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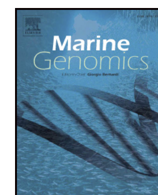

Identification and molecular characterization of *dorsal* and *dorsal-like* genes in the cyclopoid copepod *Paracyclopina nana*

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ABSTRACT

To date, knowledge of the immune system in aquatic invertebrates has been reported in only a few model organisms, even though all metazoans have an innate immune system. In particular, information on the copepod's immunity and the potential role of key genes in the innate immune systems is still unclear. In this study, we identified *dorsal* and *dorsal-like* genes in the cyclopoid copepod *Paracyclopina nana*. *In silico* analyses for identifying conserved domains and phylogenetic relationships supported their gene annotations. The transcriptional levels of both genes were slightly increased from the nauplius to copepodid stages, suggesting that these genes are putatively involved in copepodid development of *P. nana*. To examine the involvement of both genes in the innate immune response and under stressful conditions, the copepods were exposed to lipopolysaccharide (LPS), different culture densities, salinities, and temperatures. LPS significantly upregulated mRNA expressions of *dorsal* and *dorsal-like* genes, suggesting that both genes are transcriptionally sensitive in response to immune modulators. Exposure to unfavorable culture conditions also increased mRNA levels of *dorsal* and *dorsal-like* genes. These findings suggest that transcriptional regulation of the *dorsal* and *dorsal-like* genes would be associated with environmental changes in *P. nana*.

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1. Introduction

Copepods have a number of promising characteristics for invertebrate immunity studies (Huq et al., 1983; Kurtz, 2007; Raisuddin et al., 2007). However, in copepods, the innate immune system and the role of immune-relevant genes have as yet rarely been studied. Aquatic invertebrates have a primitive immune response system and also a potential role as intermediate hosts of parasites to upper predators according to their trophic position in the marine food web (Cáceres et al., 2014). Previously, the memorizing capability of the copepod defense system was observed in response to consecutive exposures to antigenically similar parasites (Kurtz and Franz, 2003) and a recent study revealed that cathepsin superfamily is conserved and responsive upon LPS exposure (Jeong et al., 2015), implicating that the host defense mechanism of copepods is a more complex system than expected (Huq et al., 1983; Kurtz, 2007).

To date, immunity studies (e.g., recognition of pathogens, cell-free or cellular responses, biomarker development against pathogens, and the integration of immune mechanisms) in aquatic invertebrates has focused on large mollusks or crustaceans including crabs, lobsters, and shrimps, as they are important economically (Mydlarz et al., 2006), while the specific role of each immune-relevant gene is not yet clearly compared to that of the mammalian immune system. In small crustaceans including copepods, several key immune components have been reported with characterization of gene/protein expression of immunity-relevant genes (Decaestecker et al., 2011; Kim et al., 2014; McTaggart et al., 2009), although the basic molecular mechanisms of innate immunity of small crustaceans are still missing and only little attention is given to these tiny species. Thus, the understanding of copepod innate immunity and its sensitivity in response to immune modulators would be comparable with those of large mollusks or crustaceans.

The NF-κB and Rel subfamily constitute the Rel/NF-κB superfamily. The Rel/NF-κB members are transcription factors involved in numerous cellular responses to diverse stimuli and function in the host defense system of invertebrates to control the expression of genes encoding immune-relevant proteins (Hetru and Hoffmann, 2009). The Rel

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homology domain (RHD) is conserved for DNA binding, dimerization, and cellular localization (Siebenlist et al., 1994). In insects and crustaceans, dorsal and dorsal-related immunity factor (Dif) proteins have a typical Rel homology domain, translocating to the nucleus to stimulate subsequent gene expression with stimulus-induced degradation of the inhibitor protein Cactus, a homolog to the vertebrate inhibitor of the NF-κB (*IκB*) gene (Belvin and Anderson, 1996). In copepods, the *Rel/NF-κB* gene (annotated as *dorsal* in this study) was recently identified in the intertidal copepod *Tigriopus japonicus* and its transcriptional response was analyzed after exposure to lipopolysaccharide (LPS) and two *Vibrio* sp. (Kim et al., 2014). However, to date, there is no additional *dorsal* gene information in copepods.

The *Rel/NF-κB* gene family functions as a central regulator of stress responses including environmental or physiological stress (Pahl, 1999). Furthermore, the *Rel/NF-κB* gene family is involved in the control of diverse signaling pathways beyond the immune response (Oeckinghaus and Ghosh, 2009). Activation of *Rel/NF-κB* gene family occurs in response to hypoxia, physical stress (i.e. UV-B, gamma radiation) or to oxidative stress (Koong et al., 1994; de Martin et al., 1999; Li and Karin, 1999; Morgan and Liu, 2011). Although the cellular role of *Rel/NF-κB* gene family upon environmental stress in vertebrates and fruitfly are well established, the gene information of *Rel/NF-κB* gene family and molecular response in response to environmental condition still remains yet unclear in aquatic invertebrates.

Paracyclopina nana Smirnov 1935 (Cyclopinidae) is a planktonic brackish water cyclopoid copepod and has been recognized as an economically important food source for higher trophic levels (i.e. developing and/or post larvae of crustaceans and fish) in the estuarine and marine environment. Moreover, the copepod has favorable characteristics such as small size, sexual dimorphism, distinctive post-embryonic developmental stages, ease of culturing, and sensitive responses to environmental changes; making *P. nana* a suitable model species for diverse experimental studies (Lee et al., 2012). In this study, we cloned and characterized the full-length cDNAs of *dorsal* and *dorsal-like* genes from the cyclopoid copepod *P. nana*. Also, we investigated their transcriptional changes at different developmental stages, LPS-exposed conditions, and environmental changes such as different culture densities, salinities, and temperatures. The results of this study are useful to better understand the potential involvement of both genes as immune modulators in copepods.

2. Materials and methods

2.1. Culture and maintenance

The cyclopoid copepod *P. nana* was maintained in a 2 L beaker with the algal diet *Tetraselmis suecica* (7.8 ± 0.8 μm) in an incubator kept at 25 °C and 15 practical salinity units (psu) under a 16 h light:8 h dark cycle. The culture medium with filtered (1 μm mesh of glass fiber filter) seawater was diluted to 15 psu using distilled water that was changed (100%) using a 50 μm sieve with fresh medium every tenth day. *T. suecica* were cultivated in 2 L transparent glass bottles with Walne's media (Walne, 1970) and diluted (15 psu) filtered seawater (1 μm mesh of glass fiber filter). The cultures were incubated at 20 °C under a 24 h light photoperiod. The algae culture was fed to copepods during their exponential growth phase.

2.2. Cloning and annotation of dorsal and dorsal-like genes

Partial sequences of *dorsal* and *dorsal-like* genes were identified in the *P. nana* RNA-seq database (Lee et al., 2015; number of contigs after trinity assembly, 125,631; length of contigs, 283,955,302 bp; average length, 2260 bp; N50 value, 4178 bp). The cDNA sequences coding for *dorsal* and *dorsal-like* genes were subjected to BLAST analysis in the GenBank non-redundant (NR; including all GenBank, EMBL, DDBJ, and PDB sequences except EST, STS, GSS, or HTGS) amino acid sequence database to confirm their identities. Both genes were subjected to 5′- and 3′-Rapid Amplification of cDNA Ends (RACE) to obtain the remaining parts with 5′- and 3′-RACE system (ver. 2.0 for 5'RACE; ver. E for 3′ RACE; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Total RNA from approximately 500 adult copepods (both sexes) was isolated with TRIzol® reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) with a tissue grinder and stored at −80 °C until use. Detailed procedure for total RNA extraction is described in Section 2.6. The 5′- and 3′-RACE systems were used to synthesize the single-stranded cDNAs for PCR amplification of the partial cDNA fragments. Gene-specific primers (GSPs) were designed for each RACE with partial cDNA sequence based on the manufacture's guideline. The universal primers for each RACE were provided from the kits. The first and nested PCR procedures were carried out with GSP1 and GSP2 primer, respectively (Table S1). A series of RACE were performed under the following conditions: 94 °C/4 min; 40 cycles of 98 °C/25 s, 55 °C/30 s, 72 °C/60 s; and 72 °C/10 min. The final PCR products were isolated from a 1% agarose/TBE gel, cloned into pCR2.1 TA vectors (Invitrogen), and sequenced with an ABI PRISM 3700 DNA analyzer (Bionics Co., Seoul, South Korea).

To validate full-length cDNA sequences of *dorsal* and *dorsal-like* genes, RT-PCR was employed with two primers: a forward primer containing a start codon, and a reverse primer containing a stop codon. RT-PCR was conducted in a reaction mixture comprising 1 μL of first strand cDNA, 5 μL of 10× PCR reaction buffer, 1 μL of 10 mM dNTPs, 10 pM concentrations of each primer, and 0.5 μL of NeoTherm™ *Taq* polymerase (GeneCraft, Köln, Germany). Reaction mixtures were subjected to amplification (1 cycle, 95 °C, 5 min; 30 cycles, 94 °C, 30 s, 55 °C, 30 s, and 72 °C, 30 s; 1 cycle, 72 °C, 7 min) using an iCycler (Bio-Rad, Hercules, CA, USA). The final PCR products were isolated from 1% agarose/Tris-Borate-EDTA (TBE) gels, cloned into pCR2.1 TA vectors (Invitrogen), and sequenced using an ABI PRISM 3700 DNA analyzer (Bionics Co., Seoul, South Korea). All the gene information was registered to the GenBank database, and accession numbers of each gene are listed in Table S1.

2.3. Conserved domain and phylogenetic analysis

Conserved domains of *dorsal* and *dorsal-like* genes such as the Rel homology domain (RHD) and Ig-like/plexin/transcription factor (IPT) were analyzed through Pfam HMM search (http://pfam.sanger.ac.uk), Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan), and web-based NCBI's Conserved Domain Database (CDD) (Marchler-Bauer et al., 2011). To place *dorsal* and *dorsal-like* genes on phylogenetic trees, we performed multiple alignments of these genes using Clustal X software (Ver. 1.83) at the level of deduced amino acid sequences with those of other species. For phylogenetic analysis, we excluded gaps and missing data matrices from the analysis. The generated data matrix was converted to nexus format, and the data matrix was analyzed with Mr. Bayes v3.1.2 program using the general time-reversible (GTR) model. A total of 1000,000 generations were conducted, and the sampling frequency was assigned as every 100 generations. After analysis, the first 10,000 generations were deleted as the burn-in process, and the consensus tree was constructed and then visualized with PHYLIP Tree View software.

2.4. Developmental stage

P. nana undergoes anamorphic development with distinct post-embryonic developmental stages by molting activity, resulting in naupliar stages (N1–6), copepodid stages (C1–5), and adults (male and female). To prepare different developmental stage samples, entire copepods were separated with three sieves (90, 150, and 200 μm). Of four separated groups (< 90, 90 ~ 150, 150 ~ 200, and >200 μm), three naupliar stages (N1–2, N3–4, N5–6; 180 individuals were separated into three groups as triplicate for each stage group), four copepod stages

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