Marine Genomics 25 (2016) 103–113

Contents lists available at ScienceDirect

Marine Genomics

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

Transcriptome mining: Multigene panel to test delousing drug response in the sea louse *Caligus rogercresseyi*

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ARTICLE INFO

Article history:
Received 20 August 2015
Received in revised form 15 December 2015
Accepted 15 December 2015
Available online 23 December 2015

Keywords:
Pyrethroids
Organophosphates
Caligus rogercresseyi
RNA-seq
RT-qPCR

ABSTRACT

Controlling infestations of copepodid ectoparasites in the salmon industry is increasingly problematic given higher instances of drug resistance or loss of sensitivity. Despite the importance of this issue, the molecular mechanisms and genes implicated in resistance/susceptibility are only scarcely understood. The objective of the present study was to identify and evaluate the expression levels of candidate genes associated with delousing drug response in the sea louse *Caligus rogercresseyi*. From RNA-seq data obtained for adult male and female sea lice, 62.48 M reads were assembled in 70,349 high-quality contigs. BLASTX analysis against UniprotKB/Swiss-Prot and the ESTs available for crustaceans in the NCBI database identified 870 transcripts previously related to genes associated with delousing drug response. Furthermore, 14 candidate genes were validated through RT-qPCR and were evaluated with deltamethrin and azamethiphos bioassays. The results evidenced an overregulation of genes involved in ion transport in salmon lice treated with deltamethrin, while those treated with azamethiphos evidenced an overregulation of genes such as *cytochrome P450*, *Carboxylesterase*, and acetylcholine receptors. The present study provides a multigene panel to test delousing drug response to pyrethroids and organophosphates in a highly prevalent pathogen of the Chilean salmon industry.

1. Introduction

Caligus rogercresseyi, a sea louse, is a widely prevalent parasite in the Chilean aquaculture industry (Bravo, 2003; Costello, 2006). This ectoparasite belongs to the Caligidae family, which includes species such as *Lernaeocera branchialis*, *Caligus clemensi*, and *Lepeophtheirus salmonis*, with this final species having greater prevalence in countries such as Scotland, Norway, Canada, and England. Not all species of salmonids cultivated in Chile are equally infected by this parasite, and while *Oncorhynchus mykiss* and *Salmo salar* are more susceptible to *C. rogercresseyi* infestation, *Oncorhynchus kisutch* is less susceptible to this same pathogen (Carvajal et al., 1998; Bravo, 2003; Hamilton-West et al., 2011).

The damages caused by this parasite to the host include epithelial loss, tissue necrosis, and the loss of protective mechanisms on both a physical and microbial level (Hamilton-West et al., 2011). Although not associated with host mortality, an infection of *C. rogercresseyi* is a highly stressful condition for fish, which is reflected by lower culture performance and a depression of the host's immune system, which thus increases susceptibility to other types of contagious diseases (Bravo, 2003; Costello, 2006; Hamilton-West et al., 2011; Oelckers et al., 2014).

Numerous chemotherapeutant treatments have been used worldwide for the control of this ectoparasite and include the application of hydrogen peroxide, avermectins, pyrethroids, and organophosphates (Torrissen et al., 2013). However, many studies suggest that the effectiveness of different treatments for sea lice principally depends on the developmental stage of the parasite (Jones et al., 1992; Grant, 2002; Bravo et al., 2010; Torrissen et al., 2013). In regards to *C. rogercresseyi* in Chile, this species was first detected in native fish and cultivated salmons in 1997. From this period until 2007, the only chemical authorized for treatment was emamectin benzoate, a policy that resulted in resistance in *C. rogercresseyi*. Following this, the Chilean governmental authorities approved the use of alternative pharmaceuticals such as deltamethrin and azamethiphos (Bravo et al., 2013).

Emamectin benzoate (EMB) is an avermectin that independently affects the developmental stages of sea lice, including the reproductive process (Grant, 2002). EMB interferes with nervous impulses by modulating voltage-gated channels, specifically the glutamate and gamma-aminobutyric acid (GABA)-gated anion channels (Grant, 2002). In the case of organophosphates, azamethiphos is effective against pre-adults and adults by interfering with the functioning of acetylcholine esterase (AChE), which provokes an over-stimulation of the nervous and muscular systems, leading to the paralysis and death of the parasite (Walsh et al., 2007). Of the pyrethroids, deltamethrin is the one most used in the Chilean industry (Arriagada et al., 2014; Helgesen et al., 2014). Distinct investigations have demonstrated that this pyrethroid affects all developmental stages of sea louse (Grant, 2002), interacting with sodium channels and depolarizing the nerve endings of the parasite (Burridge et al., 2010). It has moreover been observed that these

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http://dx.doi.org/10.1016/j.margen.2015.12.005
1874-7787/

chemotherapeutants molecularly interact with cuticle proteins, provoking a delay in the penetration of the chemical and a decrease in its bioavailability during the treatment process (Ahmad et al., 2006; Koganemaru et al., 2013).

Limited studies currently exist in invertebrates on the relation between the expression levels of candidate genes and those associated with resistance/susceptibility (R/S) to parasitic treatments. In *Anopheles gambiae* (Vontas et al., 2007) and *L. salmonis* (Carmichael et al., 2013), microarrays were used to evaluate strains that were pyrethroid or EMB R/S, respectively, with both organisms showing differentiated expressions of GABA receptors and structural genes involved in processes of detoxification, such as cytochrome P450. In a study conducted by Walsh et al. (2007) on organophosphates, 33 EST sequences were identified that presented expressional differences in the early stages of development in *L. salmonis* exposed to the trichloron drug.

Until now, no studies had evaluated transcriptomic activity in response to pyrethroids and organophosphates in one of the most prevalent ectoparasite species in the Chilean salmon industry. As such, the objective of the present study was to evaluate the response of previously described candidate genes related to delousing drug response through the use of an EST-database, as generated by Illumina sequencing of adult male and female *C. rogercresseyi*. Additionally, 14 candidate genes were validated by RT-qPCR in response to the antiparasitics deltamethrin and azamethiphos.

2. Materials and methods

2.1. Samples and bioassays

Samples from the adult male and female sea lice were collected from a commercial farm located in region X in Chile (41°40′48.5″S; 73°02′31.34″W). The permissions for the sea lice collections were authorized by Marine Harvest S.A, Ruta 226, Km. 8, Camino El Tepual, Puerto Montt, Chile. These specimens were used to compose subsequent MiSeq cDNA libraries. A pool of 15 individuals for each sex were fixed in the RNAlater® RNA Stabilization Reagent (Ambion, USA) and stored at −80 °C for subsequent RNA extraction.

The bioassays were performed according to the procedures described by the SEARCH Consortium (2006). Briefly, for the bioassays, deltamethrin (AlphaMax®, Pharmaq) was prepared via serial dilutions with seawater to four concentrations (0, 1, 2, 3 ppb). A stock solution of 10 ppm was also prepared for each bioassay by diluting 1 ml of deltamethrin in 999 ml of seawater. In regards to azamethiphos (Byelice, Bayer), a stock solution of 1 ppm diluted in methanol and three serial dilutions with seawater to four concentrations (0, 1, 3, 10 ppb) were prepared. Thirty sea lice adults (fifteen females and fifteen males) were exposed to each concentration of deltamethrin and azamethiphos in petri plates containing 50 ml of treatment seawater. Each experiment was carried out in triplicate and a control group without pesticide was also evaluated. The exposure period to either deltamethrin or azamethiphos was 30 and 60 min, respectively. During exposure, salmon lice were maintained at 12 °C ± 0.5 °C. After 24 h, the organisms were fixed in RNAlater® RNA Stabilization Reagent (Ambion, USA) and stored at −80 °C for subsequent RNA extraction. All laboratory infections and culture procedure were carried out under guidelines approved by the ethics committee of University of Concepción and appropriate veterinary supervision.

2.2. High-throughput sequencing

To identify candidates genes putatively related to delousing drug response, a transcriptome database was generated using high-throughput sequencing from adult male and female sea lice. Here, total RNA was extracted from pools containing 15 individuals for each sex using the RiboPure™ Kit (Ambion®, Life Technologies™, 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. 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 were sequenced by the MiSeq (Illumina®) platform using sequenced runs of 2 × 251 paired-end reads at the Laboratory of Biotechnology and Aquatic Genomics, Interdisciplinary Center for Aquaculture Research (INCAR), University of Concepción, Chile.

2.3. Sequence assembly and annotation

Sequence assembly was carried out using CLC Genomics Workbench software version 6.1 (CLC Bio, Denmark) using reads obtained from females and males of *C. rogercresseyi*. For this, *de novo* assembly was applied with overlap criteria of 70% and a similarity of 0.9 to exclude paralogous sequence variants (PSVs) (Renaut et al., 2010). Furthermore, the settings used were a mismatch cost = 2, deletion cost = 3, insert cost = 3, minimum contig length = 200 bp, and trimming quality score = 0.05. After the assembly process, singletons were retained in the data set as possible representatives of low-expression transcript fragments. However, the sequence redundancy of these fragments was removed using the Duplicate Finder application incorporated in the Geneious v5.1.7 software (Biomatters, Auckland, New Zealand). Consensus sequences from the *C. rogercresseyi* transcriptome were annotated according to Gene Ontology (GO) terms with the Blast2GO program (Conesa et al., 2005). Moreover, a description of the putative genes was determined with the tBLASTX algorithm and the UniprotKB/Swiss-Prot Database (http://uniprot.org) enriched with EST data for crustaceans. A cutoff E-value of 1E-05 was used. Finally, we selected contigs annotated as genes which previously had been associated with drug resistance and susceptibility (R/S) in *L. salmonis* by Carmichael et al. (Carmichael et al., 2013) and Walsh et al. (Walsh et al., 2007) (Table 1).

2.4. In silico transcription expression of candidate genes related to delousing drug response

The raw-reads were mapped against consensus contigs with a significant annotation (E-value > 10^{-5}) to the selected candidate genes annotated as gene associated with drugs R/S. The RNA-seq settings were a minimum length fraction = 0.6 and a minimum similarity fraction (long reads) = 0.5. The expression value was set as a read per kilobase of exon model (RPKM). This normalized the number of reads to the size of assembled contigs and allowed for assessing the transcripts that were overexpressed among different groups. In addition the expression values were normalized using the methods *by totals* include in CLC Genomics Workbench. In order to identify differences between sexes, RNA-seq analyses were performed separately for female and male samples of *C rogercresseyi*. The distance metric was calculated with the Manhattan method (Eisen et al., 1998), where the mean expression level in 5–6 rounds of k-means clustering was subtracted. Finally, Kal's statistical analysis test (Kal et al., 1999) were used to compare gene expression levels for larval stages and adults in terms of the log2 fold change (p = 0.0005, FDR corrected).

2.5. RT-qPCR validation

Contig sequences were used as a template for primer design with the Primer3 Tool (Rozen and Skaletsky, 1999) included in the Geneious Pro software (Drummond, 2009) (Table 2) with amplicon lengths between 120 and 150 bp. 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 synthetized using the RevertAid H Minus First