Gene 564 (2015) 73–80

Contents lists available at ScienceDirect

Gene

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

Prohibitin-2 gene reveals sex-related differences in the salmon louse *Caligus rogercresseyi*

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ARTICLE INFO

Article history:
Received 2 January 2015
Received in revised form 18 March 2015
Accepted 20 March 2015
Available online 23 March 2015

Keywords:
Prohibitin gene
Caligus rogercresseyi
Gene expression
RNA-seq
SNPs

ABSTRACT

Prohibitins are evolutionarily conserved proteins present in multiple cellular compartments, and are involved in diverse cellular processes, including steroid hormone transcription and gametogenesis. In the present study, we report for the first time the characterization of the *prohibitin-2* (*Phb2*) gene in the sea lice *Caligus rogercresseyi*. The *CrPhb2* cDNA showed a total length of 1406 bp, which contained a predicted open reading frame (ORF) of 894 base pairs (bp) encoding for 298 amino acids. Multiple sequence alignments of prohibitin proteins from other arthropods revealed a high degree of amino acid sequence conservation. In silico Illumina read counts and RT-qPCR analyses showed a sex-dependent differential expression, with mRNA levels exhibiting a 1.7-fold (RT-qPCR) increase in adult females compared with adult males. A total of nine single nucleotide polymorphisms (SNPs) were identified, three were located in the 5′ UTR of the *Phb2* messenger and six in the ORF, but no mutations associated with sex were found. These results contribute to expand the present knowledge of the reproduction-related genes in *C. rogercresseyi*, and may be useful in future experiments aimed at controlling the impacts of sea lice in fish farming.

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1. Introduction

Prohibitin-1 (Phb1) and his homologous Phb2 are evolutionarily conserved proteins present in multiple cellular compartments, and are involved in a variety of cellular processes including, mitochondrial biogenesis (Berger and Yaffe, 1998; Nijtmans et al., 2000; Artal-Sanz and Tavernarakis, 2009), cell cycle progression (Nuell et al., 1991; Cummins, 1998; Piper et al., 2002; Sharma and Qadri, 2004; Mishra et al., 2006; Theiss et al., 2007), cellular signaling (Rajalingam et al., 2005; Mishra et al., 2006) and apoptosis (Fusaro et al., 2003). Both Phb1 and Phb2 belong to a superfamily of molecules, that also include stomatin, flotillin and HflKC (Nijtmans et al., 2002). All these proteins share a common domain known as band-7 family or prohibitin domain.

Several studies have shown evidences of a sex-related role of Phb in invertebrates. Deletion of *Phb* in *Caenorhabditis elegans* led to germline defects and sterility in adults (Sanz et al., 2003). A role in spermatogenesis was suggested in *Eriocheir sinensis* (Chinese mitten crab) and *Octopus tankahkeei* spermatogenesis (Mao et al., 2012a,b), whereas the expression of *Phb2* has been reported in the giant tiger prawn *Penaeus monodon* testis and ovaries (Leelatanawit et al., 2009). Recently, a SNP marker in the *Phb2* gene was identified for the northern hemisphere salmon lice *Lepeophtheirus salmonis*, allowing for the determination of genetic sex in this species, with the gene showing a sex-dependent differential expression (Carmichael et al., 2013).

Caligus rogercresseyi is the most significant sea lice species that affects Chile's salmon industry (Rozas and Asencio, 2007; Costello, 2009; Bravo, 2010) producing annual losses estimated in over 120 million euro (Costello, 2009). In spite of the economic importance of this parasite in the country's aquaculture industry, still little is known about the molecular underpinnings involved in its reproduction. Recent studies carried out by our research group allowed the identification of several differentially expressed contigs annotating for sex-related genes, including *Prohibitins* (Farlora et al., 2014), as well as the identification of the metabolic pathways involved in the ecdysteroid synthesis in this species (Gonçalves et al., 2014). To expand the knowledge of genes putatively involved in *C. rogercresseyi* sex determination and sex differentiation, the objectives of the present work were to i) characterize a *C. rogercresseyi Phb2* homologous gene sequence (*CrPhb2*) in this species, ii) to measure the transcription expression of this gene in adult male and female individuals, and iii) to evaluate the presence of SNP's in the *CrPhb2* gene.

2. Materials and methods

2.1. Laboratory conditions for salmon lice culturing

Ovigerous specimens of *C. rogercresseyi* were collected from recently harvested fish at the AquaChile salmon processing plant in Puerto

Abbreviations: *Phb2*, *prohibitin-2*; ORF, open reading frame; bp, base pairs; SNPs, single nucleotide polymorphisms; *Phb1*, *prohibitin-1*; *CrPhb2*, *Caligus rogercresseyi Phb2*; NJ, neighbor-joining; BI, Bayesian inference; HRMA, high resolution melting analysis; HKG, housekeeping genes (HKG); RACE, rapid amplification of cDNA ends.

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http://dx.doi.org/10.1016/j.gene.2015.03.045
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Montt, Chile. Individuals were transported back to the laboratory on ice, and their egg strings were then removed and placed in culture buckets supplied with seawater flow (12 °C) and gentle aeration. Eggs were allowed to hatch and develop until the infectious copepodid stage, at which point they were harvested for RNA extraction and cDNA library construction. The culture procedure was carried out according to Bravo (2010).

2.2. High-throughput sequencing from C. rogercresseyi transcriptome

Total RNA was extracted from pools containing 10 individuals for each larval stage (nauplius I, nauplius II and copepodid), juvenile (chalimus) and adults (female and male) using the RibopureTM kit (Ambion®, Life TechnologiesTM, USA) following the manufacturer's instructions. Quantity, purity, and quality of isolated RNA were measured in TapeStation 2200 (Agilent Technologies Inc., Santa Clara, CA, USA) using the R6K reagent kit according to the manufacturer's instructions (Agilent Technologies Inc.) (Supplementary File S1). Subsequently, double-stranded cDNA libraries were constructed using the TruSeq RNA Sample Preparation kit v2 (Illumina®, San Diego, CA, USA). Two biological replicates for each sample pool (n = 12) were sequenced using the MiSeq (Illumina®) platform using sequenced runs of 2 × 250 paired-end reads at the Laboratory of Biotechnology and Aquatic Genomics, Interdisciplinary Center for Aquaculture Research (INCAR), University of Concepción, Chile.

2.3. In silico identification of Phb2 from C. rogercresseyi

The raw data for each pool of samples were separately trimmed and de novo assembled in a unique file using CLC Genomics Workbench software (Version 6.0.1, CLC Bio, Denmark). The overlap settings for this assembly were a mismatch cost of 2, an insert cost of 3, a minimum contig length of 200 bp, a similarity of 0.8, and a trimming quality score of 0.05. This assembly yielded 125,813 contigs that were annotated according to Gene Ontology terms with the Blast2Go software (Conesa et al., 2005) by mapping against protein resources. Furthermore, tBLASTn analysis was performed against EST-datasets downloaded from the NCBI for crustacean species, and specifically for EST encoding putative Phb proteins. From this, a *Phb* homologous contig of 1039 bp from *C. rogercresseyi* transcriptome was identified with an e-value of 1e − 178.

2.4. Cloning of the Phb2 gene from C. rogercresseyi

The partial sequence of the *Phb* gene was used as a template for primer design with the Primer3 tool (Rozen and Skaletsky, 2000) (Table 1) included in the Geneious Pro software (Version 6.0, Biomatters, New Zealand). For gene amplification, total RNA was isolated using the TRI reagent® (Invitrogen, Carlsbad, CA, USA) protocol. The purity was determined (ratio A260/A280) with a Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific, Copenhagen, USA), and the integrity was determined by agarose gel under denaturant conditions. From 200 ng/μl of total RNA, cDNA was synthesized using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Glen Burnie, Maryland, USA). PCR analysis was performed using 1 μl of cDNA, 10 μM of each primer (Table 1), 1.5 mM $MgCl_2$, and 0.06 U taq DNA polymerase (Thermo Scientific, Maryland, USA). PCR was performed in 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 60 s. The PCR product was analyzed by electrophoresis on 1% agarose gel and sequenced in the ABI 3730xl sequencer (Applied Biosystems, CA, USA). The sequence was analyzed using the Geneious Pro software (Version 6.0, Biomatters Ltda.). The resulting partial sequence was used for designing specific new primers (Table 1) for the amplification of the 3′ and 5′ UTR ends through the SMARTerTM RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions. The fragments obtained for the 5′ and 3′ UTR ends were cloned in the TOPO TA Cloning Kit (InvitrogenTM, Life Technologies, Carlsbad, CA, USA) and used to transform into *E. coli* JM109 electrocompetent bacteria grown in LB/amp/IPTG/Xgal plates overnight at 37 °C. The positive clones were selected and purified in order to obtain plasmid with the E.Z.N.A® Plasmid DNA Mini Kit II (Omega Bio-tek, Doraville, GA, USA). The acquired plasmids were sequenced in both directions, and their sequences were then assembled using the Geneious Pro software (Version 6.0, Biomatters Ltda.). The *Phb2* gene sequence was submitted to the NCBI Genbank database (Accession number KP203856).

Table 1
Sequences of oligonucleotide primers used.

Primer name	Sequence (5′ to 3′)	Specification
Cr-PHB_3o	ACGACGAATTCACTCTCTCCCTGA	3′-RACE
Cr-PHB_3i	ACCTGAACGCTAATACCCTGATGC	3′-RACE
Cr-PHB_5o	CAACCATGTTGTCCACACATGACG	5′-RACE
Cr-PHB_5i	AGGAAGCAAACAAAACACATGGA	5′-RACE
Cr-PHB_qF	TCATGTTCAGCCGTTTTGGA	qPCR, HRMA (466_F)
Cr-PHB_qR	TGGATGCCCGTAGTTTTCTG	qPCR, HRMA (979_R)
Cr-466_R	TAGGGTATTGGAACCAGGGG	HRMA (446_R)
Cr_979_F	AAGCCAAGCAAGAGAGACAG	HRMA (979_F)

2.5. Amino acid sequence analysis of Phb homologues

Protein alignments and the phylogenetic tree were constructed using MUSCLE included in the Geneious software according to the neighbor-joining (NJ) method, as based on the consensus sequence of *C. rogercresseyi* and other known sequences from crustaceans and insects. The data were bootstrapped 1000 times to estimate the internal stability of each node. *Homo sapiens* Phb2 was used as out-group. In addition, Bayesian inference (BI) phylogenetic analysis was performed using the implementation of MrBayes (Ronquist and Huelsenbeck, 2003) in Geneious, utilizing the default parameters.

2.6. SNP mining in the Phb2 gene

De novo assembly was applied to mine SNP variants from the transcriptome of eight pools of salmon lice using the CLC Genomics Workbench software (Version 6.0.1, CLC Bio, Denmark). The assembly parameters used were a mismatch cost of 2, a deletion cost of 3, an insert cost of 3, a minimum contig length of 200 bp, and a trimming quality score of 0.05. The de novo assembly described above was used to mine SNPs within the contig annotated as Phb using the CLC Genomic Workbench Software (Version 6.0.1, CLC Bio). To exclude paralogous sequence variants (PSVs), an overlap criterion of 70% and a similarity of 0.9 were applied (Renaut et al., 2010). Candidate SNPs were called with the following settings: window length = 11, maximum gap and mismatch count = 2, minimum average quality of surrounding bases = 15, minimum quality of central base = 20, minimum coverage = 20, minimum variant frequency (%) = 35.0, and maximum expected variations (ploidy) = 2. Further analyses, such as synonymous or non-synonymous SNPs identification and the SNPs position in the mRNA (CDS, 3′ UTR, 5′ UTR), were performed in the Geneious Software (version 6.0, Biomatter Ltda., New Zealand).

For SNP validation, high resolution melting analysis (HRMA) was performed using primers designed with the Primer3 tool (Table 1) included in the Geneious Pro software. Total DNA was isolated using the E.Z.N.A DNA extraction kit (Omega) from 20 adult salmon lice (10 females and 10 males). The PCR was carried out in 10 μl reaction with 13 ng of template DNA using the Fast EvaGreen® qPCR Master Mix (Biotum). For HRMA, thermal cycling was performed with an ECO Real Time PCR System (Ilumina Inc., USA) as follows: 2 min for enzyme activation, 40 cycles at 95 °C for 5 s, 56 °C for 5 s, and 60 °C for 25 s. HRMA data were collected between 60 and 95 °C with a temperature interval of 0.3%. Genotyping was analyzed for the presence of a discrete melting curve using the software EcoReal Time System (Illumina Inc. USA).

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