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# Molecular responses of Ostrea edulis haemocytes to an in vitro infection with Bonamia ostreae

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**Abstract:**

Bonamiosis due to the parasite Bonamia ostreae is a disease affecting the flat oyster Ostrea edulis. B. ostreae is a protozoan, affiliated to the order of haplosporidia and to the cercozoan phylum. This parasite is mainly intracellular, infecting haemocytes, cells notably involved in oyster defence mechanisms.

Suppression subtractive hybridisation (SSH) was carried out in order to identify oyster genes differentially expressed during an infection of haemocytes with B. ostreae. Forward and reverse banks allowed obtaining 1104 and 1344 clones respectively, among which 391 and 480 clones showed a differential expression between both tested conditions (haemocytes alone versus haemocytes in contact with parasites). ESTs of interest including genes involved in cytoskeleton, respiratory chain, detoxification membrane receptors, and immune system were identified. The open reading frames of two selected genes (galectin and IRF-like) were completely sequenced and characterized. Real time PCR assays were developed to study the relative expression of candidate ESTs during an in vitro infection of haemocytes by live and dead parasites.

Haemocyte infection with B. ostreae induced an increased expression of omega glutathione S-transferase (OGST), superoxide dismutase (SOD), tissue inhibitor of metalloproteinase (TIMP), galectin, interferon regulatory factor (IRF-like) and filamin genes.

**Keywords**: Bonamia ostreae; Protozoan; Ostrea edulis; Flat oyster; Haemocytes; Gene expression; Suppression subtractive hybridisation

# 1. Introduction

The parasite *Bonamia ostreae* was firstly described in France in association with mass mortality of flat oysters occurring in the late 1970's along the French coast (Pichot et al. 1979 and Comps et al. 1980). This intracellular protozoan has contributed to drastically decrease the French production of flat oysters which is now estimated at 1 900t per year (data available in the fishstat database, http://www.fao.org/fi/statist/FISOFT/FISHPLUS.asp). *Bonamia ostreae* is affiliated to the order of haplosporidia and to the phylum of cercozoan (Cavalier-Smith and Chao, 2003). The parasite is most often observed inside the haemocytes (Pichot et al. 1979; Comps et al. 1980), but it can also be observed extracellularly in the digestive gland and in the gills (Montes et al. 1994). The multiplication of the parasite is associated with haemocyte disruption. Haemocytic infiltration is observed in different tissues including digestive gland, mantle and gills (Balouet et al. 1983 ; Cochennec-Laureau et al. 2003).

Haemocytes, the circulating cells present in haemolymph, play a key role in the immune response of molluscs (Fisher, 1986). Bivalves lack specific immune system and immune memory. Their immune response relies on innate cellular and humoral mechanisms both operating in coordination to recognise and eliminate pathogens. Phagocytosis is the main cellular immune response against pathogens in molluscs (Cheng, 1981 ; Feng, 1988). Phagocytosis includes three main steps: recognition/adhesion, engulfment/internalisation and degradation (Cheng, 1981 ; Feng, 1988). However, after internalisation, various pathogens are able to survive in the phagocytes escaping post-phagocytosis mechanisms (Alavi et al. 2009). Previous studies have shown that *B. ostreae* is internalised after two hours of contact. Chagot et al. (1992) and Mourton et al. (1992) have demonstrated that the parasite *B. ostreae* was internalised after 30 min of contact with haemocytes and was not degraded after phagocytosis. Few studies have investigated the immune response of bivalve molluscs against parasites using molecular techniques (Tanguy et al. 2004; Kang et al. 2006 ; Green et al. 2009 and Prado-alvarez et al. 2009). Such studies are usually based on the suppression subtractive hybridisation (SSH) technique. This approach allows the identification of genes differentially expressed between two conditions.

Studies carried out on the interactions between haemocytes and parasites have been performed by cellular approaches (Mourton et al. 1992, Xue and Renault, 2001, Cochennec-Laureau et al. 2003 and Morga et al. 2009). Molecular responses of *Ostrea edulis* oysters against the parasite *Bonamia ostreae* have never been investigated. Moreover, few sequence data on *Ostrea edulis* genome are available in public databases (Genbank). The only sequences available have been used to determine the phylogenetic position of *Ostrea edulis* (Lopez-Flores et al. 2004).

In this context, the aim of the present study was the identification of flat oyster genes differentially expressed by haemocytes infected by the parasite *B. ostreae* compared to haemocytes alone using SSH approach. Among the obtained librairies, nine ESTs were selected according to their putative biological function: (1) detoxification and stress protein (cytochrome p450, OGST, HSP 90, SOD), (2) cell communication, membrane receptor and immune system (TIMP, galectin, IRF-like, tetraspanin) and (3) cystoskeleton structure (filamin).

In addition, RT PCR assays were developed in order to estimate expression level of ESTs of interest during an *in vitro* infection of haemocytes with live and dead parasites. Dead parasites allowed us to test the specificity of the haemocyte response induced live parasite. Finally, the open reading frames of two selected genes related to immunity (galectin and IRF-like) were completely sequenced and characterized.

# 2. Material and methods

## 2.1. Biological material

### 2.1.1. Oysters

Two-year-old flat oysters *Ostrea edulis* (n=90) were collected from Quiberon Bay (Southern Brittany, France), a bonamiosis endemic zone and were acclimatized in the quarantine facilities of IFREMER laboratory in La Tremblade (Charente-maritime, France) over 30 days. Flat oysters were maintained in 120 l raceways supplied with a constant flow of seawater enriched with phytoplankton (*Skeletonema costatum*, *Isochrisis galbana*, *Chaetoceros gracialis* and *Tetraselmis succica*).

### 2.1.2. Haemolymph collection

Haemolymph was withdrawn from the adductor muscle using a 1mL syringe equipped with a needle (0.40x90mm). Haemolymph samples were filtered on a 75 µm mesh to eliminate debris and maintained on ice to prevent cell aggregation. Haemolymph samples were pooled. Haemocyte counts were performed using a Malassey cell and cell concentration was adjusted at $2.10^6$ cells mL-1 with seawater filtered at 0.22 µm (FSW).

#### 2.1.3. Parasites

*Bonamia ostreae* was purified according to a previously published protocol (Mialhe et al. 1988). Briefly, heavily infected oysters were selected by examination of heart tissue imprints using light microscopy. After homogenization of all the organs except the adductor muscle, the parasites were concentrated by differential centrifugation on sucrose gradients and then purified by isopycnic centrifugation on a Percoll gradient. Finally, the purified parasites were suspended in FSW. *Bonamia ostreae* cells were then counted using a Malassez-cell and parasite concentration was adjusted at $10.10^7$ cells mL-1 with FSW.

For inactivation, parasites were heated at 100°C for 15 min. The efficiency of the inactivation treatment was controlled in flow cytometry using propidium iodide (Arzul et al. 2009).

## 2.2. In vitro infection protocol

A first experiment including haemocytes alone and haemocytes infected with live parasites was performed in order to construct forward and reverse SSH libraries.
The expression level of some genes identified through SSH was then evaluated during a second *in vitro* experiment including two conditions i.e. haemocytes in contact with live parasites and haemocytes in contact with dead parasites and a control consisting of haemocytes alone.
Whatever was the experiment, haemocytes were maintained in contact with purified parasites (live or dead) during 2 hours. For that purpose, 5 ml of haemocyte suspension were introduced in plastic flasks and incubated at 15°C for two hours until the formation of a cell layer. Supernatant was then withdrawn, preserved after 0.22 µm filtration and replaced by 500 µl of parasite suspension. After 2 hours, supernatant previously filtered at 0.22 µm to eliminate bacteria, was introduced again in flasks. After 12-hour incubation, cells were rinsed twice with phosphate buffer saline (PBS 1X) and were processed for RNA extraction using TRIZOL reagent (Invitrogen) following the manufacturer's instructions.

## 2.3. Suppression subtractive hybridisation (SSH)

Two micrograms of mRNA (1 µg from the haemocytes exposed to the parasite and 1 µg from the haemocytes alone) were used as templates for the SSH following the PCR-select cDNA subtraction kit procedure (Clontech) (Diatchenko et al., 1996). Forward subtraction was carried out using the haemocytes exposed to the parasite as the tester and the haemocytes alone as the driver. The opposite was performed for reverse subtraction. PCR products were cloned using TOPO TA Cloning Kit (Invitrogen). White colonies were screened by macro-arrays. Inserts were PCR amplified using TOPO F and R primers (Table 1) and one microliter of PCR product was spotted in duplicate onto nylon membrane (Roche). cDNA was digoxygenin labelled and used as probe in hybridisation experiments using the DIG-labelling and detection kit according to the manufacturer's instructions (Roche diagnostic).

## 2.4. Sequencing and sequence analysis

Clones showing a differential digoxygenin labelling intensity between infected haemocytes and haemocytes alone were selected and amplified using TempliPhi™ DNA Sequencing Template Amplification Kit (Amersham's). PCR products isolated from individual clones were sequenced in one way with TOPO F with the sequencing kit ABI BigDye® terminator version 3.1 using an ABI PRISM® 3130 XL-Avant Genetic Analyzer, a 36 cm capillary array and POP 7 polymer. Chromatograms were analyzed with Chromas 231 software. Sequences were then analysed with BlastX algorithm available from the National Center for Biotechnology Information (NCBI). EST sequences were then submitted to dbEST and GenBank databases (http://www.ncbi.nlm.nih.gov/blast/). Only E values less than $10^{-4}$ were considered significant.

## 2.5. Identification and characterization of immune-related genes

In order to obtain the complete open reading frame (ORF) of galectine, OeGal and Interferon regulatory factor like, OeIRF, RACE PCR reactions were carried out using SMART RACE cDNA Amplification Kit from Clontech according to the manufacturer's instructions. 5' and 3' primers were designed using primer 3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) (3'Galrace GACATCGACTGCAGCCACTAAACAG, 5'Galrace TATGGGCTGTGTCTCTCTTCTGCTC and 3'IRFrace TGGGTCCAGACACGGGTTCAATAC, 5'IRFrace CACCTTTGCTCTCACCCAGGTTTT). After ligation and cloning in TOPO Vector System (Invitrogen) and transformation in Top 10 competent bacteria (Invitrogen), several clones were sequenced using the same protocol as described before. Open reading frames were identified using ORF finder in NCBI. Complete sequences were deposited in GenBank and assigned under the accession numbers GU320697 (OeGal) and GU320698 (OeIRF-like). The isoelectric point and molecular mass were calculated in http://www.expasy.ch/tools/pi_tool.html.

## 2.6. Phylogenic analysis of Galectin and Interferon regulatory factor like (IRF-like)

The sequence of the complete ORF of OeGal was aligned with homologous genes available in GenBank database (*Homo sapiens sapiens* NM_006149.3, *Bos taurus* NM_001034768.1, *Mus musculus* NM_010706.1, *Oryctolagus cun*iculus NM_001082713.1, *Salmo salar* NM_001146582.1, *Xenopus laevis* NM_001085572.1, *Danio rerio* AY421706.1, *Strongylocentrus purpuratus* XM_001191059.1, *Pinctada fucata* FJ267519.1, *Venerupis phillipinarum* EU437401.1, *Biomphalaria gabralata* EF687664.1 and *Haliotis discus hannai* EF392832.1)

The sequence of the complete ORF of OeIRF-like was aligned with IRF-1, IRF-2, IRF-3 and IRF 7 genes from GenBank database (IRF-1: *Homo sapiens sapiens* ABH05670.1, *Mus musculus* P15314.1, *Oncorhynchus mykiss* NP001117765.1, *Salmo salar* AC168339.1, *Sparus aurata AAY68282.1, Scophtalmus maximus AAY68279.1, Gadus marhua ACJ06730.1, Carassius auratus ABM55678.1*, *Danio rerio* AY421706.1, *Hydra magnipapillata XP002168717.1; IRF-2* : *Homo sapiens sapiens* NP002190.2, *Mus musculus* P23906.1, *Oncorhynchus mykiss* AAK53987.1, *Salmo salar* ACI33066.1, *Chana argus* ABK63484.1, *Siniperca chuatsi* ABJ09546.1; IRF-3 : *Homo sapiens sapiens* AAH09395.1, *Mus musculus* AAH50882.1, *Salmo salar* ACN11005.1, *Danio rerio* NP001137376.1 and IRF-7: *Homo sapiens sapiens* AA136556.1, *Mus musculus* NP058546.1, *Salmo salar* NP001130020.1, *Carassius auratus* AAO18646.1).

Alignments were performed using Clustal W (Thompson et al. 1997) including in MEGA 4 (Tamura et al. 2007). A phylogenetic tree based on amino acid sequences was built using the Neighbour-Joining (Saitou and Nei, 1987) algorithm with the MEGA 4 software program. Statistical confidence on the inferred phylogenic relationships was assessed by bootstrap of 1000 replicates.

## 2.7. Expression analysis of the selected ESTs by quantitative real-time PCR

Total RNA was extracted using TRIZOL reagent (Invitrogen) following the manufacturer's instructions. Briefly, cells were lysed in TRIZOL, RNA was separated from DNA and proteins using phenol chloroform. Total RNAs were precipitated using isopropanol. The RNA pellet was washed with 75% ethanol solution and finally eluted in 22 µl of DEPC water. The RNA concentration was determined using a spectrophotometer at 260 nm and RNA quality was assessed on a 1% agarose gel. Total RNAs were treated with RQ1 RNAse-free DNAse (Promega) to remove remaining genomic DNA.
First strand cDNA was synthesized using the oligo(dT) anchor primer (5'-GAC CACGCGTATCGATGTCGACT(16)V-3') and Moloney murine leukaemia virus (M-MLV) reverse transcriptase SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen).
For each of the selected genes, forward and reverse primers were designed using primer 3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) (Table 1). Real-time quantitative PCR reactions were duplicated and performed in a total volume of 25 µL using a Mx3000 Thermocycler sequence detector (Stratagene) in 96-microwell plates. Each well (25 µL) contained 5 µL of cDNA dilution (1/30), 12.5 µL of Brilliant® SYBR® Green II PCR Master Mix (Stratagene), 2.5 µL of each diluted primer (3µM) and 2.5 µL of distilled water. Thermal cycling conditions were: 1 cycle of

activation of Hot start Taq polymerase at 95 °C for 10 min; 40 cycles of amplification at 95°C for 10 s, 60°C for 30 s; and melting temperature curve analysis at 95°C to 60°C by 0.5°C decrease of the temperature every 10 s. In all cases, negative controls (without cDNA) were included to rule out DNA contamination.
For each candidate gene, melting curve and gel picture were analysed in order to verify the specificity of the amplified products and that at a single PCR product was amplified. PCR efficacy (E) was calculated for each primer pair by determining the slopes of standard curves. These curves were generated using a serial dilutions analysis of plasmid containing the insert of interest. Amplification efficacies were calculated according to the following equation $E=10^{(1-slope)}$ [26].
The expression of the candidate genes was normalized using the elongation factor 1 alpha (EU651798) as housekeeping gene (Morga et al. unpublished data) and haemocytes alone were used as calibrator. Samples were analysed in replicate. Primers are shown in Table 1. Fold units were calculated using the method described by (Pfall, 2001).

# 3. Results

## 3.1. ESTs sequencing identification

The SSH technique was used to identify transcripts differentially expressed between haemocytes in contact with parasites and haemocytes alone. 864 clones were sequenced and 809 sequences (93% of the sequenced clones) were obtained and analysed (Table 2). These sequences ranging from approximately 175 to 600 bp in length were analysed to evaluate redundancy by multiple alignments using the Lasergene software. Finally, 330 unique ESTs were identified including 56 contigs and 274 singletons. Among these, 224 ESTs showed similarities with genes available in databases with an E value < $10^{-4}$ and clustered in 8 main categories according to their putative functions predicted by NCBI using GO (Gene Ontology) (Fig. 1): (1) detoxification, (2) cell communication, membrane receptor, immune system, (3) ribosomal protein, (4) cystoskeleton structure, (5) respiratory chain, (6) cell cycle, DNA repair, protein regulation and transcription, (7) cell metabolism and (8) hypothetical protein.

## 3.2. Identification and characterization of Ostrea edulis immune-related genes

### 3.2.1. Galectin (Oegal)

*Ostrea edulis* cDNA galectin (Oegal) shows a 1394 nucleotide (nt) sequence. The complete sequence was deposited in GenBank and assigned under the accession number GU320697. The sequence contains a short 5'-untranslated region (1-95 nt) followed by an ORF of 1103 nt (335 codons: 96-1103 nt) and finished by a 3'-untranslated region (1104-1394 nt) with a polyadenylation signal (AATAAA) starting 17bp upstream from the polyA tail (Fig. 2). No signal and extension fragment were identified, indicating that the deduced ORF corresponding to a mature protein. The complete ORF has a putative molecular mass at 36 947.07 Da and a predicted isoelectric point at 8.93. The analysis of the ORF revealed the presence of two homologous carbohydrate recognition domains (CRD), characteristic of tandem-repeat type galectin. These CRDs, CRD1 and CRD2, consist of 121 and 134 amino acids (Fig. 2) respectively.
The analysis of the CRD1 and CRD2 allowed the identification of 8 highly conserved amino acid (aa) within each CRD. These aa are known to be involved in sugar galactosyl-binding activity in invertebrate tandem-repeat type galectins (Pace et al. 2002 and Huang et al. 2007) (Fig. 2).
Phylogenic tree analysis of vertebrate and invertebrate dual-CRD or tandem-repeat type galectins grouped Oegal within the metazoan invertebrate clade and showed closest affinity to other molluscs, including pearl oyster *Pinctada fucata*, clam *Venerupis phillipinarum*, abalone *Haliotis discus hannai* and snail *Biomphalaria gabralata* (Fig. 3).

### 3.2.2. Interferon regulatory factor like (OeIRF)

*Ostrea edulis* cDNA interferon regulatory factor like (OeIRF) is composed of 1738 nucleotides (nt). The complete sequence was deposited in GenBank and assigned under the accession number GU320698. The sequence contains a short 5'-untranslated region (1-95 nt) followed by an ORF of 1076 nt (326 codons: 96-1076 nt) and finished by a 3'-untranslated region (1077-1738 nt) with a polyadenylation signal (AATAAA) starting 13 bp upstream from the polyA tail (Fig. 4). No signal and extension fragment were identified, indicating that the deduced ORF corresponding to a mature

protein. The complete ORF shows a molecular mass at 37208.09 Da and predicted isoelectric point at 5.32.
The analysis of the ORF revealed the presence of an IRF superfamilly domain, including five conserved tryptophans in the DNA binding region (Fig. 4).
Phylogenic tree analysis showed that OeIRF is closer to IRF1 and 2 than to other IRFs (Fig. 5).

### 3.3. ESTs expression patterns in haemocytes infected with live or dead Bonamia ostreae

Relative expression of the selected ESTs (Table 3) was measured in haemocytes infected with live parasites and in haemocytes infected with dead parasites. Expression levels in both conditions were normalized using haemocyte alone.
Live parasites induced a significant increase of the expression levels of OGST (1.8), SOD (3.4), TIMP (1.9), OeGal (2.8), OeIRF (4), cytochrome oxydase III (1.7) and filamin (51) (Fig. 6). Expression levels of HSP 90 and cytochrome p450 were not significantly affected by the presence of the live parasites while tetraspanin (0.5) appeared down regulated in the same condition.
Presence of dead parasites induced a significant increase of the expression of parasite cytochrome p450 (2.1) and, OeIRF (1.7) and a down regulation of OeGal (0.3) and OGST (0.4) (Fig. 6).

## 4. Discussion

Diseases are one of the major risks affecting the mollusc production worldwide and the immune responses of bivalve hosts against pathogens are often scarcely understood. However, some studies have been carried out to better understand host/pathogen interactions at a molecular level in different bivalve species including *Crassostrea gigas* (Gueguen et al. 2003), *Crassostrea virginica* (Tanguy et al. 2004), *Saccostrea glomerata* (Green et al. 2009), *Ruditapes phillipinarum* (Kang et al. 2006), *Mytilus galloprovincialis* (Pallavicini et al. 2008 and Costa et al. 2009) and *Ruditapes decussatus* (Prado-alvarez et al. 2009).
The suppression subtractive hybridization method (SSH) is a PCR-based technique that allows the identification of genes differentially expressed in response to biotic or abiotic stimuli. This method is currently used to identify genes involved in different molecular mechanisms in a large variety of species including marine invertebrates (Brown et al. 2006 ; Gestal et al. 2007 ; Meistertzheim et al. 2007 and Wang et al. 2008).
The present work constitutes the first study on *Ostrea edulis* responses to the parasite *Bonamia ostreae* based on a molecular approach, i.e. SSH. Application of this method allowed the identification of 864 ESTs differentially expressed between haemocytes alone and haemocytes infected with *B. ostreae*. Among the ESTs presenting a significant homology in Genbank, nine ESTs were selected according to their putative biological function.
The expression pattern of these ESTs was measured and compared between haemocytes in contact with live and dead parasites in order to identify the modulation specifically induced by an *in vitro* infection with *B. ostreae*. Real-time PCR analyses confirmed the differential expression of seven out of the nine tested ESTs (more than one fold difference) between the two tested conditions and the normalisator of this experiment, i.e haemocytes alone.
In our study, the putative tetraspanin gene appeared down regulated in haemocytes in contact with live parasites. On the contrary, tetraspanin was found up-regulated in response to the parasite *Perkinsus marinus* 45 days after inoculation in *C. virginica* and *C. gigas* (Tanguy et al. 2004). Members of the tetraspanin family are involved in various biological processes such as cell adhesion, migration, cell fusion, co-stimulation, signal transduction, immune system and differentiation (Boucheix and Rubinstein, 2001; Hemler, 2005 and Levy and Shoham, 2005); however, their precise function remains unknown.
The putative TIMP gene was found significantly more expressed in haemocytes exposed to live parasites in comparison with haemocytes exposed to killed parasites. Protease inhibitors such as TIMP were found over expressed in the context of infection with *Schistosoma mansoni* and *Vibrio splendidus* (Labreuche et al. 2006 and Guillou et al. 2007). *B. ostreae* like other mollusc parasites may produce proteases (Faisal et al. 1999 and Ordás et al. 2001), which may contribute to modulate the immune response of the host (Hervio et al. 1991).

The over expression of O-GST and SOD genes in presence of live parasites might be related to an increase of cytotoxic components generated during an immune response as shown by (Guillou et al. 2007) in *Biomphalaria gabralata*. SOD is known to be involved in the oxidative stress response.

Glutathione S-transferases play important role in the detoxification of endogenous and exogenous toxic compounds during an immune response (Brophy and Pritchard, 1992 and Brophy and Pritchard, 1994). A previous study showed a significant decrease of reactive oxygen species (ROS) production in *O. edulis* haemocytes after an *in vitro* infection of *O. edulis* haemocytes with live parasites versus haemocytes alone (Morga et al. 2009). These results suggest that *B. ostreae* has inhibitory effects on ROS production through the activation of genes involved in detoxification.
The putative filamin EST, a gene related to the cytoskeloton structure, was over expressed in haemocytes in contact with live parasites compared to haemocytes in contact with dead parasites. The cytoskeleton plays a central role in many cell functions such as the maintenance of cell shape, cell division, adhesion, motility, signal transduction and protein sorting. Filamins are a family of high molecular mass cytoskeletal proteins that crosslink cortical actin into a dynamic three-dimensional structure and interact with a large number of cellular proteins of great functional diversity, suggesting that they are unusually versatile signalling scaffolds **(**Feng and Walsh, 2004). The filamin over expression observed in haemocytes in contact with live parasites suggests an increase of cytoskeleton polymerisation which may result in an increased parasite internalisation. These results support the hypothesis expressed by Chagot et al., (1992). These authors demonstrated that *B. ostreae* was not engulfed when haemocytes were treated with cytochalasin B prior to parasite contact suggesting an active participation of host cells in the internalisation of the parasite. Similar results were reported by Gonzales et al. (2009) for *Toxoplasma gondii* tachyzoites and *Plasmodium berghei* sporozoites.

For the first time, we detected and fully characterized a member of the IRF family in a mollusc species. The IRF family includes nine members which regulate IFN-$\alpha/\beta$ production (De Maeyer and De Maeyer-Guignard, 1988). IRFs are generally associated with the immune response against viral infectious (Collet et al. 2003). However, they have functionally diverse roles in the regulation of the immune system particularly through Toll-like receptors and other pattern-recognition receptors (Honda and Taniguchi, 2006). In the present study, the putative OeIRF was over expressed in haemocytes exposed to live parasites. This over expression of OeIRF could translate a response of the flat oyster against the infection with parasite *B. ostreae*.
A member of the galectin family was also identified and fully characterized. Lectins act as pattern recognition receptors (PRRs) (Medzhitov and Janeway, 2002), a group of diverse, soluble and membrane-associated molecules such as Toll like receptors (TLRs), nucleotide-binding oligodimerization domains (NODs) and NK cell receptors. Galectins are able to bind glycans present on the surface of microorganisms and favour the entrance of the pathogens inside the cell. A wide range of galectin types was described in mammals (Cooper 2002). The galectin presently identified in *O. edulis* (OeGal) exhibits two CRDs joined by a linker peptide and appears closed to galectin 4 of mammals. A similar galectin was described in *Biomphalaria gabralata* (Yoshino et al. 2008) and *Pinctada fucata*. Recently, a four repeat CDRs galectin was identified in *C. virginica* (Tasumi and Vasta, 2007). These authors investigated the role of the galectin during the interactions between oyster haemocytes and the intracellular parasite *P. marinus*. *C. virginica* galectin bound bacteria, phytoplankton components and preferentially *Perkinsus spp*. trophozoites. These results suggested the direct role of the galectin in the recognition and opsonisation of potential microbial pathogens and algal food. Similar results were obtained for the tandem repeat CRDs in *B. gabralata,* the intermediate host of *S. mansoni* (Yoshino et al. 2008). In our study, OeGal expression appeared up regulated in presence of live parasites and down regulated with dead parasites. OeGal seems thus involved in the recognition of the parasite *B. ostreae*. In a previous study, phagocytosis of *B. ostreae* was not affected by the prior incubation of haemocytes with sugar (mannose, glucose, fucose, N-acethyl glucosamine and galactosamine) while a similar parasite treatment induced a significant decrease of *in vitro* infection (Chagot 1989). These results suggested that haemocyte surface lectins do not play a key role in the biding of the parasite while parasite lectins are involved as receptor or ligand during the infection process. Ours results differ from the conclusion of this previous study and suggest an involvement of the haemocyte lectins in the infection with *B. ostreae*.
The present study is the first transcriptomic investigation of interactions between haemocytes from *O. edulis* and the parasite *B. ostreae*. This work has generated new data concerning the host genome and contributes to a better understanding of the relationships between host and parasite. The analysis of expression level by real time PCR has brought new insight concerning the molecular mechanisms expressed by the haemocyte in response to the parasite internalisation. OeGal seems to be involved in the recognition of *B. ostreae* by the haemocytes. In addition the parasite seems to interact with the expression of host genes related to detoxification and immune response including SOD, OGST, TIMP and IRF-like. However, these results require further investigations.

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# Figure captions

Figure 1 Functional classification of the sequences identified in both libraries (ESTs). Genes were clustered into 8 categories according to their putative biological function. A=forward bank and B=reverse bank

Figure 2 Complete ORF nucleotide and deduced amino acid sequences of the flat oyster OeGal. ORF contains two CRD, CRD1 (galect) (96-515 nt) in red, CRD2 (galect) (675-1089 nt) in yellow. Amino acid involved in sugar galactosyl-binding activity are boxed.

Figure 3 Neighbour-joining tree showing phylogenetic analysis of galectin amino acid sequences from various species. The tree is based on 1000 bootstrap replications. The scale for branch length (0.1 substitutions/site) is shown below the tree.

Figure 4 Complete ORF nucleotide and deduced amino acid sequences of the flat oyster OeIRF. ORF contains a DNA binding domain including five conserved tryptophans (underlined in yellow) and polyadenylation signal (AATAAA) (underlined in grey).

Figure 5 Neighbour-joining tree showing phylogenetic analysis of IRF 1,2,3 and 7 amino acid sequences from various species. The tree is based on 1000 bootstrap replications. The scale for branch length (0.1 substitutions/site) is shown below the tree.

Figure 6 Relative expression by quantitative PCR of selected transcripts from SSH (Hsp 90, cytochrome p450, o-GST, SOD, TIMP, galectin, IRF, tetraspanin and fimanin) libraries. Expression levels were normalized to EF1-$\alpha$ and presented as relative expression to controls (mean ± SD, n = 2). * indicates significant differences of gene expression compared to controls

| Name | Oligonucleotide sequence (5'-3') | Concentration of Forward primer (μM) | Concentration of Reverse primer (μM) |
|---|---|---|---|
| Filamin | Forward: TGATTTAACCGACGGGAAAG<br>Reverse: CTTCATCACCGGTTTGTGG | 3 | 3 |
| TIMP | Forward: TTCGACGCATGTATGAAAGG<br>Reverse: TCTGGATTCAGGCCCCTA | 1.5 | 3 |
| OGST | Forward: GGTCGTCAGGGGTCAGTTT<br>Reverse: GGTTCCCGTTCTTGAGCA | 3 | 3 |
| CYTOP450 | Forward: GTCATCAAGCGAATGCGATA<br>Reverse: GGAGAGCTCCCTCATTTTCC | 3 | 3 |
| TETRA | Forward: TTCCATCCATTGCTGATTTG<br>Reverse: AGCTGAACTCTGCCGTGAAG | 3 | 3 |
| Hsp90 | Forward: TTTGTGGAACGGGTCAAAA<br>Reverse: AACGTCGAGCACAGTCGAG | 3 | 3 |
| OeGal | Forward: TCGGAGGTCGCCCTTAAT<br>Reverse: TTGCCGTGAACAATCAACA | 3 | 3 |
| SOD | Forward: TCGTCAATGTCAGCGTGAA<br>Reverse: AAATGTTGGGGCTGGTGA | 3 | 3 |
| OeIRF | Forward: GTGCAAGGTTTGGAGTGGTT<br>Reverse: GAAGAGGCTGGCATCTTTTG | 3 | 3 |
| ElongPCRQ5m | Forward:<br>GTCGCTCACAGAAGCTGTACC<br>Reverse: CCAGGGTGGTTCAAGATGAT | 3 | 3 |
| Topo F | GACCATGATTACGCCAAGC | | |
| Topo R | CCCAGTCACGACGTTG | | |

Table 1 Combinations of primers used in quantitative PCR assays

| | |
|---|---|
| Total number of subtracted clones | 2448 |
| Total number of clones sequenced | 864 |
| Sequences analysed | 809 |
| Total number of sequences with significant match | 226 |
| Contigs | 330 |
| Singletons | 274 |
| Redundancy | 40% |
| Average size number pb | 402 |

Table 2 General characteristics of the subtracted library and cDNA sequences from *Ostrea edulis* haemocytes.

| length | Putative homolog protein | Homolog species | e- value | Genbank Accession number |
|---|---|---|---|---|
| 361 | Cytochrome P450 | *Crassostrea gigas* | 1e-23 | ABR45717 |
| 360 | Tissue inhibitor of metalloproteinase | *Crassostrea gigas* | 3e-40 | AAW52530 |
| 475 | Omega class glutathione S-transferase | *Crassostrea gigas* | 6e-53 | CAD89618 |
| 360 | Tetraspanin | *Strongylocentrotus purpuratus* | 1e-5 | ABE27957 |
| 614 | Interferon regulatory factor | *Paralichthys olivaceus* | 2e-22 | BAA83468 |
| 359 | Heat shock protein 90 | *Crassostrea gigas* | 2e-33 | ABS18268 |
| 376 | Galectin 4-like protein transcript variant | Haliotis discus hannai | 1e-21 | ABN54798 |
| 324 | Cu/Zn superoxide dismutase | 6.1.1. Crassostrea ariakensis | 2e-54 | ABF14366 |
| 299 | Filamin | *Hirudo medicinalis* | 4e-20 | AAR36862 |

Table 3 Candidate ESTs selected for expression level analysis by quantitative real time PCR

Fig.1

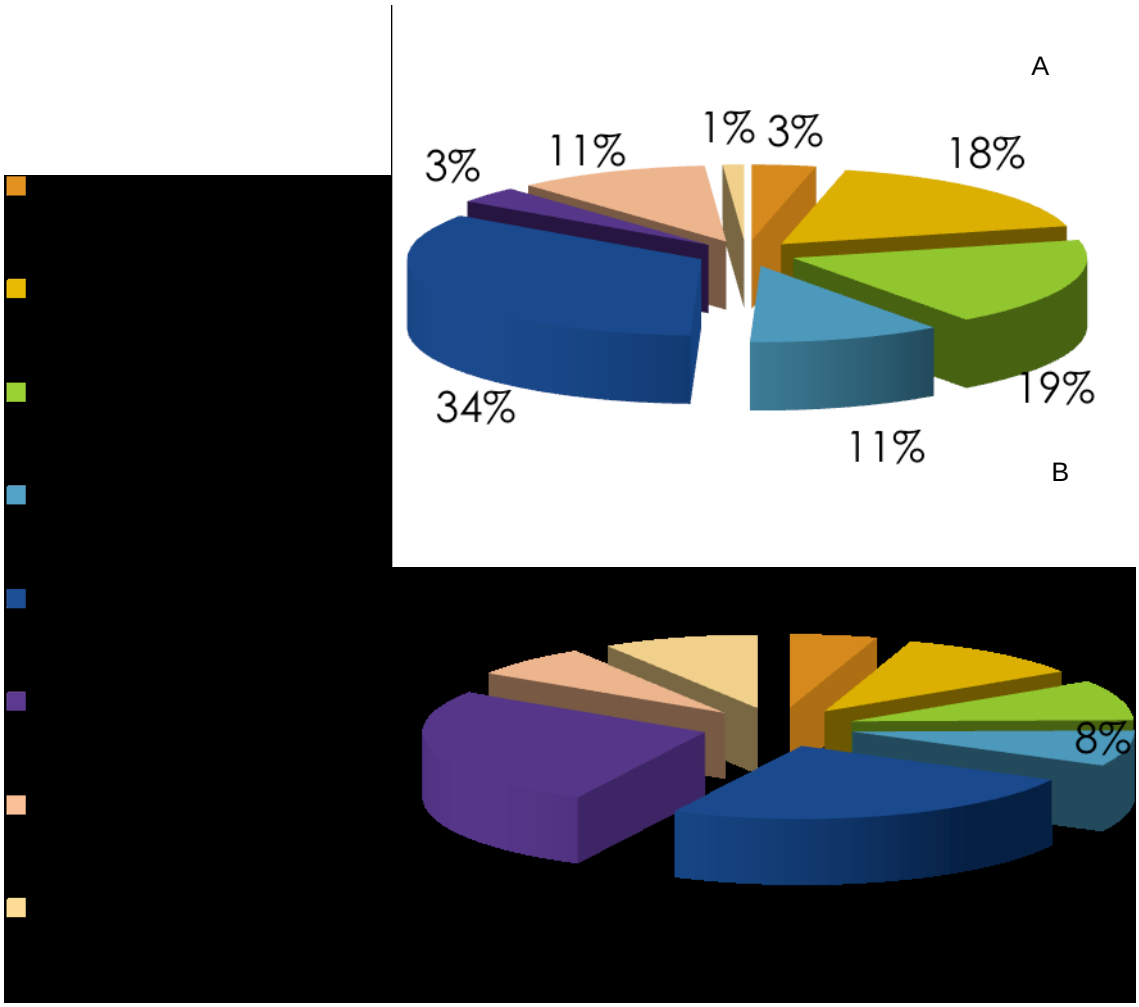


Figure 1 Functional classification of the sequences identified in both libraries (ESTs). Genes were clustered into 8 categories according to their putative biological function. A=forward bank and B=reverse bank

Fig.2

```
TCGGGGATAAGTTGGAGTGTGTGAATATTTGATAAATACCTG
CAACACAGAGCGCAGCCCTCATTTCCTTCTTCGCTGTGAAGTGAAACT
93     ATC ATG GCT ACT ATA ATG AGC CCA GGC ATT CCC TAC GTG GGA GGA
1           M   A   T   I   M   S   P   G   I   P   Y   V   G   G

138    ATC CCC GGG GGT ATG AGA GAC GGA CGC CAA ATC GTC ATC AAC GGA
45      I   P   G   G   M   R   D   G   R   Q   I   V   I   N   G

183    ATG GTG CCC CAC CAC GAG CAC AGT TTT TCG ATC AAC CTC CAA GCC
60      M   V   P   H   H   E   H   S   F   S   I   N   L   Q   A

228    GGA CCA AAC ATT AAC CCT CGG TCC AAC ACA GCT CTA CAT TTC AAC
75      G   P   N   I   N   P   R   S   N   T   A   L   H   F   N

273    CCT CGC CCG AAC GAC AAC TGT GTG GTC CGT AAT AGC TAC CAG CAC
90      P   R   P   N   D   N   C   V   V   R   N   S   Y   Q   H

318    CAC AGC TGG GGA GGG GAA GAG CGA GGA GGG TAC ATG CCG TTC CAG
105     H   S   W   G   G   E   E   R   G   G   Y   M   P   F   Q

363    AGG GGA ATG CCT TTC GAA ATA ACT GTA CTC TGC CAA CAC CAC CAT
120     R   G   M   P   F   E   I   T   V   L   C   Q   H   H   H

408    TAT AAG GTA TCA GTG AAT GGC AGA CAT TTC TGC GAT TTT CGC CAT
135     Y   K   V   S   V   N   G   R   H   F   C   D   F   R   H

453    AGA ATC GAG AAA CAT CAC GTG AAC ACG TTG ACG ATT GAG GGC GGA
150     R   I   E   K   H   H   V   N   T   L   T   I   E   G   G

498    GTA CAG ATC AGC AGT ATT CGA TTC GAT GGA GCT CAA GGT CAC GGA
165     V   Q   I   S   S   I   R   F   D   G   A   Q   G   H   G

543    CAG GGC GTA GGG GGC TTT CCT GGT CGA GTT GTT GGA GAG ATG ACG
180     Q   G   V   G   G   F   P   G   R   V   V   G   E   M   T

588    AAG GCT GCC ATG CCC ACA CCA TCT CCA CCA GCT GGG GCC TAC CCT
195     K   A   A   M   P   T   P   S   P   P   A   G   A   Y   P

633    CCG GCC GGG GGG TAC CCA GGA GGG GGA GCC CAA CCC ATG TAT AAT
210     P   A   G   G   Y   P   G   G   G   A   Q   P   M   Y   N

678    CCC CCG ATG CCA TTT ACC ACG CCC ATT TCT GGA GGA ATT TTT CCA
225     P   P   M   P   F   T   T   P   I   S   G   G   I   F   P

723    GGG AAA ATG ATT TTC ATC AGT GGA GTC CCA AAT CCG AAT GCA GAA
240     G   K   M   I   F   I   S   G   V   P   N   P   N   A   E

768    AGG TTC ACG TTG AAC CTA ATG TGT GGT CCC TAC GAC GGA AGT GAC
255     R   F   T   L   N   L   M   C   G   P   Y   D   G   S   D

813    ATT GCG CTT CAT TGT GAT GTC CGT CTG CGA GTT GGA GGT GAT TTT
270     I   A   L   H   C   D   V   R   L   R   V   G   G   D   F

858    AAT GTC GTC CTG AGA AAT TCC TGC CAG GGT GGT GGC TGG GGA GCA
285     N   V   V   L   R   N   S   C   Q   G   G   G   W   G   A

903    GAA GAG AGA CAC AGC CCA TAC TTT CCT TTC ATG CCA AAT GCC AAT
300     E   E   R   H   S   P   Y   F   P   F   M   P   N   A   N

948    TTT GAC ATG ATT ATC ATG GCA GAA CAT GAC AAA TTC AAG ATT GCC
315     F   D   M   I   I   M   A   E   H   D   K   F   K   I   A

993    GTG AAC AAT CAA CAC TTG CTC GAG TTT AGA CAT CGA CTG CAG CCA
330     V   N   N   Q   H   L   L   E   F   R   H   R   L   Q   P
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1038  CTA AAC AGA ATC GAC ACT CTA CAG ATT AAG GGC GAC CTC CGA CTG
345    L   N   R   I   D   T   L   Q   I   K   G   D   L   R   L

1083  ACT CAA GTC CGA TTC CAA TAA ATT TAT ATG TCA TTA TCA TCA TAC
360    T   Q   V   R   F   Q   *
1128  ATTCATACAAGAAACAAAATGCAGAGCAAATAATTGGTTAAAGTA
1173  TTGTACATTTGTATTTTTGTTGAAACAAATGATTATTTAATCTCT
1218  CTCTTTCACTCATATCAACATATGATATGGTACTTTCCTGCTATT
1263  TCAATACGATTATACAAATATCACTGGATAACCCAGGCAATAAAA
1308  ACACTAAGTGTATTTTAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Figure 2 Complete ORF nucleotide and deduced amino acid sequences of the flat oyster OeGal. ORF contains two CRD, CRD1 (galect) (96-515 nt) in red, CRD2 (galect) (675-1089 nt) in yellow. Amino acid involved in sugar galactosyl-binding activity are boxed.

Fig.3

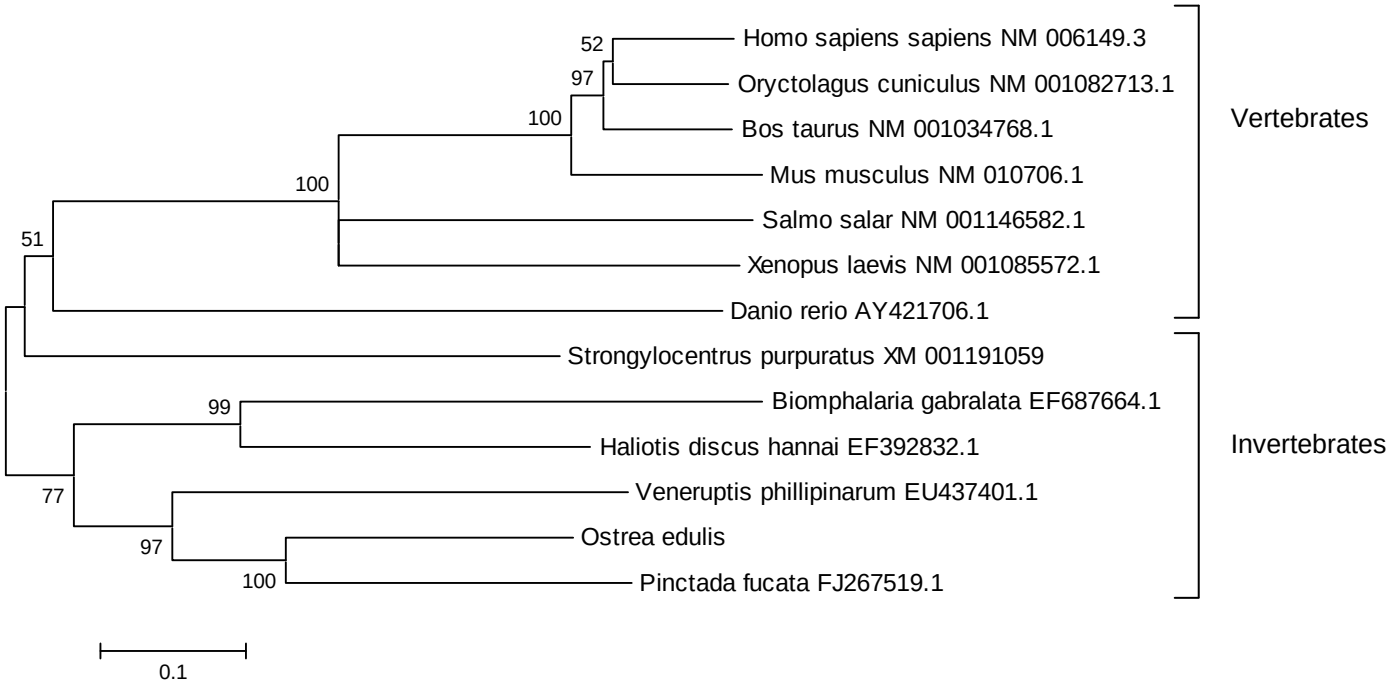


Figure 3 Neighbour-joining tree showing phylogenetic analysis of galectin amino acid sequences from various species. The tree is based on 1000 bootstrap replications. The scale for branch length (0.1 substitutions/site) is shown below the tree.

Fig.4

```
AAGTAGTGAGTGCCGGGCGTTGAGGAGGACGGACTATAGTACTGTCTCGATCATTAACATTTGTGTTTAC
TGTGTTTCCAAGGAAAACTA
91     TCA ATG GGA ATG GAC AAT ATT AGA CAG ACA GGC AGA ATA ATG ACG
1       M   G   M   D   N   I   R   Q   T   G   R   I   M   T

138    AAA AAA CGT CCG GTG GAG AGG CAG AAG ATG CGG CCG TGG ATC CAG
15      K   K   R   P   V   E   R   Q   K   M   R   P   W   I   Q

183    GAC ATG TTG GAC AAT GGG GGT GTG CAA GGT TTG GAG TGG TTC GAT
30      D   M   L   D   N   G   G   V   Q   G   L   E   W   F   D

228    AAA TCG CAG AAT CTC TTT AGA ATT AAC TGG AAG CAT GGG TCC AGA
45      K   S   Q   N   L   F   R   I   N   W   K   H   G   S   R

273    CAC GGG TTC AAT ACC ACA AAA GAT GCC AGC CTC TTC GAG AAA TAC
60      H   G   F   N   T   T   K   D   A   S   L   F   E   K   Y

318    GCC CAA CAC ACA GGA CGT TGG GAT CCG AAT GAT CTA AAC CCC AAA
75      A   Q   H   T   G   R   W   D   P   N   D   L   N   P   K

363    AAA TGG AAG GCA AAT TTC CGA TGC GCC CTG AAC AGC CTG CAA AAC
90      K   W   K   A   N   F   R   C   A   L   N   S   L   Q   N

408    GTC ATG GAG GTG AAA AAC CTG GGT GAG AGC AAA GGT GCC CAT GCG
105     V   M   E   V   K   N   L   G   E   S   K   G   A   H   A

453    TAC CGC GTT TAC CAA TTT TTG CTG GAG GAG GAA ACA AAA CCA AAA
120     Y   R   V   Y   Q   F   L   L   E   E   E   T   K   P   K

498    GAT GGG CAT CAA AGA AAA CAC AAT AAA AAG AAA CCA GAC AAA GGA
135     D   G   H   Q   R   K   H   N   K   K   K   P   D   K   G

543    AAA TCA AAA AAA CTT TGC AAA TTT GAT GAT GCA ACC GAC GAA GAG
150     K   S   K   K   L   C   K   F   D   D   A   T   D   E   E

588    CCT GAA AAA GAG ACA TTG GAA ATA AGT AAA CAA GAA ATC GAC GAA
165     P   E   K   E   T   L   E   I   S   K   Q   E   I   D   E

633    CCA ATG CCC GCA TCG GAT GAA GAG GCG GCG GCC CCA CAA CAG GAC
180     P   M   P   A   S   D   E   E   A   A   A   P   Q   Q   D

678    ACC CCC TTA CTT ACC AAG CGA CAG GTC GAT TGC TAT ACA TAT CAG
195     T   P   L   L   T   K   R   Q   V   D   C   Y   T   Y   Q

723    CAC CAT ATG GCC GGA TGC ATT GTC ATA CAG AGC CCA AGA AAG AGG
210     H   H   M   A   G   C   I   V   I   Q   S   P   R   K   R

768    CCA GCG CCA TAC TCA ACC GAG AAA GAT GAT TGT GAA GAT CCG GTG
225     P   A   P   Y   S   T   E   K   D   D   C   E   D   P   V

813    GAG ATG ACG TCA TTA GAA GAC GCC CAG CTG ATG GCA CAC GCT GCA
240     E   M   T   S   L   E   D   A   Q   L   M   A   H   A   A

858    AAG CGA AAC AGA AGT ACC GAT GAT GAC GAA AGT TTG TCG TAT TCG
255     K   R   N   R   S   T   D   D   D   E   S   L   S   Y   S

903    CAG CTG TCT GTG ACT GAC GAA TCG TCA AAC ATC TCG TGC ACT TCA
270     Q   L   S   V   T   D   E   S   S   N   I   S   C   T   S

948    GAT TCC AGT TCC TCG AGT CCA GGA GAA GAG ACA GAC AAC GTA CCG
285     D   S   S   S   S   S   P   G   E   E   T   D   N   V   P

993    CAT TTC TCC AAT CTT TTA TCA GAC AAT ACG GAT GGT GAT TGG GTT
300     H   F   S   N   L   L   S   D   N   T   D   G   D   W   V
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```
1038  ATT GAA AGC GAG GAA ACT GTA ACT ACA ACC GAC TCC TGA ACG AAC
315    I   E   S   E   E   T   V   T   T   T   D   S   *

CACTACACAAAAGTGTAACTGAATACCGCAAAAATAATGACAGTAATTAACGGCGTCTACATCAATACGATGACCGCGATCAA
CAATGCATACAGCAAGACAATGACAGTGATTAGCATTCTCTACAGCTAGACAATGACAGTGATTAGCATTCTCTACAGCTAGA
CAATGACAGTGATTAGCATTCTCTACAGCTAGACAATGACAGTGATTAGCGATGTCTACCGATAGACAACTGTGATTAGCGAT
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TATATAGTATAATTCTTTGTGTCTACATTTGCTGACTAATAGCAATGGGCAAAAAAAGAAATGTTTTTACAACCTTCGTTTTA
CATTGCACATGCATGTTCATTTATCACCCTTTTGATATTAATCATGTTTATATAAAAACAAATCGTTAACTCACCACATTGAT
TTTACTTTGTCAAAGTTCACTGCAGTGTATATAGTGCTTTGCATGATATTTTATTGTTTTTATATGCGTTTCTCAATAAATTA
TTTACTGTTGAATCATTGAATAAAAAAGCAGAGCTTTAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Figure 4 Complete ORF nucleotide and deduced amino acid sequences of the flat oyster OeIRF. ORF contains a DNA binding domain including five conserved tryptophans (underlined in yellow) and polyadenylation signal (AATAAA) (underlined in grey).

Fig.5

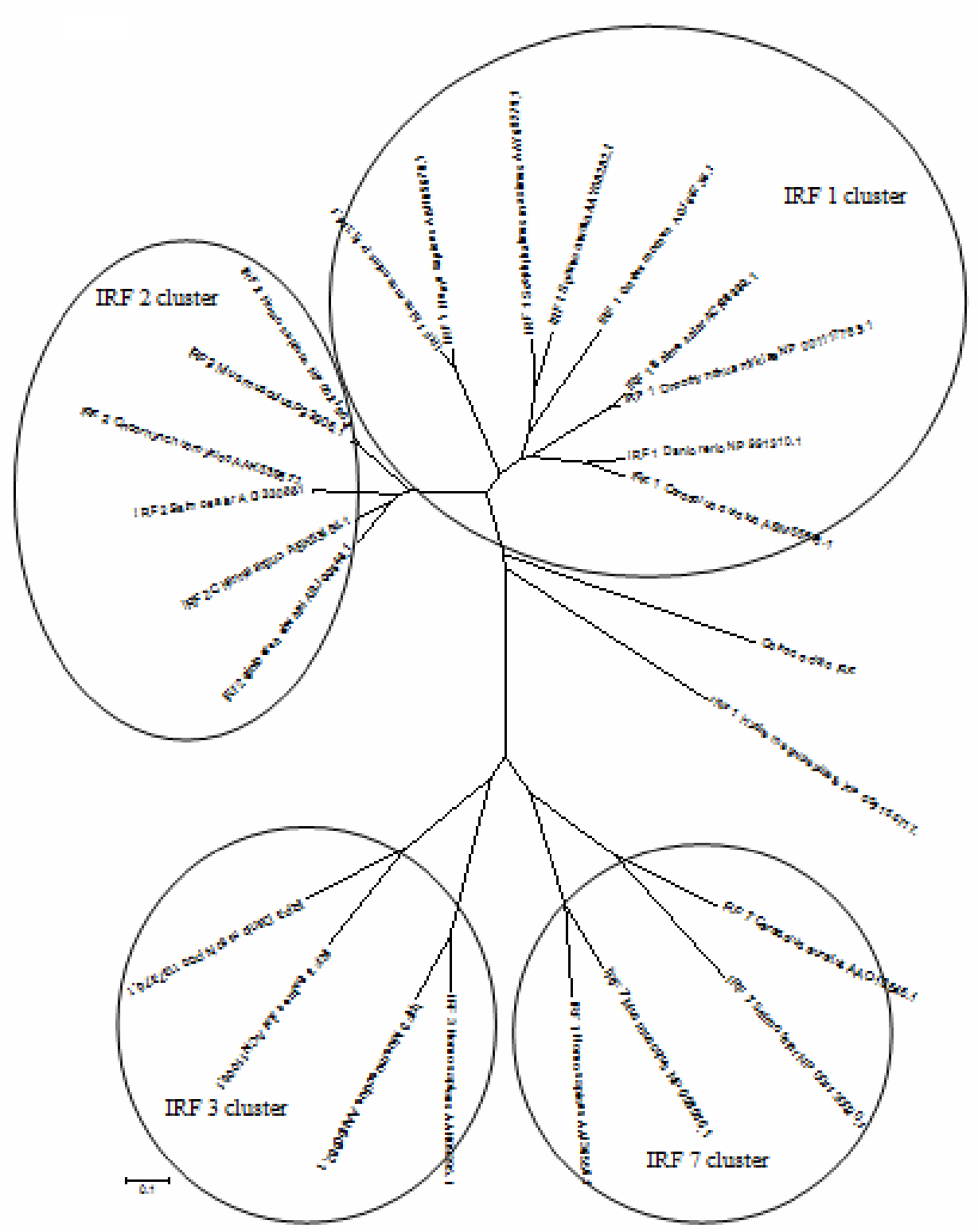


Figure 5 Neighbour-joining tree showing phylogenetic analysis of IRF 1,2,3 and 7 amino acid sequences from various species. The tree is based on 1000 bootstrap replications. The scale for branch length (0.1 substitutions/site) is shown below the tree.

Fig.6

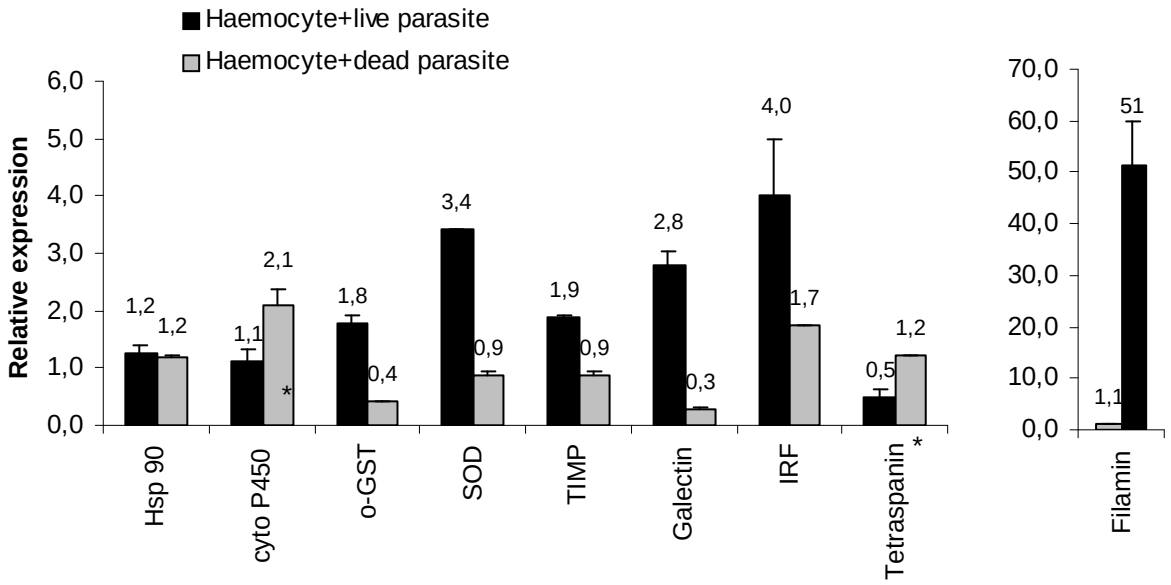


Figure 6 Relative expression by quantitative PCR of selected transcripts from SSH (Hsp 90, cytochrome p450, o-GST, SOD, TIMP, galectin, IRF, tetraspanin and fimanin) libraries. Expression levels were normalized to EF1-$\alpha$ and presented as relative expression to controls (mean ± SD, n = 2). * indicates significant differences of gene expression compared to controls