Fish & Shellfish Immunology 35 (2013) 910–917

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Concholepas concholepas Ferritin H-like subunit (*CcFer*): Molecular characterization and single nucleotide polymorphism associated to innate immune response

Jacqueline Chávez-Mardones, Valentina Valenzuela-Muñoz, Gustavo Núñez-Acuña, Waleska Maldonado-Aguayo, Cristian Gallardo-Escárate*

Laboratory of Biotechnology and Aquatic Genomics, Interdisciplinary Center for Aquaculture Research (INCAR), University of Concepción, P.O. Box 160-C, Concepción, Chile

ARTICLE INFO

Article history:
Received 6 December 2012
Received in revised form
27 June 2013
Accepted 28 June 2013
Available online 6 July 2013

Keywords:
Ferritin H subunit
Immune response
qPCR
HRMA
SNP

ABSTRACT

Ferritin has been identified as the principal protein of iron storage and iron detoxification, playing a pivotal role for the cellular homeostasis in living organisms. However, recent studies in marine invertebrates have suggested its association with innate immune system. In the present study, one Ferritin subunit was identified from the gastropod *Concholepas concholepas* (*CcFer*), which was fully characterized by Rapid Amplification of cDNA Ends technique. Simultaneously, a challenge test was performed to evaluate the immune response against *Vibrio anguillarum*. The full length of cDNA *Ccfer* was 1030 bp, containing 513 bp of open reading frame that encodes to 170 amino acid peptide, which was similar to the Ferritin H subunit described in vertebrates. Untranslated Regions (UTRs) were identified with a 5′UTR of 244 bp that contains iron responsive element (IRE), and a 3′UTR of 273 bp. The predicted molecular mass of deduced amino acid of *CcFer* was 19.66 kDa and isoelectric point of 4.92. Gene transcription analysis revealed that *CcFer* increases against infections with *V. anguillarum*, showing a peak expression at 6 h post-infection. Moreover, a single nucleotide polymorphism was detected at −64 downstream 5′UTR sequence (SNP-64). Quantitative real time analysis showed that homozygous mutant allele (TT) was significantly associated with higher expression levels of the challenged group compared to wild (CC) and heterozygous (CT) variants. Our findings suggest that *CcFer* is associated to innate immune response in *C. concholepas* and that the presence of SNPs may involve differential transcriptional expression of *CcFer*.

1. Introduction

Living organisms possess diverse mechanisms for the maintenance of optimal levels of iron, one of them being the protein Ferritin, which has a role in cellular detoxification and homeostasis, through the storage of iron in a secure and compact manner [1]. Its common molecular structure is a hollow spherical protein complex of 24 protein subunits, comprising of a thick protein shell, which can mineralize up to 4500 atoms of Fe^{+3}. In vertebrates Ferritin is composed of two types of chains or subunits, Heavy (H) and Light (L), which are coupled to form heteropolymers of different proportions and for different messenger RNA encoding [2,3]. Both subunits play different roles in the control of the iron levels in the organism, the H chain presents a molecular weight of 21 KDa, its function is the oxidation of iron, converting Fe^{+2} to Fe^{+3} by the ferroxidase center, involving 7 highly conserved residues in different taxa. In addition a tyrosine residue at position 27 which forms a complex Fe^{+3} Tyr responsible for iron biomineralization has been identified [3]. Furthermore, the L chain with a molecular weight of 19 KDa possesses amino acid residues known as the nucleation sites which provide ligands for binding Fe^{+3} [4]. Recently, a third type of ferritin designated as M subunit has been identified in lower vertebrates, which possesses both the ferroxidase center and the residues involved in iron nucleation [5]. Ferritin has been described in various organisms, including microorganisms, plants, vertebrates and invertebrates, conserving their structural characteristics [2]. Among invertebrates, mollusks have been already molecularly characterized in *Haliotis rufescens* [6], *Haliotis discus discus* [7], *Argopecten irradians* [8], *Meretrix meretrix* [9], *Crassostrea gigas* [10], *Haliotis discus hannai* [11], *Pinctada fucata* [12], *Sinonovacula constricta* [13] and *Ruditapes philippinarum* [14].

* Corresponding author. Tel.: +56 412203422.
E-mail addresses: crisgallardo@udec.cl, crisgallardo@oceanografia.udec.cl (C. Gallardo-Escárate).

1050-4648/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fsi.2013.06.028

Ferritin expression is regulated transcriptionally, as well as at post transcriptional level, dependent on intracellular iron levels, which interacts with a region called iron responsive element (IRE) and iron regulatory proteins (IRPs) [15]. In addition, previous studies suggest that the expression of Ferritin is subject to other factors such as oxidative stress [16,17], hormones and inflammatory cytokines [18], temperature [6], and heavy metals [11]. It has also been described that Ferritin is regulated by pathogen associated molecular pattern (PAMP) induction, employing the sequestration of iron to control the ROS production and pathogen proliferation [18,19].

Studies in marine genomics have gained great interest, due to the possibility of obtaining genetic information about mechanisms and candidate genes involved in immune response [20–22]. Furthermore, molecular characterization of genes involved in host defense could be used for detection of single nucleotide polymorphism (SNP), and to evaluated its association with susceptibility or resistance against pathogens [23,24]. Here, transcription level of candidate genes containing SNP variants and its effect on the immune response has been scarcely evaluated.

Concholepas concholepas is a benthic species endemic to the southeastern Pacific coast [25,26], that supports the main invertebrate small-scale fisheries in Chile. The objective of this study was to characterize *Ferritin* H-like subunit gene in *C. concholepas* (*Ccfer*) and to evaluate its association with innate immune response. Furthermore, the presence of single nucleotide polymorphisms (SNPs) were assessed, as well as their effects on *Ccfer* transcription expression in animals exposed to *Vibrio anguillarum*.

2. Materials and methods

2.1. Samples and experimental design

We obtained 36 specimens of *C. concholepas* from Puerto Oscuro, Coquimbo-Chile (31°26'S–71°36'W). The shell length of individuals was between 12 and 15 cm. These were acclimated for three days and maintained in filtered seawater at 17 °C in 100 L tanks, with food and water exchange. The 36 animals were divided into two groups with 18 individuals (control and challenged group). For the challenge *V. anguillarum* obtained from Laboratory of Biotechnology and Aquaculture Genomics was grown in Luria–Bertani (LB) and incubated at 20 °C for 24–48 h, once the concentration of 1.6×10^8 cells/mL was achieved, challenged group was injected with 100 μL of *V. anguillarum* directly in muscle, while the control group was injected with 100 μL LB medium without bacteria. Later, 3 individuals of each group were collected at 2, 4, 6, 10, 24 and 33 h post injection (p.i). The tissues sampled were muscle and gills, which were stored in RNA later RNA Stabilization Reagent (Ambion, USA) at −80 °C until RNA extraction. In addition, muscle tissue samples were fixed in absolute ethanol, for a subsequent DNA extraction using LiCl precipitation method [27].

2.2. Extraction of RNA and cDNA synthesis

Total RNA was isolated from tissues used 1 mL of Trizol Reagent (Invitrogen, Carlsbad, USA) following the manufacturer's instructions and then homogenized with liquid nitrogen. The concentration and purity of the total RNA was measured in a spectrophotometer ND 1000 (Thermo Fisher Scientific, Copenhagen, Denmark). The RNA integrity was verified by electrophoresis using 1.2% denaturing agarose gel. A 200 ng/μl of RNA were used for the first strand of cDNA synthesis using the ReverAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, MA, USA), following the manufacturer's instructions.

2.3. Cloning of the full-length CcFer cDNA

CcFer partial sequence was identified from the de novo assembly of the readings obtained from sequencing of *C. concholepas* performed with the platform 454 pyrosequencing, the sequencing results are available for download in the twentieth Digital Dryad repository (http://datadryad.org/) http://dx.doi.org/10.5061/dryad.mf00q under the access. PCR was performed using specific primers Cc-F1 and Cc-R1 (Table 1) with a final volume of 12.5 μL, which contained 1× buffer, BSA 0.2 μg/μL, 0.2 mM dNTPs, Mg^{+2} 1.5 mM, 0.5 μM of each primer, Taq polymerase 0.1 U/μL (Thermo Scientific, USA), 1 μl cDNA and water. The reactions were carried out in a Veriti thermal cycler (Applied Biosystems, USA) using the following cycle: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 51 °C for 30 s and 72 °C for 60 s, and final extension at 72 °C for 10 min. The PCR product was sequenced in Macrogen Inc. (S. Korea) in an automatic sequencer ABI3730xl (Applied Biosystems, USA). The result of the sequencing was analyzed using the Geneious 5.1 software (Biomatter, New Zealand) [28]. Further, BLASTx analysis was performed to determine the similarity of sequence with other ferritin sequences previously characterized.

Based on the identified EST sequence, four gene-specific primers (Table 1) were designed to amplify the full length cDNA of *CcFer* by rapid amplification of cDNA ends (RACE) technique. Here, First-Choice®RLM RACE kit (Ambion®, Life Technologies, USA) was used according the manufacturer's instructions. The PCR conditions were: 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, to finalize with the final extension of 72 °C for 7 min. The amplicons were visualized in 1.2% agarose gels using an Ultracam® (Model 4883) digital system. The fragments obtained for both extremes ends-UTR were inserted into the cloning vector using TOPO TA cloning Kit (Invitrogen™, Life Technologies, USA). Subsequently the vectors were transformed into electrocompetent bacteria *Escherichia coli* JM109 and cultured on agar LB/amp/IPTG/Xgal overnight at 37 °C. Positive clones were selected for plasmid isolation using E. Z. N.A ®Plasmid DNA Mini Kit II (Omega Bio-tek, Doraville, GA, USA) following the manufacturer's instructions. Finally, the *CcFer* cDNA sequence and the deduced amino acid sequences were analyzed by Geneious 5.1 software (Biomatter, New Zealand) [28].

2.4. Quantitative PCR analysis

Quantitative PCR was carried out to evaluate the *CcFer* transcription expression using the $2^{-\Delta\Delta Ct}$ method. Further, five

Table 1
Primers designed for the amplification of Ferritin (PCR, QPCR and HRM).

Primer	Sequence	Objective
Cc_F1	ATACCCATCGGCTTTTGCTT	PCR- End time
Cc_R1	GACAGAGGGTTTTCTGTGACG	
CcRACE F_E	GCCGCCAGAACTTCCACGCCGAAAGC	RACE
CcRACE F_I	GTGGCGCTGGAGGGGTTCGCCAAGT	
CcRACE R_E	GCTGTCGGCCACCTGGTGCAGGTCCA	
CcRACE R_I	ACGCTCTTCTCCAGCGCCAGGGCCA	
EF1_FCc	TGACAGTTCAGAGCGACGAC	QPCR
EF1_RCc	CATCAAGTCGGTGGAGAAGC	
EF2_FCc	AAGGACTTCGCCAAGCTGTA	
EF2_RCc	ACGCTGCTCCTTGTACCACT	
GAPDH_FCc	GTCCGTCCACAGTCTTCTGG	
GAPDH_RCc	AGGACATGAAGGTGGTCAGC	
ATub_FCc	TGCATACTGCTCTCATCACCTT	
ATub_RCc	CGTTGATTGGTGTCCAACTG	
Btub_FCc	GGTCAACATGGTGCCCTTC	
Btub_RCc	GTTCTTGGCGTCGAACATCT	
SNPCc_F	CCTCCAAAGGCAGGTCGTCGT	HRM
SNPCc_R	CTGGTCTCGTTCTCTTCTCAAAGCTG	

sequences from *C. concholepas* EST-database were selected to be evaluated as housekeeping genes (HKG): *Elongation factor 1* (EF1), *Elongation factor 2* (EF2), *glyceraldehyde-3- phosphate dehydrogenase* (GAPDH), *β-tubulin* (β tub) and *α-tubulin* (tub α) (see designed primers in Table 1). For HKG validation, qPCR analyses were performed in a StepOnePlus™ (Applied Biosystems, USA) using 10 samples randomly selected. PCR reactions were conducted using Maxima SYBR Green/ROX qPCR Master Mix (2×) kit (Thermo Scientific, USA) with a final volume of 10 μL and amplification cycle: 95 °C for 10 min (holding stage), 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The best HKG for our study was estimated by the NormFinder algorithm [29]. In addition, qPRC was used to analyze the expression levels of *CcFer* at different time points after the immune challenge experiment. The reactions were performed in triplicate in a final volume of 10 μL, with 2 μL of cDNA, 0.25 mM of primers and water, using the Maxima® SYBR Green/ROX qPCR Master Mix (2×) Kit (Thermo Scientific, USA), following manufacture's recommendations. The cycle of amplification was as follows: 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 60 °C for 1 min, followed by a dissociation curve with the same conditions listed above. The results obtained were analyzed with STATISTICA version 7.0 (StatSoft Inc., Tulsa, OK, USA) using one way ANOVA analysis where the differences in gene expression were considered significant with a *p*-value <0.05.

2.5. SNP genotyping using HRM

The SNP associated to *CcFer* gene was identified in a previous work [30], using the CLC Bioinformatics Genomics Workbench software 5.0.1 (CLC bio, Katrinebjerg, Denmark). Here, a putative SNP (C/T) was identified at 5′end-UTR. To validate the SNP variants, High-Resolution Melting Analysis (HRMA) was conducted (see Table 1 for designed primers) using genomic DNA samples obtained from the LiCl precipitation carried out in both the challenged and control groups of *C. concholepas*. HRM analysis was carried out by ECO™ Real-Time PCR System (Illumina, USA) using KAPA HRM FAST PCR Kit (Kapa Biosystem, USA), with a final volume of 10 μl. PCR condition were: 50 °C for 2 min, 95 °C for 10 min; 40 cycles of 95 °C for 15 s, and 60 °C for 30 s; followed by a melting curve from 60 °C to 95 °C.

3. Results

3.1. Characterization of CcFer

The Blastx analysis for the sequences identified from 454 database for *C. concholepas* has an E-value of 8e-118 and 3e-97 for *Reishia clavigera* (AET43963) and *C. gigas* (AAP83793) respectivement. The full length sequence obtained for *CcFer* cDNA was composed by 244 bp of 5′UTR of Ferritin gene, consisting in the iron response element (IRE) ^{63}GCTTTCAGCAGCTTTGAGAAGAGAACGA^{-36}, and a 3′UTR of 273 bp that possesses a conservative signal of polyadenylation ^{996}AATAAA^{1001}. The open reading frame (ORF) revealed 513 bp that encodes 170 amino acids. The predicted molecular mass of deduced amino acid of *CcFer* was 19.66 kDa and isoelectric point of 4.92. We identified two iron binding sites, IBRS1 (^{61}REHAEKLMKYQNTRGGR^{77}) and IBRS2 (^{124} DAQMCDFLESEYLEEQVKAMK^{144}), and also a site of N-glycosylation located between ^{108}NQAL^{111}. Further, seven residues involved in the oxidation of iron were found at the ferroxidase center ($E_{25} - Y_{32} - E_{59} - E_{60} - H_{63} - E_{105} - E_{139}$). Potential biomineralization residue (Tyr_{27}) could also be found in the derived amino acid sequence, manifesting the existence of novel *CcFer* H-like subunit (GenBank accession number KC107792) (Fig. 1).

Nucleotide sequence of *CcFer* that was analyzed by Blastn have shown a high homology with other sequences Ferritin-like in invertebrates, such as the muricid *R. clavigera* (JN413221) (96% of similitude), and the gastropod *Aplysia californica* (HM163166) (79%

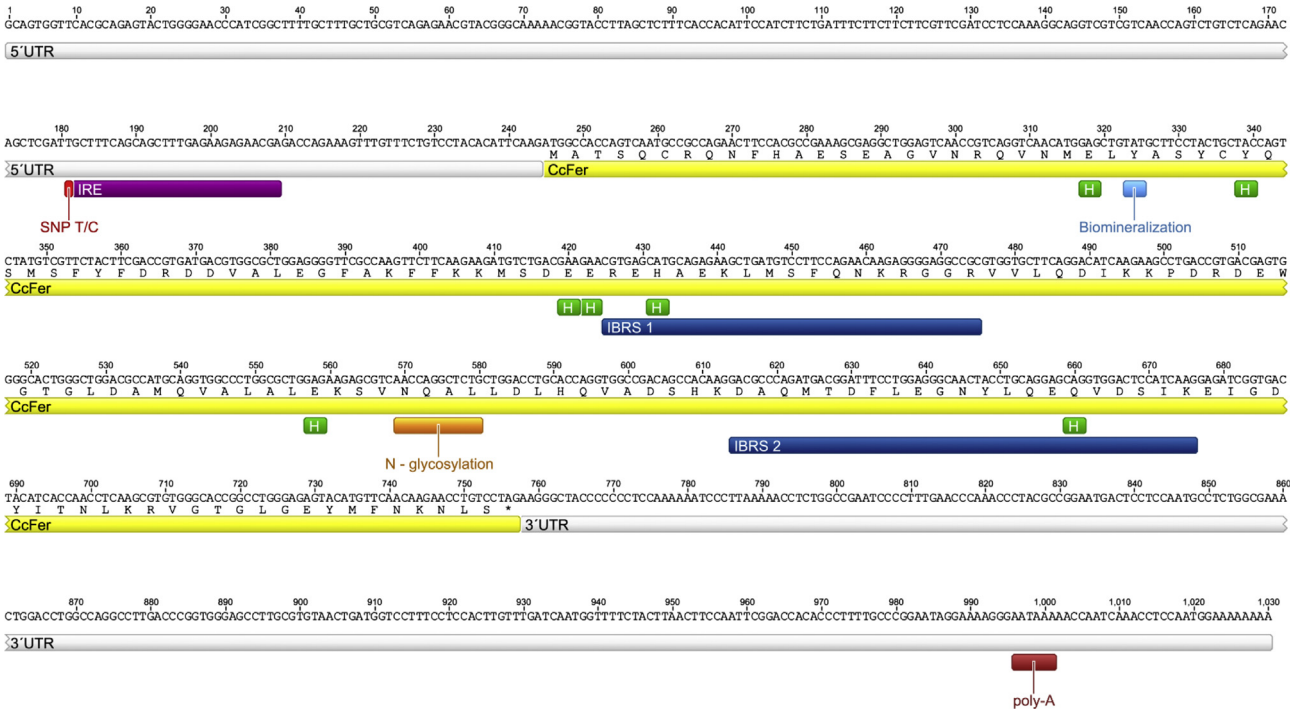

Fig. 1. Nucleotide sequence and deduction of ferritin amino acid of *C. concholepas*. The arrow indicates the position of the SNP (T/C). The solid line indicates the IRE (iron response element) structure. The boxes indicate the 7 residues responsible for the ferroxidase activity, the residue Tyr 27 in a light blue box is responsible for the iron biomineralization. The double lines correspond to the putative iron-binding regions IBRS1 and IBRS2. The residues with a orange box indicate N-Glycosylation site. Asterisk (*) indicates termination codon and the red box indicates the polyadenylation signal. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

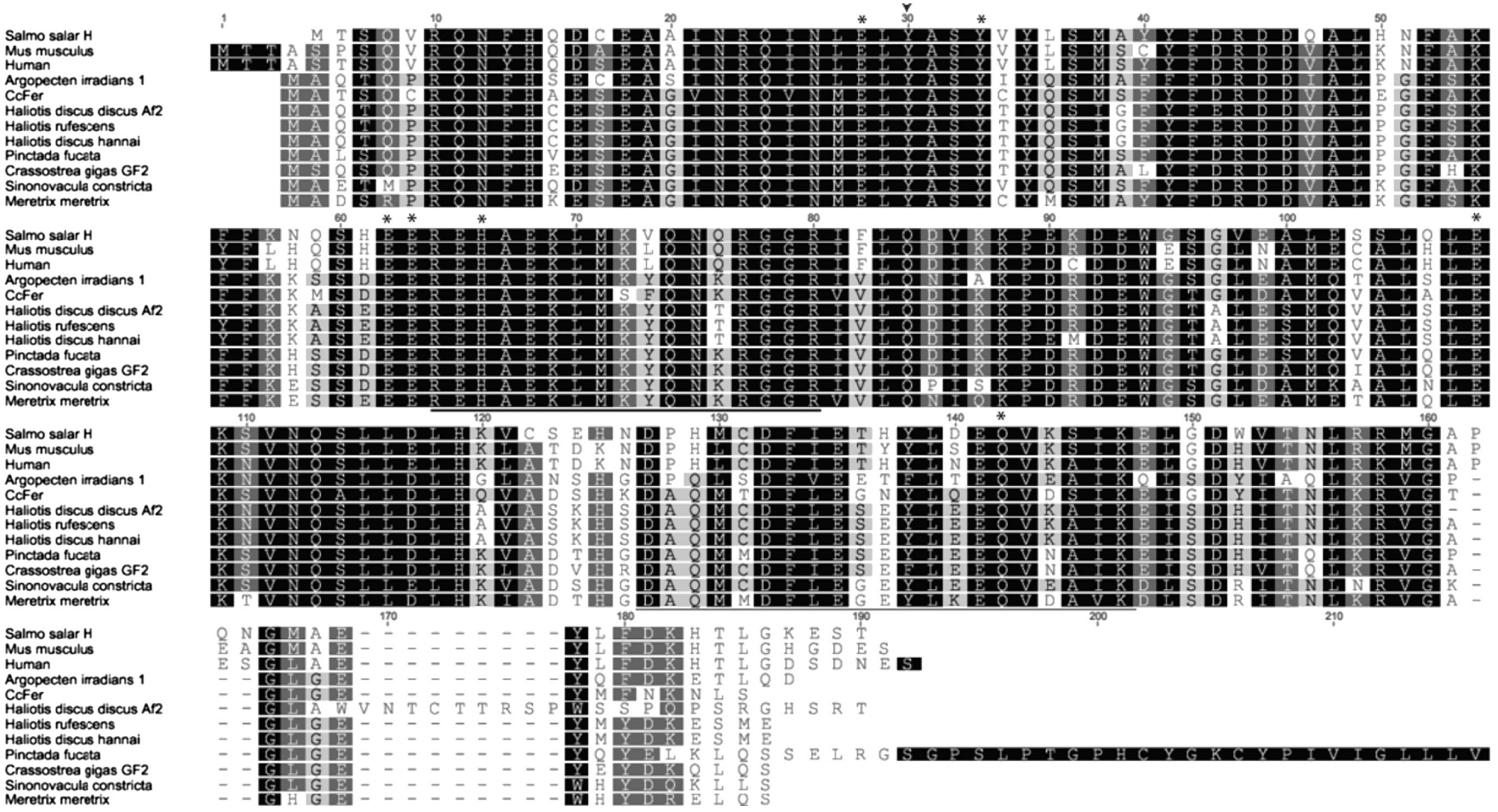

Fig. 2. Alignment of the amino acid sequence of *CcFer* with other sequences full length sequences H Ferritin chains. The access numbers in GenBank: *Haliotis discus discus* Abf2 (ABG88846) *Haliotis discus hannai* (ABH10672), *Salmo salar* (NP_001117129, partial sequence), *Sinonovacula constricta* (ACZ65230), *Haliotis rufescens* (ACZ73270), *Homo sapiens* (AAA35832), *Crassostrea gigas GF2* (AAP83794), *Pictada fucata* (AAQ12076), *Argopecten irradians* (AEN83774), *Mus musculus* (NP_034369) and *Meretrix meretrix* (AAZ20754). The asterisks indicate the 7 residues conserved in Ferritin H subunit, the arrow indicates the Tyr residue related with the biomineralization of iron. The underlined region indicates the two iron binding sites, IBRS1 and IBRS2.

of similitude). By Blastx analysis *CcFer* showed 80% identity for the *CcFer* sequence with Ferritin of the pacific oyster *C. gigas* (GF1) (AAP83793) and for *P. fucata* (AAQ12076) present 79% identity, both sequences described as H-chain Ferritins. Fig. 2 shows the degree of conservation of the relevant residues for the function of the H subunit among vertebrate organisms such as *Salmo salar* (NP_001117129), *Homo sapiens* (AAA35832) and invertebrates such as *M. meretrix* (AAZ20754), *C. gigas* (AAP83794), *A. irradians* (AEN83774) and *H. rufescens* (ACZ73270). The alignment showed 7 residues involved in the iron oxidation involved, and also the two iron binding sites IBRS1 and IBRS2. Furthermore, phylogenetic analysis was carried out using Neighbor-joining method comparing *CcFer* with different invertebrates Ferritin subunit members (Fig. 3). The tree revealed two main cluster as expected, mollusk H-like subunit clade, and *H. discus discus* Abf1 (DQ821434)//*Aterias forbesii* (AF001984) clade. This last clade was composed by a ferritin not similar to H-subunit reported from *H. discus discus* (DQ845482) and a starfish species.

3.2. Gene expression patterns of CcFer

For the ΔCt comparative method, the efficiency of the reactions for each gene target and housekeeping gene (HKG) was calculated as shown in Table 2. All set of primers and PCR conditions that achieved a reaction efficiency between 90 and 110% were sorted. Moreover, potential HKGs were evaluated by stability index using the NormFinder algorithm. Here, *Elongation factor 1* (stability index = 0.129) was selected.

3.2.1. Transcriptional response of CcFer exposed to V. anguillarum

In order to determine the tissue-specific transcriptional profile of *CcFer*, qPCR was carried out using gill and muscle tissues.

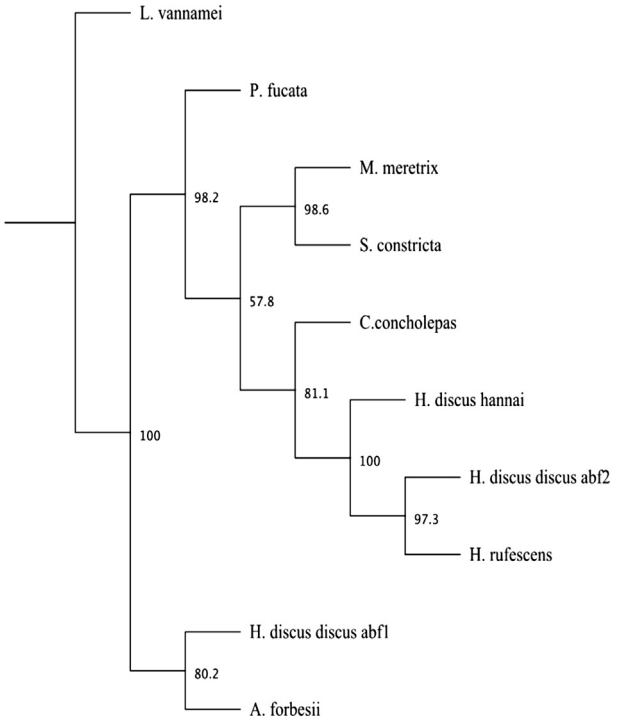

Fig. 3. Phylogenetic analysis of Ferritin in *C. concholepas*. Based on the Neighbor-joining method using nucleotide sequences available in the database of the codified regions of Ferritin, the access numbers utilized to produce the tree were: *Haliotis discus hannai* (DQ845482), subunidad *Abf2* de *Haliotis discus discus* (DQ821494), subunidad *Abf1* de *Haliotis discus discus* (DQ821434), *Haliotis rufescens* (GU191936), *Pictada fucata* (AF547223), *Aterias forbesii* (AF001984), *Litopenaeus vannamei* (AY955373), *Meretrix meretrix* (DQ069277) and *Sinonovacula constricta* (GQ906972).

Table 2
NormFinder analysis for the determination of the stability of endogenous control.

Gene name	Evaluation of stability	Standard error	Better gene
GAPDH	1.093	0.254	EF1
EF1	0.129	0.459	
EF2	0.309	0.209	

The results of this analysis are shown in Fig. 4, where *CcFer* was observed most expressed in gill with respect to muscle ($p = 0.0204$). Moreover, to evaluate the transcriptional modulation of *CcFer* upon *V. anguillarum* induction, gill tissues from challenged animals were used. The results are shown in Fig. 5, which distinguishes a basal expression of *CcFer* within two hours of post infection (hpi), and then gradually increase from 4 h to 6 h, and decrease during the 10 and 24 hpi, noting a slight increase in the expression at 33 h. These changes in the transcription of *CcFer* are statistically significant ($p = 0.0194$).

3.3. High resolution melting analysis of CcFer-SNP

The SNP identified in the sequence of *CcFer* was located 64 bp before start codon in the 5′UTR. Further, 34 *C. concholepas* individuals were genotyped by HRM analysis, showing three profiles of melt curves evidencing the different allelic variants, CC wild type and TT mutant type. The frequency archived was 35.3% of homozygous genotype CC, 29.4% of heterozygote genotype CT and 35.3% of homozygous genotype TT (Fig. 6A). In addition, we evaluated the transcriptional expression associated to each SNP genotype. Here, homozygous mutant alleles (TT) showed a greater expression in the challenged group in contrast to the wild alleles (CC) and the heterozygous (CT). However, the control animals group evidenced a significantly lower expression for the homozygous mutant alleles (TT) than the wild (CC) and heterozygous (CT) genotypes (Fig. 6B).

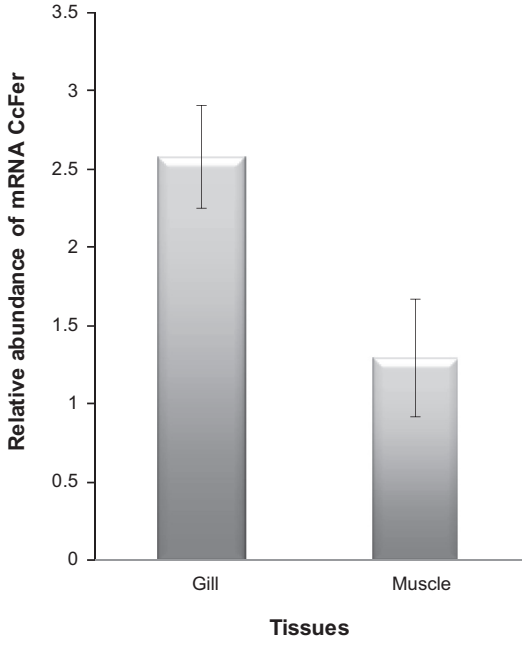

Fig. 4. Relative abundance of mRNA of *CcFer* in two tissues B: Gill, M: Muscle, normalized with *Elongation factor 1* (EF1). The statistical analysis was performed using ANOVA. The vertical bars represent ±5 standard error of the mean.

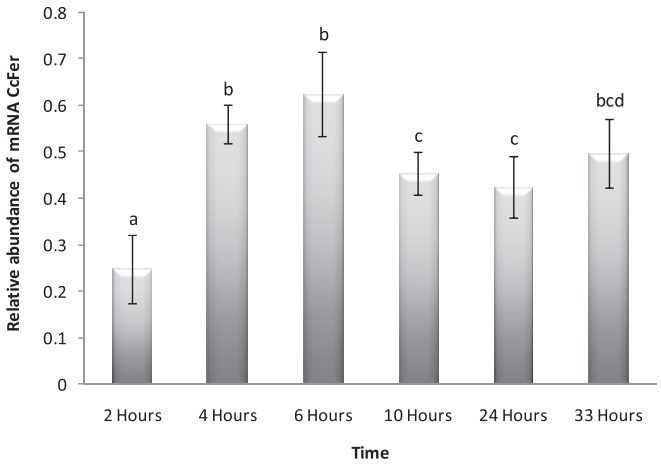

Fig. 5. Relative abundance of mRNA of *CcFer* in the gill tissue for 33 h post infection with *Vibrio anguillarum*, normalized with *Elongation factor 1* (EF1). The letters in each bar show significant differences in expression between each measurement: a) basal expression, b) relevant changes in the expression c) decrease in the expression bcd) evidence of a correlation between the expression at 4 h and 24 h, simultaneously experiencing an expression increase. Statistical analysis was performed using ANOVA. The vertical bars represent ±18 standard error of the mean.

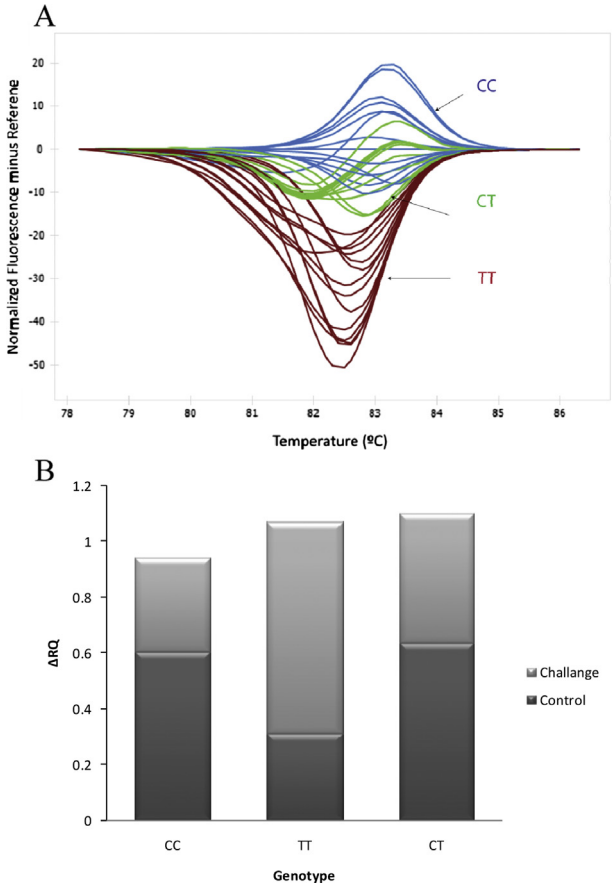

Fig. 6. A) Differential high-resolution melting analysis, applied to the SNP of type C/T *CcFer* gene. B) Delta RQ expression of *CcFer* related to SNPs genotypes in *C. concholepas*. The bars represent the differences of RQ values among control and challenged individuals for each genotype.

4. Discussion

4.1. Molecular characterization of CcFer

Ferritin protein is highly conserved across diverse taxa, characterized by its storage capacity of iron in a compact and secure manner, avoiding high metal concentrations that cause cellular damage by oxidative stress. However, due to its capacity to retain iron, the *Ferritin* gene is a potential candidate gene involved in host defense, restricting the bioavailability of iron for the invasion of pathogenic microorganisms. Consequently, the identification and characterization of *Ferritin* in marine invertebrates of commercial importance takes on added significance due to the scarce existing information. In this study we characterized the *Ferritin* gene in the gastropod *C. concholepas* and evaluated its transcriptomic expression pattern associating it with the presence of the molecular marker SNP, confirming their participation in the immune response system.

Studies regarding *Ferritin* subunits identified in marine invertebrates, demonstrate that it is constituted by a sequence between 170 and 180 amino acid residues. Our results showed that *CcFer* has an open reading frame of 513 nucleotides that encode a protein of 170 amino acid residues. These results coincides with previous reports for oysters and clams [10,14], confirming a conservative orthology with respect to other *Ferritin*-likes subunits. Herein, *CcFer* is exhibiting high identities with the clam *S. constricta* and *H. rufescens*, with 74.1% and 74.7% respectively.

Multiple sequence alignments showed that *CcFer* has a highly conserved patterns that characterize the *Ferritin* H subunit. For instance, 7 residues involved with oxidation of iron, two iron binding sites IBRS 1 and 2, and the residue of Tyr 27 were identified [31,32]. From these results and the absence of residues associated with the iron nucleation site, this reaffirms that *CcFer* corresponds to the *Ferritin* H-like subunit. The phylogenetic analysis of *Ferritin* H subunit described for invertebrates confirms the idea that *CcFer* corresponds to subunit H-like, since the tree clearly differentiates a separation between the clade of clams (*M. meretrix* and *S. constricta*) and the clade of the gastropods, the latter enters *CcFer*, along with representatives of the genus *Haliotis*.

4.2. Analysis of transcriptional expression of CcFer

The *Ferritin* gene expression in *C. concholepas* analyzed in two tissues showed higher levels of expression in the gill tissue. These are consistent with the function of this tissue, since the gill is in close contact with the surrounding seawater. Our results are consistent with a previous study that suggests that this organ could not only be important for feeding and respiration, but plays an important role in the recognition of exogenous particles from the environment, initiating the immune response at the transcriptional level [33]. These results suggest that the differential expression of *Ferritin* is subject to the species that is analyzed and the environmental conditions present at the time of the study. For instance in *H. discus hannai* the digestive gland tissue registers the highest expression of the gene in contrast to the gill tissue [11], and in the Pacific oyster (*C. gigas*) the highest expression occurs in the hemocytes, the heart and digestive gland [10]. Further, red abalone *H. rufescens*, demonstrated a tissue specific mRNA expression profile of *Ferritin*, since its expression greater in muscle, gill, foot and digestive gland, in contrast with the mantle and gonad whose expression is lower [6].

Regarding the utilization the *V. anguillarum* for the challenged of *C. concholepas* it is important emphasize some previous reports that have shown the modulation of the expression of the different components of the innate immune response in marine

invertebrates triggered by *Vibrio* spp., such as in *C. gigas* ECSIT (evolutionarily conserved signaling intermediate in toll pathways) where its expression was modified in hemolymph exposed to *V. anguillarum*, postulating that ECSIT in *C. gigas* must be a potential factor in the defense system of the Pacific oyster [34]. Moreover, that the pathogens possess siderophores, which are low-molecular-mass molecules that have a high specificity for chelating or binding iron to be used as bacterial nutrient [35]. These mechanisms could play an important role to capture the iron of the intracellular environment before the microorganism uses it. Furthermore, *Ferritin* prevents the excessive formation of reactive oxygen species via the Fenton reaction [18,36]. Quantitative analysis of *CcFer* expression was performed in the gill tissue of *C. concholepas* individuals challenged with *V. anguillarum* and evaluated during 33 h. Here, a peak of gene expression was archived at 6 h post induction with the pathogen, which then dropped up to 33 h. These results coincide with those observed in other studies for marine invertebrates, where transcription of *Ferritin* gene increases in the presence of a stressor. For instance, a study conducted using *Haliotis tuberculata*, the profile of 12 expression genes were analyzed after two successive infections with *Vibrio harveyi*. The results showed that *Ferritin* increases in the surviving organisms after the first and second infection, suggesting that this mollusk sequesters the iron available to limit the proliferation of *V. harveyi* [37]. In vertebrates, such as the red drum fish (*Sciaenops ocellatus*), exposed to the pathogen *Edwardsiella tarda* and *Streptococcus iniae*, the liver experiences an increase in the expression of *ferritin* from 4 h up to 48 h post infection. This is mainly due to the fact that the liver plays an important role in the iron metabolism in vertebrates [38].

4.3. Single nucleotide polymorphism associated to CcFer

A single nucleotide polymorphism (SNP) is one of the forms of common genetic variation in the genome, consisting of a base change in a nucleotide sequence, finding more frequently in the non-coding regions [39]. When present in the coding region SNPs are classified into two types: the SNP-synonymous altering the amino acid sequence of proteins and synonymous SNP, or so called silent mutations, which do not affect primary structure of the protein [40]. The presence of SNPs in non-coding and coding regions of candidate genes may be related to the regulation of the innate immune response of marine invertebrates, specifically the resistance or susceptibility of exposed individuals response to pathogens. For instance, a study carried out in *Crassostrea virginica* evidenced that a polymorphism in the serine protease inhibitor (*cvSI-1*) gene was associated with improved survival after disease-caused mortalities and in disease resistant eastern oyster strains [41]. Interestingly, the C allele of the SNP198 (C/T), consistently increased in frequency after mortalities that were caused primarily by Dermo and possibly also by MSX. Its frequency in the disease-resistant strain was significantly higher than that in the susceptible strains and the base population from which the selected strains were derived. According to the authors, SNP198 is a synonymous mutation, and its association with disease resistance may be due to its close linkage to a functional polymorphism nearby. Previous studies in *A. irradians* showed a non-synonymous change of Thr to Lys generated by an SNP C/A in *SOD* genes, it granted higher susceptibility to organisms with the C allele, which generated the change to Thr, presents a challenge by *V. anguillarum* [24]. We preliminarily identified three SNPs however only characterized one, which could have implications in transcription regulation due to its location in the 3′UTR. Besides, it is important emphasize that this SNP is located 64 bp before the transcriptional start site and prior to the iron regulatory element (IRE). We suggest that it might be altering the stability of the mRNA, affecting the connection between IRE and iron regulatory protein (IRPs). On the other hand, the other two polymorphisms were not consistent for HRM analysis. The association between the transcriptional profile of *CcFer* and SNP, demonstrated that individuals with the TT genotype (mutant) experience a greater expression of *CcFer* in response to the challenge with *V. anguillarum*, in contrast to the individuals with genotypes CC (wild) and CT (heterozygous) that do not show a significant difference between the control and challenged group. This suggests that the challenged individuals experience a differential response after an induction with pathogens, which is reflected in the increased expression of *CcFer* in individuals possessing the mutant SNP genotype. It is important this is the first time that described an SNP marker in Ferritin and presents association with its mRNA abundance levels in response to a marine pathogen.

5. Conclusion

This work provides a new and relevant information concerning the response at a transcriptional level of *Ferritin* against pathogens in one species of gastropod sparsely studied such as *C. concholepas*. In addition, it demonstrates how a variation in a nucleotide sequence, can trigger changes in gene transcription levels in *CcFer* in response to pathogens.

Acknowledgments

This research has been made possible thanks to funding provided by FONDEF grant D09I1065 and FONDAP project 15110027 (CONICYT-Chile).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2013.06.028.

References

[1] Orino K, Watanabe K. Molecular, physiological and clinical aspects of the iron storage protein ferritin. The Veterinary Journal 2008;178:191–201.
[2] Andrews SC, Arosio P, Bottke W, Briat Jean-Franqois, von Darl M, Harrison P, et al. Structure, function, and evolution of ferritins. Journal of Inorganic Biochemistry 1992;47:161–74.
[3] Harrison PM, Arosio P. The ferritins: molecular properties, iron storage function and cellular regulation. Biochimica et Biophysica Acta 1996;1275:161–203.
[4] Santambrogio P, Levi S, Arosio P, Palagi L, Vecchio G, Lawson DM, et al. Evidence that a salt bridge in the light chain contributes to the physical stability difference between heavy and light human ferritins. The American Society for Biochemistry and Molecular Biology 1992;267:14077–83.
[5] Giorgi A, Mignogna G, Bellapadrona G, Gattoni M, Chiaraluce R, Consalvi V, et al. The unusual co-assembly of H- and M-chains in the ferritin molecule from the Antarctic teleosts *Trematomus bernacchii* and *Trematomus newnesi*. Archives of Biochemistry and Biophysics 2008;478:69–74.
[6] Salinas-Clarot K, Gutiérrez A, Núñez-Acuña G, Gallardo-Escárate C. Molecular characterization and gene expression of ferritin in red abalone (*Haliotis rufescens*). Fish and Shellfish Immunology 2011;30:430–3.
[7] Zoysa MD, Lee J. Two ferritin subunits from disk abalone (*Haliotis discus discus*): cloning, characterization and expression analysis. Fish and Shellfish Immunology 2007;23:624–35.
[8] Li J, Li L, Zhabg S, Li J, Zhang G. Three ferritin subunits involved in immune defense from bay scallop *Argopecten irradians*. Fish and Shellfish Immunology 2012;32:368–72.
[9] Wang X, Liu B, Xiang J. Cloning, characterization and expression of ferritin subunit from clam *Meretrix meretrix* in different larval stages. Comparative Biochemistry and Physiology, Part B Biochemistry and Molecular Biology 2009;154:12–6.
[10] Durand J-P, Goudard F, Pieri J, Escoubas J-M, Schreiber N, Cadoret J-P. *Crassostrea gigas* ferritin: cDNA sequence analysis for two heavy chain type subunits and protein purification. An International Journal on Genes and Genomes 2004;338:187–95.
[11] Wu C, Zhang W, Mai K, Xu W, Wang X, Ma H, et al. Transcriptional up-regulation of a novel ferritin homolog in abalone *Haliotis discus hannai* Ino

by dietary iron. Comparative Biochemistry and Physiology, Part C 2010;152:424–32.
[12] Zhang Y, Meng Q, Jiang T, Wang H, Xie L, Zhang R. A novel ferritin subunit involved in shell formation from the pearl oyster (*Pinctada fucata*). Comparative Biochemistry and Physiology, Part B Biochemistry and Molecular Biology 2003;135:43–54.
[13] Li C, Li H, Su X, Li T. Identification and characterization of a clam ferritin from *Sinonovacula constricta*. Fish and Shellfish Immunology 2011;30:1147–51.
[14] Kim H, Elvitigala DAS, Lee Y, Lee S, Whang I, Lee J. Ferritin H-like subunit from Manila clam (*Ruditapes philippinarum*): molecular insights as a potent player in host antibacterial defense. Fish and Shellfish Immunology 2012;33:926–36.
[15] Torti FM, Torti SV. Regulation of ferritin genes and protein. The American Society of Hematology 2002;99:3505–16.
[16] Zheng W-j, Hu Y-h, Sun L. Identification and analysis of a *Scophthalmus maximus* ferritin that is regulated at transcription level by oxidative stress and bacterial infection. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 2010;156:222–8.
[17] Tsuji Y, Ayaki H, Whitman S, Morrow C, Torti S, Torti F. Coordinate transcriptional and translational regulation of ferritin in response to oxidative stress. Molecular and Cellular Biology 2000;20:5818–27.
[18] Ong ST, Ho JZS, Ho B, Ding JL. Iron-withholding strategy in innate immunity. Immunobiology 2006;211:295–314.
[19] Recalcati S, Invernizzi P, Arosio P, Cairo G. New functions for an iron storage protein: the role of ferritin in immunity and autoimmunity. Journal of Autoimmunity 2008;30:84–9.
[20] Rhee J-S, Kim B-M, Jeong C-B, Horiguchi T, Lee Y-M, Kim I-C, et al. Immune gene mining by pyrosequencing in the rockshell, *Thais clavigera*. Fish and Shellfish Immunology 2012;32:700–10.
[21] Moreira R, Balserio P, Planas JV, Fuste B, Beltran S, Novoa B, et al. Transcriptomics of in vitro immune-stimulated hemocytes from the manila clam *Ruditapes philippinarum* using high-throughput sequencing. PLoS One 2012;7:1–16.
[22] Venier P, Varotto L, Rosani U, Millino C, Celegato B, Bernante F, et al. Insights into the innate immunity of the Mediterranean mussel *Mytilus galloprovincialis*. BioMed Central Genomics 2011;12:1–19.
[23] Núñez-Acuña G, Aguilar-Espinoza A, Chávez-Mardones J, Gallardo-Escárate C. Ubiquitin-conjugating enzyme E2-like gene associated to pathogen response in *Concholepas concholepas*: SNP identification and transcription expression. Fish and Shellfish Immunology 2012;33:1065–8.
[24] Bao Y, Li L, Zhang G. Polymorphism of the superoxide dismutase gene family in the bay scallop (*Argopecten irradians*) and its association with resistance/susceptibility to *Vibrio anguillarum*. Developmental and Comparative Immunology 2010;34:553–61.
[25] Castilla J. Pesquería de moluscos gastrópodos en Chile: *Concholepas concholepas*, un caso de estudio. Monografías Biológicas 1982;2:199–212.
[26] Cárdenas L, Sánchez R, Gomez D, Fuenzalida G, Gallardo-Escárate C, Tanguy A. Transcriptome analysis in *Concholepas concholepas* (Gastropoda, Muricidae): mining and characterization of new genomic and molecular markers. Marine Genomics 2011;4:197–205.
[27] Aljanabi SM, Martinez I. Universal and rapid salt-extraction of high genomic DNA for PCR- based techniques. Nucleic Acids Research 1997;25:4692–3.
[28] Drummond A. Geneious. v. 5.1. Available from: http://www.geneious.com; 2009.
[29] Andersen CL, Jesen JL, Orntoft TF. Normalization of real - time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data set. Cancer Research 2004;64:5245–50.
[30] Gallardo-Escárate C, Núñez-Acuña G, Valenzuela-Muñoz V. SNP discovery in the marine gastropod *Concholepas concholepas* by high-throughput transcriptome sequencing. Conservation Genetics Resources 2013, in press.
[31] Constanzo F, Colombo M, Staempfli S, Santoro C, Marone M, Frank R, et al. Structure of gene an pseudogenes of human apoferritin H. Nucleic Acids Research 1986;14:721–36.
[32] Leong JS, Jantzen SG, von Shalburg KR, Cooper GA, Messmer AM, Liao NY, et al. *Salmo salar* and *Esox lucius* full-length cDNA sequences reveal changes in evolutionary pressures on a post-tetraploidization genome. BioMed Central Genomics 2010;11:1–17.
[33] Calvo JAM. La perfusión de Branquias como modelo de valoración de la calidad de agua y su relación con la producción intensiva de truchas. Universidad Complutense de Madrid; 1996.
[34] Zhang L, Li L, Zhang G. The first identification of molluscan Ecsit in the Pacific oyster, *Crassostrea gigas*, and its expression against bacterial challenge. Aquaculture Research 2012;43:1071–80.
[35] Butler A, Theisen RM. Iron (III)–siderophore coordination chemistry: reactive of marine siderophores. Coordination Chemistry Reviews 2010;254:288–96.
[36] Arosio P, Ingrassia R, Cavadini P. Ferritins: a family molecules for iron storage, antioxidation and more. Biochimica et Biophysica Acta 2009;1790:589–99.
[37] Travers M-A, Maistertzheim A-L, Cardinaud M, Friedman CS, Huchette S, Moraga D, et al. Gene expression patterns of abalone, *Haliotis tuberculata*, during successive infections by the pathogen *Vibrio harveyi*. Journal of Invertebrate Pathology 2010;105:289–97.
[38] Hu Y-h, Zheng W-j, Sun L. Identification and molecular analysis of a ferritin subunit from red drum (*Sciaenops ocellatus*). Fish and Shellfish Immunology 2010;28:678–86.
[39] Seeb JE, Carvalho G, Hauser L, Naish K, Roberts S, Seeb L. Single-nucleotide polymorphism (SNP) discovery and applications of SNP genotyping in non-model organisms. Molecular Ecology Resources 2011;11:1–8.
[40] Liao P-Y, Lee KH. From SNPs to functional polymorphism: the insight into biotechnology applications. Biochemical Engineering Journal 2010;49:149–58.
[41] Yu H, He Y, Wang X, Zhang Q, Bao Z, Guo X. Polymorphism in a serine protease inhibitor gene and its association with disease resistance in the eastern oyster (*Crassostrea virginica Gmelin*). Fish and Shellfish Immunology 2011;30:757–62.