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Heat Shock Protein 90 of *Bonamia ostreae*: Characterization and Possible Correlation with Infection of the Flat Oyster, *Ostrea edulis*

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Abstract :

In this study, we described the cytosolic HSP90 of *Bonamia ostreae*, an intracellular parasite of *Ostrea edulis* hemocytes. The complete open reading frame was assembled by Rapid Amplification cDNA Ends reactions on cDNA of *B. ostreae*-infected hemocytes. *HSP90* amplification was corroborated in infected oysters and *B. ostreae* purified cells. The functionality of the HSP90, studied by inhibitory assays with radicicol, suggests that this protein may play a role in hemocyte invasion. Our results inform the molecular basis that governs *B. ostreae*–*O. edulis* interactions.

Keywords : Bivalve hemocytes ; Gene ; Haplosporidia ; Pathogen ; Protist ; radicicol

Bonamia ostreae (Haplosporidia) is a parasitic protist that has caused important oyster production losses during last decades (Pichot et al. 1979). Its natural host is the European flat oyster *Ostrea edulis* and it has been reported in Europe and in the East coast of North America (Bucke et al. 1984; Elston et al. 1986; McArdle et al. 1991; Montes 1990; Narcisi et al. 2010; Van Banning 1985). *Bonamia ostreae* parasitizes larvae, seeds, juveniles and adult organisms. However oysters older than 2 years are more susceptible to the infection (Arzul et al. 2011; Cáceres-Martínez et al. 1983; Culloty and Mulcahy 1996). Although *B. ostreae* is considered an intrahaemocytic parasite it can be found extracellularly on gills and in connective tissue (Comps et al. 1980; Montes et al. 1994; Van Banning 1990). The complete life cycle of the parasite is not known. However, it seems that intermediate hosts are not needed since infected oysters can transmit the disease to healthy organisms under experimental conditions (Elston et al. 1986). One of the main inconveniences in the study of this parasite is the absence of optimal conditions for maintaining an in vitro cell culture. To date, *B. ostreae* cells can only be obtained by a process of purification from heavily infected oysters (Mialhe et al. 1988). Purified *B. ostreae* has been used to study its effect on the immune system of the oyster *O. edulis*. Both in vitro contact with haemocytes and inoculation to healthy oysters revealed that the parasite modifies the immune status of the host to induce the up-regulation of immune related genes and the activation of cellular parameters (Comesaña et al. 2012; Morga et al. 2009; Morga et al. 2011a, b). However, the process of infection and the parasite mechanisms to evade host defenses still remain unclear. Although parasite genomic data could help to better understand these mechanisms, genomes of Haplosporidia are poorly documented. Actin is currently the typical functional gene described in these protists that has been used in phylogenetic

studies (Burki et al. 2010; López-Flores et al. 2007). Among protists that parasitize vertebrates, the heat shock protein 90 (HSP90) has been one of those most commonly studied. HSP90 is a molecular chaperone involved in numerous biological processes including folding and stabilization of many proteins such as kinases, transcription factors and signalling molecules (Hahn 2009; Pratt and Toft 2003; Yahara 1998).

From an evolutionary point of view, heat shock proteins are considered very useful in phylogenetic studies because they are conserved throughout prokaryotes and eukaryotes (Chen et al. 2006; Fast et al. 2002). Higher eukaryotes have two cytosolic HSP90 isoforms that are inducible, and three organelle-specific HSP90 in chloroplasts, mitochondria and the endoplasmic reticulum. Parasite HSP90 proteins are involved in the development of infective stages and cell invasion by *Leishmania*, *Plasmodium*, *Toxoplasma* and *Eimeria* (Ahn et al. 2003; Banumathy et al. 2003; Echeverria et al. 2005; Péroval et al. 2006; Wiesgigl and Clos 2001). Due to its involvement in infective processes, HSP90 has been proposed as a good target for therapy (Neckers and Tatu 2008).

In the present work, the complete *B. ostreae HSP90* gene has been amplified and characterized from the cDNA of infected *O. edulis* haemocytes. *HSP90* detection has been tested in a set of infected and non-infected samples as well as in purified parasites. The involvement of this protein in the infection process of *O. edulis* haemocytes has also been investigated through in vitro experiments.

MATERIALS AND METHODS

Animals and haemolymph. *Ostrea edulis* oysters were collected in Quiberon Bay (Southern Brittany, France) and maintained in quarantine facilities consisting of an open circuit with 1-µm filtered sea water (FSW) supplemented with *Skeletonema costatum*, *Isochrisis galbana*, *Chaetoceros gracialis* and *Tetraselmis suecica*.

Haemolymph was withdrawn using sterile syringes and needles after drilling a hole in the valve. Individual haemolymph samples were filtered through a mesh of 75 µm and maintained on ice to avoid cell aggregation. Cell counts were performed using a Malassez chamber.

Parasite purification. Infected oysters were selected after light microscopic visualization of *B. ostreae* in heart imprints. The degree of infection was assigned as described before (Robert et al. 2009). Parasites were purified from highly infected oysters following Mialhe et al. (1988) with slight modifications: the 40%-20% sucrose gradient was replaced by one of 60%-20%, and the Percoll gradient step was removed. Purified parasites were suspended in 0.22-µm FSW and counted on Malassez chamber.

Rapid Amplification of cDNA Ends (RACE). Expressed Sequence Tags (ESTs) with high similarity to parasite genes were previously identified in a Suppression Subtraction Hybridisation (SSH) of *B. ostreae* infected- and non-infected haemocytes (Morga et al. 2011b). Among these, an EST showing similarity with *Monosiga brevicolis* HSP90 (e-value 5e-38) was selected for further characterization.

The open reading frame (ORF) of the *HSP90* was completed using the SMART RACE cDNA Amplification kit (Clontech) on infected oyster cDNA synthesized from the SSH kit (Clontech). Sense and antisense primers were designed on the known sequence following manufacturer´s instructions (Table 1). Once the total ORF was fully characterized by RACE PCR, the pair of primers HSP-F and HSP-R was designed to amplify the sequence (Table 1). PCR reactions were performed in a volume of 50 µl containing PCR buffer (1X), 0.2 mM nucleotides, 0.5 µM of each primer, 7.5 U of Taq polymerase (New England Biolabs) and 1 µl of cDNA previously used for the RACE reactions. Thermal cycling was 94 ºC for 5 min, 35 cycles of 94 ºC for 1 min of denaturing, 58 ºC for 1 min of annealing and 72 ºC for 2.5 min of extension, followed

by a 10 min final extension at 72 ºC. The resulting product was subcloned in several fragments using internal primers (Table 1). The pairs of primers utilized and each corresponding annealing temperature utilized were: HSP-F/156-R (58 ºC), 156-F/HSPseq-R (60 ºC), HSP90-II3/HSPseq-R (60 ºC) and HSP90-II3/HSP-R (60 ºC). The total PCR product was diluted 1:10 in distilled water and used as template. The thermal cycle and the PCR mix were the same as described above.

Cloning and sequence analysis. PCR products were cloned using the TOPO TA Cloning kit (Invitrogen) and were transformed in Top 10F competent bacteria (Invitrogen). Inserts were analyzed by PCR using TOPO-F and TOPO-R primers (Table 1) before being sequenced from both ends with the same primers using a BigDye terminator Cycle Sequencing Ready Reaction kit and a 3100 Avant Genetic analyzer ABI Prism (Applied Biosystem). Raw chromatograms were analysed with Chromas 231 software (Technelysium). Sequence assembly, translation, multiple alignment and searches of homology were performed using ExPaSy tools (http://us.expasy.org/tools), and the GenBank database Blast algorithm (http://ncbi.nlm.nih.gov/blast/).

Phylogenetic analysis. Available HSP90 amino acid sequences were downloaded from GenBank and were included in phylogenetic analyses of the deduced BoHSP90 amino acid sequence obtained in the present study. Multiple sequence alignments were performed using Clustal W (Thompson et al. 1997). Phylogenetic analyses were conducted using the Maximum Likelihood method under MEGA5 software (Tamura et al. 2011). Statistical confidence of the inferred phylogenetic relationships was assessed by bootstraps of 1,000 replicates.

Genomic DNA extraction, status of samples regarding *B. ostreae* and *HSP90* amplification. Genomic DNA was extracted from 25-mg gill tissues collected from infected and non- infected oysters or from purified *B. ostreae* using the QIAamp DNA

Mini-kit (Qiagen). Infected and non- infected samples were identified by PCR using the BO-BOAS primer pair according to Cochennec et al. (2000). The same samples as well as DNA from purified parasites were tested by PCR using primers 156-F/156-R (Table 1) amplifying 154 bp of *HSP90* gene. PCR reaction was performed in a volume of 50 µl containing PCR buffer (1X), 0.2 mM nucleotides, 1 µM of each primer, 2.5 U of Taq polymerase (New England Biolabs) and 1 µl of genomic material containing 100 ng/µl. Thermal cycling was at 94 ºC for 5 min, 40 cycles of 94 ºC for 1 min of denaturing, 58 ºC for 1 min of annealing and 72 ºC for 1 min of extension, followed by 10 min of final extension at 72 ºC.

In vitro exposure of haemocytes to *B. ostreae* preincubated with radicicol. The HSP90 inhibitor radicicol (SigmaAldrich), a macrocyclic antifungal drug, was suspended in 1 mM dimethyl sulfoxide (DMSO). This parasite suspension was exposed for one hour at room temperature to increased concentrations (1, 5, 10 and 50 µM) radicicol diluted in FSW. Negative control parasites were exposed to FSW alone. The volume of DMSO used to dilute the highest concentration of radicicol was added to FSW and used as toxicity control (DMSO alone). Haemocytes were permitted to attach to the bottom of the 1.5-ml Eppendorf tube for 30 min. Then, a *B. ostreae* suspension was added to them at a ratio of 5:1 (parasite: haemocyte). Contact experiments were conducted at room temperature for one hour. Approximately 100 µl of the challenged haemocytes were then carefully resuspended by collecting with a pipette and cytocentrifuging at 100*g* and 4 ºC for 1 min. Slides were fixed in ethanol and stained with Hemacolor (Merck) before light microscopic visualization. A total of 100 haemocytes containing at least one internalized parasite were counted. Results are presented by indexing the percentages of infection with the corresponding control. The

experiment was repeated three times with a total of 10 oysters. Statistical differences were assessed using Students *t*-test.

RESULTS

Characterization of *B. ostreae HSP90*. The *HSP90* ORF was characterized by RACE-PCR and confirmed by PCR. It includes 2184 nucleotides encoding a protein of 728 amino acids (Fig. 1). This new gene was named *BoHSP90* and deposited on the GenBank database with the accession number JX235927. The sequence did not contain signal peptide. The protein has a theoretical isoelectric point of 5.09 and a molecular weight of 83.46 KDa. Two characteristic domains were identified in *BoHSP90* including an ATP binding domain (Fig. 1, single underline) and HSP90 domain (Fig. 1, double underline). The ATP binding domain is located between amino acids 37 and 182 and shows conserved residues involved in ATP binding and geldanamicyn (GA), a benzoquinone antibiotic, recognition. The HSP90 region starts at the residue 191 and contains a variable region of 68 residues with a high proportion of lysine and glutamic acid residues. The alignment of BoHSP90 with the 65 conserved amino acids comprising the 12 characteristic motifs of cytosolic HSP90 in other eukaryotes, including humans, are concordant with the idea that the HSP90 of *B. ostreae* is cytosolic. Among these, four motifs were exactly conserved in BoHSP90 and the remaining were one or two amino acids different in *B. ostreae* (Fig. 2). In total, 52 of the 65 conserved amino acids of cytosolic HSP90 perfectly aligned with the *B. ostreae* sequence. The MEEVD motif at the end of the C-terminal region, distinctive of cytosolic HSP90, was also conserved. However, the first glutamic acid residue in other eukaryotes was substituted by an aspartic acid residue in BoHSP90.

Phylogenetic analysis. BoHSP90 was compared with representatives of the cytosolic HSP90 subfamily. Results of similarity obtained after searching BlastP

databases are shown in Table 2. BoHSP90 displayed 56% identity with *M. brevicolis* (XP_001744222.1) and *Hydra magnipapillata* (XP_002165028.1). The phylogenetic tree obtained with complete HSP90 amino acid sequences (Fig. 3A) included a total of 14 sequences and 663 positions. BoHSP90 grouped with yeast in a sister group to other protist representatives of the groups Alveolata and Heterokonta. Metazoan representatives were used as outgroup. The sequences of near relatives of *Bonamia ostreae* were selected in the GeneBank to construct a more complete phylogenetic tree with 24 amino acid sequences (Fig. 3B). In this case the total residues analyzed were 256 since most of the sequences were incomplete and positions containing gaps were removed from the analysis. Alveolata and Heterokonta representatives grouped in the same branch although with a low support. Rhizaria resolved as a sister group to Alveolata and Heterokonta. Among Rhizaria representatives, *B. ostreae* grouped into Cercozoa with a bootstrap value of 47% with its closer relative *Gymnophrys sp*.

PCR amplification of *BoHSP90* in gills of *O. edulis*. Genomic DNA of gill tissues of *O. edulis* oysters were tested regarding the presence of *B. ostreae* using a PCR targeting the *SSU rDNA* fragment (Cochennec et al. 2000). The same samples were tested by PCR using 156-F/156-R primers (Table 1) that amplify *BoHSP90* gene. Specific *BoHSP90* primers yielded a product of 154 bp only in the oysters found positive using BO-BOAS (Fig. 4A, B) as well as in DNA from purified *B. ostreae* parasites (Fig. 4C).

Effect of radicicol on the phagocytosis of *B. ostreae*. Purified parasites were preincubated with increased concentrations of radicicol before being used for contact experiments with *O. edulis* haemocytes. The internalization index was calculated for each radicicol concentration and oyster. The mean ± standard deviation of 10 analyzed oysters are represented in Fig. 5. No differences were observed between controls and

DMSO samples, indicating that the volume of DMSO used had no effect on haemocyte phagocytosis ability. The treatment of *B. ostreae* with 10 µM and 50 µM of radicicol significantly decreased the number of infected haemocytes compared to control (p value < 0.01). Haemocyte samples exposed to *B. ostreae* preincubated with 50 µM radicicol had significantly fewer infected cells than samples exposed to 1, 5 or 10 µM radicicol. After *B. ostreae* incubation in 50 µM radicicol, the number of infected haemocytes decreased more than 40% compared to controls.

DISCUSSION

Bonamia ostreae has been described as the causative agent of mass mortality events in natural stocks of the flat oyster *O. edulis* during last decades in France (Pichot et al. 1979). Although the immune response of the flat oyster to *B. ostreae* has been recently studied (Comesaña et al. 2012; Morga et al. 2009; Morga et al. 2011a), the information about the molecular basis that governs *B. ostreae* infection remains unknown. The impossibility of cultivating this parasitic protist and the difficulty in obtaining sufficient concentration of pure DNA hamper critical genomic analyses. In a previous study, some genes differentially expressed in *B. ostreae* infected haemocytes were identified using a SSH (Morga et al. 2011b). Among the identified ESTs, some transcripts were suspected to belong to the parasite. More particularly, one of these ESTs had homology with HSP90 from *M. brevicolis* and was used as a starting point of the present study.

HSP90 is a molecular chaperone expressed in all eukaryotic organisms and is divided into several subfamilies (Chen et al. 2006; Yahara et al. 1998). HSP90 is highly represented in most cells, reaching 1--2% of total cytoplasmatic proteins (Csermely et al. 1998). HSP90 is involved in a variety of cell processes by maintaining the structure and integrity of “client” proteins (Csermely et al. 1998; Hao et al. 2010; Yahara et al.

1998). Most of the information on protist HSP90 concerns those parasitic in mammals such as *Plasmodium falciparum*, *Toxoplasma gondii* and *Theileria parva*, (Ahn et al. 2003; Banumathy et al. 2003; Echeverria et al. 2010; Gerhards et al. 1994; Roy et al. 2012; Son et al. 2001). That the host environment is hostile for intracellular parasites and that HSP90 may help ameliorate this effect has been proposed (Csermely et al. 1998; Maresca and Carratù 1992; Neckers and Tatu, 2008). Furthermore, in *P. falciparum* the expression of HSP90 is up-regulated during infection and has been used as a marker for the physiological state of the parasite (Pallavi et al. 2010). Together, these data suggest a role for HSP90 in host-parasite interactions.

In the present work, HSP90 ORF was completed by RACE in cDNA of *B. ostreae* infected oysters. In order to confirm that the *HSP90* gene belongs to *B. ostreae*, we tested the amplification of *HSP90* in infected and non-infected oysters as well as in purified *B. ostreae*. We observed a positive correlation between *HSP90* amplification and infected oysters detected by *SSU rDNA* amplification with BO-BOAS primers (Cochennec et al. 2000). A positive band of the expected size was also obtained in purified parasites. This fact leads us to verify that the *HSP90* gene did not belong to the host.

Representatives of two subfamilies of HSP90 were described in protists, the cytosolic HSP90, and the HSP90 with a signal peptide that is found in the endoplasmic reticulum (Chen et al. 2006). *BoHSP90* lacks signal peptide and is composed of 728 amino acids including specific residues of cytosolic HSP90 proteins. An exhaustive study based on the characterization of HSP90 across all kingdoms revealed the existence of 12 conserved motifs in cytosolic HSP90 (Chen et al. 2006). The presence of these 12 conserved motifs in BoHSP90 confirms that it belongs to the group of cytosolic HSP90. However, BoHSP90 showed some peculiarities. Among these

conserved motifs, four were exactly conserved in BoHSP90 while the remaining motifs displayed one or two amino acid differences. Two of these amino acids differences were also observed in other protists included in our study (Fig. 2). For example, in *B. ostreae, Trypanosoma cruzi* and *Dictyostelium discoideum,* the second glutamic acid residue of the MEEVD motif, located at the C-terminal region, appeared to be substituted in those three species by glutamine, lysine, or aspartic acid, respectively. The MEEVD motif is involved in protein-protein interactions and mediates the recognition of tetratrico peptide repeat domains found in co-chaperones (D´Andrea and Regan 2003). Residues responsible for the ATPase activity that confers the chaperone function to HSP90, are located in the amino terminal domain (Prodromou et al. 1997a, b) although there are also ATP binding sites in the carboxy terminal domain (Garnier et al. 2002). A total of 17 residues of the amino terminal domain are involved in ATP binding (Dutta and Inouve 2000; Prodromou et al. 1997a, b) including the characteristic GxG pockets (Obermann et al. 1998). BoHSP90 presented 15 conserved residues of the ATP binding sites (Fig. 1). The valine and threonine residues at positions 172 and 174 in human HSP90 appeared to be replaced in BoHSP90 by isoleucine and aspartic acid, respectively; and the valine residue at position 189 is shifted one amino acid upstream in BoHSP90.

The N-terminal ATP binding sites are also recognized by the benzoquinone antibiotic geldanamycin (GA), the first described HSP90-specific inhibitor (Whitesell et al. 1994). The binding of GA to HSP90 disrupts the chaperone function by inhibiting the ATP binding that favours the conformational changes necessary for the folding of client proteins (Hao et al. 2010). Radicicol is a macrocyclic antifungal that shares the geldanamycin binding sites and also interferes with HSP90 functions (Schulte et al. 1998; Sharma et al. 1998). In order to investigate the involvement of HSP90 during

haemocyte infection with *B. ostreae* we carried out in vitro experiments with parasites exposed to radicicol. The percentage of infected haemocytes drastically decreased after incubation of *B. ostreae* with this compound. To date, infective forms and entry mechanism of *B. ostreae* remain unknown. However, a study developed on haemocytes exposed to cytochalasin B revealed that the infection is a result of host phagocytosis and active parasite internalization (Chagot et al. 1992). Other studies have shown that the percent of infected haemocytes did not vary after exposure to live or dead *B. ostreae* (Morga et al. 2009). Therefore, the significant diminution of infected haemocytes observed in the current study suggests a possible role of radicicol in BoHSP90 inactivation. A similar inhibitory effect on cell invasion was previously observed in the intracellular parasite *Eimeria tenella* exposed to GA (Péroval et al. 2006). Moreover, intracellular growth and cell life cycle of other parasites like *T. gondii* and *P. falciparum* were also disturbed after treatment with GA (Ahn et al. 2003; Banumathy et al. 2003). Perhaps is not surprising that both radicicol and GA may interfere with the function of parasitic protist HSP90, since they share an ATP binding domain between amino acids 37 and 182 on this protein.

Because HSP90 is a highly conserved protein of eukaryotes and prokaryotes (Hao et al. 2010), it has been useful in understanding the evolution of all eukaryotes (Breglia et al. 2007; Chen et al. 2006; Parfrey et al. 2010), but most especially the phylogenetic position of dinoflagellates and alveolates (Fast et al. 2002; Harper et al. 2005; Hoppenrath and Leander 2010; Leander and Keeling 2004; Shalchian-Tabrizi et al. 2006). Previous studies using ribosomal DNA and actin have placed *B. ostreae* amongst Haplosporidia (Carnegie et al. 2000; Cochennec et al. 2000; López-Flores et al. 2007; Reece et al. 2004). Here, we first used complete HSP90 GeneBank sequences in an attempt to better define the placement of BoHSP90, but this strategy was not terribly

informative and placed the parasite gene with yeasts in a distinct branch inside the protist cluster. We attribute this in part to the lack of complete HSP90 sequences of rhizarian representatives which are near relatives of *B. ostreae* (Burki et al. 2010; Harper et al. 2005; Yoon et al. 2008). When a second strategy using partial HSP90 sequences was employed (even though it reduced by half the number of informative positions in the phylogenetic matrix), BoHSP90 grouped with rhizarian representatives in a sister branch of the cluster of Alveolata and Heterokonta. Using multiprotein phylogenetic analyses (Burki et al. 2007; Hackett et al. 2007; Parfrey et al. 2010; Yoon et al. 2008), the relationship of Rhizaria to Alveoltata plus Heterokonta has been previously discussed and the term "Harosa" proposed to include them (Cavalier-Smith 2010; Cavalier-Smith and Chao 2012). In this study, BoHSP90 is resolved with moderate support (47%) within the rhizarian cluster that includes haplosporidian members of the Cercozoa and very near to a species of *Gymnophrys* (Fig. 3B). These data are congruent with the earlier placement of haplosporidians within the group of Cercozoa (Cavalier-Smith 2002; Cavalier-Smith and Chao 2003). *More importantly, these data for BoHSP90 describe for first time a complete cytosolic HSP90 gene in Rhizaria that can be used to extend hitherto less informative parts of the eukaryotic tree.*

In the present work, the HSP90 of the Haplosporidia *B. ostreae* has been fully characterized, increasing the data available on protist parasite genes. We succeeded in amplifying the complete *BoHSP90* from cDNA of infected oysters. Since obtaining genomic material on some protists is a difficult task, the use of infected samples could be a good alternative to increase parasite genomic data. *BoHSP90* amplification was corroborated in a set of infected and non-infected oysters and also in purified parasites. Inhibitory studies with radicicol lead us to hypothesize that BoHSP90 could be involved

in the infection process of *O. edulis* haemocytes. The characterization of the new *B. ostreae HSP90* gene coding for a functional protein might have great interest for future studies on this species.

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FIGURE LEGEND

Fig. **1:** Complete ORF and deduced amino acid sequence of *BoHSP90*. The initial and stop codons are marked in grey. Conserved domains appear underlined: ATP binding domain is simple underlined, the HSP90 domain is double underlined. Residues of the variable region are indicated in grey. Residues involved in ATP binding are marked in black.

Fig. **2:** Multiple alignment of HSP90 amino acid sequence of *Bonamia ostreae* with other representatives of the cytosolic HSP90 subfamily. Variable region is

enclosed by arrows. Conserved motifs of cytosolic HSP90 are marked in grey and the MEEVD motif at the C-terminal end is indicated in black. The conserved GxG boxes are also pointed out in the alignment. Sequences of HSP90 were obtained from the GeneBank Database: *Mus musculus* (BAE27553.1), *Homo sapiens* (NP_005339.3), *Perkinsus marinus* (XP_002775585.1), *Toxoplasma gondii* (XP_002368278.1), *Babesia bovis* (XP_001611554.1), *Achlya ambisexualis* (AAM90674.1), *Candida albicans* (XP_721353.1). The following consensus symbols were used in the alignment: (*) residues identical in all sequences, (:) conserved substitutions and (.) semi-conserved substitutions.

Fig. **3:** Maximum Likelihood tree of the amino acid sequences showing the phylogenetic relationships among HSP90 complete amino acid sequences (**A**) and partial amino acid sequences (**B**). Bootstrap of 1,000 repetitions. Sequences of HSP90 were obtained from the GeneBank Database: *Theileria parva* (AAA30132.1), *Plasmodium falciparum* (AAA66179.1), *Saccharomyces cerevisiae* (AAA02743.1), *Hydra magnipapillata* (XP_002165028.1), *Dugesia japonica* (ACM91724.1), *Crassostrea gigas* (ABS18268.1), *Philodina roseola* (ACC43981.1), *Microplitis mediator* (ABV55506.1), *Phytopythium helicoides* (BAI94555.1), *Phytophthora phasoli* (ABU99530.1), *Pseudopedinella elastica* (ACM78547.1), *Nannochloropsis gaditana* (ACM78546.1), *Thraustochytrium aureum* (ACM78551.1), *Spumella uniguttata* (AAR27540.1), *Prorocentrum micans* (AAR27546.1), *Massisteria marina* (ADK90079.1), *Larcopyle butschlii* (BAK61732.1), *Thaumatomonas sp.* (ABJ80964.1), *Bigelowiella natans* (XP_001712819.1), *Gymnnophrys sp.* (ADK90077.1).

Fig. **4:** **A**. Detection of *HSP90* with specific primers 156-F/156-R. Lanes 1 and 30, 100 bp DNA ladder (New England BioLabs), lanes 2--28, DNA from gills of infected and non-infected oysters. Negative control (non-infected oyster) in lane 29. **B**.

Detection of the *SSU rDNA* with BO-BOAS primers in the same samples. **C**. Detection of *HSP90* in DNA from purified *Bonamia ostreae* (lane 2). Positive and negative controls are DNA of infected and non-infected oysters (lanes 3, 4) and no-DNA control (lane 5). Lanes 1 and 6 DNA ladder (SmartLadder, Eurogentec).

Fig. **5:** *Bonamia ostreae* internalization index after incubation with increased concentrations of radicicol. (*) Significant differences between values indicated with lines (p value < 0.01, n=10).

Figure 1

```
1     tcggggattgcgtaaaaattagctttactaaataaatggttgcagttaatgaatctgttaaa   62
                                        -  M  V  A  V  N  E  S  V  K
63    ggagagcgctacgcttttccgcggacatcaacaatctgctcggcttgataatcaacacg   122
       G  E  R  Y  A  F  S  A  D  I  N  N  L  L  G  L  I  I  N  T
123   ttctattcgaacaacgatgtcttcctcagggaactcatctccaattcgtctgacgcaata   182
       F  Y  S  N  N  D  V  F  L  R  E  L  I  S  N  S  S  D  A  I
183   gacaagataaagttcaagtcgcttacagactcttcggtgctcggcgatgaaacagaattt   242
       D  K  I  K  F  K  S  L  T  D  S  S  V  L  G  D  E  T  E  F
243   aaaatccaaatcgtccctaacaaggaagccaaaacgctcactatccgtgacaacggaatt   302
       K  I  Q  I  V  P  N  K  E  A  K  T  L  T  I  R  D  N  G  I
303   ggcatgacgaaagacgaaatggtcaaaaatctaggaacaatcgccacgtccggaactaaa   362
       G  M  T  K  D  E  M  V  K  N  L  G  T  I  A  T  S  G  T  K
363   gctttcatggacgctattcaagcgggcgccgatattcaaatgatcggccaattcggtgtc   422
       A  F  M  D  A  I  Q  A  G  A  D  I  Q  M  I  G  Q  F  G  V
423   ggattctattccgcgttcctcgtttcagatctcgtcgaagtcagatcaaagagtaacgac   482
       G  F  Y  S  A  F  L  V  S  D  L  V  E  V  R  S  K  S  N  D
483   ggcaagcaatttgtctggacctccacggcttcgggcgaattccacatttatgacgactcc   542
       G  K  Q  F  V  W  T  S  T  A  S  G  E  F  H  I  Y  D  D  S
543   gaaaacgagcaccagctcaaacgcggcaccgaaatcgttttgcacttgaaagacggccag   602
       E  N  E  H  Q  L  K  R  G  T  E  I  V  L  H  L  K  D  G  Q
603   acggagtacctcgaagaaaacaagatcaaagagatcgtgcacaaacacagcgaattcgtt   662
       T  E  Y  L  E  E  N  K  I  K  E  I  V  H  K  H  S  E  F  V
663   ggtcctcagatcctcctctggacttcgaaagaggtcgaaaaagaagtcagcgacgacgaa   722
       G  P  Q  I  L  L  W  T  S  K  E  V  E  K  E  V  S  D  D  E
723   gacgagaagaaagaagacgagaagaaggactctgataagaaaaaagatgaagactcggac   782
       D  E  K  K  E  D  E  K  K  D  S  D  K  K  K  D  E  D  S  D
783   gaaaagaaagaagacaaatcggacgaagtcgcggtgaaagatgtttcagatgctccggag   842
       E  K  K  E  D  K  S  D  E  V  A  V  K  D  V  S  D  A  P  E
843   ccgaagaaggaaaagaaaaaagtcactgaaatcgtcgaagagttcactcgcgtcaacagc   902
       P  K  K  E  K  K  K  V  T  E  I  V  E  E  F  T  R  V  N  S
903   accaaacctctgtgggcgatgaagccggaagacatcaagaaggaagagtacaccgcgttc   962
       T  K  P  L  W  A  M  K  P  E  D  I  K  K  E  E  Y  T  A  F
963   tacaagtccatctcgaacgactgggaagaaccgttggcttacaagcacttccacgtcgaa   1022
       Y  K  S  I  S  N  D  W  E  E  P  L  A  Y  K  H  F  H  V  E
1023  gggcaagtcgagttcactgtcattctcttcgttccgaagagaatgcctttcgatctcttc   1082
       G  Q  V  E  F  T  V  I  L  F  V  P  K  R  M  P  F  D  L  F
1083  aataacaataaggatccaagcaacatcaagcttttcgtccgtcgtgttctgatcaacgaa   1142
       N  N  N  K  D  P  S  N  I  K  L  F  V  R  R  V  L  I  N  E
1143  caagcgaaagagctttgtcctgaatatttgagcttcatcaaaggagttgttgacagtgaa   1202
       Q  A  K  E  L  C  P  E  Y  L  S  F  I  K  G  V  V  D  S  E
1203  gaaatgccactcaatatttcaagagagaccttgcagaagaacacaataatgaaagtgatc   1262
       E  M  P  L  N  I  S  R  E  T  L  Q  K  N  T  I  M  K  V  I
1263  cgcaacaacatcgccaaaaaagccatcgaactcttcttcgaaatctctgaagacaaagag   1322
       R  N  N  I  A  K  K  A  I  E  L  F  F  E  I  S  E  D  K  E
1323  cttttcaagaccttctacaagaacttttccaaaaacatcaaatatggcatccacgaagat   1382
       L  F  K  T  F  Y  K  N  F  S  K  N  I  K  Y  G  I  H  E  D
1383  tcgacgaaccgcaagaagctcgcagatctcattcgcttcaactccacctccagtgggaag   1442
       S  T  N  R  K  K  L  A  D  L  I  R  F  N  S  T  S  S  G  K
1443  gaacaacggtcgctcaaagattatgtcgagtcgatgcctgaacaccaaaaggatatttac   1502
       E  Q  R  S  L  K  D  Y  V  E  S  M  P  E  H  Q  K  D  I  Y
1503  tacatcaccggcgactcccaagaatcgctggagaacatgcccttcctcgaagtcctcaaa   1562
       Y  I  T  G  D  S  Q  E  S  L  E  N  M  P  F  L  E  V  L  K
1563  aagaaaaagctcgaggttctttttatgaccgacccgatcgacgagtactttgtgcagcag   1622
       K  K  K  L  E  V  L  F  M  T  D  P  I  D  E  Y  F  V  Q  Q
1623  atgagcgaatacgaaggacacaagttggtgagcgtcaccaaagaaggactcgagttgccg   1682
       M  S  E  Y  E  G  H  K  L  V  S  V  T  K  E  G  L  E  L  P
1683  caaacagacgaagagaagaaaaagcacgaagaggtgaagaaggagttcgagcccttctgc   1742
       Q  T  D  E  E  K  K  K  H  E  E  V  K  K  E  F  E  P  F  C
1743  aaacgcgtgaaggagatccttggagcgcgcgtcgagaaagtcgaggtgagcaatcgtatg   1802
       K  R  V  K  E  I  L  G  A  R  V  E  K  V  E  V  S  N  R  M
1803  accgagtcgccgtgctacatttccaccggcaaattcgggtggagttccaggatggggcag   1862
       T  E  S  P  C  Y  I  S  T  G  K  F  G  W  S  S  R  M  G  Q
1863  atcatgaagaaccaagcgctgcgcgacaccaccttcagctcccacatggccgccaaaaag   1922
       I  M  K  N  Q  A  L  R  D  T  T  F  S  S  H  M  A  A  K  K
1923  atcttggagatcaacccgcatcacgccgtcgtggaggagatggcgagacgcctgaaggaa   1982
       I  L  E  I  N  P  H  H  A  V  V  E  E  M  A  R  R  L  K  E
1983  aacgtgaacgacgcgatggcgaaggatctggtgtggctcatatacgaaacggctgtgctc   2042
       N  V  N  D  A  M  A  K  D  L  V  W  L  I  Y  E  T  A  V  L
2043  acttcgggcggtctctctttggagaacccggtcgagtacgcaggcagaatctataagctg   2102
       T  S  G  G  L  S  L  E  N  P  V  E  Y  A  G  R  I  Y  K  L
2103  acgcgtcttggactggggatcgtcaccgagaaggacgctggcgaagatttgccggacctg   2162
       T  R  L  G  L  G  I  V  T  E  K  D  A  G  E  D  L  P  D  L
2163  gaacctgctgatggagagaaggagaacgaagctacggcagagatggaagatgtcgattga   2222
       E  P  A  D  G  E  K  E  N  E  A  T  A  E  M  E  D  V  D  -
2223  ttagtaaagcgtgtcgcgacaaaaaaaaaaaaaaaaaaaaaaaaaaaaaagtactctgc   2282
2283  gttgataccactgcttgccctatagtgagtcgtattagaagccgaattctgcagatatcc   2342
2343  atcacactggcggccgctcgagcatgcatctagagggccca
```

Figure 2

Figure 3

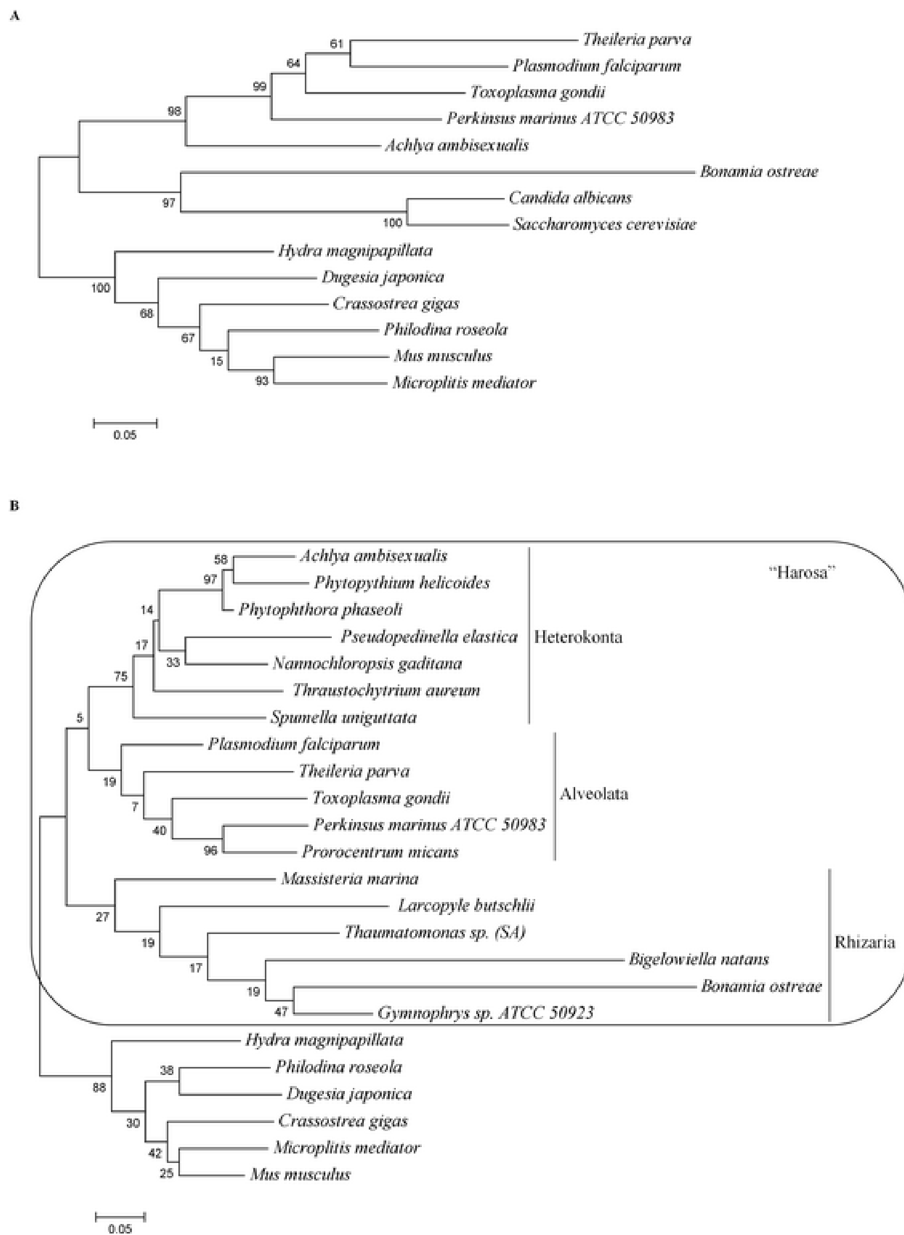

Figure 4

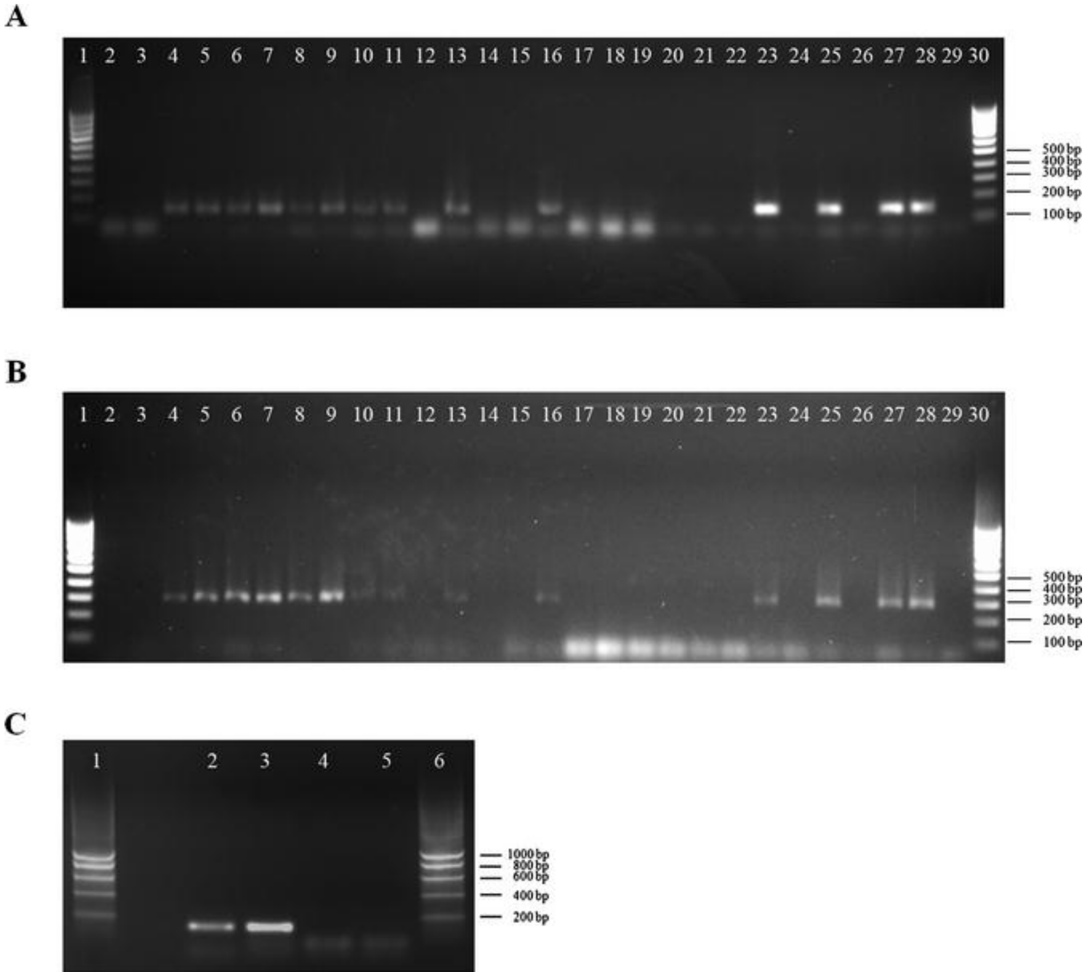

Figure 5