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RESEARCH REPORT

# Identification, cloning and environmental factors modulation of a αβ defensin from the Lessepsian invasive mussel *Brachidontes pharaonis* (Bivalvia: Mytilidae)

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**Abstract**

Immunological effectors of invasive species playing a role in addressing new colonization are still poorly studied. In the present study the cDNA sequence of the defensin from a Lessepsian invasive species, the Red Sea mussel *Brachidontes pharaonis,* was cloned using RACE method. Defensins are a class of widely known antimicrobial peptides (AMPs), oligopeptides with a broad spectrum of targeted organisms ranging from viruses to parasites. Analysis of BpDef sequence (262 bp) revealed the presence of an ORF coding for 81 amino acids. The full-length amino acid sequence showed the highest similarity to antimicrobial peptides MGD1 and MGD2 sequence from *Mytilus galloprovincialis*. Phylogenetic analysis suggested that BpDef belongs to the αβ defensin AMPs with a typical domain structurally characterized by a α helix and two β sheets. BpDef mRNA is located in circulating hemocytes with small intra-cytoplasmic granules and with large granules. The transcription of defensin gene was modulated by the stress from temperatures and oxygenation condition. Temperatures of 20 °C did not stimulate a BpDef transcription over a short time. At 30 °C the kinetics of BpDef gene transcription showed up regulation after one day, while it was down regulated after six days, both under normoxia and hypoxia conditions.

**Key Words**: *Brachidontes pharaonis*; Antimicrobial peptides (AMPs); defensin; Lessepsian mussel; environmental stress effect

## Introduction

Biological invasions are considered one of the main concerns worldwide they elicit ecological modifications (mostly detrimental) to ecosystem dynamics with tangible economic rebounds on local societies (Ojaveer *et al.,* 2015). Invaders act by competing for space with native species, often intercepting trophic resources (Carlton, 1992, 1999; Garci *et al*., 2007). Nevertheless, also invasive species, once arrived in a new recipient site, need to cope with new, often stressful, environmental conditions. Exactly like all the other species, invaders need to deploy all possible arms to cope with new recipient conditions; their success within the naïve community will depend on their ability to adapt and keep up individual fitness as high as possible (Lockwood *et al.,* 2009, Pascual *et al.,* 2010; Barbarro and Abad, 2013). Barbarro and Comeau (2014) reported adaptation capacity of *Xenostrobus securis* that ensure its dissemination within estuarine environments. Most studies have examined the ability of invaders to occupy a new site by analyzing the role of functional and life-history traits (Sarà *et al.,* 2013) and several other ecological features, mainly through their effect on density-dependent processes affecting the persistence of invasive populations over time (Lockwood *et al.,* 2005). The shift in dominance of some specie (Gestoso *et al*., 2013) can alter habitat structure and complexity with effects on trophic web relations. In contrast, few data have been gathered on sub-organismal (*e.g.,* cellular) "arms" of invasive species that often represent the first line of adaptation which increases the probability of colonization of new geographic areas.

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In particular, scantly data has been produced investigating the role of ecoimmunology in invasion biology; moreover, how immunological effectors of invasive species play a role in addressing new colonization is still poorly studied (Lee and Klasing, 2004). When an organism is subjected to stressful conditions as might be expected in a novel recipient site (thousands of miles far from the core of its biogeographic area), the first line of response aims to cope with new variable conditions by modifying immunological, biochemical, physiological and behavioral traits. Here we chose the Pharaonic mussel *Brachidontes pharaonis* as a model species to study the ecoimmunology of invasive species. The Pharaonic mussel is one of the 100 most aggressive alien 75 species in the Mediterranean Sea: it is a Lessepsian (*i.e.,* coming from the Red Sea through the Suez Canal) invasive species and it is currently spreading through the shallow intertidal zones of the western Mediterranean Basin (Sarà *et al.,* 2013). This species is a reliable model to infer on the role of immunological traits in coping with highly variable abiotic conditions of novel recipient habitats. In shallow waters, this species should be able to cope with extremely varying thermal conditions (from 5 °C to 32 °C) and water oxygen levels, often changing from normoxia to anoxia in only a few hours during nighttime and calm wind (Sarà, 2009).

Here, we focused on the immunological response by antimicrobial peptides (AMPs). AMPs are very important components of natural defenses in most living organisms. These peptides are gene-encoded, relatively small (< 10 kDa), amphipathic (Boman, 2003; Sang and Blecha, 2008). AMPs constitute the first line of innate immunity for mollusks exposed to various potential pathogens in aquatic environments. AMPs have been isolated from a wide variety of animals and plants (Reddy *et al.,* 2004) as well as bacteria and fungi, and they exhibit a broad-spectrum activity against gram-positive and gram-negative bacteria, protozoa, yeasts, fungi, and viruses. In molluscs, AMPs have been characterized mainly in marine bivalves, such as the mussels *Mytilus edulis* and *Mytilus galloprovincialis* (Charlet *et al.,* 1996; Mitta *et al.,* 2000a). Using biochemical approaches and molecular cloning, four families of cysteine-rich AMPs have been identified in *Mytilus* spp.: defensins, myticins, mytilins, and mytimycin (Mitta *et al.,* 2000b; Balseiro *et al*., 2011; Li *et al.,* 2011). Defensins are relatively short antimicrobial peptides (12 - 100 amino acids in length) that are widely distributed in single-celled microorganisms, plants, invertebrate and vertebrate species (Jenssen *et al.,* 2006). Defensins respond rapidly to invading microorganisms by forming pores or otherwise disrupting the microbial membrane (Tincu and Taylor, 2004). They can interact with the microbial DNA or RNA after penetrating the microbial membrane (Boman *et al.,* 1993; Park *et al.,* 1998). Cationic defensins involved in host defense have been linked to acute inflammation (Tincu and Taylor, 2004). Many defensins with a toxic action against prokaryotes appear relatively nontoxic in eukaryotes and some of them also have antitumor and antiviral activities (Chen *et al.,* 2001; Zang *et al.,* 2002). Defensins are very attractive as potential therapeutic agents for pharmaceutical or agricultural applications because of their small size, which allows easy synthesis, their mode of action, broad spectrum activity, highly selective toxicity and their ability to generally circumvent bacterial resistance-development mechanisms (Jenssen *et al.,* 2006).

Thus, the main objectives of the present study were to: (i) clone the full-length cDNA of the defensin from *B. pharaonis* (BpDef), (ii) examine the structural characteristics of the deduced amino acid sequence and investigate the cellular localization of mRNA for BpDef, and (iii) evaluate the quantitative gene expression profile of gill BpDef under thermal stress and hypoxia conditions.

## Materials and Methods

*Sampling, animal maintenance, experimental treatments and cell preparation*

About 300 adult *Brachidontes pharaonis* (2.3 ± 0.2 cm umbonal length) were collected in May 2014 from ponds in Western Sicily (Salina Ettore; Lat.12°27’34” E; Long. 37°51’42”N). Once brought back to the laboratory, animals were acclimated for one month in a flow-through system of oxygenated (by aeration; dissolved oxygen [DO] about 8 mg l-1) seawater at 20 °C, before starting experiments mirroring the seawater features of the sampling location (Ezgeta-Balic *et al.,* 2013). Water in the tanks was continuously aerated and changed daily, and specimens were fed regularly (3 times a day) with monoalgal culture of *Isochrysis galbana*. Laboratory procedures were designed to evaluate the immunological effects of sudden oxygen depletion at two different temperatures that *B. pharaonis* could experience in late spring and summer in shallow ponds at these latitudes. Two different temperatures were applied in our experiments: 20 °C as a control temperature (mirroring that of pond seawater at the time of sampling and coincident with the water temperature in the acclimatization tanks) and 30 °C, which represents the mean of the maximal water temperatures recorded in the pond in late summer (Sarà *et al.,* 2000). Thus, animals were divided into four groups: two groups were left at 20 °C, and two were moved to separated aquariums in which the water temperature was increased (through submergible heaters; Tetratec HT - 200W) by about 1 °C for 6 - 8 h to reach 30 °C in about three to four days. Once 30 °C was reached, animals were acclimated at this temperature for at least two weeks. In the combination of hypoxia and 30 °C we have registered 4 % of mortality after 1 week. In the pond, and generally in shallow waters at this latitude, DO can suddenly drop from normoxia (7 - 9 mg l-1) to anoxic and hypoxic conditions (0 - 2 mg l-1; Sarà G, pers. obs.), particularly during the night and calm wind conditions, but usually hypoxic events are never longer than 8 - 9 h. Accordingly, two aquariums out of four (one at 20 °C and one at 30 °C) were exposed to a sudden decrease of DO, by insufflating gaseous nitrogen ($N_2$). Thus, the DO concentration was slowly moved from normoxia to hypoxia (to about 1.8 - 2.1 mg l-1) in not more than 2 h. The hypoxia event was maintained for at least 8

h. Then the aquaria water was aerated again and the normoxia conditions quickly restored. The other two aquariums (one at 20 °C and one at 30 °C) were left with their initial unaltered normoxic conditions. At 30 °C the control values are referred to the sample acclimatized to 21 °C and moved for 8 h at 30 °C. Temperature was measured with Type-G thermo-loggers (iButton; AlphaMAc, Inc Canada) while DO was controlled by the oxygen sensor four times a day. To carry out immunological analyses (see below for details), we sampled hemolymph and gill (see below) from animals (n = 5) from all tanks (treated and controls) into three successive temporal steps: (1) at the end of the hypoxia period - about 8 h after the beginning of treatment, this point is considered as "0"; (2) at 24 h after the beginning of treatment and lastly (3) 168 h after the beginning of treatment. Hemolymph (about 0.3 ml per mussel) was collected from the posterior adductor muscle with a 1 ml syringe containing 0.2 ml of the anti-coagulant modified Alsever's solution buffer (NaCl 0.42 g, Sodium-citrate·$2H_2O$ 0.8 g, Citric acid·$H_2O$ 0.055 g, D-glucose 2.05 g, DW 100 ml). Hemocytes were pelleted by 10 min centrifugation at 800*g*, 4 °C, suspended in MS (12 mM $CaCl_2$, 11mM KCl, 26 mM $MgCl_2$, 43 mM TrisHCl, 0.4 M NaCl, pH 8.0) and maintained in ice until *in situ* hybridization was performed. Gills dissected from specimens were immediately soaked in RNA later Tissue collection (Ambion, Austin, TX), and stored at -80 °C until use. The amount of peptides recovered from bivalve gills suggest that most likely gills, and perhaps other epithelial tissues, naturally express endogenous AMPs (Mercado *et al.*, 2005). *B. pharaonis* body is too small to recover sufficient material to extract RNA from hemocytes or digestive gland. Instead mussel gills represent roughly 13 % of the smooth body weight portion. Thus, this tissue is a reasonable source for new antimicrobial molecules.

*Cloning and sequence analysis*

Total RNA from gills was isolated using an RNAqueous™-Midi Kit purification system (Ambion, Austin, TX) and reverse transcribed by the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen). Amplification was performed with primers (Table1) designed from coding sequences from *Mytilus galloprovincialis* of Defensin MGD2b (Def:AF177539.1) (DEF-F1: TATGAAAGCAGTATTCGTCTTG; DEF-R1: CTCCACATCGTCCCTTCTCA). The end-point PCR mix contained 1 µl of template, 0.4 µM of each primer, 0.8 mM of dNTPs and 0.625 U of Gotaq polymerase (Promega) in 25 µl final volume PCR program started with initial denaturation at 95 °C for 2 min, followed by 40 cycles including 30 sec at 95 °C, 30 sec annealing at 60 °C, 30 s elongation at 72 °C, and 5 min final elongation at 72 °C. A single band of 164 bp in size was detected. The amplicon was cloned into the pCR™II vector (TA cloning Kit, Invitrogen) and its sequence revealed 94 % homology with the MGD2 of *M. galloprovincialis.* The full-length cDNA sequence obtained with Rapid Amplification cDNA ends (RACE) technology, using the GeneRacer kit (Invitrogen, USA). The kit allows only the amplification of full-length transcripts and the elimination of truncated mRNA from the amplification process. The primers used to study the defensin AMP of Brachidontes pharaonic are showed in Table 1.

*Phylogenetic analysis*

Similarity searches were performed using the FASTA algorithm (http://www.ebi.ac.uk/ Tools/fasta/). Sequences were subjected to multiple alignments using CLC workbench 6.4. A phylogenetic tree was made by the Neighbor-Joining method (NJ) after

**Table 1** Primers used to study the defensin AMP of *Brachidontes pharaonis*

| Name | Sequence (5'-3') | PCR objective |
|---|---|---|
| Gene Racer 5' | CGACTGGAGCACGAGGACACTGA | 5' Race |
| Def-Bp 5' RACE | CCACCGCATCGTCCTGGAATGGACT | 5' Race |
| Gene Racer N 5' | GGACACTGACATGGACTGAAGGAGTA | 5' Nested |
| Def-Bp 5'NESTED | TTTGGACAGCCAAACCCAGCAGACG | 5' Nested |
| Def-Bp 3' RACE | TGTGGCGTCTGCTGGGTTTGG | 3' Race |
| GeneRacer 3' | GCTGTCAACGATACGCTACGTAACG | 3' Race |
| Def-Bp 3' NESTED | GCGTCTGCTGGGTTTGGCTGT | 3' Nested |
| GeneRacer N 3' | CGCTACGTAACGGCATGACAGTG | 3' Nested |

1000 bootstrap iterations by using CLC workbench 6.4. Distances were corrected for multiple substitutions and gap positions were excluded. The accession numbers are as follows: *Mytilus galloprovincialis* MGD1, AAD45117.1; MGD2, AAD45118.1; MGD2b, AAD52660.1; *Ruditapes philippinarum*, AEK78067.1; *Ornithodoros moubata,* BAC10304.1; *Ornithodoros moubata DEF-B*, BAB41027.1; *Crassostrea gigas*, CAJ19280.1; *Drosophila melanogaster*, AAF58855.1; *Bombus terrestris,* ACI14287.1; *Apis cerana*, ACH96412.1; *Anopheles gambiae*, ABB00987.1; *Branchiostoma belcheri*,Q86QN6.1; *Ornithodoros puertoricensis*, ACJ04430.1; *Homo sapiens* alpha1, AAT68878.1; *Homo sapiens* beta, EAW80482.1; *Oncorhynchusmykiss beta4*, CBB12549.1; *Oncorhynchus mykiss* beta3, CBB12548.1; *Oncorhynchus mykiss* beta2, CBB12547.1; *Oncorhynchus mykiss beta 1,* CBB12546.1; *Danio rerio* beta 2, 001075023; *Danio rerio* beta3, 001075024.1.

*In situ hybridization assay*

Digoxigenin-11-UTP-labeled riboprobes (DIG-riboprobe) were produced according to the instructions of the manufacturer (Roche Diagnostic, Mannheim, Germany), and used at a final concentration of 1 µg/ml . The probe was generated by PCR amplifying a cDNA fragment of 177 bp of cDNA using the forward primer (GGATACTGTGGTGGATGGCA) and the reverse primer (CGTCTGTTAATAGAGCTGATCCA). After sampling and centrifugation to remove anticoagulant, the cells were resuspended at $5.10^5$/ml final concentration in Marine Solution (MS: 12 mM $CaCl_2$; 11 mM KCl; 26 mM $MgCl_2$; 45 mM Tris; 38 mM HCl; 0.45 M NaCl; pH 7.4). Then 100 µl of cell suspension was layered on a slide in replicate, and they were fixed in Bouin’s fluid (saturated picric acid:formaldehyde:acetic acid 15:5:1) for 30 min in a humid chamber. After washing in PBST (PBS containing 0.1 % Tween 20), the hybridization was carried out overnight with hybridization buffer (50 % formamide, 5X standard saline citrate (SSC), 50 µg/ml heparin, 500 µg/ml yeast tRNA, and 0.1 % Tween 20). Incubation temperature was at 42 °C. After washings with PBST followed by 0.3 % SSC/1 % Tween 20 (twice for 10 min each), the anti-DIG Fab-AP (Roche Diagnostic) 1:500 was added, and, after 1 h incubation at room temperature, washed with PBST. Finally, the cells were incubated in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma-Aldrich) to reveal the presence of the riboprobe.

*Real-time PCR analysis of BpDef expression*

Animals were held in tanks at temperatures of 21 °C and 30 °C, and at each temperature two groups of animals were held under normoxia and hypoxia (> 2 pp of $O_2$) conditions. The experimental design consisted of measurements over one week, due to a limited number of animals. Gills were therefore taken at 0, 24 and 168 h after the heat and hypoxic treatment. Gill tissue expression of the *BpDef* gene was examined by real-time PCR analysis with the Sybr-Green method (Applied Biosystems 7500 real-time PCR system). Tissue expression was performed in a 25-µl PCR containing 2 µl cDNA converted from 250 ng total RNA, 300 nM *BpDef* forward (5’-GTGGCGTCTGCTGGGTTT-3’) and *BpDef* reverse primers (5’-GAATGGACTTACAATGTCGATGACA-3’), 300 nM 28S forward (5’-CACCCGACCCGTCTTGTAA-3’) and 28S reverse (5’-CCATGACTTGCGCACATGTT-3’) primers, and 12.5 µl Power Sybr-Green PCR Master Mix (Applied Biosystems). The 50 cycles of the two-step PCR program consisted of initial polymerase activation for 3 min at 95 °C followed by a denaturing step at 95 °C for 15 s, and then annealing/extension was carried out at 60 °C for 45 s when the fluorescent signal was detected. Each set of samples was run three times, and each plate contained quadruplicate cDNA samples and negative controls. The specificity of amplification was tested by real-time PCR melting analysis. To obtain sample quantification, the 2-ΔΔCt method was used, and the relative changes in gene expression were analyzed as described in the Applied Biosystems Use Bulletin N.2 (P/N 4303859). The amount of *BpDef* transcript from the various tissues was normalized to partial sequence of 28S in order to compensate for variations in input RNA amounts The 28S sequence was cloned from *B. pharaonis* using primers 28 SFc ATCCGTAAAGACCCGCCTG and 28SRc (CAGAATCGCTACGGACCTCC). Relative *BpDef* expression was determined by dividing the normalized value of the target gene in each tissue by the normalized value obtained from the untreated tissue.

*Statistical analysis*

qRT-PCR data are presented as the means ± SE. Differences among tested groups and the control groups were analyzed by a one-way ANOVA with post-hoc Student-Newman-Keuls test. Significance was accepted at the level of $p < 0.05$.

## Results

*cDNA cloning and sequencing of the BpDef in B. pharaonis*

A fragment of 164 bp was obtained after amplification and cloning using primers previously designed on the sequenceMGD2 (AF177539.1 205) of *M. galloprovincialis.* The full-length cDNA of *BpDef* was obtained by the rapid amplification of cDNA ends (RACE) method (Fig. 1). Analysis of the sequence of 262 nucleotides revealed the presence of an ORF coding for 81 amino acids with a predicted molecular weight of 9.054 kDa. Analyses carried out with the program Signal P.4 server NN show a potential signal peptide of 23 amino acids (a.a.) in the amino terminal portion followed by the mature peptide from a.a. 25 to a.a. 60 and a carboxy-terminal extension.This peptide has eight cysteine involved in 4 disulfide bridges, which hold the molecule in its active form. Calculation of Peptide Parameters through the APD database Prediction indicate a 44 % of total hydrophobic ratio

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1    ATGAAAGCAGCATTCGTCTTGTTGGTGGTTGGTTTGTGCATCATGACGGACGTGGCGTCT   60
      M  K  A  A  F  V  L  L  V  V  G  L  C  I  M  T  D  V  A  S
61   GCTGGGTTTGGCTGTCCAAACAATTATCAATGTCATCGACATTGTAAGTCCATTCCAGGA  120
      A  G  F  G  C  P  N  N  Y  Q  C  H  R  H  C  K  S  I  P  G
121  CGATGCGGTGGATACTGTGGTGGATGGCACAGATTGAGGTGCACTTGTTATAGATGCGGT  180
      R  C  G  G  Y  C  G  G  W  H  R  L  R  C  T  C  Y  R  C  G
181  GGGAGAAGGGAAGATGCGGAGGATATTTTTGATATCTTTGATAATGAAGTAGCAGATCGC  240
      G  R  R  E  D  A  E  D  I  F  D  I  F  D  N  E  V  A  D  R
241  TTCTGAAtttgatggatatggtttgaaacgaaagatgttgaaaatggatcagctctatta  300
      F  *
301  acagacgtttttctttctgatactaaatcaatatatttttcaatgactttttttacttt   360
361  aaatgttcttctgtctagtacttaacatgttagaaaaaagtaaaaaataaactctttagc  420
421  atcaaaaaaaaaaaaa
```

**Fig. 1** Nucleotide and deduced amino acid sequence of the defensin from *Brachidontes pharaonis* gills. 3'UTR regions are described in lower case letters; the coding region is in upper case letters; the STOP codon is indicated by asterisk. Signal peptides from 1 to 23 a. a. positions are indicated in bold. Mid-gray shading mature peptide is found at positions 25 - 60 and the eight cysteine conserved residue are in bold and underlined. Arrows indicate 2 bactericidal stretches calculated by a server for prediction of protein antimicrobial regions: the first found in 29 to 41 and the second found in 43 to 63 a.a. positions.

and + 1 of the total net charge. The Grand Average hydropathy value of the peptide (GRAVY) is equal to -0.106 and the Wimley-White whole residue hydrophobicity of the peptide is 10.74 kcal/mol. The antimicrobial character of the molecule is suggested by the value of the Protein-binding Potential (Boman index) of 1.74 kcal/mol. Analysis carried out by an automated web server for prediction of protein antimicrobial regions (AMPA) revealed a mean antimicrobial value of 0.244 and 2 bactericidal stretches, the first found in 29 to 41 and the second found in 43 to 63 a.a. positions (Fig. 1).

The identity of nucleotide and a.a. sequences was confirmed using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The results of the comparison and a alignment with the sequences of *M. galloprovincialis* indicate an identity of 93 % and a similarity of 96 % with the antimicrobial peptide MGD1 and an identity of 78 % and a similarity of 84 % with the antimicrobial peptide of the same MGD2 Mediterranean mussel (Fig. 2).

Sequence identity and the common conserved sequence characteristics indicate that BpDef belongs to the αβ defensin family of AMPs. Alignment of a.a. sequences shows the domain typical of αβ defensins structurally characterized by a helix and two sheets αβ (Fig. 2).

*Phylogenetic analysis*

The construction of the phylogenetic tree by the NJ method and Bootstrap analysis showed three different clusters (Fig. 3). The first included αβ defensins of bivalve and gastropods (*C. gigas*, *R. philippinarum, M. galloprovincialis, C. puortoricensis, O. moubata*). The second included the α defensins of arthropods and some vertebrates (*A. cerana, B. terrestris, D. melanogaster, A. gambiae, B. belcheri, P. japonicus, R. norvegicus M. musculus, H. sapiens*) were organized into two related groups. The third included β defensins of vertebrates (D*. rerio, O. mykiss, R. norvegicus M. musculus, C. hircus, H. sapiens*). The BpDef sequence presented the closest relationships with the αβ defensins.

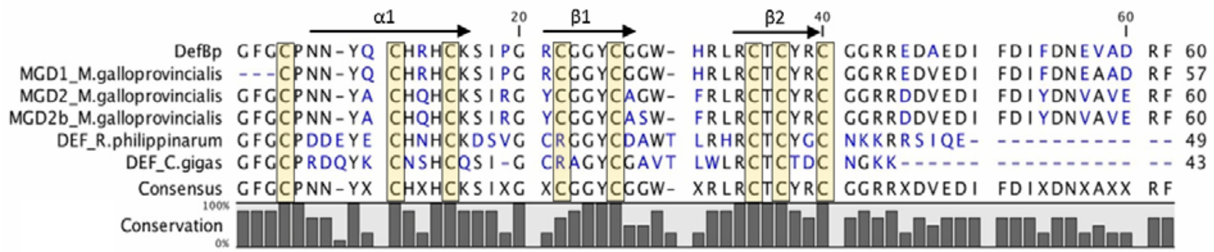


**Fig. 2** Sequence alignment and structural organization representative of the bivalve molluscs defensin family AMP. The conserved and identical residues in AMP proteic domain are reported in black and conservative substitutions are reported in gray. Arrows indicate the location of the hydro-phobic and positively charged residues in a domain typical of αβ defensins structurally characterized by a helix and two sheets. The eight conserved cysteine residues involved in disulfide bridges are represented by yellow shading.

*BpDef mRNA localization*

To investigate the cellular localization of BpDef in *B. pharaonis* mRNA, *in situ* hybridization assays were performed on circulating hemocytes obtained from the posterior abductor muscle of *B. pharaonis* specimens. Two cell categories mainly based on size of intracellular granules were detected as follows: granulocytes with (i) small and (ii) large intra-cytoplasmic granules. Both hemocytes with small intra-cytoplasmic granules (B, D) and with large granules (F) express BpDef mRNA (Fig. 4). Controls with the sense strand probe were negative (A, C, E).

*BpDef expression under environmental stressors*

Real-time RT-PCR enabled us to quantify *Defensin gene* expression kinetics from *B. pharaonis* gills under thermal and hypoxia stressors.

In all the condition under hypoxia the values are downregulated and reduced respect the normoxia condition from 5 to 20 times.

Timing of BpDef was shown as the following scheme (Fig. 5): at 21 °C, under normoxic conditions, BpDef transcription declined to the lowest value after 1 day from T0, but reached at the control values (CNT) after 6 days. Under hypoxia at 21 °C, the gene expression was down regulated 1 day after the treatment and this condition was maintained until day 6. At 30 °C the CNT values are referred to the sample acclimatized to 20 °C and moved for 8 hour at 30 °C. The kinetics of BpDef gene expression showed an increase to the control values after one day, while it was down regulated after six days, both under normoxia and hypoxia conditions.

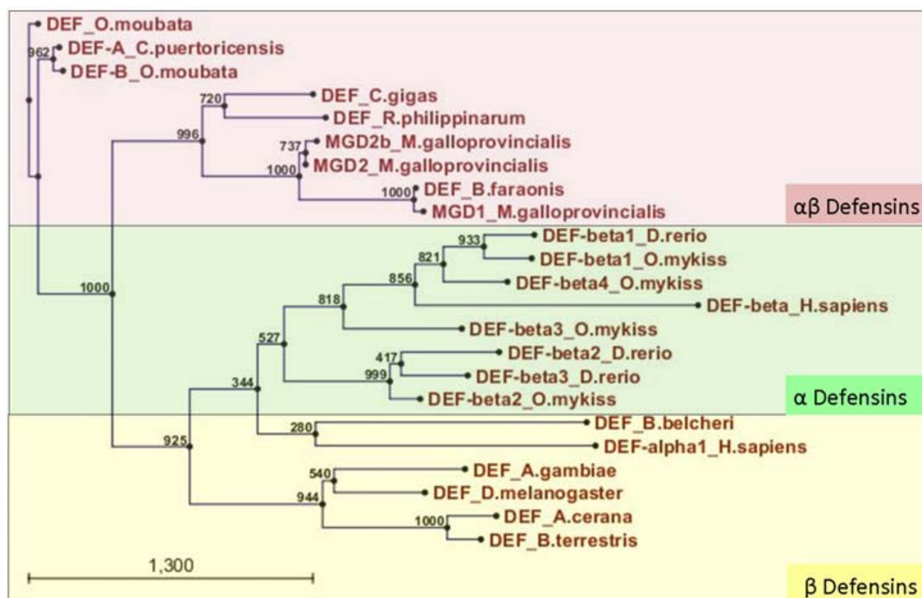


**Fig. 3** Phylogenetic tree of defensins belonging to invertebrates (bivalve molluscs and insect) and vertebrates (fish and mammals). The tree was constructed by the NJ method and bootstrap analysis. Numbers represent the percentages of 1,000 bootstrap replicates in which the same internal branch was recovered. Bars indicate the number of amino acid residue substitutions for site. The sequences are separated into three specific clusters: the molluscs αβ defensins, the vertebrate α defensins including humans, and αβ defensins of arthropods.

## Discussion

In molluscs, hemocytes are the primary immune cells engaged in several innate immune reactions including phagocytosis, clotting, encapsulation, and the synthesis of several immune-related molecules. Among these molecules, the antimicrobial peptides (AMP) are characterized by their extremely heterogenic structures (Bulet *et al.,* 2004; Li *et al*., 2011).

Based on primary structure and consensus cysteine array, defensin, mytilin, myticin and mytimycin AMP families have been identified in the mussels *M. edulis* (Charlet *et al.,* 1996) and *M. galloprovincialis* (Hubert *et al.,* 1996; Mitta *et al.,* 1999a).

In this work, the putative antimicrobial peptide of αβ defensins family, BpDEF was cloned and characterized in the Lessepsian *B. pharaonis.* Outcompeting with the indigenous mussel *Mytilaster minimus*, it had strong negative effects on survival and growth of the native species.

Like all the invasive species, it expanded its range, due to selection favoring strongly growth and reproduction, traits that may compete against immune function (Carrington *et al.,* 2015).

The cDNA of BpDEF encodes a 81 a.a peptide. The putative propeptide contains a signal peptide of 23 a.a. and a mature peptide of 40 a.a.. The sequence contains features common to other AMPs: α-helices, overall positive charge, and strong hydrophobic regions. Sequence alignment with other known big defensins showed the presence of 8 conserved cysteine residues forming the typical defensin consensus pattern. The Boman index value, that estimates the potential or a protein to bind to a wide range of proteins, indicated the strong antibacterial feature of *B. pharaonis* defensin. Two predicted bactericidal stretches were found in correspondence of the mature peptide.

The arrangement of the cysteines, their neighboring a.a., the spacing between the cysteines and the high identity with the antimicrobial peptide MGD from *M. galloprovicialis,* further support the hypothesis that BpDef is a member of the defensin family. The structural homology study carried out relating to the crystallographic structure of MGD1 and the phylogenetic analysis indicates that the molecule belongs to the category of αβ defensins.

Using In *situ* ibridation (ISH), cells expressing the defensin transcript have been identified: granulocytes with large and small cytoplasmic granules.

AMPs as Defensins, Mytilin B, and Myticins A and B, are produced as precursor molecules processed to active compounds within the hemocytes (Mitta *et al.,* 1999 a, b, 2000b). Furthermore, previous studies have shown that defensins and mytilins are stored in granulocytes.

In *M. galloprovincialis* immunocytochemistry at the ultrastructural level showed that defensins (i) are predominantly located in vesicles of a granulocyte subclass containing small granules, but (ii) are also found in large granules of the other granulocyte subclass containing large granules (Mitta *et al.,* 1999a, 1999b; Cantet *et al*., 2012). In addition, no hyalinocyte resulted positive for AMP mRNA..

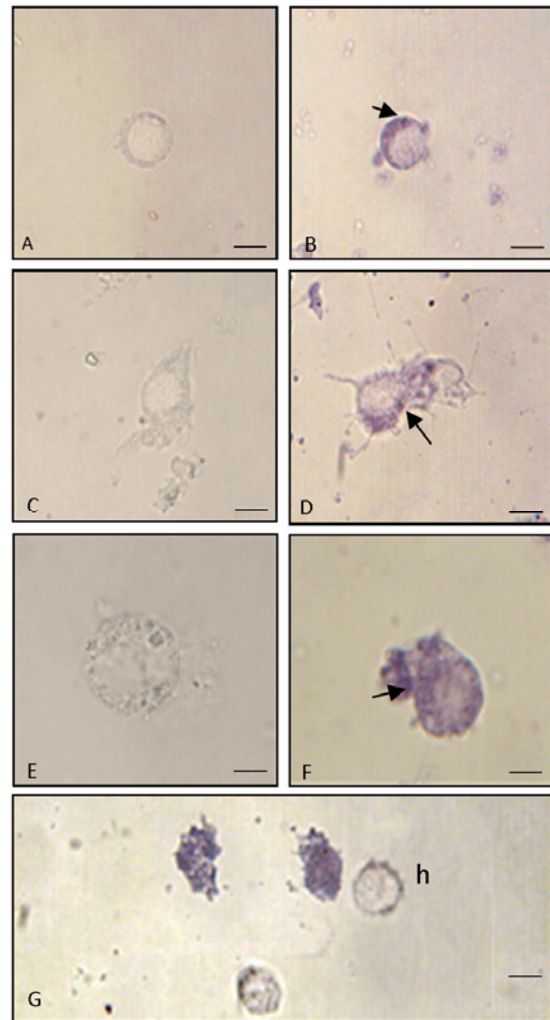


**Fig. 4** *In situ* hybridization assay: detection of transcripts encoding of BpDef in hemocytes withdrawn from abductor muscle of *B. pharaonis.* Granulocytes with small cytoplasmic granules (A-B); granulocytes with large cytoplasmic granules (C, D, E, F) . Hemocytes treated with BpDef antisense DIG-riboprobe (A, C, E). Control: hemocytes treated with BpDef sense DIG-riboprobe (B, D, F and G). Arrows indicate granules positive to riboprobe. (h) negative hyalinocyte. Bar: 10 µm. In Fig. 4G hyalinocytes cells (h) not showed transcript of BpDef contrary to granulocytes resulted positive to BpDef antisense DIG-riboprobe.

In *Mytilus spp.* the mRNAs encoding the AMPs are first translated into pro-peptides that share a common structure including a signal peptide in the amino terminal portion followed by the mature peptide and a carboxy-terminal extension position (Parisi *et al.*, 2009). The pro-peptides undergo intra-hemocyte maturation and are stored in granules (Li *et al.,* 2011).

Environmental factors including temperature, oxygen, food availability, and contaminants can affect immune parameters of molluscs (Fisher, 1988).

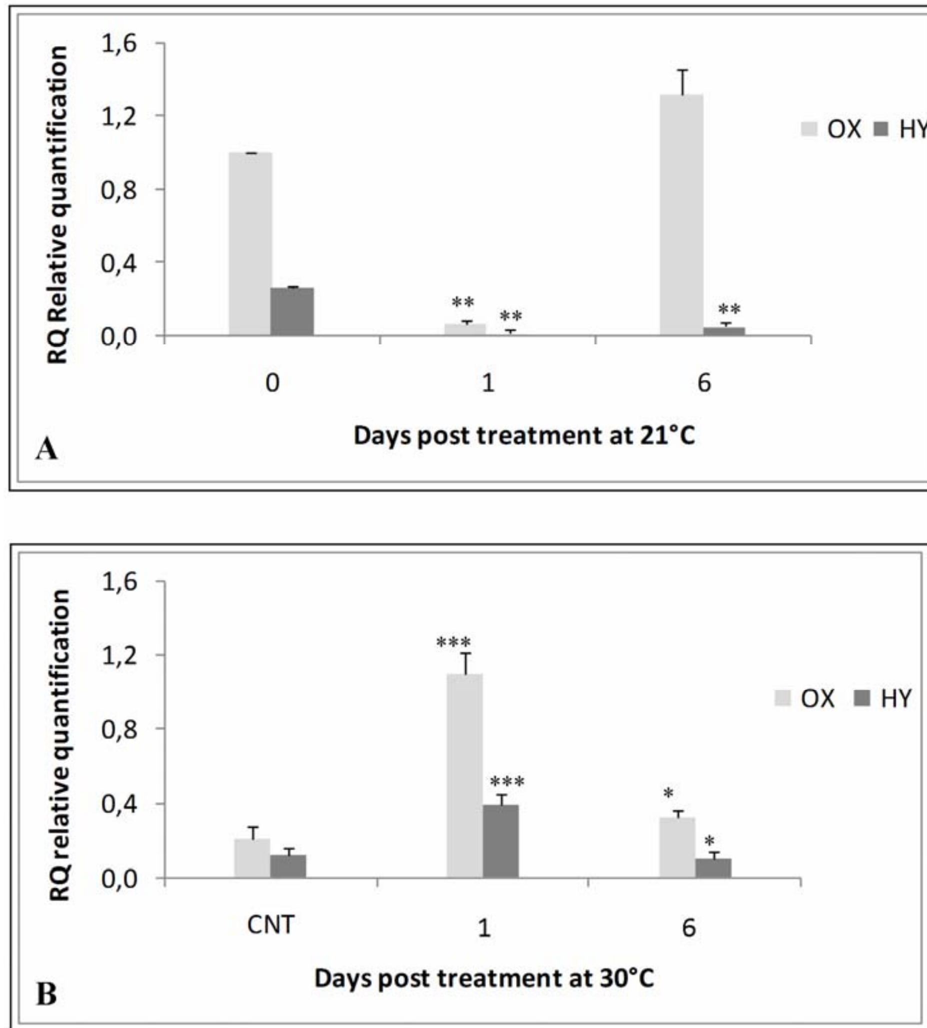


**Fig. 5** Quantitative expression profile of *BpDef* in the gill at 21 °C and hypoxia treatment (A) and 30 °C and hypoxia treatment (B). The relative expression was calculated based on the standard curve and normalized to the 28S mRNA level in gill at 0, 1, and 6 days. CNT values are referred to the sample acclimatized to 21 °C and moved for 8 h at 30 °C. Data from qRT-PCR are presented as mean ± SE. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ significant level against control (0 and CNT). OX: normal oxygenation condition; HY: hypoxia treatment with $O_2 <$ 2 ppm.

Numerous studies have demonstrated that changes in environmental factors strongly influence mollusc immune parameters, even if temperature effects should differ among species. However, most studies carried out in the last decades have investigated the effects of single environmental factors, whereas a limited number of studies assess the multiple effects of abiotic factors are scant. At the immunological level, the ability of an organism to maintain the immuno-surveillance unaltered under changing environmental conditions may preserve its survival. The immune system is highly integrated with other systems and their effectors can respond to the stress resulting from widely fluctuating temperatures. Here, the temperature of 21 °C did not stimulate a BpDef transcription over a short time. However, under normoxia, the persistence in the mesocosm at 21 °C maintain the production of BpDef mRNA, which is greatly depressed under the unfavorable condition of hypoxia. The decrease of the expression of defensin gene could result from the mobilization of cells from tissues to hemolymph, in order to reserve a response to stress (Matozzo and Marin, 2011; Cantet *et al.*, 2012).

At 30 °C, depleted oxygen seems also to contribute to the down-regulation of BpDef production, only after six days the values seem to be comparable to the beginning of the stimulation (CTN condition). Effects of environmental variables on immune functions of mussels have been extensively studied due to the use as sentinel (Shaw *et al.,* 2011). Roche (2001) demonstrated by Northern Blot that AMP genes, defensin, mytilin B and myticin B were constitutively expressed in winter, while the expression of only mytilin and myticin B was detectable in summer. In addition,

heat-shock resulted in no change in mytilin B expression but in suppression of defensin in cold weather condition and its induction in hot weather condition.

In conclusion, here we report for the first time the presence of mRNA for an AMP of a defensin family in a *Lessepsian B. pharaonis.* The results enrich the basic research on the AMP gene family in mussels, increasing our understanding of the immune system and laying a theoretical foundation for further practical application in studies of ecological immunology for understand immune function in the context of life-history traits. The assessment of gene transcription of antimicrobial peptides (AMPs) could provide new evidences for the ecological success of one specie in favor to others.

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**References**

Balseiro P, Falcó A, Romero A, Dios S, Martínez-López A, Figueras A, *et al. Mytilus galloprovincialis* myticin C: a chemotactic molecule with antiviral activity and immunoregulatory properties. PLoS ONE 6(8): 23140, 2011.

Barbarro JM and Abad MJ. Co-existence of two mytilid species in a heterogeneous environment: mortality, growth and strength of shell and byssus attachment. MEPS 476: 115-128, 2013.

Barbarro JM and Comed L. Byssus attachment strength of two mytilids in mono specific and mixed-species mussel beds. Biofouling 30: 975-985, 2014.

Barbieri M, Maltagliati F, Di Giuseppe G, Cossu P, Lardicci C, Castelli A. *Xenostrobus securis* (Bivalvia: Mytilidae) in brackish-water biotopes of the western Mediterranean provide evidence of its invasive potential. Mar. Biodiv. Rec. 4: 1-4, 2011.

Boman HG, Agerberth B, Boman A. Mechanisms of action on *Escherichia coli* of cecropin P1 and PR-39, two antibacterial peptides from pig intestine. Infect. Immun. 61: 2978-2984, 1993.

Boman HG. Antibacterial peptides: basic facts and emerging concepts. J. Intern. Med. 254: 197-215, 2003.

Bulet P, Stöcklin R, Menin L. Anti-microbial peptides: from invertebrates to vertebrates. Immunol. Rev. 198: 169-184, 2004.

Cantet F, Toubiana M, Parisi MG, Sonthi M, Cammarata M, Roch P. Individual variability of mytimycin gene expression in mussel. Fish Shellfish Immunol. 33: 641-644, 2012.

Carlton JT. The scale and ecological consequences of biological invasions in the world's oceans. In: Sandlund OT, Schei PJ, Viken A (eds), Invasive species and biodiversity management, Kluwer Academic Publishers, Dordrecht, pp 195-212, 1999.

Carrington E, Waite JH, Sarà G, Sebens K P. Mussels as a model system for integrative ecomechanics. Annu. Rev. Mar. Sci. 7: 443-469, 2015.

Charlet M, Chernysh S, Philippe H, Hetru C, Hoffmann JA, Bulet P. Innate immunity. Isolation of several cysteine-rich antimicrobial peptides from the blood of a mollusc, *Mytilus edulis*. J. Biol. Chem. 271: 21808-21813, 1996.

Cheng LL, Bartholomay LC, Olson KE, Lowenberger C, Vizioli J, Higgs S, *et al.* Characterization of an endogenous gene expressed in *Aedes aegypti* using an orally infectious recombinant sindbis virus. J. Insect Sci. 1: 10, 2001.

Garci M, Trigo J, Pascual S, Gonza A, Rocha F, Guerra A. *Xenostrobus securis* (Lamarck, 1819) (Mollusca: Bivalvia): first report of an introduced species in Galician waters. Aquacult. Int. 15:19-24, 2007.

Gestoso I, Arenas F, Rubal M, Veiga P, Peña M, Olabarria C. Shifts from native to non-indigenous mussels: Enhanced habitat complexity and its effects on faunal assemblages. Mar. Environ. Res. 90: 85-95, 2013.

Hernroth B. The influence of temperature and dose on antibacterial peptide response against lipopolysaccharide in the blue mussel, *Mytilus edulis.* Fish Shellfish Immunol. 14: 25-37, 2003.

Jenssen H, Hamill P, Hancock RE. Peptide antimicrobial agents. Clin. Microbiol. Rev. 19: 491-511, 2006.

Lee KA, Klasing KC. A role for immunology in invasion biology. Trends Ecol. Evol. 10: 523-529, 2004.

Li H, Parisi MG, Parrinello N, Cammarata M, Roch P. Molluscan antimicrobial peptides, a review from activity-based evidences to computer-assisted sequences. Inv. Surv. J. 8: 85-97, 2011.

Lockwood JL, Cassey P, Blackburn TM. The role of propagule pressure in explaining species invasions. Trends Ecol. Evol. 20: 223-228, 2005.

Lockwood JL, Cassey P, Blackburn TM. The more you introduce the more you get: the role of colonization pressure and propagule pressure in invasion ecology. Div. Distr. 15: 904-910, 2009.

Matozzo V, Marin MG. Bivalve immune responses and climate changes: is there a relationship? Inv. Surv. J. 8: 70-77, 2011.

Mitta G, Hubert F, Dyrynda EA, Roch P. Mytilin B and MGD2, two antimicrobial peptides of marine mussels: gene structure and expression analysis. Dev. Comp. Immunol., 24: 381-393, 2000a.

Mitta G, Vandenbulcke F, Noel T, Romestand B, Beauvillain JC, Salzet M. Differential distribution and defence involvement of antimicrobial peptides in mussel. J. Cell Sci. 113: 2759-2769, 2000b.

Mitta G, Hubert F, Noel T, Roch P. Myticin, a novel cysteine-rich antimicrobial peptide isolated from haemocytes and plasma of the mussel *Mytilus*

*galloprovincialis*. Eur. J. Biochem. 265: 71-78, 1999a.

Mitta G, Vandenbulcke F, Hubert F, Roch P. Mussel defensins are synthesised and processed in granulocytes then released into the plasma after bacterial challenge. J. Cell Sci. 112: 4233-4242, 1999b.

Ojaveer H, Galil BS, Campbell ML, Carlton JT, Canning-Clode J, Cook Ej, *et al.* Classification of non-Indigenous species based on their impacts: considerations for application in marine management. PLoSBiol, 13(4): e1002130, 2015.

Park Cb, Kim HS, Kim SC. Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. Biochem. Biophys. Res. Commun. 244: 253-257, 1998.

Parisi MG, Li H, Toubiana M, Parrinello N, Cammarata M, Roch P. Polymorphism of mytilin B mRNA is not translated into maturepeptide. Mol. Immunol. 46: 384-392, 2009.

Pascual S, Villalba A, Abollo E, Garci M, Gonzalez A, Nombela M, *et al.* The mussel *Xenostrobus securis*: a well-established alien invader in the Ria de Vigo (Spain, NE Atlantic). Biological Invasions 12: 2091-2103, 2010.

Reddy KV, Yedery RD, Aranha C. Antimicrobial peptides: premises and promises. Int. J. Antimicrob. 24: 536-547, 2004.

Roch P. Behaviour of defense peptides in environmentally stressed mussels. Revue Médicale Vétérinaire153: 517-520, 2001.

Sang Y, Blecha F. Antimicrobial peptides and bacteriocins: alternatives to traditional antibiotics. Anim. Health Res. Rev. 9: 227-235, 2008.

Sarà G. Palmeri V, Rinaldi A, Montalto V, Helmuth B. Predicting biological invasions in marine habitats through ecophysiological mechanistic models: a study case with the bivalve *Brachidontes pharaonis*. Diversity Distribution 19: 1235-1247, 2013.

Sarà G, Romano C, Caruso M, Mazzola A. The new Lessepsian entry *Brachidontes pharaonis* (Fischer P.,1870) (Bivalvia, Mytilidae) in the western Mediterranean: a physiological analysis under varying natural conditions. J. Shellfish Res. 19: 967-977, 2000.

Sarà G, Romano C, Mazzola A. A new Lessepsian species in the western Mediterranean (*Brachidontes pharaonis* Bivalvia: Mytilidae): density, resource allocation and biomass. Mar. Biodiv. Rec. I8, 2008.

Shaw JP, Dondero F, Moore MN, Negri A, Dagnino A, Readman JW, *et al.* Integration of biochemical, histochemical and toxicogenomic indices for the assessment of health status of mussels from the Tamar Estuary, UK. Mar. Environ. Res. 72: 13-24, 2011.

Tincu JA, Taylor SW. Antimicrobial peptides from marine invertebrates. Antimicrob. Angents Chemother. 48: 3645-3654, 2004.

Zhang L, Yu W, He T, Yu J, Caffrey RE, Dalmasso EA, Fu S, *et al.* Contribution Of Human Alpha-Defensin 1, 2, And 3 To The Anti-Hiv-1 Activity Of Cd8 Antiviral Factor. Sci. 298: 995-1000, 2002.