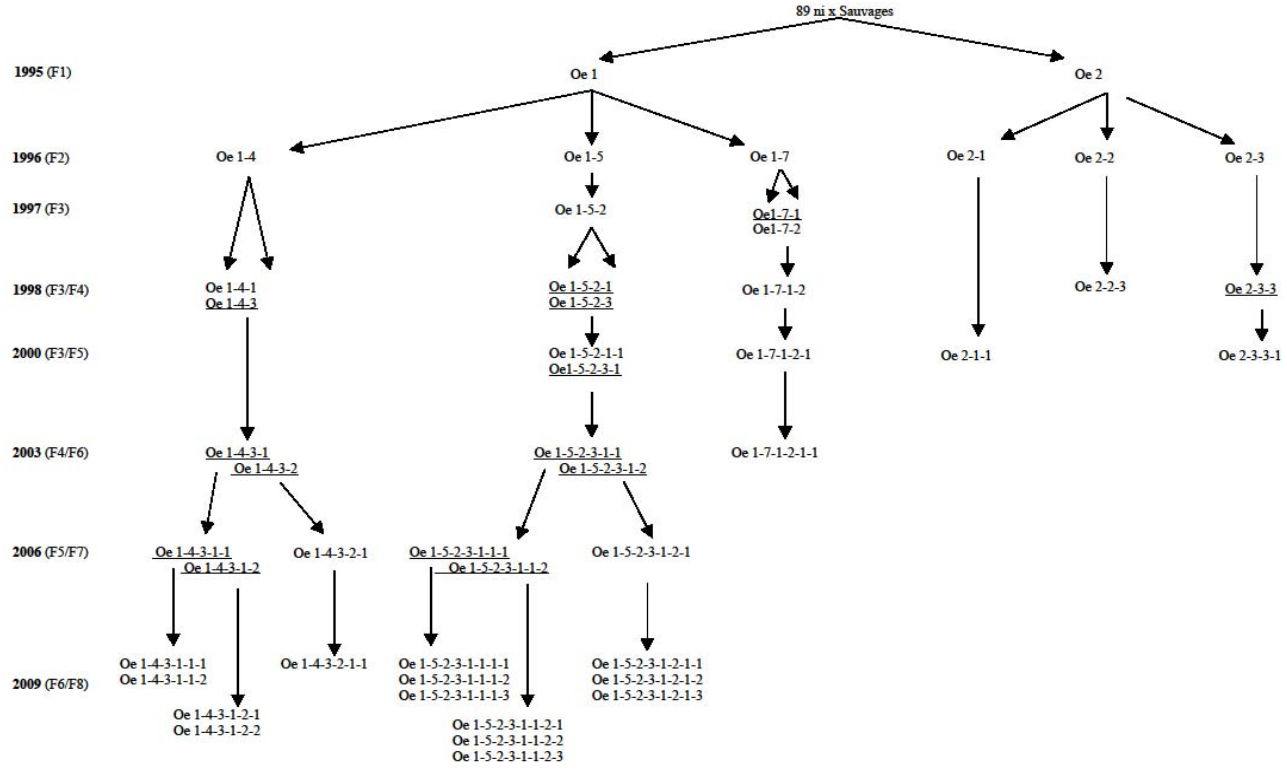

Figure 16. Généalogie des lignées consanguines issues du programme de sélection.

5.2. Reproduction d'animaux issus d'expérimentations de sélection en mer

Une autre reproduction en mélange d'animaux sélectionnés a aussi été réalisée à partir de 75 descendants d'animaux reproduits à l'écloserie d'Argenton en 2007 et ayant précédemment participé et survécu en 2003 et 2004 à une expérimentation en mer en baie de Quiberon dans le cadre d'une convention OFIMER sur la faisabilité technique d'une filière de production commerciale d'huîtres plates *Ostrea edulis* tolérantes à *Bonamia ostreae*.

Cette reproduction 2009 avait pour objectif de maintenir un effectif suffisant de géniteurs pour produire les animaux nécessaires aux besoins de nouvelles expérimentations en mer.

Figure 17. Dispositif mis en place pour la ponte en mélange

La reproduction en mélange des géniteurs sélectionnés (Figure 17) n'a permis de récolter que 7 pontes dont 3 n'ont pas survécu. Ce résultat est peu conforme au nombre de pontes espérées et aux résultats d'élevages larvaires d'années antérieures de production pour des reproductions du même type. Les 4 lots survivants ont été conservés.

6. Le test d'épreuve à la Bonamiose de 2010

Les familles F2 produites pour le test d'épreuve à *Bonamia ostreae* n'ont pas été choisies en fonction de leurs caractéristiques, mais des effectifs disponibles par famille suite aux mortalités majeures déjà évoquées. Ainsi, les familles suivantes ont été retenues : OEF20904, OEF20909 et OEF20967. Prévu initialement en début d'année 2010, le test d'épreuve n'a pu être mis en place qu'en avril en raison des difficultés à obtenir suffisamment de parasite à purifier pour les besoins expérimentaux.

Le test d'épreuve expérimental par cohabitation (Figure 18) a demandé la mise en place de 8 bacs d'élevage, chaque bac contenant 2 clayettes de 50 animaux des familles F2 évoquées précédemment et 2 clayettes de 25 animaux sauvages ayant subi une injection de parasite, chaque clayette de F2 supportant une clayette d'animaux sauvages pour optimiser les conditions d'infection. Le renouvellement en eau réduit et les apports phytoplanctoniques ont été déterminés en tenant compte d'expérimentations antérieures sur le même parasite.

Cette étude, réalisée en collaboration entre les équipes génétique et pathologie demande un suivi quotidien des mortalités et devrait permettre d'approfondir les interactions entre l'huître plate *Ostrea edulis* et le parasite *Bonamia ostreae*.

Figure 18. Système expérimental pour la cohabitation des lots sélectionnés et sauvages.

Une autre expérimentation par injection de parasites chez des individus issus de la famille OEF20909, l'une des familles retenues pour le test d'épreuve par cohabitation, est également en cours. Cette étude, qui consiste à injecter une quantité connue de parasites à chaque huître, permettra de combiner l'étude de différents paramètres (caractéristiques hémocytaires, transcriptomiques, génétiques, portage en parasite) et constitue la poursuite de l'approche développée au laboratoire dans l'étude des interactions entre un bivalve et un de ses parasites. En effet, la thèse de Benjamin Morga (2007-2010) a déjà permis d'obtenir des résultats particulièrement intéressants sur ces interactions.

Pour réaliser cette expérimentation, 6 bacs contenant 52 animaux ayant reçu une injection de *Bonamia ostreae* connue et 2 bacs contenant un nombre équivalent d'animaux témoins ayant reçu une injection d'eau de mer filtrée ont été mis en place.

Un mois et demi après le début de l'expérimentation, et un suivi des mortalités, les animaux ont été sacrifiés à des fins d'analyses qui seront réalisées ultérieurement.

7. Etat des stocks à La Tremblade en 2010

Au mois d'août 2010, un état des stocks d'animaux présents à l'écloserie de la Tremblade a été effectué (Tableau 2). L'objectif pour l'année 2011 est de conserver dans de bonnes conditions de croissance l'ensemble des lots, pour en faire de futurs géniteurs et maintenir ainsi les différentes lignées du programme de sélection.

7.1.1.1..1.1.1.1 Intitulé du lot	7.1.1.1..1.1.2 Nombre d'animaux triés
OECS0901	**630**
OECS0905	**285**
OECS0917	**245**
OECS0922	**250**
OECS0923	**400**
OECS0927	**360**
OECS0935	**145**
OECS0943	**230**
OECS0948	**140**
OECS0950	**50**
OECS0954bis	**110**
OECS0955	**120**
OECS0956	**360**
OECS0963	**35**
OESS0919	**250**
OESS0929	**300**
OESS0947	**225**
OESS0963	**330**
OEF20902	**5**
OEF20904	**117**
OEF20907	**74**
OEF20909	**425**
OEF20910	**34**
OEF20913	**209**
OEF20936	**33**
OEF20946	**17**
OEF20967	**15**

Tableau 2. Détail des lots présents à l'écloserie de La Tremblade le 03 Août 2010

8. Conclusion

Au cours des 3 années de production décrites dans ce rapport, nous avons dû faire face aux contraintes techniques de l'écloserie : variation de la qualité d'eau, des apports phytoplanctoniques et de la température de maturation des géniteurs, et aussi tenir compte une nouvelle fois de la particularité d'un modèle biologique, l'huître plate *Ostrea edulis*, dont les femelles incubent les larves pendant une semaine à 10 jours, ce qui exclut toute fécondation externe après collecte des gamètes, comme cela se pratique couramment sur d'autres modèles comme l'huître creuse.

Malgré ces difficultés techniques et le fait que les années de production soient difficilement comparables, les lots reproduits n'ayant pas le même parcours zootechnique et la même généalogie, il ressort qu'en moyenne 50 % des couples mis en conditionnement se sont reproduits, donnant ainsi une descendance suffisante pour maintenir les différentes familles et lignées et garantir la réalisation d'expérimentations sur la résistance de l'huître plate au parasite *Bonamia ostreae* dans le cadre des différents projets auxquels participe le LGP au sein du programme P7 « Aquaculture durable ».

Ces dernières années, la station expérimentale d'Argenton s'est intéressée à améliorer les conditions de reproduction et d'élevage de l'huître plate en écloserie. Les

résultats de ces travaux ont montré que, par une bonne maîtrise de la prophylaxie aux différentes étapes de la production (conditionnement des géniteurs, qualité d'eau, cultures phytoplanctoniques, élevages larvaires et post-larvaires), il était possible de réduire significativement les épisodes de mortalité. Une démarche du même type est en réflexion à l'écloserie de La Tremblade et devrait permettre à moyen terme une amélioration progressive des résultats des productions chez cette espèce.

9. Annexes

9.1. Annexe 1. Article Lallias et al. (2007)

D. Lallias, A.R. Beaumont, C.S. Haley, P. Boudry, S. Heurtebise, S. Lapègue. 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.

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A first-generation genetic linkage map of the European flat oyster *Ostrea edulis* (L.) based on AFLP and microsatellite markers

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Summary

This study presents the first genetic linkage map for the European flat oyster *Ostrea edulis*. Two hundred and forty-six AFLP and 20 microsatellite markers were genotyped in a three-generation pedigree comprising two grandparents, two parents and 92 progeny. Chi-square goodness-of-fit tests revealed high segregation distortion, which was significant for 32.8% of markers. Sixteen microsatellites and 235 AFLPs (170 type 1:1 AFLPs and 65 type 3:1 AFLPs) were used to build sex-specific linkage maps using CRIMAP software. The first parental map (P_1) consisted of 104 markers grouped in nine linkage groups, and spanned 471.2 cM with an average spacing of 4.86 cM. The second parental map (P_2) consisted of 117 markers grouped in 10 linkage groups (which equals the haploid chromosome number), and covered 450.0 cM with an average spacing of 4.21 cM. The estimated coverage of the genome was 82.4% for the P_1 map and 84.2% for the P_2 map. Eight linkage groups that were probably homologous between the two parents contained the same microsatellites and 3:1 AFLPs (segregating through both parents). Distorted markers were not randomly distributed across the genome and tended to cluster in a few linkage groups. Sex-specific differences in recombination rates were evident. This first-generation genetic linkage map for *O. edulis* represents a major step towards the mapping of QTL such as resistance to bonamiasis, a parasitosis that has drastically decreased populations of flat oysters since the 1960s.

Keywords amplified fragment length polymorphism, flat oyster, genetic linkage map, microsatellite, *Ostrea edulis*.

Introduction

The European flat oyster or 'native' oyster, *Ostrea edulis*, is endemic to the Atlantic and Mediterranean coasts of Europe. Natural populations are found in eastern North America from Maine to Rhode Island, following intentional introductions in the 1940s and 1950s (Jaziri 1990). *Ostrea edulis* exhibits interesting reproductive characteristics such as sequential protandrous hermaphroditism, with the possibility of changing sex several times in the same reproductive season, and brooding of eggs and early larvae in the mantle cavity (Yonge 1960; Le Dantec & Marteil 1976).

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The flat oyster industry was of considerable economical importance in the 19th century in France and Britain (Neild 1995). Massive mortalities occurred around the turn of the 20th century from which the industry has never recovered. Oyster aquaculture production fell further from 30 000 t in 1970 to 6000 t [Food and Agriculture Organization of the United Nations (FAO) 2006] because of two parasitic diseases, marteiliasis (caused by *Marteilia refringens*) and bonamiasis (caused by *Bonamia ostreae*).

Since 1985, Ifremer (French Research Institute for Exploitation of the Sea) has been undertaking a selective breeding programme for resistance to bonamiasis with the main aim of producing families of oysters tolerant to the protozoan parasite *B. ostreae* (Haplosporidian protist, Carnegie *et al.* 2000). A similar approach was also used in Ireland (Culloty *et al.* 2004). In France, two improved oyster strains (S85 and S89) were produced by individual selection in mass spawning progenies (Naciri-Graven *et al.*

1998) and represent a valuable genetic resource for QTL mapping of this trait.

Genetic linkage maps have been established for almost all major aquaculturally important species, including tilapia (e.g. Agresti *et al.* 2000), catfish (e.g. Waldbieser *et al.* 2001), salmon (e.g. Moen *et al.* 2004), rainbow trout (e.g. Nichols *et al.* 2003), abalone (e.g. Baranski *et al.* 2006) and shrimp (e.g. Li *et al.* 2003). A few studies reported the construction of genetic maps in bivalves, including the Pacific oyster *Crassostrea gigas* (Hubert & Hedgecock 2004; Li & Guo 2004), the Eastern oyster *Crassostrea virginica* (Yu & Guo 2003), the blue mussel (Lallias *et al.* 2007) and the Zhikong scallop *Chlamys farreri* (Wang *et al.* 2004, 2005; Li *et al.* 2005). QTL have been mapped in a few shellfish species, including disease resistance in *C. virginica* (Yu & Guo 2006) and production traits in the Kuruma prawn (Li *et al.* 2006a). A genetic linkage map for the flat oyster is a first step towards the identification of QTL for resistance to bonamiasis and the eventual development of marker-assisted selection (MAS) in this species.

Materials and methods

Mapping family

The first stage of the selective breeding programme initiated by Ifremer in 1985 consisted of the production of two improved oyster strains (S85 and S89) by mass selection (Naciri-Graven *et al.* 1998). Selection was applied both through inoculation tests (Mialhe *et al.* 1988) in an experimental hatchery and by field testing in natural conditions. In parallel to the selection programme, several generations of inbred lines have been produced since 1995. These inbred lines were initiated by crossing the selected oyster strain S89 and a wild-type oyster, followed by successive full-sib matings. A sixth-generation inbred line, OELL2000-set2, has had zero mortality from bonamiasis in the field since 2000.

The mapping family used in this study was initiated in 2003 by crossing a wild-type oyster (W102) and an oyster from the inbred line OELL2000-set2 (L002-53). Two full-sibs from this F_1 family were then crossed to make the mapping family (OE.F2.04.63). The mapping family consisted of two grandparents (F_0; L002-53 and W102), two parents (F_1; 23–31 and 23–32) and 92 progeny (F_2). The mapping family was sampled when the progeny were about 15 months old.

In order to achieve bi-parental crosses, oysters were held in pairs in small aquaria. Each aquarium was individually supplied with filtered sea water and the outlet pipe of each aquarium was placed above a 100-μm mesh sieve for the collection of late larvae. It is not possible to non-destructively identify female oysters even when they are brooding, so the respective sexes of the two F_1 parents were not determined. The F_1 parent 23–31 was referred to as 'Parent 1' or 'P_1' and the F_1 parent 23–32 as 'Parent 2' or 'P_2'.

Genotyping

DNA was extracted from gill tissue using a standard chloroform extraction followed by purification with the Wizard® DNA Clean-Up System (Promega) (Wilding *et al.* 2001). Quality and concentration of each DNA sample was assessed using a spectrophotometer and by running a small amount on a 2% agarose gel.

Twenty microsatellite markers selected from those developed by Naciri *et al.* (1995), Launey (1998), Morgan *et al.* (2000), Morgan & Rogers (2001), Sobolewska *et al.* (2001) and Launey *et al.* (2002) were amplified by PCR according to the authors' protocols. Markers informative in the parents were genotyped across the full family.

AFLP analysis was performed using a modified version of Vos *et al.* (1995); the protocol followed Wilding *et al.* (2001), but digestion and ligation were achieved in the same mix by incubating for 16 h at 16 °C. Sixty AFLP primer pairs were genotyped in the mapping family (Table S1). Electrophoresis and data collection were carried out on an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). Electrophoresis parameters were set at injection for 15 s at 15 kV, and running at 60 °C for 25 min at 15 kV with POP4 polymer. Data were analysed with GENEMAPPER® software version 3.7, and individuals were scored for the presence [*A*] or absence [*a*] of the amplified AFLP fragment (peak). A peak-absent marker phenotype was assumed to be the homozygote genotype *aa*.

Distortion of segregation ratios

Segregation distortion analysis was performed using the chi-square goodness-of-fit statistical test between the F_1 parents and the F_2 progeny. For the codominant microsatellites, three types of segregation could be observed depending on the number of alleles present in the two parents: genotypic distributions of 1:1:1:1; 1:2:1 and 1:1. For the dominant AFLP markers, there were only two genotypic classes: presence or absence of the peak. Two types of segregation could be observed, either 1:1 (when only one of the two parents exhibited the peak) or 3:1 (when both parents exhibited the peak). Inference of AFLP genotypes from the phenotype (presence or absence of peak) allowed determination of the following AFLP transmissions:

- Type I, for which only one F_0 parent and one F_1 parent exhibited the peak. Each phenotype could be assigned to a genotype (*Aa* for presence of the peak or *aa* for absence of the peak) and the grandparental and parental origins of AFLP alleles could be tracked without ambiguity (1:1 segregation type).
- Type II, for which both grandparents were *Aa* and only one F_1 parent exhibited the peak. Each phenotype could be assigned to a genotype (*Aa* or *aa*) but it was not possible to assign the grandparental origin of the *A* allele (1:1 segregation type).

• Type III, for which only one F_0 parent and the two F_1 parents exhibited the peak. The presence of a peak in the F_2 progeny led to an ambiguous genotype *A_* (either *AA* or *Aa*), but the grandparental origin of the *A* allele could be tracked (3:1 segregation type).

Linkage analysis

CRIMAP software (Green *et al.* 1990) was used for the construction of genetic linkage maps. First, the Two-Point option was used to estimate recombination frequencies between each pair of markers for each of the two F_1 parents, at a LOD score ≥3.0. These two-point data were used to construct parent-specific linkage groups. The second step consisted of ordering markers within each linkage group using the Build command, with sequential incorporation of loci starting with the most informative pair of markers. The Fixed and All commands were used to add the unplaced markers after Build into the map, by decreasing order of informativeness. After the addition of a new marker to a sequence of ordered markers, the new order was tested against alternative order using the Flips command. Markers that led to an ambiguous map position (i.e. markers that had two or more alternative map positions with a small difference in their likelihood) were discarded from further analysis. Finally, the Chrompic command was used to display the number and location of recombinations on each chromosome, highlighting candidate data errors.

The mapping of 20 microsatellite markers and type III AFLPs (segregating through both Parent 1 and Parent 2) in the two parental genetic linkage maps P_1 and P_2 allowed the identification of probable homology groups. Homology groups were assumed when at least two markers (microsatellites and/or AFLPs) were linked in two linkage groups (P_1 and P_2) and when several markers in the same linkage group were common to both the P_1 and P_2 maps. Indeed, some common markers were linked but could not be assigned to the two parental maps because they had ambiguous map positions and were therefore discarded.

Genome size and coverage

Average marker spacing of each map was calculated by dividing the total length of the map by the number of intervals. The average marker spacing for each linkage group was calculated by dividing the length of each linkage group by the number of intervals on that linkage group. The expected length of the genome was estimated using method 4 of Chakravarti *et al.* (1991). Genome coverage estimates were determined by dividing the observed genome length by the expected length of the genome.

Recombination frequency

Differences in recombination frequencies between the two parents were estimated using *G*-tests of independence that compared parental and recombinant genotypes for each parent for each pair of linked markers (with LOD score >3.0).

Results

Segregation distortion

High segregation distortion was evident in the mapping family. Only 25% of the microsatellites (four out of 16 informative markers: *Oe1/47*, *Oe3/37*, *Oedu.HA21* and *Oedu.B11*) appeared to segregate according to Mendelian rules. Sixty-one per cent of the 1:1 AFLPs (107 out of 175 markers) and 92% of the 3:1 AFLPs (65 out of 71 markers) exhibited Mendelian segregation. Overall, 69% of the AFLP markers (172 out of 246 markers) were considered to have Mendelian inheritance. The high percentage of Mendelian 3:1 AFLPs compared with the 1:1 AFLPs was probably due to the fact that selection of 3:1 markers was based on a preliminary scoring of 48 F_2 individuals after which highly distorted 3:1 AFLPs were discarded. In total, 16 microsatellites and 235 AFLPs (170 type 1:1 AFLPs and 65 Mendelian type 3:1 AFLPs) for a total of 251 markers were included in the final linkage analysis. Distorted microsatellites and 1:1 AFLPs were included in the analysis after mapping the non-distorted markers.

Parent-specific linkage maps

The P_1 genetic linkage map was based on 16 microsatellites and 145 AFLPs segregating in this parent. The AFLPs consisted of 71 markers of type I (31 from L002-53, 40 from W102), nine of type II and 65 of type III (37 from L002-53, 28 from W102). The resulting map consisted of 104 markers (64.6% of available markers), comprising 14 microsatellites (87.5%), 62 type I AFLPs (87.3%), seven type II AFLPs (77.8%) and 21 type III AFLPs (32.3%). Nine linkage groups were set up for the P_1 map covering 471.2 cM (Fig. 1). The sizes of the linkage groups ranged from 23.6 to 95.8 cM. The number of markers per linkage group varied from 4 to 22. The average distance between two loci ranged from 3.16 cM (P_1_3) to 10.1 cM (P_1_8), with an average spacing of 4.86 cM. The largest interval varied from 9.7 cM (P_1_3) to 35.3 cM (P_1_4) (Table 1). The observed map length was 471.2 cM for the P_1 map, and the estimated genome length was 571.7 cM. The observed coverage was therefore 82.4% for the P_1 map.

The P_2 genetic linkage map was based on 16 microsatellites and 154 AFLPs segregating in this parent. The

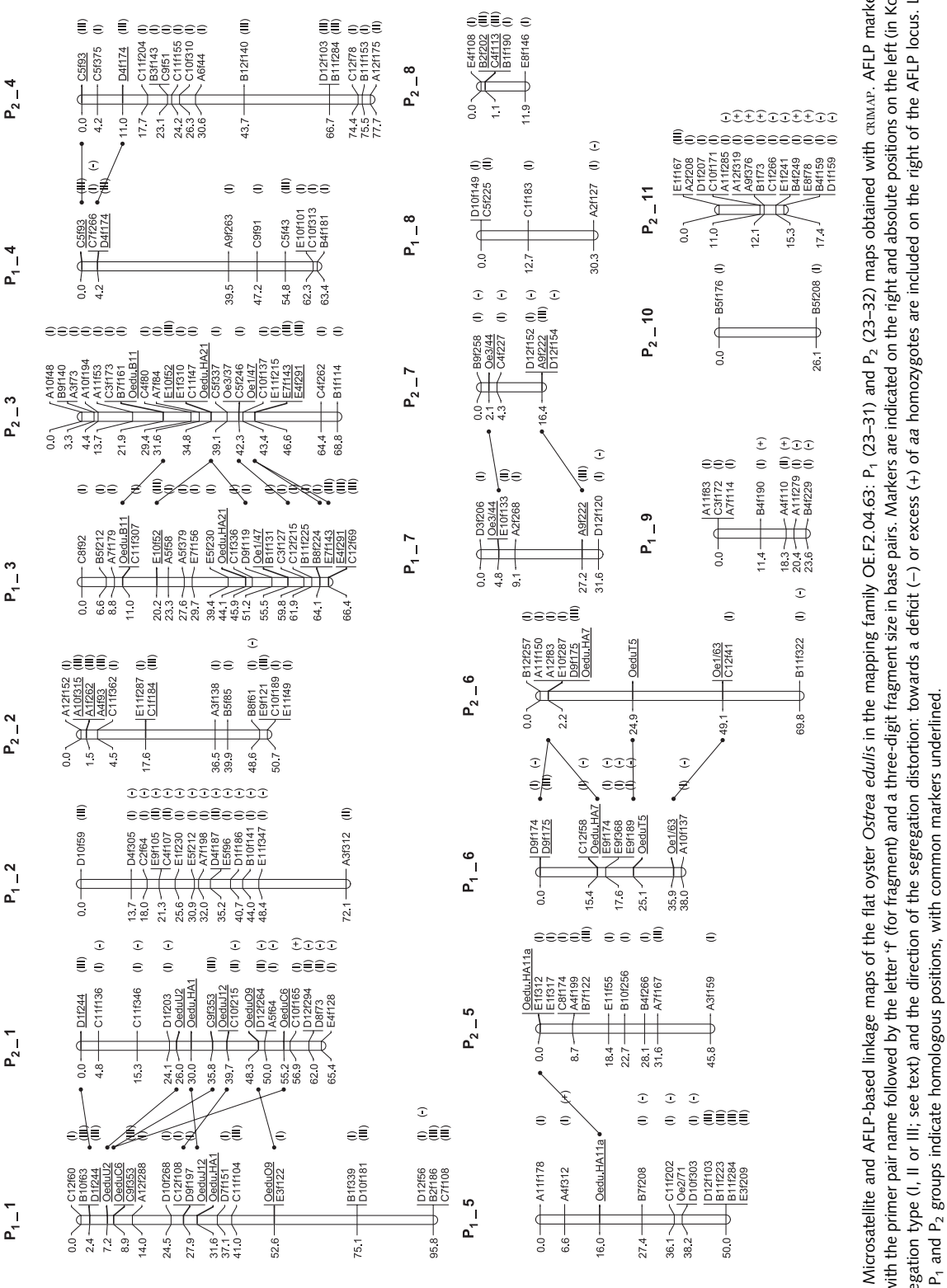

Figure 1 Microsatellite and AFLP-based linkage maps of the flat oyster *Ostrea edulis* in the mapping family OE.F2.04.63: P_1 (23–31) and P_2 (23–32) maps obtained with CRIMAP. AFLP markers are labelled with the primer pair name followed by the letter 'f' (for fragment) and a three-digit fragment size in base pairs. Markers are indicated on the right and absolute positions on the left (in Kosambi cM). The segregation type (I, II or III; see text) and the direction of the segregation distortion: towards a deficit (–) or excess (+) of *aa* homozygotes are included on the right of the AFLP locus. Lines between P_1 and P_2 groups indicate homologous positions, with common markers underlined.

Table 1 Statistics for homology linkage groups for the Parent 1 and Parent 2 genetic maps of the European flat oyster *Ostrea edulis*.

		Parent 1				Parent 2			
Linkage group	Common markers	Length (cM)	No. markers	Marker spacing (cM)	Largest interval (cM)	Length (cM)	No. markers	Marker spacing (cM)	Largest interval (cM)
1	17 (7)	95.8	21	4.79	22.5	65.4	17	4.09	10.5
2	4 (4 in P2)	72.1	14	4.81	23.7	50.7	13	4.23	18.9
3	7 (6)	66.4	22	3.16	9.7	68.8	24	2.99	17.8
4	3 (2)	63.4	9	7.93	35.3	77.7	15	5.55	23.0
5	1 (1)	50.0	11	5.00	11.8	45.8	11	4.58	14.2
6	4 (4)	38.0	10	4.22	15.4	69.8	10	7.76	24.2
7	7 (2)	31.6	6	6.32	18.1	16.4	6	3.28	12.1
8	2 (2 in P2)	30.3	4	10.10	17.6	11.9	5	2.98	10.8
9	0 (0)	23.6	7	3.93	11.4	–	–	–	–
10	0 (0)	–	–	–	–	26.1	2	26.10	26.1
11	0 (0)	–	–	–	–	17.4	14	1.34	11.0
Total	45 (28)	471.2	104	4.86	35.3	450.0	117	4.21	26.1

In brackets are the number of common markers that were mapped in both P_1 and P_2 maps (some common markers were linked to a group but could not be mapped).

AFLPs consisted of 84 markers of type I (38 from L002-53, 46 from W102), five of type II and 65 of type III (37 from L002-53, 28 from W102). The resulting map consisted of 117 markers (76.0% of available markers), comprising 14 microsatellites (87.5%), 76 type I AFLPs (90.5%), five type II AFLPs (100%) and 22 type III AFLPs (33.8%). Ten linkage groups were set up for the P_2 map, covering 450.0 cM (Fig. 1). The sizes of the linkage groups ranged from 11.9 to 77.7 cM. The number of markers per linkage group varied from 2 to 24. The average distance between two loci ranged from 1.34 cM (P_2_11) to 26.1 cM (P_2_10), with an average spacing of 4.21 cM. The largest interval varied from 10.5 cM (P_2_1) to 26.1 cM (P_2_10) (Table 1). For the P_2 map, the observed map was 450.0 cM and the estimated genome length 575.8 cM. The observed coverage was 84.2% for the P_2 map.

Eight probable homology groups were identified. No homology group was found for P_1_9, P_2_10 and P_2_11. Two pairs of homology groups (P_1_2 and P_2_2; P_1_8 and P_2_8) were found based on linkage of several markers that were mapped in only one of the two parental maps: P_1_2 and P_2_2 have four common markers that could be mapped only in P_2_2; P_1_8 and P_2_8 have two common markers that could be mapped only in P_2_8 (Table 1 and Fig. 1). Comparison of orders of markers between the two parental maps was possible for the six homology groups in which common markers were mapped in both P_1 and P_2 maps. Marker order seemed conserved for most homology groups. The greatest discrepancy occurred for group 1 where five microsatellites were not in the same order in the two maps. The lengths of homology groups 3 and 5 were similar between the P_1 and P_2 maps but in most cases there was a discrepancy in homology group lengths between the two maps, particularly for group 1 and group 6 (Fig. 1).

Distorted AFLP markers showed non-random distribution or clustering in both genetic maps (P_1 and P_2). In the P_1 map, the 30 mapped distorted AFLPs (type 1:1) were mainly located on four linkage groups, P_1_2 (containing 12 *aa* homozygote deficiency markers in a 35-cM segment), P_1_5 (containing three *aa* homozygote deficiency markers and one *aa* homozygote excess marker), P_1_6 (containing six *aa* homozygote deficiency markers in a 38-cM segment) and P_1_9 (containing two markers with *aa* homozygote deficiency and two with *aa* homozygote excess). In addition, four other groups contained each only one distorted marker showing *aa* homozygote deficiency: P_1_1, P_1_4, P_1_7 and P_1_8. In the P_2 map, the 26 mapped distorted AFLPs were concentrated on three linkage groups: P_2_1 (containing nine *aa* homozygote deficiency markers and one *aa* homozygote excess marker), P_2_11 (containing five markers with *aa* homozygote deficiency and five with *aa* homozygote excess in a 5-cM segment) and P_2_7 (containing four *aa* homozygote deficiency markers in a 16-cM segment). In addition, two other groups, P_2_2 and P_2_6, each contained one distorted marker showing *aa* homozygote deficiency (Fig. 1).

Distribution of markers

The assumption of a random distribution of AFLP markers in the genome was tested by Spearman correlation coefficients and chi-squared test for departure from a Poisson distribution following Barreneche *et al.* (1998). Spearman correlation coefficients (r_s) between genetic length and number of markers per group were 0.85 for Parent 1 (u_c = 2.40, $P < 0.05$) and 0.61 (u_c = 1.84, $P > 0.05$) for Parent 2. Therefore, AFLP markers were generally randomly distributed in the linkage groups of the P_1 map but not in

the P_2 map because of significant clustering of markers. Observed and expected distributions of AFLPs were compared for 20-cM intervals in both the P_1 and P_2 maps. The mean of the Poisson distribution was 4.2 for P_1 and 4.95 for P_2. No significant departure from the Poisson distribution was observed for Parent 1 ($\chi^2 = 3.1$, 6 d.f., $P = 0.796$). However, this goodness-of-fit test was highly significant for Parent 2 ($\chi^2 = 81.04$, 13 d.f., $P = 0.000$), mostly due to three intervals of 20 cM containing only one marker (large interval gaps remained to be filled in P_2_4, P_2_6 and P_2_10) and to one interval of 20 cM containing 14 markers (high clustering in P_2_11).

Parent-specific recombination differences

Differences in recombination frequencies were observed between the two parents. Eighty-six pairs of markers were segregating in both parents (17 pairs of microsatellites and 69 pairs of microsatellites/AFLPs). Forty-four pairs of markers showed statistically different recombination frequencies between Parent 1 and Parent 2; 12 of these were associated with higher recombination in Parent 2 and 32 with higher recombination in Parent 1 (Fig. 2).

Discussion

Mapping family and experimental design

The mapping family consisted of a three-generation pedigree (grandparents, parents and offspring) that did not come from truly inbred lines (homozygous for all loci), but from a cross between a sixth-generation inbred line and a wild oyster. This mating scheme is unusual for a mapping family in a shellfish species. Indeed, the classical mating schemes in experimental populations where inbred lines are available generally involve the analysis of either backcross or F_2 progeny. Some mapping panels reported in the literature consist of three-generation pedigrees of backcross families (Naruse *et al.* 2000; Sakamoto *et al.* 2000; Li & Guo 2004) or F_2 families (Shimoda *et al.* 1999; Li *et al.* 2003). Other mapping panels consisted of two-generation pedigrees (parents and offspring) (Coimbra *et al.* 2003, Lallias *et al.* 2007). However, when studying natural populations, or when inbred lines are not available, individuals can be taken from the population, genotyped and mated in pairs to yield a number of full-sib families. In a particular family, any pair of segregating loci will represent either an F_2 (if both parents are heterozygous for the pair of markers) or a backcross (if only one parent is heterozygous whereas the other is homozygous) (Kearsey & Pooni 1998). This strategy has been used in several studies (Waldbieser *et al.* 2001; Yu & Guo 2003; Wang *et al.* 2005; Li *et al.* 2006b; Lallias *et al.* 2007) and is the most commonly used experimental design in shellfish species.

However, in the context of QTL mapping, a three-generation pedigree was chosen for our mapping family in *O. edulis*. This experimental design proved to be efficient for the mapping of microsatellites (87.5% mapped in both parents) and type I AFLPs (87.3% mapped in P_1 and 90.5% mapped in P_2). However, as expected according to the informativeness of the markers (Ritter *et al.* 1990), the mapping of type III AFLPs (3:1 segregation) in an F_2-type family was less powerful because only 32.3% and 33.8% were mapped in the two parental maps.

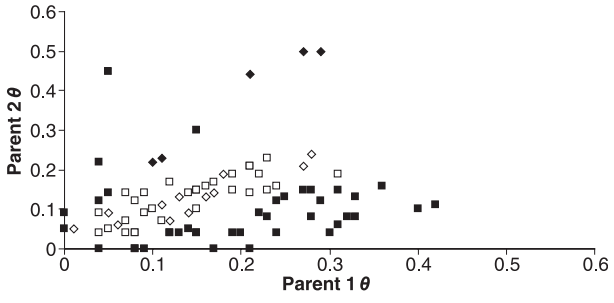

Figure 2 OE.F2.04.63 Parent 1 vs. Parent 2 recombination fractions for 86 pairs of markers segregating from both parents. Recombination fractions (θ) between two microsatellite markers (diamond) or between one AFLP marker and one microsatellite (square) were plotted. Open symbols are cases in which θ was statistically homogeneous between the two parents; solid symbols are cases in which θ was significantly heterogeneous between the two parents ($P < 0.05$).

Segregation distortion

High segregation distortion was evident in our mapping family of *O. edulis*. Overall, 32.8% of the markers were distorted with microsatellites being commonly so (75%) and AFLPs much less so (31%). The range of segregation distortion reported in this study was similar or slightly higher than that reported in another oyster species, *C. gigas*: 31% with allozymes (McGoldrick & Hedgecock 1997), 20.9% with microsatellites (Launey & Hedgecock 2001) and 26.9% with AFLPs (Li & Guo 2004). In our study, the high proportion (85.1%) of distorted AFLP markers that showed an *aa* homozygote deficiency could be explained by a high genetic load that has previously been reported in bivalves (McGoldrick & Hedgecock 1997; Bierne *et al.* 1998; Launey & Hedgecock 2001). The mapping family came from crossing into a selected oyster strain that had been through a strong population bottleneck with a small effective number of breeders (Launey *et al.* 2001). In addition, the mapping family originated from six generations of full-sib matings that would certainly have undergone some inbreeding depression. Therefore, assuming that purging of deleterious genes by full-sib crosses was not complete by the sixth generation, it is probable that the high segregation distortion observed was because of linkage of markers with lethal or deleterious genes in the recessive state.

The mapping of distorted markers may help understand the distribution of deleterious recessive genes in the genome.

Indeed, Yu & Guo (2003) reported in *C. virginica* the clustering of six distorted markers in an area spanning 5 cM, which could potentially correspond to the presence of a deleterious gene nearby. In the same way, Li & Guo (2004) reported the mapping of at least four major deleterious recessive genes in the female map of the Pacific oyster, highlighted by the clustering of markers with segregation distortion in the same direction. In the rainbow trout (*Oncorhynchus mykiss*) map, large regions of linkage groups contained blocks of distorted markers that could be linked to sub-lethal genes (Young *et al.* 1998). In our study, distorted markers tended to cluster on specific linkage groups and sometimes to a small segment of a linkage group (Fig. 1). These clusters of distorted markers could therefore correspond to the location of potential deleterious genes in *O. edulis*.

However, markers with *aa* homozygote deficiency and with *aa* homozygote excess could be found on the same linkage groups (P_2_1, P_1_5, P_1_9 and P_2_11; see Fig. 1). With type I AFLP segregation, in which one grandparent and one parent carries a DNA fragment, the grandparental and parental origins of AFLP alleles could be tracked without ambiguity. Indeed, consider the grandparental cross A_1a_1 by a_2a_2, where the numbers are used to track pedigree. The two F_1 parents have to be A_1a_2 and a_1a_2, so that the progeny are A_1a_1, A_1a_2, a_1a_2 and a_2a_2. Thus, *Aa* (marker-presence phenotype) and *aa* (marker-absent phenotype) progeny are each of two possible types, one of which is grandparental and therefore potentially homozygous identical by descent (IBD) for a linked recessive deleterious mutation. Excesses of *aa* homozygotes can still be attributable to IBD for a linked mutation. This is the likely explanation for the deficiency of *Aa* heterozygotes within a region of *Aa* excesses on P_2_11, for example, all the more so because the *Aa* grandparent in these cases was L002-53, which has an elevated likelihood of being homozygous for a deleterious recessive mutation.

Linkage map and genome coverage

This study presents the first genetic linkage map for the European flat oyster *O. edulis* and the first linkage map in any flat oyster species. The genome coverage achieved in *O. edulis* was good, above 82%, and compared favourably with the ones established in cupped oyster species which were in the range of 70–90% depending on the study (Yu & Guo 2003; Hubert & Hedgecock 2004; Li & Guo 2004). Moreover, the number of linkage groups in Parent 2 matched the haploid number of 10 chromosomes in this species (Thiriot-Quiévreux & Ayraud 1982; Thiriot-Quiévreux 1984) although only nine linkage groups could be clearly identified in Parent 1. This discrepancy for Parent 1 suggests that gaps remain to be filled and that more markers should be added to the maps for a better coverage of the genome. This was confirmed by the fact that only eight probable homology groups were found, and that no clear homology could be found for three linkage groups, P_1_9, P_2_10 and P_2_11. Finally, some of the linkage groups consisted of only two markers or spanned a small genetic distance (<20 cM). Therefore, these groups may in fact belong to the same chromosome and may coalesce by adding more markers.

Recombination differences between the sexes

Our study reported higher recombination rates in the Parent 1 with 32 significant pairwise recombination rate differences (out of 44) compared with Parent 2 (12 out of 44 significant pairwise comparisons) (Fig. 2). Unfortunately, because of the brooding behaviour of *O. edulis*, the sex of our individual F_1 oyster parents could not be determined. However, large sex-specific differences in recombination rates have been reported in several studies. Higher recombination rates in females were found in rainbow trout (Sakamoto *et al.* 2000), channel catfish (Waldbieser *et al.* 2001), zebrafish (Knapik *et al.* 1998), *C. virginica* (Yu & Guo 2003), *C. gigas* (Hubert & Hedgecock 2004) and *Penaeus monodon* (Wilson *et al.* 2002). Therefore, these potential sex-specific differences in recombination rates in *O. edulis* should be confirmed and investigated further by mapping more markers.

Future uses

The development of genetic linkage maps is particularly useful for the mapping of QTL and for MAS. Several studies have highlighted the potential for MAS in breeding programmes in fisheries (Ward *et al.* 2000; Perry *et al.* 2001; Liu & Cordes 2004). MAS has a huge potential in aquaculture breeding programme, especially for traits difficult to phenotype, but so far no successfully applied MAS has been reported in fish or shellfish species. Although disease resistance generally seems to have a low heritability in some species (Gjedrem 2000), it is nevertheless an ideal trait for the application of MAS, because of the economic significance of high survival in aquaculture. Moreover, MAS would reduce the time of selection between generations. Several studies have reported the location of QTL for disease resistance in rainbow trout based on the classical approach for QTL mapping using interval mapping, the ANOVA-based approach or bulk segregant analysis (Palti *et al.* 1999; Ozaki *et al.* 2001; Rodriguez *et al.* 2004). Disease resistance is of particular interest for the flat oyster, which has suffered such a huge decline from parasitic diseases, and MAS for disease resistance could be an important tool in the regeneration of oyster aquaculture. The *O. edulis* genetic map described here represents a first step towards the search for QTL in this species.

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Supplementary material

The following supplementary material is available for this article online from http://www.blackwell-synergy.com/doi/full/10.1111/j.1365-2052.2007.01647.x

Table S1 Primer pairs used for scoring AFLPs.

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9.2. Annexe 2. Article Lallias et al. (2008)

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Aquatic Living Resources

Bonamia ostreae-induced mortalities in one-year old European flat oysters *Ostrea edulis*: experimental infection by cohabitation challenge

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Abstract – Bonamiosis is a parasitic disease (causative agent: *Bonamia ostreae*) affecting the European flat oyster *Ostrea edulis*, responsible for a drastic decline in its aquaculture production. Selective breeding programs for resistance to bonamiosis have been undertaken to counter this disease. In the present study, a 6-month cohabitation challenge experiment was performed in order to transmit the disease from wild oysters injected with the parasite to two tested families of oysters (20 and 8-month old at the beginning of the experiment, with different genetic backgrounds) originating from a selective breeding program developed by IFREMER in France. Mortalities were checked daily and ventricular heart smears were performed on dying or moribund oysters to detect the level of infection by *B. ostreae*. Mortality started after 4 months of cohabitation in the tested oysters. The cumulative mortalities after 6 months were 58% for the wild oysters, 9% for Family 1 (20-month old) and 20% for Family 2 (8-month old). In the dying oysters, the parasite could be detected in 67% of the wild oysters, 68% of Family 1 and 89% of Family 2. It was detected in only 11% of the surviving oysters of Family 2. The mortality and the level of infection by the parasite were significantly higher in Family 2 than in Family 1. Our results demonstrate that prespawning oysters as young as 1 year-old can become infected with the parasite and, most importantly, can die from bonamiosis. This result is inconsistent with the commonly accepted critical age of 2 years-old for the disease development. Additionally, no clear relationship between shell length and level of infection was observed. We also review the different methods for infection of the European flat oyster *O. edulis* with *B. ostreae* under experimental conditions and their main results.

Key words: *Ostrea edulis* / *Bonamia ostreae* / Cohabitation experiment / Transmission / Heart smear

Résumé – Mortalités induites par *Bonamia ostreae* chez des huîtres plates européennes *Ostrea edulis* âgées d'un an : infection expérimentale par cohabitation. La bonamiose est une maladie parasitaire (agent causal : *Bonamia ostreae*) affectant l'huître plate européenne *Ostrea edulis*, responsable d'un déclin drastique de sa production aquacole. Des programmes de sélection pour la résistance à la bonamiose ont été entrepris pour contrer cette maladie. Dans cette étude, une expérience de 6 mois d'infection par cohabitation a été réalisée de manière à transmettre la maladie à partir d'huîtres sauvages injectées avec le parasite vers deux familles testées d'huîtres (âgées de 20 et 8 mois en début d'expérience, avec des origines génétiques différentes) issues du programme de sélection développé par IFREMER en France. Les mortalités ont été vérifiées quotidiennement et des frottis de cœur ventriculaire réalisés sur les huîtres mortes ou moribondes pour détecter le niveau d'infection par *B. ostreae*. La mortalité a commencé chez les huîtres testées après 4 mois de cohabitation. Les mortalités cumulées après 6 mois étaient de 58 % chez les huîtres sauvages, 9 % chez la Famille 1 (âgées de 20 mois) et 20 % chez la Famille 2 (âgées de 8 mois). Chez les huîtres mourantes, le parasite a pu être détecté chez 67 % des huîtres sauvages, 68 % de la Famille 1 et 89 % de la Famille 2. Il n'a pu être détecté que chez 11 % des huîtres survivantes de la Famille 2. La mortalité et le niveau d'infection par le parasite étaient significativement plus élevés chez la Famille 2 que chez la Famille 1. Nos résultats démontrent que des huîtres âgées de un an peuvent devenir infectées par le parasite et surtout, peuvent mourir de bonamiose. Ce résultat

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contraste avec l'âge critique de développement de la maladie communément accepté de 2 ans. De plus, aucune relation claire entre la longueur de la coquille et le niveau d'infection n'a été observée. Nous faisons également la revue des différentes méthodes d'infection de l'huître plate européenne *O. edulis* avec *B. ostreae* en conditions expérimentales et leurs principaux résultats.

1 Introduction

The European flat oyster *Ostrea edulis* is a species endemic to European and North African coasts and can be found from Norway to Morocco as well as in the whole Mediterranean Basin (Jaziri 1985). It is a sequential protandrous hermaphroditic oyster which can change sex within one breeding season, being asynchronously male or female although selfing has been recorded under experimental conditions (Lallias 2007) and which broods eggs and early larvae in the mantle cavity of the female (Yonge 1960; Le Dantec and Marteil 1976).

Following overexploitation of European oyster populations during the 19th century, massive mortalities occurred in France in the 1920's. Some populations later recovered but many were replaced by the Portuguese oyster *Crassostrea angulata* that had been introduced accidentally into France in the 1860's and later by the Pacific oyster *Crassostrea gigas* in 1970's (Comps and Duthoit 1976). Over the last forty years, European production of *O. edulis* has shown a drastic decline from a peak output of nearly 30 000 tons in 1961, mainly due to the rapid spread and strong impact of two parasitic diseases, due to *Marteilia refringens* and *Bonamia ostreae*. In 2006, 61% of the production was in Spain (3200 tons) and 19% in France (1000 tons) (FAO 2007).

Bonamiosis is an intrahaemocytic parasitosis caused by the protozoans *Bonamia ostreae* and *Bonamia exitiosa*. The parasite *B. ostreae* was first described in Brittany (France) by Pichot et al. (1979) and Comps et al. (1980). It is an intracellular parasite (2–5 μm) that infects haemocytes and exhibits two distinct cellular types, either dense or clear cells. Its inclusion into the phylum Haplosporidia has recently been confirmed (Carnegie et al. 2000; Cochennec et al. 2000). This disease was later reported in the Netherlands (Van Banning 1985, 1991), Denmark (Elston et al. 1987), Spain (Montes and Melendez 1987; Figueras 1991), England (Bucke et al. 1984), Ireland (McArdle et al. 1991) and North America (Elston et al. 1986; Friedman et al. 1989; Barber and Davis 1994; Friedman and Perkins 1994). The disease has been correlated with haemocyte destruction and haemocytic infiltration of the connective tissue of the gills, mantle and digestive gland due to the proliferation of *B. ostreae* (Balouet et al. 1983; Cochennec-Laureau et al. 2003). The first mortalities are generally observed when the oysters reach two-years old (Culloty and Mulcahy 1996) although younger individuals (0+ and 1+) have been shown to be susceptible to the infection (Lynch et al. 2005). Some studies reported a seasonal pattern of prevalence and mortality, with highest levels occurring in autumn-winter (Grizel 1985; Montes 1990; Van Banning 1991; Culloty and Mulcahy 1996).

Numerous studies have aimed to clarify the mode of transmission and dynamics of the disease (see Annexes 1 to 4). Most of them were based on field testing, deploying naïve oysters (i.e. never exposed to the parasite) in a contaminated area, recording the time to the first infections and their evolution over time (Poder et al. 1982; Tigé and Grizel 1984; Montes 1991; Martin et al. 1993; Cáceres-Martínez et al. 1995; Culloty and Mulcahy 1996; Naciri-Graven et al. 1998; Culloty et al. 1999; Montes et al. 2003; Culloty et al. 2004; Lynch et al. 2005). Other studies were based on experimental cohabitation between oysters sampled in contaminated areas and naïve oysters, in order to analyze the evolution of the disease (Elston et al. 1986, 1987; Martin et al. 1993; Culloty et al. 1999). The development of a protocol to purify *B. ostreae* from infected oysters (Mialhe et al. 1988) enabled experimental infections by injecting the parasite into flat oysters (Mialhe et al. 1988; Martin et al. 1993; Hervio et al. 1995; Culloty et al. 1999) or by the cohabitation between source oysters injected with a known number of cells of *B. ostreae* and naïve oysters (Culloty et al. 1999). Finally, several studies focused on the role of intermediate hosts or vectors in the transmission of the disease, in particular the role of the macrofauna (Culloty et al. 1999; Lynch et al. 2007).

Despite new management practices (e.g. reducing stocking densities under suspension culture or selling oysters at a lower weight before significant *B. ostreae*-induced mortalities occur), the production of *O. edulis* in Europe has remained low due to bonamiosis. Selective breeding programs were initiated in Ireland and France, with the main objective of producing flat oysters tolerant to bonamiosis (Culloty et al. 2004; Lapègue et al. 2004). In France, this was first initiated in 1985 by IFREMER, producing two oyster strains (S85 and S89) by mass spawning (Naciri-Graven et al. 1998). Individual selection was applied through inoculation tests and field testing, the surviving oysters being used to produce the next generation. Three generations of selection were carried out for S85 and two for S89. At that stage, microsatellite analyses showed that these strains exhibited a low genetic diversity due to population bottlenecks, leading to small effective population sizes and subsequent inbreeding (Launey et al. 2001). As a result, the second stage of this selective breeding program consisted of the production of bi-parental families combined with within-family selection. Families produced in 1995 consisted of within-strain crosses. Then, in order to maximize genetic variability, families produced in 1998 were issued from among-strain crosses between S85 and S89 (Launey 1998). These families showed enhanced survival and lower prevalence of the parasite compared with control wild-type oysters in *B. ostreae*-contaminated areas (Lapègue et al. 2004).

The aim of the present study was to undertake a challenge experiment by cohabitation in order to transmit the disease from wild oysters (injected with the parasite) to two families of oysters originating from the IFREMER selective breeding program and to follow the dynamics of mortality in association with the detection of *B. ostreae*. We also review the different methods for infection of the European flat oyster *O. edulis* with *B. ostreae* under experimental conditions and their main results.

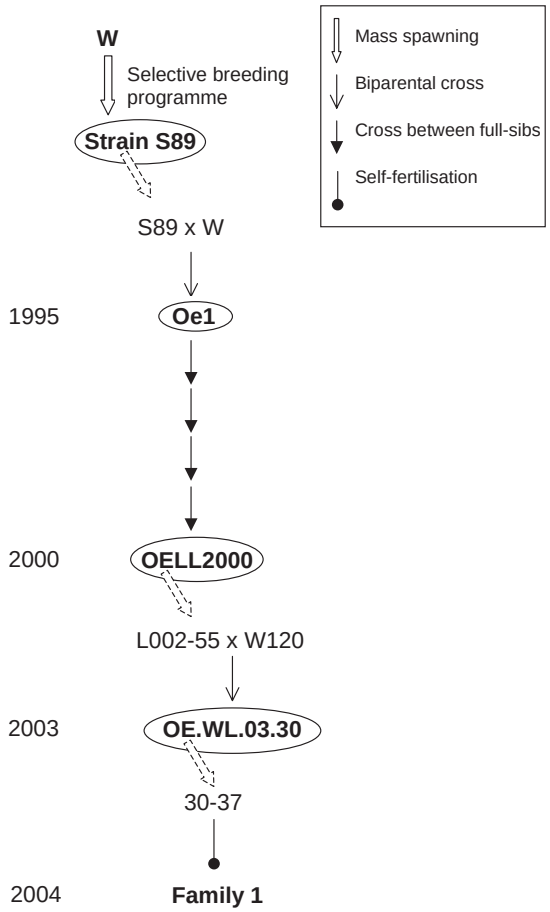

Fig. 1. Production of the experimental Family 1. W: wild oysters; S: selected strain; L: inbred line. OELL2000 is an inbred line of 4th generation.

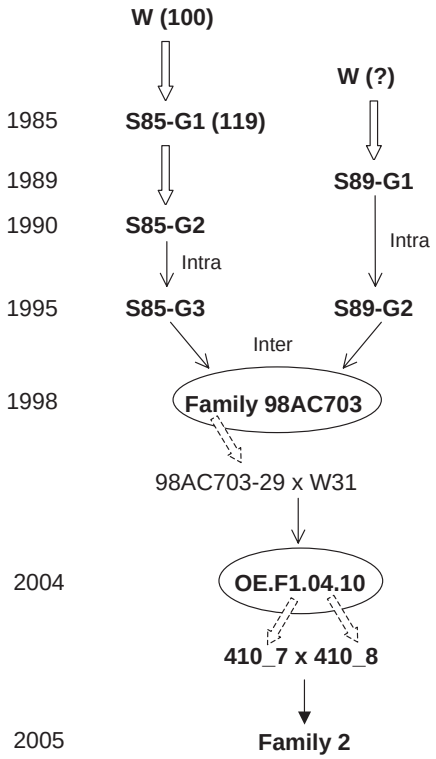

Fig. 2. Production of the experimental Family 2. W: wild oysters. In bracket is stated the number of progenitors used to produce the next generation by mass spawning ?: unknown number of progenitors. S85-G1, S85-G2 and S85-G3 represent the three generations of selection of the selected strain S85. S89-G1 and S89-G2 represent the two generations of selection of the selected strain S89. Intra: intra-strain biparental crosses; Inter: inter-strain biparental crosses.

2 Materials and methods

2.1 Wild oysters

Wild oysters weighing 45–50 g (n = 462) were collected in January 2006 from Quiberon Bay (Brittany, France), a bonamiosis-infected zone since 1980. Prevalence reported in Quiberon Bay in October 2005 was estimated to be 4% (data from REPAMO, French network for the surveillance of mollusc diseases).

2.2 Biparental families

Because flat oysters are alternating hermaphrodites and females brood their larvae in their mantle cavity, it was not possible to determine in advance the sex of the oysters or to strip spawn the gonads for collecting gametes. Therefore, all biparental crosses were achieved by putting pairs of oysters in 2l aquaria, each aquarium being individually supplied with filtered sea water. The outlet water pipe of each aquarium was placed above a 100 μm-mesh sieve for the collection of larvae. Sieves were checked daily for the presence of larvae and larvae and spat were cultured following a protocol based on that originally described by Walne (1974).

The first experimental family, Family 1 (Fig. 1), was initiated in 2003 by crossing a wild-type oyster and an oyster from the inbred line OELL2000 to make an F1 family. This inbred line was derived from a cross between an oyster from the IFREMER selected strain S89 (see above) and a wild oyster, followed by 4 generations of biparental crosses between full-sibs. Two full-sibs from this F1 family ("30–37" and "30–38") were then placed in an aquarium together in 2004 to obtain the F2 generation. However, microsatellite-based parentage analyses later revealed that all of the F2 offspring came from a self-fertilisation of the F1 parent 30-37 (Lallias 2007).

The second experimental family, Family 2 (Fig. 2), was initiated in 2004 by firstly crossing a wild oyster with an oyster from one of the selected families produced in 1998 during the selective breeding program to produce an F1 family. A second-generation was obtained in 2005 by crossing two full-sibs from this F1 family. Parentage was confirmed by microsatellite analysis (Lallias 2007).

Families 1 and 2 were 20 and 8-month old at the beginning of the experiment.

2.3 Bonamia challenge experiment

Our experimental design consisted of 10 raceways, 5 containing Family 1 with 100 oysters per raceway and

5 containing Family 2 with 110 oysters per raceway. Each raceway contained 4 trays in stacks of two, the 2 upper trays containing the wild oysters and the 2 lower trays the tested oysters. Phytoplankton-enriched sea water was delivered at 150 L h^{-1} for each raceway. Raceways were fully emptied and cleaned only once a week, assuming that it would favor the transmission of the parasite between oysters. The position of each stack was changed at each cleaning: the stack furthest from the water inflow was exchanged with the stack closest to the water inflow. Purification of *B. ostreae* was performed as described in Mialhe et al. (1988) and led to the collection of 443×10^6 parasites from a single heavily-infected oyster. This amount of parasites enabled the injection using syringes of 1×10^6 parasites into the heart cavity of 330 wild oysters after their anesthesia using $MgCl_2$ (Culloty and Mulcahy 1992). For each raceway, 16-17 injected wild oysters and 5-6 non-injected wild oysters were put in each upper tray, the lower tray containing 50 tested oysters.

2.4 Detection of Bonamia ostreae

Mortality was checked daily from January until July 2006. The 4 trays were lifted out from the water to drain for at least 30 min before inspecting the oysters and removing any that were gaping, characteristic of dead or dying individuals. A heart imprint was performed on each gaping oyster that consisted of dissecting out the ventricle, drying it on paper and then performing several imprints on a slide. After air drying for 5 min, the slide was then stained with Hemacolor® (Merck). Slides were observed under the light microscope (× 1000) and the level of infection by the parasite was characterized according to Hervio et al. (1995) into the following categories:

- negative results ($B0^-$) when no parasite was detected after 5 min of screening heart imprints from an individual oyster,
- low infections ($B0^+$) when 10 or fewer parasites were observed during 5 min screening
- moderate infections ($B0^{++}$) when around one parasite per microscopic field of view was detected,
- heavy infections ($B0^{+++}$) when several or numerous parasites were observed in each microscopic field of view.

Total shell length (from hinge to outer shell edge) of each dead oyster was measured. Cumulative mortality curves were computed to follow the dynamics of mortality as well as the dynamics of appearance of *B. ostreae* in each raceway.

At the end of the experiment, all remaining oysters from Family 2 were analyzed using heart imprints and the level of infection of the parasite determined as explained above. Oysters from Family 1 were kept alive for further experiments.

2.5 Statistical analyses

Comparisons of mortality and detection frequency were performed by χ^2 test of independence at the end of the challenge experiment. For comparisons of detection frequency among raceways within each family, 2 classes were considered: no parasite infection ($B0^-$) versus parasite infection ($B0^+$, $B0^{++}$ and $B0^{+++}$ pooled). For comparison of detection frequency between the 2 families, the 4 classes were considered.

Differences in the total length of the dead oysters according to the level of infection by the parasite ($B0^-$, $B0^+$, $B0^{++}$, $B0^{+++}$) were tested for the 2 families by single classification analysis of variance (ANOVA) or by Kruskall-Wallis test (when the data were not normal but the variances were equal). All pairwise comparisons were performed, using Dunn's procedure after Kruskall-Wallis and Bonferroni's method after the ANOVA (MINITAB® Release 14 Statistical Software). In all tests a p-value <0.05 was considered to be statistically significant.

3 Results

3.1 Mortality and level of infection in the two families

Cumulative overall mortalities for the wild and tested oysters of the Family 1 and 2 were analyzed for each of the raceways, as well as the cumulative mortalities of oysters that died containing the parasite (*Bonamia*-positive oysters $B0^+$, $B0^{++}$ and $B0^{+++}$ pooled). All the raceways showed a similar pattern, with the first mortalities appearing a few days after the beginning of the experiment in the wild oysters but most mortalities occurring in June and July (140 days post challenge) in the tested oysters. For the two families, no significant differences were found in the number of dead wild oysters nor the detection frequency of the parasite at the end of the experiment among the 5 raceways. Similarly, the number of dead tested oysters and the parasite detection frequencies were homogeneous among the raceways. As a result, data from all 5 raceways were pooled for each tested family.

Over the 6 month period, the cumulative mortality of the wild oysters reached 58.4%. In the last 40 days of the experiment, mortality rate of the wild oysters decreased and the cumulative mortality begins to plateau (Fig. 3). Overall, 66.8% of the wild oysters that died during the challenge experiment were confirmed, by heart imprint, to have the parasite present. Mortality in the tested families began in April (i.e. 70 days post challenge) and mortality of oysters that were *Bonamia*-positive occurred in June and July (Fig. 3). In total, 52 oysters of Family 1 died (9.4%). Reliable heart imprints could not be performed for 11.5% of these oysters because of the degree of tissue decay; 28.8% were diagnosed $B0^-$, 28.9% $B0^+$, 23.1% $B0^{++}$ and 7.7% were $B0^{+++}$. Among the dead oysters of Family 1 for which a heart imprint could be performed (n = 46), the parasite *B. ostreae* could be detected in 67.5%. For Family 2, 105 oysters in total died over the 6 month period (19.1%). Imprints could not be performed for 9.5% of them; 9.5% were $B0^-$, 16.2% $B0^+$, 22.9% $B0^{++}$ and 41.9% were $B0^{+++}$. Among the dead oysters of Family 2 for which a heart imprint could be performed (n = 95), the parasite *B. ostreae* could be detected in 89%. The mortality was significantly higher in Family 2 than in Family 1 (χ^2 = 20.87, $p < 0.001$, 1 d.f.) as well as the intensity of infection by the parasite found in heart imprint (χ^2 = 24.34, $p < 0.001$, 4 d.f.).

Mean mortalities and levels of infection over 10-day periods are presented to illustrate their temporal trends (Fig. 4).

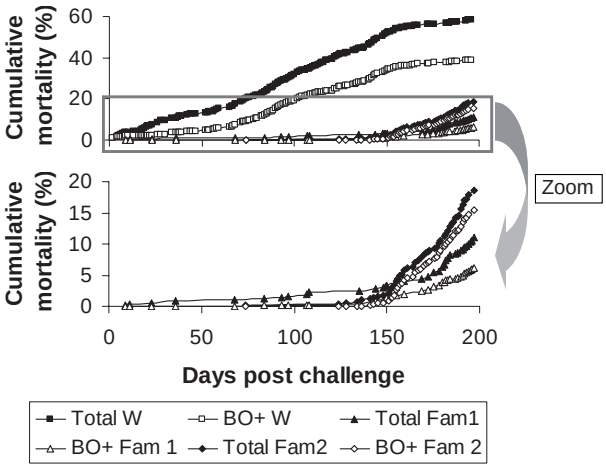

Fig. 3. Cumulative mortalities for the 6 month trial of the challenge experiment. The two upper curves (square) represent the cumulative total mortality (in black = Total W) and the cumulative mortality of oysters infected with the parasite (in white = BO^+ W) for the wild oysters (upper trays). The 4 lower curves represent the total cumulative mortality (in black = Total) and the cumulative mortality of the *Bonamia*-positive oysters (in white = $B0^+$) respectively for the family 1 (triangle) and the family 2 (diamond). Data were pooled across all raceways.

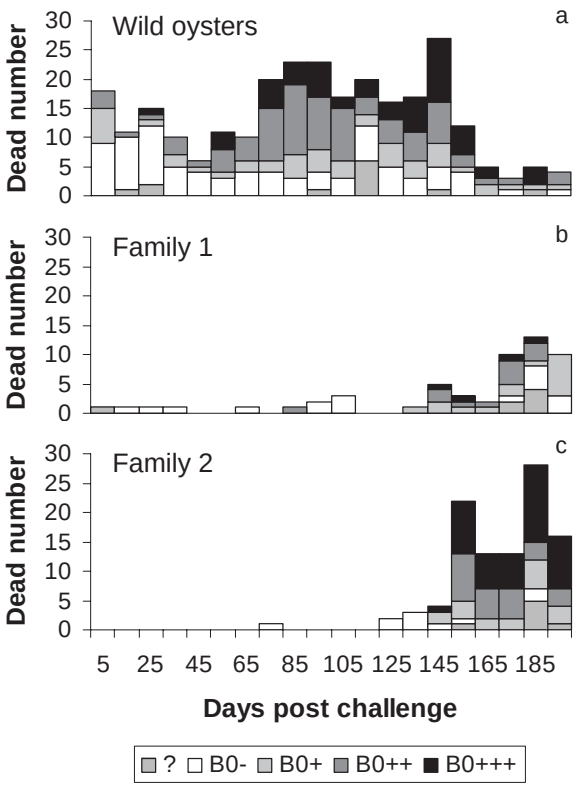

Fig. 4. Kinetics of bonamiosis development for the batch of wild oysters and the two tested families (Family 1 and Family 2). Mortalities were monitored daily and reported every 10 days. *Bonamia* infection intensities (negative ($B0^-$), low ($B0^+$), moderate ($B0^{++}$) and heavy ($B0^{+++}$)) were determined by examination of heart tissue imprints. ? corresponds to dead oysters for which the infection intensity could not be determined because of tissue decay.

Most of the wild dead oysters that died after the first 50 days of the experiment were moderately to heavily infected with the parasite. It can be seen that the wild oysters died during the whole course of the experiment, potentially leading to a significant source of *Bonamia* infection for the two tested families. In the last 40 days of the experiment, the number of wild oysters that died decreased (Fig. 4a). For Family 1, the few oysters (but one) dying in the first 130 days of the experiment were negative for the presence of the parasite. After that date, the dead oysters mainly exhibited a low to moderate level of infection and only a few heavily infected oysters could be detected (Fig. 4b). For Family 2, only one oyster, negative for the parasite presence, died during the first 80 days of the experiment and the remaining mortalities began 120 days (4 months) after the beginning of the challenge experiment. Contrary to Family 1, most of the dead oysters of Family 2 were moderately to heavily infected with the parasite (Fig. 4c).

3.2 Relationship between shell length and level of infection

Table 1a shows the total length (cm) of the oysters of Family 1 that died during the challenge experiment, depending on their level of infection to the parasite *B. ostreae* (revealed by heart imprint). The mean length ranged from 5.51 cm for the heavily infected oysters ($B0^{+++}$) to 5.92 cm for the lightly infected ones ($B0^+$). The data were normally distributed (Anderson-Darling $a^2 = 0.681$, $p = 0.070$) and with similar variances (Bartlett's test statistic = 4.23, $p = 0.238$; Levene's test statistic = 1.05, $p = 0.381$) so were analyzed by single classification ANOVA (Table 1b). No significant difference in the total length of the oysters in the 4 groups of parasite level infection could be observed ($F_{3,41} = 0.53$, $p = 0.666$) for Family 1.

The mean shell lengths of Family 2 oysters that died during the challenge experiment ranged from 4.28 cm for the non-infected oysters ($B0^-$) to 5.09 cm for the heavily infected oysters ($B0^{+++}$) (Table 2a). The data were not normally distributed (Anderson-Darling $a^2 = 1.908$, $p < 0.005$) and could not be made so by log-transformation but exhibited similar variances (Levene's test statistic = 1.16, $p = 0.328$). Therefore the data were analyzed by a Kruskal-Wallis test. Significant difference in the total length of the oysters in the 4 groups of parasite level infection was observed for Family 2 ($H = 8.13$, 3 d.f., $p = 0.043$). However, Dunn's method for all pairwise comparisons between treatment medians revealed no significant differences although the greatest pairwise difference in shell lengths was between $B0^{+++}$ and $B0^-$ (Table 2b).

3.3 Comparison of the level of infection between the dead and surviving oysters of the Family 2

All the surviving oysters of Family 2 were analyzed by heart imprint after 6 months. Overall, 444 oysters were analyzed, 94 in raceway 8, 85 in raceway 11, 87 in raceway 12,

Table 1. The results and analysis of *Ostrea edulis* total length (cm) at date of death according to their level of infection to the parasite *Bonamia ostreae* during a 6-month challenge experiment (Family 1).

(a) Means, medians and standard deviations of oyster total length (Family 1)

Infection level	*N*	Mean length	Median length	SD
$B0^{-}$	14	5.58	5.64	1.05
$B0^{+}$	15	5.92	5.94	0.74
$B0^{++}$	12	5.75	5.79	0.57
$B0^{+++}$	4	5.51	5.64	0.73

(b) Analysis of variance table

Source	*DF*	SeqSS	*MS*	*F*	*p*
Infection level	3	1.05	0.3488	0.53	0.666
Error	41	27.15	0.6623		
Total	44	28.20			

Table 2. The results and analysis of *Ostrea edulis* total length (cm) at date of death according to their level of infection to the parasite *Bonamia ostreae* during a 6-month challenge experiment (Family 2).

(a) Means, medians and standard deviations of oyster total length (Family 2)

Infection level	*N*	Mean length	Median length	*S D*
$B0^{-}$	9	4.28	4.50	1.09
$B0^{+}$	17	4.71	4.66	0.84
$B0^{++}$	23	5.05	5.15	0.52
$B0^{+++}$	44	5.09	5.02	0.88

(b) Multiple comparisons between median total lengths using Dunn's procedure after Kruskal-Wallis analysis. Any rank difference, divided by its standard deviation, which is greater than the given value of 2.63 (SE of difference) is significant at the 5% level. Columns are substracted from rows.

Infection level			
$B0^{+}$	0.53		
$B0^{++}$	1.81	1.54	
$B0^{+++}$	2.25	2.11	0.43
	$B0^{-}$	$B0^{+}$	$B0^{++}$

95 in raceway 13 and 83 in raceway 14. Most of them were $B0^{-}$ (86.2% for raceway 8, 89.4% for raceway 11, 93.1% for raceway 12, 87.4% for raceway 13 and 88.0% for raceway 14) and almost no $B0^{+++}$ oysters were found (0% in raceways 8, 11, 12 and 13; 1.2% in raceway 14). No significant differences between raceways were found for the heart imprint results achieved on the surviving oysters ($\chi^2 = 3.92$, $p = 0.864$, 8 d.f. after pooling $B0^{++}$ and $B0^{+++}$; $\chi^2 = 2.55$, $p = 0.636$, 4 d.f. after pooling $B0^{+}$, $B0^{++}$ and $B0^{+++}$). Therefore, data among the 5 raceways were pooled. Overall, 88.7% of the surviving oysters were not infected with the parasite ($B0^{-}$), 8.4% were slightly infected ($B0^{+}$), 2.7% were moderately infected ($B0^{++}$) and only 0.2% were heavily infected ($B0^{+++}$). The difference in the detection frequency between the 2 groups of oysters (dead, surviving) in Family 2 was striking: 89% against 11% of

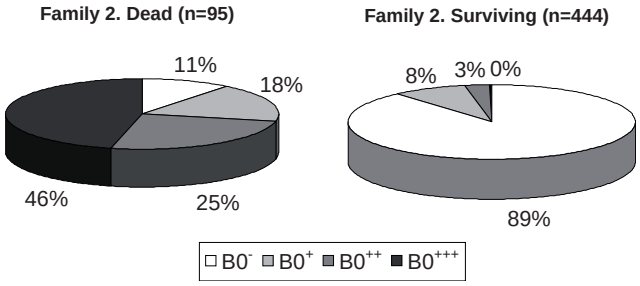

Fig. 5. Comparison of the level of infection of the parasite (after heart imprint) between the two groups of oysters of Family 2, the 95 that died during the 6-month trial (for which a heart imprint could be performed) and the 444 that survived the challenge experiment.

Bonamia-positive in the dead or surviving groups, respectively (Fig. 5).

4 Discussion

4.1 Challenge experiment

Field testing and experimental infection by injection of purified parasite have been widely used for epidemiological studies or in the context of a selective breeding program for resistance to bonamiosis. These methods proved efficient to transmit bonamiosis to flat oysters, leading in some cases to a high percentage of mortality associated with a high prevalence of the parasite (e.g. Tigé and Grizel 1984; Elston et al. 1986; Mialhe et al. 1988; Naciri-Graven et al. 1998).

Cohabitation was chosen in our study because it is likely to mimic the mode of transmission of the disease in the wild. Challenge by injection creates stress and tissue lesion, and would bypass the natural pathway of entry of the parasite into the oyster. Most cohabitation experiments performed so far did not involve the injection of purified parasite in the source oysters (Elston et al. 1986; Elston et al. 1987; Martin et al. 1993; Culloty et al. 1999). In our study, however, wild oysters were injected with the parasite in order to increase the parasite burden in each raceway and to create a "*Bonamia* pump". It was anticipated that injected wild oysters would infect the non-injected ones before their own death and that this would maintain a high parasite pressure during the course of the experiment. *Bonamia*-positive wild oysters were detected throughout the challenge experiment, indicating the usefulness of this approach (Figs. 3 and 4). Finally, this approach allows some control on the parasite pressure applied because a known number of purified parasites is injected in a known number of oysters, and has proven efficient to infect naïve flat oysters (Culloty et al. 1999).

4.2 Detection of Bonamia ostreae

Several diagnostic tools have been used so far for the detection of the parasite *B. ostreae*, either based on light microscopy observation (tissue imprints, haemolymph cell monolayers and histological sections) or PCR-based techniques by amplifying

portions of the 18S rDNA or the ITS of the parasite (Carnegie et al. 2000; Cochennec et al. 2000). In this study, we chose to perform heart imprint for the detection of *B. ostreae* because this methodology is easy, quick, performed at a low cost and allows high throughput of the data. Heart imprints have been used as a diagnostic tool for the detection of *B. ostreae* in numerous studies (Martin et al. 1993; Hervio et al. 1995; Culloty and Mulcahy 1996; Naciri-Graven et al. 1998; Culloty et al. 1999; Culloty et al. 2004; Lynch et al. 2005) (Annex 1). However, Balseiro et al. (2006) reported that the PCR methodology showed a higher sensitivity than histological and cytological studies particularly at the early stages of infection. An advantage of the heart imprint method is that a semi-quantitative scale for the level of infection with the parasite can be used ($B0^-$, $B0^+$, $B0^{++}$ or $B0^{+++}$) contrary to a PCR assay in which the band is either present or absent.

Several studies have reported the use of quantitative PCR for the detection of *Bonamia* species. Corbeil et al. (2006), Marty et al. (2006) employed a real-time PCR assay (18S rDNA gene) but the detection did not succeed in being quantitative. Nevertheless, this methodology was faster and increased the sensitivity at low prevalence and severity. A reliable Q-PCR assay for a quantitative estimation of the number of copies of the parasite requires further optimization and standardization (Corbeil et al. 2006).

4.3 Factors controlling transmission of the disease

The first mortalities and infections in the tested oysters occurred in May, i.e. after 4 months of cohabitation (Fig. 3). This compares favorably with the previously reported period of 3 to 6 months for transmission (Poder et al. 1982; Tigé and Grizel 1984; Grizel 1985; Elston et al. 1986; Montes 1991; Hervio et al. 1995; Culloty and Mulcahy 1996; Culloty et al. 2004) (Annex 1). However, some studies of field testing reported a longer time for the transmission of the disease, from 6 to 12 months (Culloty et al. 1999; Montes et al. 2003) (Annex 1). In contrast, experimental infection by direct injection of purified parasite could lead to the first detection of the parasite after only 2 months (Mialhe et al. 1988; Hervio et al. 1995) (Annex 4).

The parallel evolution of the total cumulative mortality and the *Bonamia*-positive cumulative mortality (Fig. 3) led us to conclude that the mortalities observed were most probably due to the parasite *B. ostreae*. However, there was a delay in the detection of the first infections. For example, the wild oysters dying in the first 50 days of the challenge experiment were mainly negative for the parasite or slightly infected (Fig. 4a). This early mortality among the wild oysters was probably due to the stress associated with their transport from Quiberon to La Tremblade, or could be due to tissue damage during the injection process. In the same way, the tested oysters dying first were negative for the presence of the parasite, which could reflect the prepatent period (Fig. 4b,c). However we cannot exclude the hypothesis that some negative ($B0^-$) individuals might correspond to "false negative" results due to the low sensitivity of heart imprints.

Successful transmission of the disease occurred in both families, but mortalities occurred faster in Family 2 (8 month-old at the beginning of the experiment) than in Family 1 (20 month-old at the beginning of the experiment) (χ^2 = 20.87, $p < 0.001$, 1 d.f.). Also, the infection frequency was significantly lower in Family 1 than in Family 2 (χ^2 = 24.34, $p < 0.001$, 4 d.f.). The cumulative mortalities observed in the 2 families were relatively low (9% in Family 1 and 20% in Family 2, Fig. 3) compared with the study of Culloty et al. (1999) where a 6-month cohabitation trial (injected oysters/naive oysters) resulted in a 32% cumulative mortality in the group of oysters from an area free of *B. ostreae*. A lower parasite pressure in our study could explain the lower cumulative mortality observed in our tested families. Indeed, only 1×10^6 parasites were injected in each of 160 source oysters (for 500 tested oysters), compared with 5×10^6 parasites in each of 100 source oysters (for 150 tested oysters) in Culloty et al. (1999) (Annex 3). However, this lower cumulative mortality might reflect the genetic background of our oysters for which one parent came from a selected strain.

Transmission of the disease also depends on environmental factors such as temperature, salinity or physical manipulation. Indeed, Hine et al. (2002) showed that extreme environmental factors and physical manipulation increased the transmission of *Bonamia exitiosa* to *Ostrea chilensis*, as does crowding among *B. exitiosa*-infected oysters. Audemard et al. (2008) performed laboratory experiments suggesting that warm temperature and high salinity (30 psu) increased *Bonamia* sp. pathogenicity in the Asian oyster *Crassostrea ariakensis*. However, in our study, the two families were held in the same environmental conditions and were subjected to the same manual handling, so non-environmental factors are likely to be responsible for the differences between the two families in the level of the transmission of the disease.

Eight month-old oysters, Family 2, presented higher mortality and detection frequency to the parasite than the 20 month-old oysters, Family 1. This result was inconsistent with the findings of Culloty and Mulcahy (1996) that suggested that 2 year-old was the critical age for the disease development. Therefore, age may not be the key factor in development of the disease, but one of several genetic or environmental factors (Cacéres-Martínez et al. 1995). Indeed, Lynch et al. (2005) followed the prevalence and intensity of infection in young prespawning oysters (1–3 month-old to 18 month-old) by heart imprint and PCR techniques and confirmed that such young oysters were susceptible to bonamiosis although they did not report mortality data: after 6 months in the field, prevalence was less than 10% in oysters being 1–3 month-old at the beginning of the experiment (Annex 1). We have shown both susceptibility of young oysters to the disease and mortalities in our experimental conditions.

The most probable cause of the discrepancy in the development of the disease between the 2 tested families was a difference in their genetic background. Both families originated from a cross between a wild oyster and an oyster derived from the IFREMER selective breeding program. However, only Family 2 was directly derived from a selected family (family 98AC703 produced in 1998) (Fig. 2) that had been tested for its resistance to bonamiosis both in the field and by injection. In contrast, Family 1 originated from a 6^{th} generation inbred line that showed no mortality in the field but

whose resistance was not tested by inoculations (Fig. 1). Moreover, because the two families issued from crosses involving two different wild oysters, differences in the rate of infection between the two families could be due to differences in the genomes of the wild oyster parents.

Finally, the heart imprints performed on the surviving oysters of Family 2 (Fig. 5) revealed that 89% of the surviving oysters were not infected by *B. ostreae*. Even if some of those $B0^-$ oysters might be slightly infected (due to the low sensitivity of the heart imprint technique), the majority of them are probably truly not infected. Therefore, these results suggest a resistance rather than a tolerance of infection by the parasite.

4.4 Potential implications for the management of flat oysters stocks

Producers commonly observe that mortalities occur in the field in 2.5–3 year-old flat oysters (i.e. when they reach market size). However, our experiment demonstrates for the first time that one year-old oysters can die due to *B. ostreae* infection. Even if no mortality in the spat has so far been reported in the natural environment, our results suggest that specific conditions (such as close contact of spat with infected adult oysters or density) can induce mortalities in such young oysters in relation with the infection to *Bonamia ostreae*. Therefore the potential impact of *B. ostreae* on the dynamics of flat oyster stocks should be further investigated, as early mortality has a more important impact than later one when individuals have already reproduced. However, such phenomenon might favor the natural selection of the more resistant oysters, since the fitness difference between "susceptible" and "resistant" genotypes would be increased in case of earlier mortality.

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Annexes 1 to 4. Review of the different methods used to infect the European flat oyster *Ostrea edulis* with *Bonamia ostreae*; mo: month.

Annex 1. Field testing.

Reference	Technical information				Results				
	N tested oysters	Age tested oysters	Duration	Diagnostic method	% mortality	% parasite detection	Time before first mortalities	Level of observed infection	Relation with age/size
Poder et al. (1982)	1500 healthy immersed in contaminated areas; 4 different sites; 15 oysters tested each month	3–4 years old	8 mo	histology	only infection data because sampling of only	7–80% after 8 mo, depending on the site	3–7 mo before first infections	-	-
Tigé and Grizel (1984)	7 batches of 800 oysters. Each batch put in the field at a different time of the year (march to november)	Unknown	12 mo	histology	25–60% after 7 mo; 70–90% after 10–12 mo	6.6–52% after 5-6 mo, depending on the time of immersion	4–5 mo	-	-
Montes (1991)	sampling after 3 and 6 months in 3 estuaries; density 600 oysters per rope (rafts of 500 m^2 with 500 ropes); total of 1418 oysters tested	Unknown	6 mo	histology and imprint	-	0–9% after 3 months; 9–31% after 6 mo	3 mo before first infections	-	-
Martin et al. (1993)	150 oysters per bag, 3 to 5 bags per lot; oysters from programme of selection to bonamiosis (selected) and control (not selected)	21 mo old	7 mo	heart and digestive gland imprint	34% (control); 7% (selected)	47% (control) and 9% (selected) after g 7 mo in surviving	only final sampling	-	-
Cáceres-Martínez et al. (1995)	3 size groups of oysters (small, medium, large) placed in baskets on rafts; 20–30 oysters of each group collected monthly	18 mo old	8 mo	histology	>40% (small and medium); 32% (large)	20–30% at the last sampling date, no cumulative data	0-2-4 mo (large, medium and small respectively) for first infections	-	positive relation between Bonamia presence and mean total length

Annex 1. Continued.

Reference	Technical information				Results				
	N tested oysters	Age tested oysters	Duration	Diagnostic method	% mortality	% parasite detection	Time before first mortalities	Level of observed infection	Relation with age/size
Culloty and Mulcahy (1996)	2 age groups, oyster beds, no initial prevalence; mortality recorded, sampling of 58 live oysters of each group at each sampling time (every months for 1.5 y-o group; 11 times for 2.5 y-o group)	1.5–2.5 years old		histology and heart imprint	8–58% (1.5 years-old stock, 10–64% (2.5 years-old stock) depending on sampling month	0–65% (1.5 years-old stock); 18–70%(2.5 years-old stock depending on sampling month	2.5 years-old stock already infected at the beginning of the experiment; first infections after 6 months for 1.5 years-old stock	0–9% (1.5 y-o); 0–28%(2.5 y-o) of +++ depending on the sampling month	relation with age (first infections when age 2-year-old)
Naciri-Graven et al. (1998)	3 bags containing each 263 oysters 3rd generation selected strain S85 (selected); 3 bags containing each 288 oysters (control)	4 mo old	20 mo	heart or gill imprints	40.9% (selected); 86.5% (control)	22.7% (selected); 48.9% (control) at the end of experiment, on experiment, on surviving oysters	mortality <10% until 8th month	-	tendency towards higher weights in selected strains
	3 bags containing each 239 oysters 2nd generation selected strain S89 (selected); 3 bags containing each 231 oysters (control)	11 mo old	19 mo	heart or gill imprints	46.6% (selected); 51.7% (control)	12.5% (selected); 21.1% (control) at the end of experiment, on surviving oysters	-	-	
	12 bags containing each 223 oysters cross between two selected strains S85 and S89 (selected); 12 bags containing each 269 oysters (control)	5 mo old	22 mo	heart or gill imprints	60.6% (selected); 83.9% (control)	47.2% (selected); 64% (control) at the end of experiment, on surviving oysters	-	-	
Culloty et al. (1999)	327 oysters placed in a bag (1 × 0.5 m) on trestle, area exposed at low spring tide only, bag 40 cm off the bed; 30 oysters tested after 0, 1, 2, 5 or 10 months; contaminated area	Unknown	10 mo	heart imprint	14% after 2 mo, 24.7% after 10 mo	25% after 10 mo, on surviving oysters	6–10 before first infections	-	-

Annex 1. Continued.

Reference	Technical information				Results				
	N tested oysters	Age tested oysters	Duration	Diagnostic method	% mortality	% parasite detection	Time before first mortalities	Level of observed infection	Relation with age/size
Montes et al. (2003)	3 hatchery-produced stocks cultivated on rafts in a contaminated area; sampling of at least 30 oysters of each stock every 3 months	4 mo old	24 mo	histology	8–28% after 9 mo; 32–55% 15 mo; 74–100% after 21 mo	13–27% after 15 mo;33–60% after 21 mo	12 mo before first	-	-
Culloty et al. (2004)	1 selected stock (S) and 4 stocks from parasite-free area (I); 700 oysters, 70 oysters per bag; lower shore, oyster bags on trestles 40 cm off bed; exposed only at spring tide; 30 oysters of each months. 7.7–17.6 °C. Site 1	mean weight 57–67 g	22 mo	heart imprint	20%, 44%, 86% after 6, 12 or 19 mo (S); 5–16%, 38–73%, 96–100% after 6, 12 or 19 mo (I)	18% after 6 mo, 25% after 12 mo, 50% after 19 mo (S); 4–79% after 6 mo, 77–100% after 12 mo, 50–100% after 19 mo (I)	3–6 mo before first infections (I); selected stock infected at beginning of experiment	0–14% after 6 mo; 7–63% after 12 mo; 0–33% after 19 mo of +++	-
	1 selected stock (S), 1 stock from contaminated area (C) and 4 stocks from parasite-free area (I); 700 oysters, 70 oysters per bag; oyster bags on trestles; continually covered; 30 oysters of each stock tested every 3 months. 3.1-20.4 °C. Site 2	mean weight 57–67 g	12 mo	heart imprint	44% or 84% after 6 or 12 mo (S); 40% or 82% after 6 or 12 mo (C); 70–98% or 100% after 6 or 12 mo (I)	41% or 55% after 6 or 12 mo (S); 77% or 8% after 6 12 mo (C); 63–100% or 22% after 6 or 12 mo (I)	3–6 mo before first infections (I and C); selected stock infected at beginning of experiment	0–30% after 6 mo; 0–9% 12 mo of +++	
	1 selected stock (S), 1 stock from contaminated area (C) and 4 stocks from parasite-free area (I); 700 oysters, 70 oysters per bag; oyster bags on trestles; continually covered; 30 oysters of each stock tested every 3 months. 9.9–16.3 °C. Site 3	mean weight 57–67 g	16 mo	heart imprint	12%, 72% or 78% after 6, 12 or 16 mo (S); 56%, 98% or 56%, 98% or 12 or 16 mo (C); 12–50%, 38–96% or 94–100% after 6, 12 or 16 mo (I)	14%, 6% or 28% after 6, 12 or 16 mo (S); 29% or 14% after 6 or 12 14% after 6 or 12 mo (C); 0–12%, 0– after 6, 12 or 16 mo (I)	3 mo before first infections (I and C); selected stock selected stock infected at experiment	most infections observed were light were light	-

Annex 1. Continued.

Reference	Technical information					Results			
	N tested oysters	Age tested oysters	Duration	Diagnostic method	% mortality	% parasite detection	Time before first mortalities	Level of observed infection	Relation with age/size
Lynch et al. (2005)	1000 oysters deployed in two different sites; stock from parasite-free area; 200 oysters per bag; bags placed intertidally on trestles 40 cm off bed; 60 oysters tested every 2s months	1 mo old	6 mo	heart mprint and PCR	- -	2–3% imprint) or 0–7% (PCR) after 4 mo; 9% (heart imprint) or 3–9% (PCR) after 6 mo	2–4 mo before first infections depending on the site	+ until 4 mo, ++ after 6 mo	older oysters with higher prevalence of infection and higher intensity of infection
	200 oysters deployed in one site; stock from contaminated area; 200 oysters per bag; bags placed intertidally on trestles 40 cm off bed; 60 oysters tested every 2 months	2–3 mo old	6 mo	heart imprint and PCR	-	5% (heart imprint) or 10–17% (PCR) after 4 mo; 6% (heart imprint) or 0–12% (PCR) after 6 mo	initial sample with low frequency of the parasite (0–) 6%)	+	
	1000 oysters deployed in two different sites; 1 stock from parasite-free area (I) and 1 stock from contaminated area (C); 200 oysters per bag; bags placed intertidally on trestles 40 cm off bed; 60 oysters tested every 2 months	18 mo old	6 mo	heart imprint and PCR	- -	5–14% (heart imprint) or 6–54% (PCR) after 6 mo (I); 7–26% (heart imprint) or 14–50% (PCR) after 6 mo (C)	2–4 mo before first infections	+, ++, +++	

-: missing information or result; +: light infection, ++: moderate infection, +++: heavy infection.

Annex 2. Cohabitation experiment without injection of the parasite (source oysters come from a contaminated area).

Reference	Technical information						Results		
	N source oysters (initial prevalence)	*N* tested oysters (initial prevalence)	Experimental conditions	Age	Duration	Diagnostic method	% mortality	% parasite detection	Time before first mortalities
Elston et al. (1986)	55 (unknown)	30 (zero)	Unknown	Unknown	9 mo	histology	100%	83%	3–4 months
Elston et al. (1987)	58 (at least 30%)	58 (zero)	50-L tank, flow of 2 l/min seawater, 11–17 °C	Unknown	11 mo 11 mo	histology	26% (source), 98% (tested) at the end of experiment	12% (source), 12% (source), the end of experiment	4 weeks (<10%) until week 28)
Martin et al. (1993)	150 (40–50%) per batch	2 batches (control and selected); 250 per batch (0% selected; 8% control)	15–18 °C; fed with 4.10^9 cells/h/100 l tank; system; water renewed during 1 h every 24 h	21 mo old	7 mo	heart and digestive gland imprint	30% (control); 10% (selected)	50% (control) 44% (selected) after 7 months in surviving	week 2 for selected (<5% until week 17); week 5 for control (<10% until week 20)
Culloty et. (1999)	87 (25%)	150 (parasite-free area)	500 L static tank, aerated, sea water changed 3 times a week, 8–15 °C, daily food	Unknown	6 mo	heart imprint	91.6% (source oysters); 19.5% (tested oysters)	66.7% (source oysters); 46.6% (tested oysters) at the end of 6 months	-

-: missing information or result.

Annex 3. Cohabitation experiment with injection of the parasite (source oysters injected with *Bonamia ostreae*).

Reference	Technical information						Results			
	N source oysters (Bonamia injected)	*N* tested oysters (initial prevalence)	Experimental conditions	Age	Duration	Diagnostic method	% mortality	% parasite detection	Time before first mortalities	Level of observed infection
Culloty et al. (1999)	100 (5×10^6 cells injected in each oyster)	150 (zero)	500-L static tank, aerated, sea water changed 3 times a week, 8–15 °C, daily food	Unknown	6 mo	heart imprint	53.3% (source 32% (tested oysters)	31.2% (source) and 12.5% moribund oysters; 80.4% (source) and 22.1% (tested) in the surviving oysters	-	- -
This study	220 (1×10^6 cells injected in each of 160 oysters)	500 Family 1 500 Family 1	5×150-L tanks; each tank containing 100 tested oysters and 44 source oysters (32 injected); water flow 150 L h^{-1}; daily food; sea water changed once a week	20 mo old	6 mo	heart t imprint	58% (source); 9.4% (tested)	67.5% in the moribund oysters (tested)	4 months 4 before first infections (tested)	33% of +, 26% of ++ and 9% of +++ after 6 mo in the dead oysters (tested)
	220 (1×10^6 cells injected in each of 170 oysters)	550 Family 2	5×150-L tanks; each tank containing 110 tested oysters and 44 source oysters (34 injected); water flow 150 L h^{-1}; daily food; sea water changed once a week	8 mo old	6 mo	heart imprint	58% (source) 19.1% (tested)	89.5% in the moribund oysters (tested); 11% in the surviving oysters (tested)	4 months before first infections (tested)	18% of +, 25% of ++ and 46% of +++ after 6 mo in the dead oysters (tested); 8 8% of + and 3% of ++ in the surviving oysters (tested)

-: missing information or result; +: light infection, ++: moderate infection, +++: heavy infection.

Annex 4. Experimental infection by injection of purified parasite.

Reference	Technical information							Results			
	N oysters	Experimental conditions	Inoculation location	*N* Bonamia injected	Age	Duration	Diagnostic method	% mortality	% parasite detection	Time before first mortalities	Level of observed infection
Mialhe et al. (1988)	3 batches of 30 oysters (parasite--free area)	-	digestive gland	-	Unknown	4 mo	gill tissue imprint	100% after 4 mo after 4 mo	50% after 2 mo, 100%	- -	- -
Martin et al. (1993)	2 batches (control and selected); 250 per batch; initial prevalence 0% (selected) and 8% (control)	15–18 °C; fed with 4×10^9 cell h^{-1}100 L^{-1} tank; closed system; water renewed during 1 h every 24 h	heart (after anaesthesia with $MgCl_2$)	50 000 cells per oyster	21 mo old	7 mo	heart and digestive gland imprint	52.4% (control); 28% (selected)	68% (control) and 51% (selected) after 7 mo in surviving	week 15 for selected <10% until week 23); week 1 for control (<10% until week 19)	
Hervio et al. (1995)	30 (zero)	50-L tank, digestive recirculating filtered sea-water (10 μm) changed weekly, 18 °C	10 000 gland	3 years	4 mo old	heart imprint 4 mo	26% after 4 mo	34% after 4 mo	4 months 4 mo	+, ++ before first infections	+, ++
	43 (zero)			1 000 000				43% after 4 mo	69% after before +++	2 months first infections	+, ++,
Hervio et al. (1995)	100 (zero)	200-L tank, recirculating filtered sea-water (10 μm) changed weekly, 18 °C	digestive gland	220 000	3 years old	4 mo	heart imprint	43% after 4 mo	52% after 4 mo	2 months before first infections	+, ++, +++

Annex 4. Continued.

Reference	Technical information							Results			
	N oysters	Experimental conditions	Inoculation location	*N* Bonamia injected	Age	Duration	Diagnostic method	% mortality	% parasite detection	Time before first mortalities	Level of observed infection
Hervio et al. (1995)	4 groups containing each 160, 43, 97 or 61 oysters (2 different locations)	200-L tank, recirculating filtered sea-water (10 μm) changed weekly, 18 °C	digestive land	100 000	3–4 years old	6 mo	heart imprint	32–79% after 6 mo, depending on the group (32% for "resistant" group) group)	20–69% after 6 mo, depending on the group (20% for "resistant"	2–4 months months before first infections	+, ++, +++ (14–51% of +++ after 6 mo)
Culloty et al. (1999)	180 (zero); 30 oysters tested after 2 weeks and remaining ones after 6 months	500-L static tank, aerated, sea water changed 3 times a week, 8–15 °C, daily food	mantle cavity	5 000 000	Unknown	6 mo	heart imprint	53% after 6 mo	31.2% 6 mo in moribund; 80.4% surviving oysters	-	-
	19 (zero); hemolymph tested after 7 weeks; remaining ones after 6 months	75-L tank, aerated, daily food, filtered and UV treated sea water	mantle cavity	4 150 000	Unknown	6 mo	hemolymph imprint and histology	5.5% after 6 mo	100% after 6 mo in moribund; 29.4% in in surviving oysters		

-: missing information or result; +: light infection, ++: moderate infection, +++: heavy infection

9.3. Annexe 3. Article Lallias et al. (2009)

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ORIGINAL ARTICLE

Combining Two-Stage Testing and Interval Mapping Strategies to Detect QTL for Resistance to Bonamiosis in the European Flat Oyster *Ostrea edulis*

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Abstract We have identified quantitative trait loci (QTL) in the flat oyster (*Ostrea edulis*) for resistance to *Bonamia ostreae*, a parasite responsible for the dramatic reduction in the aquaculture of this species. An F_2 family from a cross between a wild oyster and an individual from a family selected for resistance to bonamiosis was cultured with wild oysters injected with the parasite, leading to 20% cumulative mortality. Selective genotyping of 92 out of a total of 550 F_2 progeny (i.e., 46 heavily infected oysters that died and 46 parasite-free oysters that survived) was performed using 20 microsatellites and 34 amplification fragment length polymorphism primer pairs. Both a two-stage testing strategy and QTL interval mapping methods were used. The two-stage detection strategy had a high power with a low rate of false positives and identified nine and six probable markers linked to genes of resistance and susceptibility, respectively. Parent-specific genetic linkage maps were built for the family, spanning ten linkage groups (n=10) with an observed genome coverage of 69–84%. Three QTL were identified by interval mapping in the first parental map and two in the second. Good concordance was observed between the results obtained after the two-stage testing strategy and QTL mapping.

Keywords *Ostrea edulis* · Disease resistance · QTL mapping · Survival analysis · Statistical power

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Introduction

Genetic and genomic tools, such as quantitative trait loci (QTL) and candidate genes, increasingly contribute to improve the efficiency of selective breeding programs in aquaculture species (Liu and Cordes 2004). In cultured bivalves, genetic maps have been established for the Pacific oyster *Crassostrea gigas* (Hubert and Hedgecock 2004; Li and Guo 2004), the Zhikong scallop *Chlamys farreri* (e.g. Li et al. 2005), the blue mussel *Mytilus edulis* (Lallias et al. 2007a) and the European flat oyster *Ostrea edulis* (Lallias et al. 2007b). However, the mapping of QTL onto those genetic maps has rarely been achieved in bivalves (e.g. in *Crassostrea virginica*, Yu and Guo 2006).

The flat oyster *O. edulis* is a species endemic to European coasts, both Atlantic and Mediterranean. It has been introduced in the USA, Canada and Japan (Ruesink et

al. 2005), but most of its production is located in Europe. Its worldwide aquaculture production decreased from around 30,000 tons in the 1960s to 6,000 tons today mainly due to two parasitic diseases, marteiliosis and bonamiosis. Bonamiosis is an intrahaemocytic parasitosis due to the protist *Bonamia ostreae* of the phylum Cercozoa (Cavalier-Smith and Chao 2003). In Europe, it was first observed in Brittany (France) and was then reported in other European countries.

Since 1985, Ifremer (Institut français de recherche pour l'exploitation de la mer) has been undertaking a selective breeding program for resistance to bonamiosis (Naciri-Graven et al. 1998). Similarly, selective breeding was also initiated in Ireland (Culloty et al. 2004). The families selected in France showed enhanced survival and a lower prevalence of the parasite compared with control oysters in *Bonamia*-contaminated areas; the relative performances of the selected, back-crossed and control families suggested an additive genetic component for the trait (Bédier et al. 2001). However, the heritability of this trait remains to be estimated because of the technical difficulties of required experiments. In this context, the identification of QTL of resistance/susceptibility to bonamiosis would contribute to a better understanding of the genetic basis of this trait and enable an estimation of the potential of marker-assisted selection (MAS). MAS would be a valuable tool to accelerate the selective breeding process by increasing the trait response between two generations of selection.

The primary aim of our study was to identify QTL for resistance or susceptibility to bonamiosis in *O. edulis*. This relied on a 6-month trial challenge experiment in which wild oysters (injected with high concentrations of purified *B. ostreae*) were cultured with the tested oysters (F_2 segregating family) in order to transmit bonamiosis from the wild oysters to the tested oysters. Two extreme phenotypic classes were scored at microsatellite and amplification fragment length polymorphism (AFLP) markers: heavily infected oysters that died during the challenge experiment (susceptible to the disease) and oysters that survived and in which no parasite could be detected (non-susceptible to the disease).

A two-stage testing strategy was firstly performed (Moen et al. 2004). It is based on a transmission disequilibrium test (TDT) on animals susceptible to the disease followed by a survival analysis test using all animals (susceptible and non-susceptible). This method can reduce the rate of false positives (detection of a QTL when there is none) whilst reducing the genotyping effort by genotyping only the susceptible ones. However, Moen et al. (2004) did not explore the impact of their two-stage testing strategy on statistical power of detecting a QTL. Moreover, in most settings, multiple loci are mapped, and it is of interest to know the power for detection of multiple susceptibility loci, as well as the rate of false positives when carrying out multiple testing. Therefore, power and rate of false positives were computed for two-stage selection strategies in multi-testing schemes aimed at mapping multiple susceptibility genes.

Finally, a regression interval mapping analysis (Haley and Knott 1992) was performed for identifying and mapping QTL of resistance or susceptibility to the disease. Both analyses (two-stage testing strategy and regression interval mapping), aim to link genotypes (different alleles of molecular markers) to phenotypes (rapidity of death, level of infection to the parasite). Results obtained with those two approaches were compared and the value of each discussed in the context of QTL mapping for disease resistance in aquaculture species.

Materials and Methods

Segregating Family

The family used in this study was initiated in 2004 by crossing a wild-type oyster (W31) and an oyster (98AC703-29) from one of the selected families that were produced by Ifremer during the selective breeding program to bonamiosis. Two full-sibs from this F_1 family were then crossed to make the F_2 segregating family (OE.F2.05.04). This family was chosen, among several similar families, based on DNA polymorphism of microsatellite markers for the grandparents and F_1 parents (410_7 and 410_8) and after parentage checking. Female flat oysters are brooding their larvae into the mantle cavity. Therefore, controlled crosses can only be performed by putting two oysters in a tank and collecting the larvae on a sieve. Therefore the female and male parents were not identified.

B. ostreae Challenge Experiment

The experimental design consisted of five raceways each containing 110 oysters (8-month old at the beginning of the experiment) from the segregating family and 44 wild oysters. A cohabitation experiment was chosen because it mimics the mode of the disease transmission in nature (Lallias et al. 2008). The 6-month cohabitation experiment was started in mid-January 2006 by injecting 1×10^6 cells of purified parasites into the heart cavity of the wild oysters (from Quiberon bay, Brittany, France). Mortality was checked daily and total shell length (from hinge to outer shell edge) of each dead oyster was measured. Heart smears were performed on the dead oysters, and the level of parasitic infection was characterised: no infection ($B0^-$), low infections ($B0^+$), moderate infections ($B0^{++}$) or heavy infections ($B0^{+++}$). The challenge experiment was terminated

in August 2006 when heart smears were carried out on all of the surviving oysters from the segregating family. Full details of the cohabitation challenge experiment are detailed in Lallias et al. (2008).

In addition to assessing parasitic infection levels by heart smear, polymerase chain reaction (PCR) tests were carried out on a random subset of oysters by using the primer pairs BO/BOAS (Cochennec et al. 2000) that amplify a portion of the 18S rDNA of the parasite. Agarose gel (2%) electrophoresis was performed on PCR products alongside to a 100-bp molecular weight standard. Samples containing *B. ostreae* exhibited a 300-bp band.

Genotyping

Samples from the mapping family consisted of the two grandparents (98AC703-29 and W31), the two F_1 parents (410_7 and 410_8) together with 46 F_2 progeny that died highly infected with the parasite (heart smear $B0^{+++}$, PCR positive) and 46 F_2 progeny that survived (heart smear $B0^{-}$, PCR negative).

DNA was extracted from gill tissue using a standard chloroform extraction (Sambrook et al. 1989) followed by purification with the Wizard® DNA Clean-Up System (Promega). Quality and concentration of DNA was assessed using a spectrophotometer and by running a small sample on a 2% agarose gel.

Twenty microsatellite markers selected from those developed by Naciri et al. (1995), Morgan et al. (2000), Morgan and Rogers (2001), Sobolewska et al. (2001) and Launey et al. (2002) were amplified by PCR according to the authors' protocols.

AFLP analysis was performed by using a modified version of Vos et al. (1995), in which digestion and ligation were achieved in the same mix being incubated for 16 h at 16°C. Thirty-four AFLP primer pairs were genotyped in the mapping family. Electrophoresis and data collection were carried out on an ABI 3100-Avant (Applied Biosystems). Electrophoresis parameters were set at injection for 15 s at 15 kV, running for 25 min at 15 kV and 60°C, with POP4 polymer. Data were analysed with GeneMapper® software version 3.7, and individuals were scored for the presence [A] or absence [a] of the amplified AFLP fragment (peak). The peak-absent marker phenotype is considered to be the homozygote genotype *aa* (whereas the peak-present marker phenotype corresponds to the genotypes *Aa* or *AA*).

Distortion of Segregation Ratios

Segregation distortion analysis was performed using the chi-square goodness-of-fit statistical test between the F_1 parents and the F_2 progeny as detailed in Lallias et al. (2007b).

Power for Detection of Susceptibility Genes in Single- and Two-stage Selection Strategies

Two-stage selection strategy consists of (1) carrying out a TDT on susceptible offspring to the disease and (2) testing only significant markers for susceptible and non-susceptible offspring by survival analysis. The advantage of two-stage selection strategy versus one-stage testing is in the reduced genotyping cost. In this section, we investigate how power is affected by using two versus one stage selection strategies. We assume dominant markers such as AFLPs. The susceptibility locus (*S*, *s*) and the marker locus (*A*, *a*) are assumed in full linkage disequilibrium. There are three possible parents: (a) homozygotes for the locus for susceptibility to the disease and banded (*SA*/*SA*), (b) heterozygotes for the locus for susceptibility to the disease and banded (*SA*/*sa*) and (c) homozygotes for the locus for non-susceptibility (or resistance) to the disease and not banded (*sa*/*sa*). This gives three possible mating types with at least one segregating parent in the full family: (1) *SA/sa*×*SA/SA*, (2) *SA/sa*×*SA/sa* and (3) *SA/sa*×*sa/sa*. Matings *SA/sa*×*SA/SA* and *SA/sa*×*sa/sa* correspond to type 1:1 segregations, whereas *SA/sa*×*SA/sa* corresponds to type 3:1 segregation. However, the mating type *SA/sa*×*SA/SA* led to offspring that were all banded and therefore not segregating. Therefore, only two mating types were considered: *SA/sa*×*sa/sa* and *SA/sa*×*SA/sa*. To simplify, the marker alleles are removed, giving *Ss*×*ss* and *Ss*×*Ss*. We define Ψ_{SS}, Ψ_{Ss} and Ψ_{ss} as the probabilities of developing the disease when animals have genotype *SS*, *Ss*, and *ss*, respectively. These are equivalent to "penetrance", used for hereditary diseases (Ott 1999). This parameter (Ψ) is used to account for genes other than *S/s* that may influence the disease. Table 1 illustrates the probabilities of inheritance of alleles linked to susceptibility to the disease for the two mating types.

Table 1 Probabilities of susceptible and non-susceptible offspring with different genotypes for different types of mating Ss×Ss and Ss×ss in a full sib family

Mating	Offspring genotype	Probability	
		Susceptible	Non-susceptible
Ss×Ss	SS	$1/4\Psi_{SS}$	$1/4\ (1-\Psi_{SS})$
	Ss	$1/4\Psi_{Ss}$	$1/4\ (1-\Psi_{Ss})$
	sS	$1/4\Psi_{Ss}$	$1/4\ (1-\Psi_{Ss})$
	ss	$1/4\Psi_{ss}$	$1/4\ (1-\Psi_{ss})$
Ss×ss	Ss	$1/2\Psi_{Ss}$	$1/2\ (1-\Psi_{Ss})$
	ss	$1/2\Psi_{ss}$	$1/2\ (1-\Psi_{ss})$

Ψ_{SS}, Ψ_{Ss} and Ψ_{ss} are the probabilities of developing the disease when animals have genotype *SS*, *Ss* and *ss*, respectively

Power and Expected Rate of False Positives in Single-Stage Detection of Susceptibility Loci In order to compute power, a χ^2 test for contingency tables was modeled (Cohen 1988). Power in single-stage detection of susceptibility loci was noted $P_{1,\alpha}$ (Appendix A). Power for detection of multiple susceptibility loci (n_l) under multiple testing of m markers (each marker represents one test) is given by $P^A = \left(P_{1,\alpha}\right)^{n_l}$, which assumes that the probability of detection is independent for each tested locus, and therefore, susceptibility loci are not linked. The corresponding expected number of false positives is $EFP^A=\alpha(m-n_l)$. The aim of a sound experimental design is to cover as much as possible of the genome with the highest P^A and the lowest EFP^A.

Power and Expected Rate of False Positives in Two-Stage Detection of Susceptibility Loci Moen et al. (2004) proposed a two-stage selection strategy based on first stage detection using only TDT with susceptible animals and second-stage detection using all animals (susceptible and non-susceptible) and a survival analysis. TDT (Spielman et al. 1993) compares the number of times that a marker allele is transmitted or not transmitted from a heterozygous parent to an affected offspring, and therefore, only the affected offspring are considered. Their strategy also required a Mendelian segregation test (MST) to avoid markers that are not inherited in a Mendelian fashion. Use of the normal approximation to the binomial distribution was used to compute power for a TDT, P_{TDT} (Appendix B). Power for detection of multiple susceptibility loci and expected number of false positives are $P^{B1} = \left(P_{TDT,\alpha}\right)^{n_l}$ and $EFP^{B1}=\alpha(m-n_l)$.

Only significant markers will be tested in the second stage. It is assumed that tests in the two stages are fully independent of each other. Power in the second stage was computed using the same formulae as for single-stage selection (with performance for all animals). The overall power of detection in two-stage strategy for detection of multiple susceptibility loci is $P^B=P^{B1}\cdot P^A$. The expected number of false positives is $EFP^B=\alpha^2(m-n_l)$, which assumes that the same significant level, α, is used in either testing stage.

From the above formulae, it can be observed that (1) power is always greater for single-stage testing but at a higher rate of false positives; (2) TDT depends on the transmission parameters and, therefore, may yield non-significant results for dominant susceptibility loci. A transmission parameter (ν) is the probability of transmission of one of the parental alleles to its offspring. For example, in mating type, $Ss\times ss$, if the probability of susceptibility to the disease is the same for carriers of either one or two copies of the S allele, then $\nu_{Ss\times ss}=1/2$ as it is under the null hypothesis and (3) the expected rate of false positives is always smaller for two-stage selection strategies, as proposed by Moen et al. (2004).

We computed power at significance level $\alpha=0.05$ in one- and two-stage QTL detection strategies and for all loci having $\Psi_{SS}=0.70$, $\Psi_{Ss}=0.25$ and $\Psi_{ss}=0.05$. Note that with the relative small family size, linkage disequilibrium between alleles at the eight loci would be expected for those significant markers. The number of offspring used was 92 for single-stage detection of susceptibility loci and 46 for the TDT. Additionally, we computed power for single-stage detection of susceptibility loci using the Bonferroni correction so the significance level became α/number of tests. The total number of markers tested was either 100 or 200 for each QTL detection strategy type.

QTL analysis

Firstly, a two-stage testing strategy was performed AFLP marker by AFLP marker to identify potential QTLs, as described in Moen et al. (2004). A TDT was applied on the 46 affected offspring (oysters that died heavily infected with the parasite) for all the AFLP markers segregating in that family. A MST (test of Mendelian inheritance using all offspring) was applied to the whole dataset (affected and non-affected offspring) on markers significant after the TDT. The second test was the survival analysis itself. Survival analysis is used to describe and compare the survival times of two or more groups. Only markers that were significant after the TDT and not significant after the MST were kept for the survival analysis. Survival of two groups of offspring was compared: offspring with the peak-present marker phenotype and offspring with the peak-absent marker phenotype. With the Kaplan–Meier method, survival is recalculated every time a member of the group dies. To calculate the fraction of individuals who survived on a particular day, the number alive at the end of the day is divided by the number alive at the beginning of the day. Kaplan–Meier survival curves were constructed for both groups and the hazard ratio (h) computed as described in Moen et al. (2004).

Secondly, a genetic linkage map was built for the F_2 family with CriMap software as described in Lallias et al. (2007b). All microsatellites, all AFLPs of type 1:1 and the Mendelian 3:1 AFLPs were considered for linkage analysis. Two parental maps were constructed, one for each F_1 parent.

Finally, a QTL mapping approach was performed with the QTL express software (Seaton et al. 2002) (http://qtl.cap.ed.ac.uk). In our study, one fixed effect was tested, the raceway in which the oysters were kept (five different raceways), and one covariate, the total length at the time of death (in centimeters). The trait analysed was binary: The oysters that survived the challenge experiment were coded "0", and the ones that died during the challenge experiment were coded "1". The module "Large Single Full-Sib Family

Analysis (Tree)" was chosen because it is designed for the case of a family with two parents that are not assumed to come from a cross between two distinct and different genetic lines. The analysis makes a comparison between the two gametes carried by the male parent (the paternal component) and the two gametes carried by the female parent (the maternal component). The finding of QTL used a regression interval mapping approach (Haley and Knott 1992). Chromosome-wide significance threshold (which takes into account multiple testing on a specific chromosome) was estimated after performing 1,000 permutations according to Churchill and Doerge (1994). Separate analyses were performed for each F_1 parent, using the parent-specific maps established with CriMap. For the parent 410_7, the "pat" model was used to fit the paternal component and to find QTL. For the parent 410_8, the "mat" model was used to fit the maternal component and to find QTL. The paternal component relating to 410_7 and the maternal component to 410_8 were arbitrary (sex of the parental oysters unknown).

Results

Power for Detection of Susceptibility Genes in Single- and Two-stage Selection Strategies

Power for detection of susceptibility genes in one- and two-stage selection strategies are given in Table 2. For the offspring size of 92, power to detect multiple susceptibility loci was rather high (between 0.93 and 1) irrespective of the mating type. Power in the two-stage detection strategy was high but marginally lower than power in one-stage detection for all mating types.

The expected number of false positives for each type of mating is given in Table 3. As expected, single-stage detection had the highest number of false positives. For one true susceptibility locus and 200 markers (tests), the number of false positives was high (~10). Bonferroni correction had the lowest number of false positives. The two-stage detection strategy had also very small expected number of false positives.

Survival Analysis and the Search for Potential QTL of Resistance/Susceptibility to Bonamiosis

The 34 AFLP primer pairs produced 309 markers, 201 AFLPs of type 1:1 (peak present in only one of the two parents) and 108 of type 3:1 (peak present in both parents). After the TDT, 144 markers were significant and kept for the MST: 83 of type 1:1 (22 with $p<0.05$; 20 with $p<0.01$; 41 with $p<0.001$) and 61 of type 3:1 (16 with $p<0.05$; 18 with $p<0.01$; 27 with $p<0.001$). Of the 144 markers significant after the TDT, only 26 were not significant after the MST: five markers segregated through the parent 410_7 (1:1 type), ten through the parent 410_8 (1:1 type) and 11 through both parents (3:1 type) (Table 4).

Of the 26 markers kept for survival analysis, 15 were significant (four with $p<0.05$, two with $p<0.01$ and nine with $p<0.001$). The peak-present marker phenotype corresponded

Table 2 Power to detect multiple susceptibility loci in one- or two-stage testing strategies

True SL	Power		
	Single stage, $\alpha=0.05$	Single stage, $\alpha=0.05/100$	Two stages, $\alpha=0.05$
Mating type Ss×Ss			
8	0.9696	0.3396	0.9692
7	0.9734	0.3887	0.9730
6	0.9771	0.4449	0.9768
5	0.9809	0.5092	0.9806
4	0.9847	0.5828	0.9845
3	0.9885	0.6670	0.9883
2	0.9923	0.7634	0.9922
1	0.9962	0.8737	0.9961
Mating type Ss×ss			
8	1.0000	0.9982	0.9976
7	1.0000	0.9984	0.9979
6	1.0000	0.9986	0.9982
5	1.0000	0.9988	0.9985
4	1.0000	0.9991	0.9988
3	1.0000	0.9993	0.9991
2	1.0000	0.9995	0.9994
1	1.0000	0.9998	0.9997

True SL number of true QTL segregating in the population; α= significance level. $\Psi_{SS}=0.70$; $\Psi_{Ss}=0.25$; $\Psi_{ss}=0.05$

Table 3 Expected number of false positives detected in one- or two-stage QTL detection strategies

True SL	Single stage, $\alpha=0.05$		Single stage, $\alpha=0.05/100$		Two stages, $\alpha=0.05$	
	Number of tests		Number of tests		Number of tests	
	100	200	100	200	100	200
8	4.60	9.60	0.05	0.10	0.23	0.48
7	4.65	9.65	0.05	0.10	0.23	0.48
6	4.70	9.70	0.05	0.10	0.24	0.49
5	4.75	9.75	0.05	0.10	0.24	0.49
4	4.80	9.80	0.05	0.10	0.24	0.49
3	4.85	9.85	0.05	0.10	0.24	0.49
2	4.90	9.90	0.05	0.10	0.25	0.50
1	4.95	9.95	0.05	0.10	0.25	0.50

True SL number of true QTL segregating in the population; α= significance level. $\Psi_{SS}=0.70$; $\Psi_{Ss}=0.25$; $\Psi_{ss}=0.05$

Table 4 TDT and MST for the 26 markers that were kept for survival analysis (significant after TDT among susceptible progeny and non-significant after MST in the whole progeny)

Marker	Parental genotypes		No. of susceptible offspring (dead)		No. of resistant offspring (surviving)		TDT	MST
	410-7	410-8	Present	Absent	Present	Absent		
A1f150	aa	Aa	33	12	21	25	9.8**	3.1
E1f43	Aa	Aa	25	21	38	8	9.2**	2.1
A3f73	aa	Aa	12	34	25	21	10.5**	3.5
A3f165	Aa	Aa	42	4	35	11	8.1**	3.7
E3f169	aa	Aa	10	36	27	19	14.7***	3.5
E3f255	Aa	Aa	25	21	38	8	9.2**	2.1
E4f291	Aa	aa	16	30	23	23	4.3*	2.1
A5f225	aa	Aa	16	30	21	25	4.3*	3.5
E5f126	Aa	Aa	40	6	33	13	4.0*	0.9
E5f157	aa	Aa	13	33	24	22	8.7**	3.5
B8f234	Aa	Aa	43	3	32	14	10.9***	2.1
E9f147	Aa	Aa	40	6	37	9	4.0*	3.7
E9f368	aa	Aa	9	37	28	18	17.0***	3.5
E9f371	Aa	aa	31	15	22	24	5.6*	2.1
A12f288	Aa	Aa	43	3	33	13	10.9***	2.8
A12f429	Aa	Aa	25	21	38	8	9.2**	2.1
B12f52	aa	Aa	16	30	21	25	4.3*	3.5
B12f243	aa	Aa	37	9	17	29	17.0***	2.8
B12f478	Aa	aa	31	15	22	24	5.6*	2.1
C1f99	aa	Aa	37	9	18	28	17.0***	3.5
D1f129	Aa	Aa	42	4	33	13	8.1**	2.1
D1f162	Aa	aa	31	15	23	23	5.6*	2.8
D1f203	Aa	Aa	22	24	40	6	15.5***	2.8
D1f328	aa	Aa	12	34	30	16	10.5**	0.7
C5f112	Aa	aa	31	15	24	22	5.6*	3.5
D5f203	Aa	Aa	40	6	37	9	4.0*	3.7

Present band-present phenotype (Aa or A?), *absent* band-absent phenotype (aa), *TDT* transmission disequilibrium test, *MST* Mendelian segregation test

*$p<0.05$, **$p<0.01$, ***$p<0.001$

to a resistance allele in nine cases and to a susceptible allele in six cases. Hazard ratios were in the range 0.24–0.45: Inheriting the peak-present allele reduced (or increased) the mortality by ~24% to 45% for a resistant (or susceptible) marker (Table 5). Kaplan–Meier survival curves were constructed for the markers with $p<0.01$; these were eight AFLPs ($p<0.01$) for which the peak-present phenotype corresponded to a resistant allele (Fig. 1) and three AFLPs ($p<0.01$) for which the peak-present phenotype corresponded to a susceptible allele (Fig. 2).

Genetic Linkage Mapping

Overall, 25% of the markers were distorted, 47.4% of the microsatellites (nine out of 19) and 23.6% of the AFLPs (73 out of 309).

The 410_7 parental genetic linkage map was based on the 17 microsatellites that were informative for this parent and 157 AFLPs segregating through this parent. The AFLPs consisted of 112 markers of type 1:1 (43 from 98AC703-29, 55 from W31 and 14 from both grand-parents) and 45 markers of type 3:1 (29 from 98AC703-29 and 16 from W31). The resulting map consisted of 127 markers (73.0%), comprising 16 microsatellites (of 17: 94.1%), 94 type 1:1 AFLPs (of 112, 83.9%), 17 type 3:1 AFLPs (of 45, 37.8%). Ten linkage groups were established for the 410_7 map covering 465.6 cM (Fig. 3). The estimated genome length was 553.37 cM according to method 4 of Chakravarti et al. (1991). The observed coverage was therefore 84.1% for the 410_7 parental map. Features of the genetic linkage map are shown in Table 6. Distorted markers tended to cluster in specific linkage groups (LG3_410_7, LG4_410_7, LG7_410_7, LG8_410_7, LG9_410_7 and LG10_410_7).

The 410_8 parental genetic linkage map was based on the 18 microsatellites that were informative for this parent and 124 AFLPs segregating through this parent. The AFLPs consisted of 79 markers of type 1:1 (31 from

Table 5 Survival analysis results, hazard ratio and LRANK

Marker	Origin	*R*/*S*	O_a	E_a	O_p	E_p	Hazard ratio (*h*)	LRANK
A1f150	W31, 410-8	S	12	21.8	33	24.2	0.40	7.7**
E1f43	703-29, 410-7, 410-8	R	21	10.0	25	36.0	0.33	15.5***
A3f73	703-29, 410-8	R	34	24.2	12	21.8	0.39	8.4**
E3f169	W31, 410-8	R	36	24.1	10	21.9	0.31	12.3***
E3f255	703-29, 410-7, 410-8	R	21	9.5	25	36.5	0.31	17.6***
E5f157	703-29, 410-8	R	33	24.5	13	21.5	0.45	6.4*
B8f234	703-29, 410-7, 410-8	S	3	10.1	43	35.9	0.25	6.4*
E9f368	W31, 410-8	R	37	23.6	9	22.4	0.26	15.6***
A12f288	703-29, 410-7, 410-8	S	3	9.5	43	36.5	0.27	5.5*
A12f429	703-29, 410-7, 410-8	R	21	10.0	25	36.0	0.33	15.5***
B12f243	703-29, 410-8	S	9	23.1	37	22.9	0.24	17.3***
C1f99	703-29, 410-8	S	9	22.4	37	23.6	0.26	15.6***
D1f129	703-29, 410-7, 410-8	S	4	9.7	42	36.3	0.36	4.3*
D1f203	W31, 410-7, 410-8	R	24	12.6	22	33.4	0.34	14.3***
D1f328	W31, 410-8	R	34	21.5	12	24.5	0.31	13.7***

R resistance marker, *S* susceptible marker, O_a total number of dead offspring in the band-absent marker phenotype, E_a its relative expected count, O_p total number of dead offspring in the band-present marker phenotype, E_p its relative expected count
*$p<0.05$, **$p<0.01$, ***$p<0.001$

98AC703-29, 39 from W31 and nine from both grandparents) and 45 markers of type 3:1 (29 from 98AC703-29 and 16 from W31). The resulting map consisted of 98 markers (70.0%), comprising 14 microsatellites (of 18, 77.8%), 71 type 1:1 AFLPs (of 79, 89.9%), 13 type 3:1 AFLPs (of 45, 28.9%). Ten linkage groups were established for the 410_8 map covering 386.7 cM (Fig. 4). The estimated genome length was 556.91 cM. The observed coverage was therefore 69.4% for the 410_8 map. Features of the genetic linkage map are shown in Table 7. Distorted markers towards a deficit (−) or excess (+) in *aa* homozygotes could be mapped on six different linkage groups: LG1_410_8, LG3_410_8, LG4_410_8, LG6_410_8, LG8_410_8 and LG10_410_8. Generally, distorted markers in the same direction and with the same level of statistical significance tended to cluster and to be mapped over a very short distance (e.g. 2 or 3 (+) markers in LG1_410_8, LG6_410_8, LG8_410_8 or LG10_410_8; 4 (−) markers in LG3_410_8).

Of the seven markers that remained significant after the survival analysis and that segregated through the parent 410_7, four could be mapped on the 410_7 map, three resistant markers and one susceptible marker: D1f203 on LG2_410_7; E3f255, E1f43 and A12f429 on LG4_410_7. The three markers on LG4_410_7 were mapped in a 14-cM area, and interestingly, they have the same grand-parental origin (98AC703-29), while the fourth marker (D1f203) mapped in another linkage group came from the second grandparent (W31) (Fig. 3).

For the 410_8 parent, of the 15 markers significant after the survival analysis, 12 of those could be mapped on the 410_8 map, eight resistant markers and four susceptible markers. These 12 markers were distributed on four linkage groups: D1f203 on LG2_410_8; E5f157, A3f73 and A1f150 on LG3_410_8; E1f43, E3f255 and A12f429 on LG4_410_8; D1f328, E3f169, E9f368, B12f243 and C1f99 on LG6_410_8. The three markers on LG3_410_8 mapped in a 9-cM area, the markers on LG4_410_8 clustered altogether and the five markers on LG6_410_8 mapped in a 18-cM area. Moreover, it is interesting to note that resistant markers on the same linkage group came from the same grandparent, while the susceptible markers came from the other grandparent (LG3_410_8 and LG6_410_8; Fig. 4).

We also compared the mapping of the markers that were significant after the survival analysis in the two parental maps. The marker D1f203 mapped in the two parental maps at the same end of the linkage groups (LG2_410_8 and LG2_410_7), very close to microsatellite *Oedu*U2. Moreover, the three markers E1f43, E3f255 and A12f429 were mapped in the two parental maps, in the terminal part of the linkage groups: They were clustered at the end of group LG4_410_8, while in LG4_410_7, they were mapped in a 14-cM terminal area with two of them being clustered (E1f43 and A12f429).

QTL Mapping

No significant effects were found for the fixed effect (raceway) or the covariate (length of oyster at the time of death). Consequently, they were not included in the QTL model.

For the 410_7 parent, four linkage groups exhibited a significant paternal estimate, meaning that the two different gametes carried alleles of different effect for the QTL. The

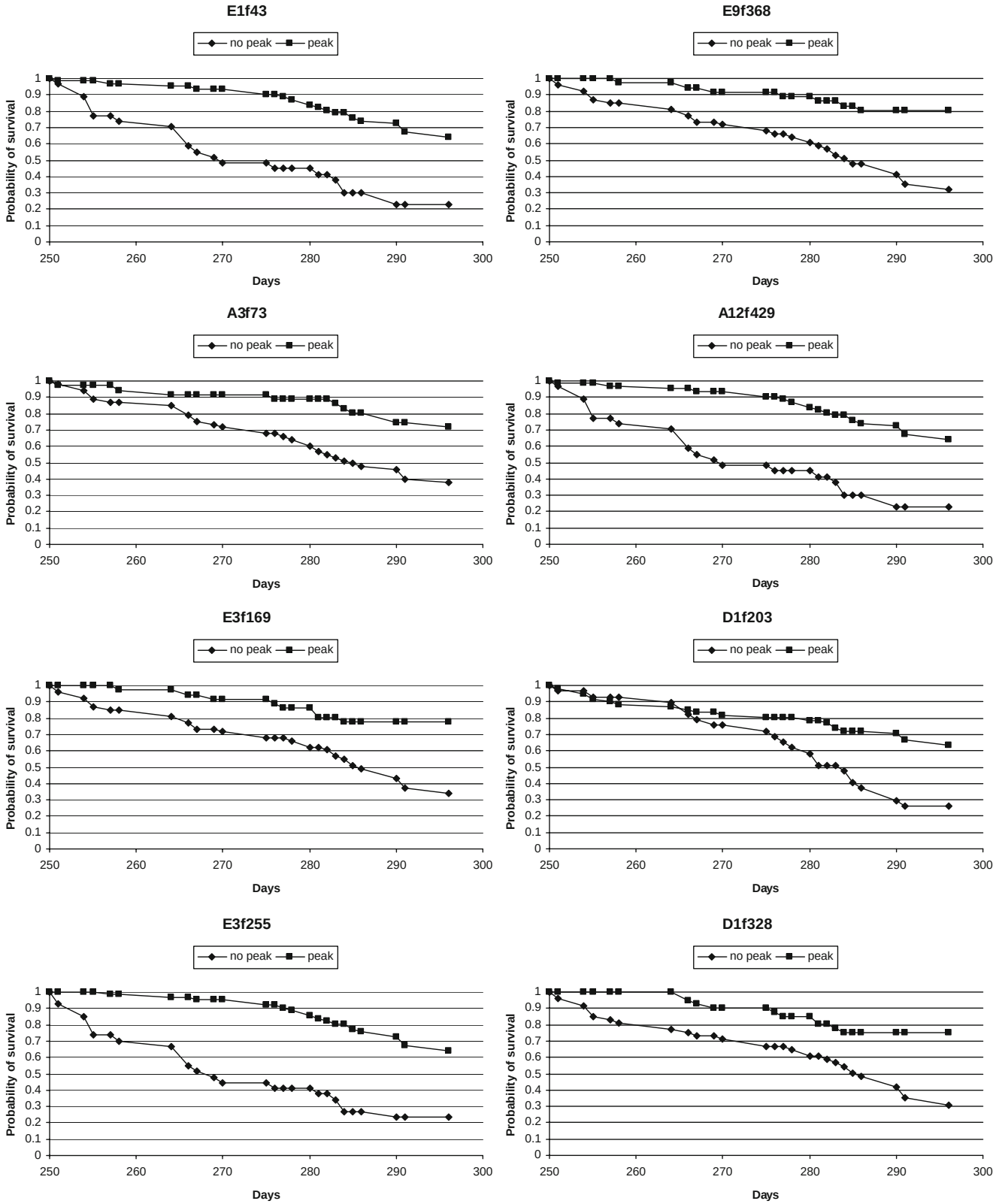

Fig. 1 Kaplan–Meier survival curves for eight AFLP markers ($p<0.01$) for which the peak-present phenotype corresponded to a resistant allele. *Days* number of days after the beginning of the challenge experiment

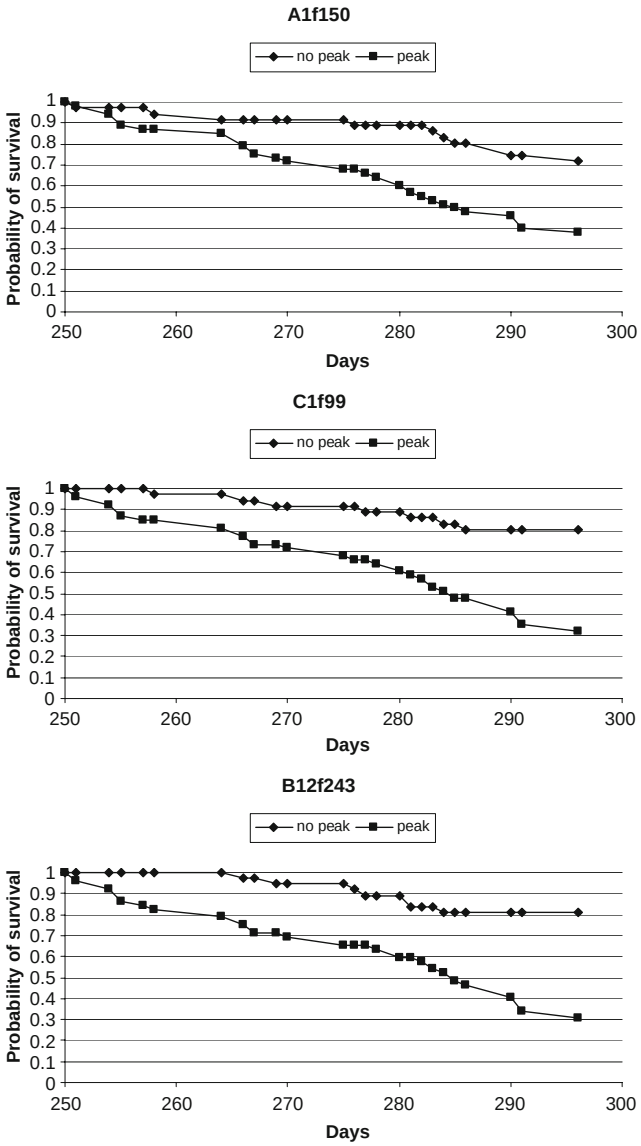

Fig. 2 Kaplan–Meier survival curves for three AFLP markers ($p < 0.01$) for which the peak-present phenotype corresponded to a susceptible allele. *Days* number of days after the beginning of the challenge experiment

best estimate of the location for the QTL was 0 cM for LG2_410_7, 0 cM for LG3_410_7, 24 cM for LG4_410_7 and 8 cM for LG6_410_7. Only linkage group LG2_410_7 attained significance at the chromosome-wide 0.01 level. The two linkage groups LG4_410_7 and LG6_410_7 attained significance at the chromosome-wide 0.05 level, but only just (Table 8).

For the 410_8 parent, two linkage groups exhibited a significant maternal estimate. The best estimate of location for the QTL was 61 cM for LG3_410_8 and 17 cM for LG6_410_8. Linkage group LG3_410_8 attained significance at the chromosome-wide 0.05 level and LG6_410_8 at the chromosome-wide 0.01 level (Table 9).

Discussion

Segregation Distortion

Relatively high segregation distortion was reported in the mapping family OE.F2.05.04, averaging 25% of overall markers. The range of segregation distortion reported in this study was similar to that reported in oysters (Launey and Hedgecock 2001; Li and Guo 2004; Lallias et al. 2007b). Such a high level of segregation distortion is presumed to be due to the extremely high genetic load estimated in oysters (Bierne et al. 1998; Launey and Hedgecock 2001). Moreover, the distribution of distorted markers was not random in the genetic linkage maps produced and tended to form clusters of distorted markers that were restricted to a few linkage groups (e.g. LG3_410_7, LG8_410_7 or LG3_410_8; Figs. 3 and 4). These clusters of distorted markers could therefore correspond to the location of potential deleterious genes in *O. edulis*, similarly to the mapping of potential deleterious genes in the rainbow trout (Young et al. 1998) or the Pacific oyster *Crassostrea gigas* (Li and Guo 2004).

In the second stage of the two-stage selection strategy (Moen et al. 2004), we excluded markers departing from Mendelian segregation in the first stage. Distortion may be caused by recessive lethal genes at larval stages (Bierne et al. 1998), which may include susceptibility to bonamiosis. Null alleles and other problems can also lead to such departures from Mendelian segregation. Therefore, those markers were ignored in the second-stage analyses because it is very difficult with the available data to figure out the causes underlying the observed departures from Mendelian segregation.

Linkage Map and Genome Coverage

Genome coverage was above 82% for the 410_7 parental map and 69.4% for the 410_8 parental map. Those genome coverage estimates compared favorably with the ones established in cupped and flat oysters' species, which were in the range of 70–90% depending on the study (Hubert and Hedgecock 2004; Li and Guo 2004; Lallias et al. 2007b).

Moreover, the number of linkage groups in the two parental maps matched the haploid number of ten chromosomes in this species (Thiriot-Quiévreux and Ayraud 1982). However, more markers should be added to increase the genome coverage. Indeed, some of the linkage groups consisted of only two markers or spanned a small genetic distance (<20 cM). Therefore, these groups may in fact belong to the same chromosome and may fuse by adding more markers. Despite this, the average marker spacing (4 cM) was suitable for the search of QTL (Erickson et al. 2004).

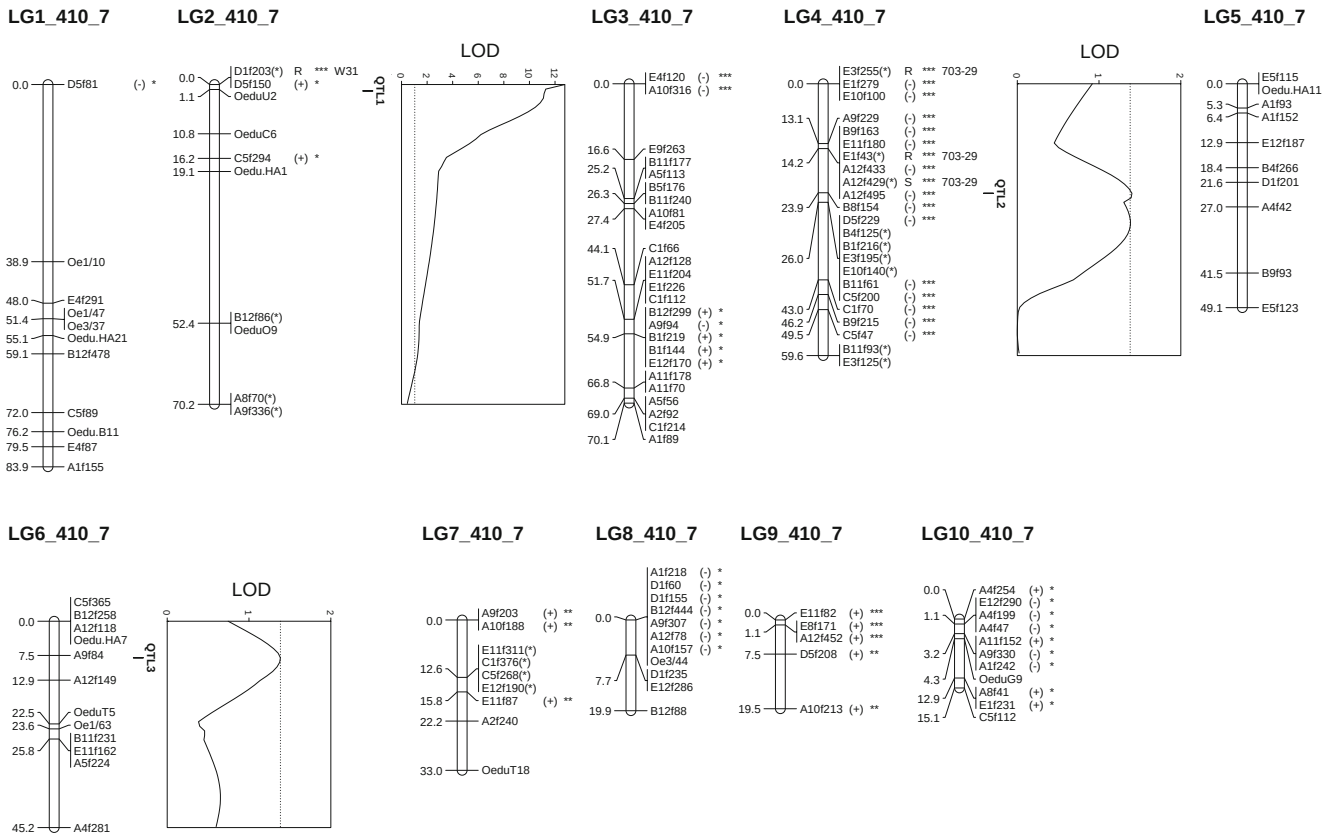

Fig. 3 Microsatellite and AFLP-based linkage map of the flat oyster *O. edulis* in the mapping family OE.F2.05.04: 410_7 parental map obtained with CriMap, 127 markers, 466 cM. AFLP markers are labelled with the primer pair name followed by the letter *f* (for fragment) and a three-digit fragment size in base pairs. Markers are indicated on the *right*; and absolute positions on the *left* (in Kosambi cM). On the right of AFLP locus names is specified the direction of the segregation distortion: towards a deficit (−) or excess (+) of null homozygotes with the statistical significance (*$p<0.05$, **$p<0.01$ and ***$p<0.001$). *R* resistant marker, *S* susceptible marker (detected by the approach described in Moen et al. 2004), with the statistical significance and grandparental origin. Locus names immediately followed by an *asterisk* correspond to 3:1 type AFLP. Interval mapping results for bonamiosis resistance/susceptibility are shown for linkage groups G2_410_7, G4_410_7 and G6_410_7: the LOD score is plotted against the position (cM) along the linkage group, with the *dotted line* representing the chromosome-wide significance threshold

QTL Mapping of Resistance/Susceptibility to a Disease

Several studies have highlighted the potential for MAS in breeding programs in fisheries and probably shellfisheries in the future (Liu and Cordes 2004; Rothschild and Ruvinsky 2007). MAS has a huge potential in aquaculture breeding programs, especially for traits that are difficult to quantify phenotypically and would increase the response of the trait to the selection by increasing the accuracy of selection.

Although disease resistance generally seems to have a low heritability in some species, it is nevertheless an ideal trait for the application of MAS, due to the economic significance of high survival in aquaculture. Traditionally, the QTL mapping approach was designed for continuous variable quantitative traits. However, it has been shown that this analysis is robust for binary traits, such as resistance/susceptibility to a disease (death/alive trait coded as “0” or “1”; Visscher et al. 1996). Therefore, we decided to couple

Table 6 Length, number of markers, average spacing and largest interval between markers in linkage groups of the 410_7 parental map in *O. edulis* established with CriMap

Linkage group	Length (cM)	No. of markers	Average spacing (cM)	Largest interval (cM)
LG1_410_7	83.9	11	8.39	38.9
LG2_410_7	70.2	10	7.8	33.3
LG3_410_7	70.1	25	2.92	16.7
LG4_410_7	59.6	23	2.71	17.0
LG5_410_7	49.1	10	5.46	14.5
LG6_410_7	45.2	12	4.11	19.4
LG7_410_7	33.0	9	4.13	10.8
LG8_410_7	19.9	11	1.99	12.2
LG9_410_7	19.5	5	4.88	12.0
LG10_410_7	15.1	11	1.51	8.6
Total	465.6	127		

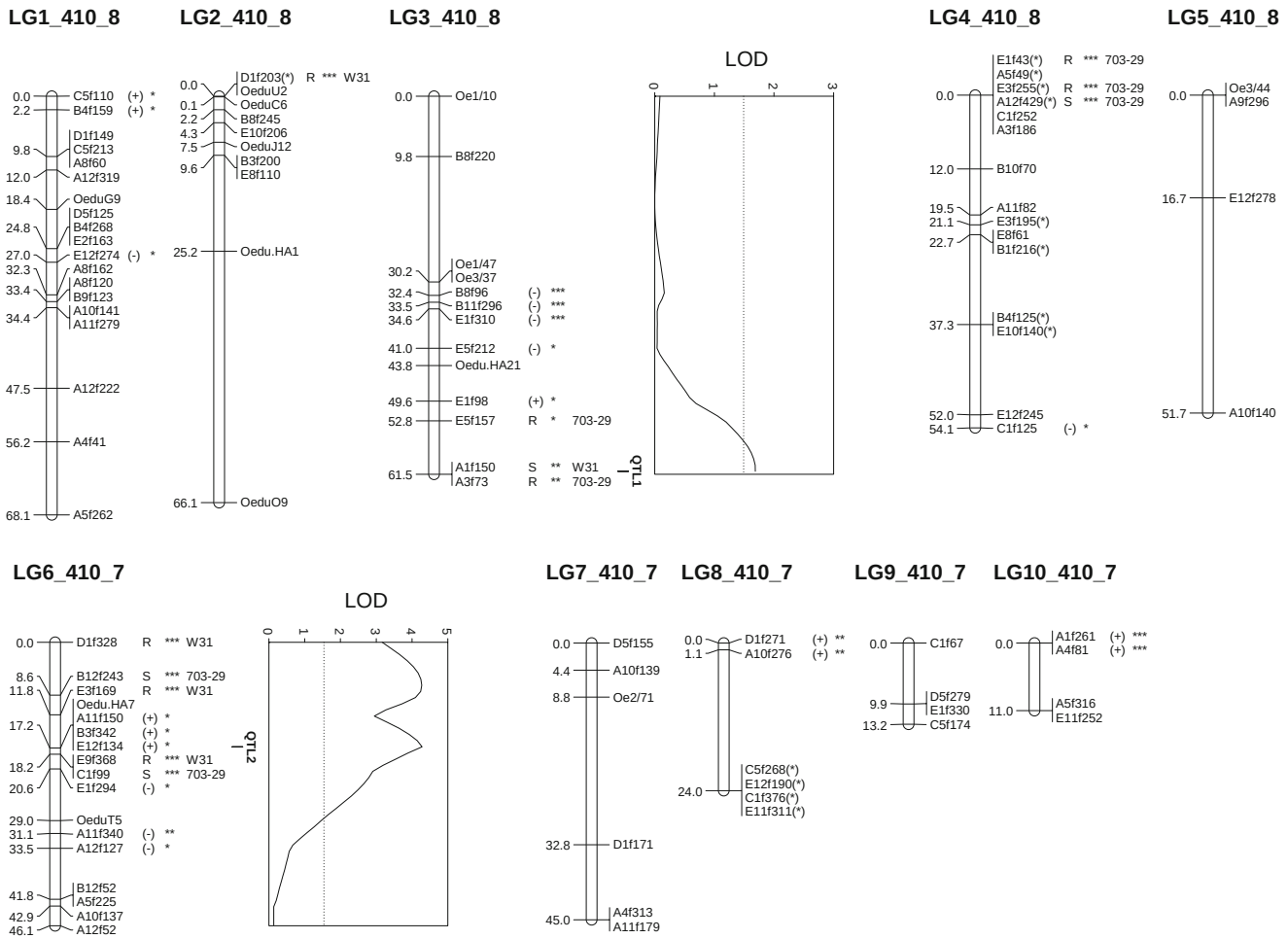

Fig. 4 Microsatellite and AFLP-based linkage map of the flat oyster *O. edulis* in the mapping family OE.F2.05.04: 410_8 parental map obtained with CriMap, 98 markers, 387 cM. Interval mapping results for bonamiosis resistance/susceptibility are shown for linkage groups G3_410_8 and G6_410_8: the LOD score is plotted against the position (cM) along the linkage group, with the *dotted line* representing the chromosome-wide significance threshold. See Figure 3 for abbreviations

Table 7 Length, number of markers, average spacing and largest interval between markers in linkage groups of the 410_8 parental map in *O. edulis* established with CriMap

Linkage group	Length (cM)	No. of markers	Average spacing (cM)	Largest interval (cM)
LG1_410_8	68.1	19	3.78	13.1
LG2_410_8	66.1	10	7.34	40.9
LG3_410_8	61.5	13	5.12	20.4
LG4_410_8	54.1	15	3.86	14.7
LG5_410_8	51.7	4	17.20	35.0
LG6_410_8	46.1	17	2.88	8.6
LG7_410_8	45.0	6	9.00	24.0
LG8_410_8	24.0	6	4.80	22.9
LG9_410_8	13.2	4	4.40	9.9
LG10_410_8	11.0	4	3.67	11.0
Total	98	386.7		

the marker by marker approach with a traditional QTL mapping approach in order to compare the results obtained with these two alternative approaches. The finding of QTL was achieved by using a two-stage testing strategy (Moen et al. 2004) and a regression interval mapping approach (QTL express software, Seaton et al. 2002).

Statistic power of the two-stage testing strategy and rate of false positives were computed by simulations. Power calculations were carried out assuming that multiple QTL are unlinked. If two QTL are closely linked and the alleles increasing susceptibility are on the same homologous chromosome in a parent donating meiosis then the power calculation would be similar to one QTL with increased penetrance respect to either single QTL. The opposite would occur when alleles at two QTL with high and low penetrances are on the same homologous chromosome. On the other hand, the two-stage testing strategy assumed that

Table 8 Results from fitting a single QTL for the parent 410_7 (QTL express software)

Linkage group	F ratios			Location (cM)	Paternal estimate (standard error)
	Threshold, p 0.05	Threshold, p 0.01	Observed		
LG2_410_7	6.78	9.87	83.65	0	0.3574 (0.039)
LG3_410_7	7.97	12.14	5.57	0	−0.134 (0.057)
LG4_410_7	6.63	11.72	6.73	24	−0.1601 (0.062)
LG6_410_7	6.65	10.65	6.65	8	−0.1329 (0.051)

Threshold p 0.05 and threshold p 0.01 correspond to chromosome-wide significance thresholds at α=5% and 1% after performing 1,000 permutations

TDT performed on susceptible animals and the survival analyses used on the whole data set are independent tests. This assumption is not fully correct, since there may be some dependence between the variables used (affected/resistant and number of days survived). As pointed out by Moen et al. (2004), this assumption is supported by the fact that animals tested in the TDT are only a subset of the animals tested in the survival analysis.

False negatives are missing true linkages, whereas false positives are false linkage claims. Mapping in genome scans should minimise both. For the two-stage strategy, a total of 309 markers were tested with nine significant results at $P<0.001$ after the survival analyses. This implies that, on average, less than one of the significant results is spurious (false positive). False negatives would be given by the type I error and would also be less than one for the 309 markers.

Power for detection of multiple susceptibility loci in single-stage detection was the highest at the expense of a high rate of false positives. The use of a Bonferroni correction to control the false positive rate resulted in a dramatic reduction of power for some mating types when detecting multiple susceptibility loci. The two-stage detection strategy had a high power with a low rate of false positives (Tables 2 and 3). The two-stage testing strategy was a powerful and robust method for identifying QTL of resistance/susceptibility to a disease and allowed us to identify 15 probable AFLP markers linked to genes of resistance (nine of them) or susceptibility (six of them) to the disease. Considering all the above, the two-stage selection strategy might be useful in aquaculture species with high family sizes and incomplete genetic maps.

The QTL mapping approach chosen was a regression interval mapping (interval mapping based on least-squares regression methods; Haley and Knott 1992). Indeed, an interval mapping approach is based on information from two linked flanking markers and has been shown to be more powerful than a single marker analysis particularly for medium-density maps (with markers around 20–35 cM apart) and to increase the accuracy of parameters estimation (Darvasi et al. 1993). The analysis was based on a single full-sib family experimental design (and not a F_2 analysis) because the mapping family was a three-generation outbred family whose grandparents were not issued from different genetic lines fixed for different alleles at the QTL. The analysis was interpreted in terms of paternal and maternal components, i.e. whether the two gametes of each parent carried alleles of different effect for the QTL. However, no estimation of the interaction component (and therefore dominance of the QTL) could be performed because of the lack of codominant loci (e.g. microsatellites). Furthermore, a consensus map could not be built because most markers were segregating in only one of the two parents, and the estimation of the interaction component implies that the map is the same in the two parents. Moreover, because only 92 progeny were genotyped, a one-QTL model was fitted but not a two-QTL model. The results obtained after fitting the one-QTL model should be interpreted with caution because the role of neighbouring QTL in biasing the estimation of location and gene effect of a QTL has been

Table 9 Results from fitting a single QTL for the parent 410_8 (QTL express software)

Linkage group	F ratios			Location (cM)	Maternal estimate (standard error)
	Threshold, p 0.05	Threshold, p 0.01	Observed		
LG3_410_8	7.22	12.08	8.17	61	−0.149 (0.052)
LG6_410_8	7.96	12.14	22.19	17	0.229 (0.049)

Threshold p 0.05 and threshold p 0.01 correspond to chromosome-wide significance thresholds at α=5% and 1% after performing 1,000 permutations

widely assessed (Haley and Knott 1992; Jansen 1993). The results of the one-QTL model can be misleading when there are in fact two linked QTL segregating on the same linkage group.

Despite some limitations, our study has made major progress towards the identification of genetic resistance/susceptibility to bonamiosis. Several potential markers of interest were identified, and there was good concordance between the results obtained after the two-stage testing strategy, genetic mapping and QTL mapping. Identified markers tended to cluster or were restricted to a few groups: In the 410_7 map, three markers were mapped in a 15-cM area in LG4_410_7 group, and a fourth marker was mapped in LG2_410_7 group (Fig. 3); in the 410_8 map, three markers were mapped in a 9-cM area in LG3_410_8, five markers were mapped in a 17 cM area in LG6_410_8, three markers were clustered at the end of LG4_410_8 and one marker was mapped in LG2_410_8 (Fig. 4). Moreover, the significant QTL found after the regression interval mapping approach were mapped in the same area as the markers that were significant after the survival analysis. Therefore, this study clearly demonstrates for the first time the usefulness of combining different approaches for the search of QTL in aquaculture species, associated with a high statistical power.

Hazard ratios in this study were very high, with single alleles inheritance affecting survival of the offspring bearing it by as much as 24% to 45%. We may wonder how such variation can be maintained against purifying selection. The effects may vary between families for the same alleles and their related causal genes due to different genetic backgrounds and non-additive effects. Important G×E (genotype×environment) interactions might also occur. Therefore, QTL analysis in a single family is limiting, and QTL analysis should be extended to several families in order to test these hypotheses.

Several studies have reported the location of QTL for disease resistance in rainbow trout, based on the classical approach for QTL mapping using interval mapping, the ANOVA-based approach or bulk segregant analysis (e.g. Ozaki et al. 2001; Rodriguez et al. 2004). The results obtained in this study, even if preliminary, are promising and represent a first step towards MAS in the flat oyster. However, before implementation of MAS in a selective breeding program, the role of epistasis and genomic background should be assessed (Danzmann et al. 1999; Perry et al. 2003). Moreover, screening natural populations for outlier levels of differentiation at QTL loci (Rogers and Bernatchez 2005) could add value in terms of robustness and universality of the QTL identified. The QTL identified could be scored in wild and selected populations. One should expect an average increase in gene frequencies of QTL markers for resistance in the selected populations and a respective decrease in the QTL for susceptibility to the disease.

The addition of codominant markers (such as microsatellites or SNPs) is critical to increase the accuracy of the genetic maps obtained, the power of detection of the QTL and the accuracy of the estimation of the QTL effects. Moreover, before implementation of MAS in *O. edulis*, fine QTL mapping should be achieved in order to restrict the region of interest to a more narrow area. A further step would be to map candidate genes involved in the resistance to the disease that were recently identified after performing a suppression subtractive hybridisation library (Morga et al., manuscript in preparation). This would help to corroborate QTL with candidate genes and would represent a further step into the understanding of the genetic component of the resistance/susceptibility of *O. edulis* to *B. ostreae*.

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Appendix A. Power in Single-Stage Detection of Susceptibility Loci

In order to compute power, a χ^2 test for contingency tables was modeled (Cohen 1988). There are four types of offspring possible for each type of mating (Table 1): (1) banded and with the disease, (2) banded without the disease, (3) not banded and with the disease and (4) not banded without the disease. Under the null hypothesis (the AFLP locus is not linked to the susceptibility locus), $\Psi_{SS}=\Psi_{Ss}=\Psi_{ss}=1/2$. Let t_α be the value in a χ^2 test with 1 degree of freedom equal or higher than for expected by chance at significance level (α). Power is $P_{1,\alpha}=1-\beta$ with $\beta=\int_{t_\alpha}^{\infty} f(x)dx$, being $f_x \sim \chi^2(\lambda, df)$, where λ is the non-centrality parameter of a non-central χ^2 distribution with df=1 degrees of freedom. The non-central parameter of the χ^2 distribution is

$$\lambda = N\sum_{i=1}^{4}\frac{[p_0(i)-p_A(i)]^2}{p_o(i)}$$

where N is the total number of individuals in the experiment, $p_o(i)$ and $p_A(i)$ are the probabilities of each type of offspring (i=1 to 4) under the null and alternative hypothesis, respectively. For the mating type $Ss\times ss$: $p_o(i)$

$=1/4$ $(i=1,4)$; $p_A(1)=1/2\Psi_{Ss}$; $p_A(2)=1/2$ $(1-\Psi_{Ss})$; $p_A(3)=1/2\Psi_{ss}$; and $p_A(4)=1/2$ $(1-\Psi_{ss})$. For the mating type $Ss\times Ss$: $p_o(1)=3/8$, $p_o(2)=3/8$, $p_o(3)=1/8$ and $p_o(4)=1/8$; $p_A(1)=1/4\Psi_{SS}+1/2\Psi_{Ss}$; $p_A(2)=1/4$ $(3-\Psi_{SS}+2\Psi_{Ss})$; $p_A(3)=1/4\Psi_{ss}$; and $p_A(4)=1/4$ $(1-\Psi_{ss})$.

Appendix B. Power in Two-Stage Detection of Susceptibility Loci

Under the null hypothesis (the AFLP is not linked to the susceptibility locus), for a sire *Ss*, the probability of transmission of the allele *S* and being banded is 1/2 and 3/4 for segregation of mating types 1:1 and 3:1, respectively. At a significance level of $\alpha=0.05$, the maximum number of offspring inheriting one of the markers alleles by chance is $Nx=\frac{n+1.96\sqrt{n}}{2}$ and $Nx=\frac{3n+1.96\sqrt{3n}}{4}$. In the above formula, n is the number of offspring in the full sib family used for TDT test, and therefore, $n=N/2$. This is a two-sided probability to account that either marker allele could be inherited in a distorted fashion. Under the alternative hypothesis, we assume that the distorted frequency of segregation of marker alleles is the transmission disequilibrium parameter, ν (Gomez-Raya 2001). This parameter has different values for the different mating types:

$$\nu_{SsxSs}=\frac{\frac{1}{4}\psi_{SS}+\frac{1}{2}\psi_{Ss}}{\frac{1}{4}\psi_{SS}+\frac{1}{2}\psi_{Ss}+\frac{1}{4}\psi_{ss}},$$

$$\nu_{Ssxss}=\frac{\frac{1}{2}\psi_{Ss}}{\frac{1}{2}\psi_{Ss}+\frac{1}{2}\psi_{ss}}$$

Transmission parameter $\nu_{Ss\times ss}$ corresponds to segregation 1:1, whereas transmission parameter $\nu_{Ss\times Ss}$ corresponds to segregation 3:1. The probability of Nx or more individuals inheriting the given marker allele under ν is the power of the test: $P_{TDT}=\int_{-z}^{\infty}f(x)dx$, where $f(x)$ is the normal density, with $z=\frac{\frac{n+1.96\sqrt{n}}{2}-n\nu}{\sqrt{n\nu(1-\nu)}}$ and $z=\frac{\frac{3n+1.96\sqrt{3n}}{4}-n\nu}{\sqrt{n\nu(1-\nu)}}$ for mating with segregation types 1:1 and 3:1, respectively.

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