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Differential transcriptome analysis of the common shrimp *Crangon crangon*: Special focus on the nuclear receptors and RNAi-related genes

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ABSTRACT

The decapod *Crangon crangon* is one of the most valuable European fisheries commodities. Despite its economic importance, little sequence data is available for this shrimp species. In this paper, we report the transcriptome sequencing for five different stages of *C. crangon* (early embryo, late embryo, larva, female adults and male adults) and the annotation and stage-specific expression analysis of nuclear receptors (NRs) and RNA interference (RNAi)-related genes. The NRs are transcription factors that play an essential role in growth, development, cell differentiation, molting/metamorphosis and reproduction, while the RNAi-related genes are very important for internal gene expression regulation and in antiviral defense. We discovered a NR in the female *C. crangon* which is either a very rapidly evolved homolog of HR10, or a novel NR altogether. This new NR could act as a biological marker for sex determination as it is not expressed in male adults. Most RNAi-related genes were present in *C. crangon*, proving that the requirements for successful RNAi is present in this decapod shrimp. RNAi-based applications in *Crangon* such as its use in functional genomics or as antiviral therapeutics could become very important in the near future.

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1. Introduction

The common shrimp *Crangon crangon*, belonging to the decapod Crustacea, is found in coastal areas all around the British Isles (Henderson, 1987), the Baltic Sea (Dornheim, 1969), the southern North Sea, as well as in large parts of the Mediterranean and the Black Sea (Labat, 1977; Luttikhuizen et al., 2008). Economically, this shrimp is one of the most valuable commodities in the European fishery. The commercial value of the whole North Sea landings alone represents about €100 million (Verhaegen, 2012). Despite its importance, available sequence data for this shrimp is very limited. In GenBank, 186 coding DNA sequence (CDS) entries were available in the genus *Crangon* as of January 2014, representing less than 15 different proteins. With the development of the 'next generation' sequencing technologies, such as Illumina sequencing, we have cost-effective ways at our disposal to generate large amounts of genomic and transcriptomic data for non-model species. This transcriptome could therefore be a huge step forward for all research on this shrimp. In this paper, we report on the sequencing and analysis of the *C. crangon* transcriptome which provides us with a considerable amount of sequence information for this species. Additionally, the annotation and stage-specific expression analysis of the nuclear receptors as well as the RNA interference (RNAi) related genes will be presented.

The nuclear receptor (NR) superfamily is a group of transcription factors which is present in all metazoans. They are involved in a vast array of essential biological processes such as molting and metamorphosis (Ashburner, 1973), embryonic development (King-Jones and Thummel, 2005), cell differentiation (Siaussat et al., 2007) and reproduction (Raikhel et al., 1999). Transcriptional regulation of many of these NRs is dependent on the binding of receptor ligands, which are usually small, lipophilic compounds such as steroids, retinoids, thyroid hormones and fatty acids. These NRs are therefore a direct link between signaling molecules that control the biological processes and the transcriptional responses. The NRs have a typical structure consisting of five or six regions which have a modular character. These domains are each responsible for one or more specific molecular functions. The N terminal domain is the highly variable A/B domain. Further downstream we find the much conserved C domain or DNA binding domain (DBD), the D domain which acts as

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a hinge between the C domain and the E domain or ligand binding domain (LBD). Some NRs, such as the ecdysone receptor (EcR) in *Drosophila*, also have a variable C terminal F domain of which the function is unknown still (Koelle et al., 1991).

In this project, we aim to report on the identification of the set of NRs in the transcriptome of the common shrimp *C. crangon* and on the comparison of expression of these receptors between data collected from embryonic, larval, adult male and adult female stage shrimp RNA. These findings can then lead to the use of some of these nuclear receptors as biological markers for development, molting, sex determination and reproduction. This can be of use in ecological, ecotoxicological and aquacultural applications. Furthermore, since the available knowledge and sequence data on this important class of receptors in non-insect arthropods is rather scarce, the annotation of this crustacean set of NRs is also of great importance.

Additionally, we aim to annotate the RNAi-related genes and analyze these as well over the different developmental stages. Contrary to the NRs, these genes are not members of the same protein superfamily and thus do not all share a common architecture. What they do share is that they all have a certain role in one or more RNAi pathways. RNAi is a gene silencing process present in all Eukaryota. The mechanism was first described in the nematode *Caenorhabditis elegans* by Fire and Mello, who, in 2006, both received the Nobel Prize in Medicine for their work on RNAi (Fire et al., 1998). To date, the RNAi technique has become a widely used functional genomics tool to unravel the function of genes with unknown function(s), and this technique also shows great potential in both medicine and crop protection as well.

Three major RNAi pathways have been described so far: the small interfering RNA (siRNA) pathway, the microRNA (miRNA) pathway and the piwiRNA (piRNA) pathway. All 3 pathways have similar working mechanisms and although some components might be shared and cross-talk between the pathways is observed, different genes are involved in the 3 pathways. In general, the machinery is initiated by double-stranded RNA (dsRNA). Upon entry into the cell, this dsRNA is processed into smaller RNA duplexes (e.g. siRNAs, piRNAs, etc.) by a ribonuclease type-III, named Dicer. These small dsRNAs are then incorporated into a protein complex called the RNA-induced silencing complex (RISC) which then mediates the specific degradation of target mRNA.

The siRNA pathway is mainly involved in gene silencing after introduction of foreign dsRNA into the cell and is therefore considered very important in the defense of eukaryotic cells against viruses. The miRNA pathway is used as an internal gene-regulation mechanism and the piRNA seems to play a role in the protection of the genome from retrotransposons. Reviews on these RNAi pathways, their components and their functions are available (Huvenne and Smagghe, 2010; Ketting, 2011; Liu and Paroo, 2010; Scott et al., 2013).

The presence of a RNAi mechanism in shrimps was first demonstrated when Robalino et al. (2005) performed the first successful antiviral RNAi experiments injecting dsRNA into the penaeid shrimp *Litopenaeus vannamei*. Subsequently, the presence of some RNAi core genes was also confirmed in *Penaeus monodon* (Dechklar et al., 2008; Su et al., 2008; Unajak et al., 2006). Since then, many studies involving RNAi in gene function research have followed.

Besides the importance of RNAi for functional genomics, in shrimps as well as in other arthropods, more insights in this mechanism could lead to other applications as well. Because viral infections such as the white spot syndrome (WSS) in shrimps is a serious problem in aquaculture (Bateman et al., 2012; Stentiford et al., 2009, 2012), new insights in these RNAi mechanisms and their role in viral defense could lead to new applications in disease control and subsequently result in higher production rates of cultured shrimps.

In this paper, we aim to provide a first set of RNAi-related genes for a shrimp species which can be the first step in further research in this field. Although some sequence data on these RNAi-related genes is already available in penaeid shrimps *P. monodon*, *L. vannamei* and *Marsupenaeus japonicas*, it is still fragmentary. Nonetheless, the data on penaeid shrimps can help us with the annotation of this group of genes in *C. crangon* as they are all decapod shrimp species.

2. Material and methods

2.1. RNA extraction and sequencing

RNA was extracted from early developing eggs (E0), eggs that were almost ready to hatch (E1), *C. crangon* larvae two days post hatching (L2), one male (M) and one female shrimp (F). The extraction was carried out using the TRIzol Plus RNA Purification Kit (Invitrogen, Merelbeke, Belgium) following the manufacturer's instructions. The adult shrimps as well as the eggs used in this transcriptome sequencing were caught at sea at low tide (Raversijde, Belgium) and directly processed. The larvae were hatched and reared at ILVO (Oostende) and originated from eggs from the same location. RNA quantifications were performed using Qubit fluorimetry (Life technologies, Carlsbad, CA). For each of the 5 samples, 100 ng of total RNA was used to prepare a directional sequencing library using the NuGEN Encore Complete RNA-Seq DR kit (NuGEN, San Carlos, CA). A gel-based size selection was performed on the resulting libraries to obtain libraries with inserts longer than 200 bp. The 5 mRNA libraries were equimolarly pooled and sequenced in one lane of an Illumina HiSeq 2000 flowcell, generating 2×100 bp reads. After sequencing, the data was demultiplexed using the sample specific nucleotide barcodes.

2.2. Assembly

After sequencing, the raw sequence data was assembled into contigs (stretches of overlapping DNA, supposedly representing a single consensus RNA transcript). A *de novo* transcriptome was assembled for each sample with Trinity Release-2011-08-20 (Gnerre et al., 2011). One extra assembly was made with all sample data taken together.

2.3. Bowtie and GO annotation

Bowtie (Langmead et al., 2009) was used for mapping the sample reads back to the Trinity *de novo* assembly and counting the number of reads that matched to each contig. For each sample the top 1000 most expressed contigs were selected for a general gene ontology (GO) analysis with the Blast2GO suite (Gotz et al., 2008). Default settings were used to BLAST those sequences, map them to GO terms and annotate.

2.4. Searching and annotating NRs and RNAi genes

Each assembly was converted to a BLAST nucleotide database. A protein nuclear receptor (NR) FASTA file and protein RNAi genes FASTA of known genes, were blasted (tblastn) with those databases to find contigs related to genes of interest. These results were then further analyzed and annotated using blasts against the GenBank protein database, alignments were made with possible orthologs and phylogenetic analysis was performed. Amino acid sequences for the NRs and RNAi genes in other arthropod species were collected from the GenBank database. Selected sequences were aligned by CLUSTALW2/CLUSTALX2 (Larkin et al., 2007). LBD-sequences were used to build most NR trees. In case only a DBD-containing sequence was retrieved from the transcriptome database, a DBD tree was built. The trees were made by the neighbor-joining method using MEGA 5 software (Tamura et al.,

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