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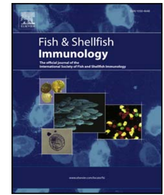

Short communication

Characterization and expression analysis of g- and c-type lysozymes in Dabry's sturgeon (*Acipenser dabryanus*)

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ABSTRACT

Dabry's sturgeon (*Acipenser dabryanus*) is mainly distributed in the upper Yangtze River. Although extensively farmed, little information is available on its innate immune system. In this study, we conducted de novo transcriptome assembly of the head kidney to create a comprehensive dataset for *A. dabryanus*. A total of 51,324,686 high quality reads were obtained from head kidney cDNA library by the Illumina sequencing platform and 131,261 unigenes were determined to contain complete ORFs. The complete coding sequences of g- and c-type lysozymes were identified from unigenes, and designated as ADLysG and ADLysC. *Aeromonas hydrophila* infection of Dabry's sturgeon caused a significant increase ($P < 0.05$) in blood for both lysozyme types, confirming their active defensive role against bacterial infections. This research provides the first characterization of these enzymes in an ancestral chondrostean. These data suggest that ADLysG and ADLysC have the potential for immune defense system against bacterial infection.

1. Introduction

The polyploidy fish, Dabry's sturgeon (*Acipenser dabryanus* Dumeril, 1868) is endemic to China where is restricted to the Yangtze River system and in particular to its upper main stream, after being recently extirpated from the lower part of the river in the Sichuan Province. It also enters main tributaries [1]. The natural population drastically declined over the last 50 years, mainly due to overfishing, [1]. Since 1996 the species is listed in the IUCN red list as Critically endangered and harvesting from natural populations is banned. Despite this protection measure, after 20 years the status of the species in the wild is further worsened, due to illegal poaching and to habitat degradations, such as pollution and damming that interrupt reproductive migrations. This species has been intensively reared in aquaculture over the past decades for commercial and conservation purposes. Dabry's sturgeon is considered by some authors as the landlocked ecotype of the Chinese sturgeon in the Yangtze river [2]. The reproductive distinction between the two species would be guaranteed by the different spawning times with occur in Winter for the Chinese and in Spring for the Dabry's sturgeon. However, in spite of this evolutionary proximity, and in spite of the sympatry of the two species, when raised in an artificial system Dabry's sturgeon has been observed to be relatively resilient to diseases compared to, *A. sinensis*. Indeed, *A. sinensis* is more sensitive to some pathogens such as *Aeromonas hydrophila* and *non-tuberculous mycobacteria* [3,4]. Currently (end-January 2018), only 217 Dabry's sturgeon nucleotide sequences are recorded in the NCBI database, most of which reporting microsatellite loci (116) or mitochondrial COI sequences used for barcoding (55). Sequence information about genes involved in the immunoresponse of *A. dabryanus* are limited to TRIMs (unpublished) and to Interferon Regulatory Factors [5]. Head kidney is an important immune organ in fish, its role is equivalent to those of the mammalian bone marrow [6]. The innate immune system plays an essential role in the early defense against pathogen infection, and lysozymes are crucial in this response. Lysozymes are ubiquitous antimicrobial enzymes that promote the hydrolysis of 1,4 beta linkage between N-acetylmuramic acid and N-acetylglucosamine of peptidoglycan, a major component of bacterial cell wall [7,8]. Based on differences in structural, catalytic, and immunological characteristics, lysozymes in the animal kingdom are generally classified into three main types: i) c-type, mainly present in Chordata and in some Arthropoda; ii) g-type, also present within

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Chordata and detected in some Mollusca) and i-type, widely spread across invertebrate phyla and absent from Chordata [9–11]. The characterization of c- and g-type lysozymes in fish is currently limited to bony fish (Teleosts) [9,12–14] and no information is available for Chondrostean.

In this study, based on the first transcriptome characterization of Dabry's sturgeon head kidney, performed by Illumina sequencing, two types of lysozyme genes were characterized and their expression profiles was evaluated in different tissues following infection with *A. hydrophila*.

2. Materials and methods

2.1. Experimental fish and sample collection

All fish handling and experimental procedures were approved by the Animal Care and Use Committee of the Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences (YFI). All animals used in the present study were produced trough controlled reproduction of aquaculture animals. Four Dabry's sturgeon (500 ± 10 g) were sampled for transcriptome sequencing. After anaesthetization by 0.05% MS-222 (Sigma, USA), head kidney tissues were collected, immediately immersed in RNAlater (Biological Industries, USA) and then transferred to −80 °C until RNA preparation. A total of 65 F2 Dabry's sturgeon (35 ± 2 g) were cultured for tissue distribution and bacterial challenges in Taihu hatchery. Fish were maintained in aerated water tanks at 25 °C for a week to ensure good health prior to sampling. A virulent strain of *Aeromonas hydrophila,* pathogenic to Dabry's sturgeon, was isolated from diseased Chinese sturgeon (*A. sinensis*) in a previous study and stored in YFI (unpublished).

2.2. RNA isolation, library construction, illumina sequencing, and de novo assembly of sequencing reads

Total RNA for library preparation was independently isolated from head kidney of four animals with the RNeasy Plus Mini Kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions. The RNA concentration was measured by the Nanodrop-2000 spectrophotometer (Thermo, USA) while integrity was assessed by Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). Removal of genomic DNA contamination in RNA preparations was done using RNase-free DNase I (Qiagen) for 30 min at 37 °C. The sample with the highest RNA Integrity Number (RIN = 9.6) was selected for library preparation. The mRNA-seq library was constructed with the mRNA-seq Sample Preparation Kit (Illumina, San Diego, CA) as previously described [15]. Finally, the cDNA library was obtained by PCR amplification and purification, and was sequenced over one lane with the Illumina HiSeq 2000 as external service (Beijing Berry Genomics Co., Ltd, China). After filtering out adaptor-only and low quality reads (reads with more than 50% of bases having a Q-value ≤20) by the softwares WipeAdapter.pl (Biomarker Technologies Co., Ltd, Beijing, China) and Fastq_filter (Biomarker Technologies Co., Ltd, Beijing, China) respectively, clean reads were assembled with the assembling program Trinity, version Trinityrnaseq_r2012-06-08 [16], with the parameters set at a similarity of 90%, as better described by Yue and colleagues (2015) [17].

2.3. Transcriptome annotation and ontology

Open reading frames (ORFs) of transcript and unigene sequences were predicted using TransDecoder 1.0 package (http://transdecoder.sourceforge.net/) from the Trinity package, with the minimum ORF length set at 100 bp. Unigene sequences were assigned to the NCBI Nt database (BLASTn), the NCBI non-redundant (Nr) protein databases and the Swiss-Prot protein database all downloaded in August 2013.

2.4. Sequence and phylogenetic analyses

The complete coding sequence of g- and c-type lysozymes were identified from transcriptome sequencing, and designated as ADLysG and ADLysC, respectively. The cDNA sequence was translated into protein sequence by the Translate tool (http://www.expasy.org/tools/dna.html). Analysis of ORF in cDNA sequences was performed using a web service ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). The putative signal peptides were analyzed using SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). . The deduced amino acid sequences of two lysozyme genes were aligned with the corresponding sequences from various animals using the ClustalW software (http://www.ebi.ac.uk/Tools/msa/clustalw2/). To this purpose we have used the same sequences previously used by Ye et al., 2010 [13] and added the ones of 6 mammal species retrieved from GenBank and Ensembl database. Additionally, lysozyme sequences of two other sturgeon species were retrieved by blasting our sequences against the transcriptomes of the Chinese sturgeon (*A. sinensis*) and the Adriatic sturgeon (*A. naccarii*), the first available as unpublished data in our laboratory and the second searchable by blast at http://compgen.bio.unipd.it/anaccariibase. A cluster analyses was performed by Neighbor-joining (NJ) method [18] and Maximum Likelihood (ML), implemented in MEGA7 software [19]. Bootstrap analysis [20] was used with 1000 replicates to test the significance of branches. The trees are shown in Fig. S2.

2.5. Tissue distribution and immune challenge in A. dabryanus by quantitative real-time PCR

To examine the tissue specificity of the two lysozymes, nine healthy Dabry's sturgeons were sacrificed and twelve tissues removed for RNA extraction, including gills, skin, muscle, liver, spleen, head kidney, mid-kidney, intestine, eye, brain, heart, and blood. To evaluate the immune responses of ADLysG and ADLysC, 56 fish held at 25 °C were randomly divided into two groups: one group was injected intraperitoneally with 200 μl *A. hydrophila* suspension in sterile PBS (1.9×10^7 cfu/ml), and the other injected intraperitoneally with 200 μl sterile PBS (0.1 M, pH = 7.2) as a control. Four tissues fragments (blood, liver, mid-kidney, and spleen) from four animals explanted at various timepoints (3, 12, 24, and 36 h post injection [hpi]) were immediately soaked in RNA save Tissue storage solution (Biological Industries, USA) and stored at −80 °C.

The tissues and organs described above were used for total RNA extraction. All cDNA samples were prepared with the first strand cDNA synthesis kit (Fermentas, Canada). The specific primers (Table S1) were designed to detect the corresponding gene. The KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems) and a Step-one Plus real-time PCR system (ABI) were used to measure the expression of ADLysG and ADLysC as described previously [21]. The Dabry's sturgeon β-actin gene was used as the internal reference gene. The total reaction mixture and reaction procedure for RT-qPCR were performed as described previously [21]. In brief, for comparison, a standard was constructed using a mixture of equal mole amounts of purified PCR products of each gene amplified from cDNA. A serial dilution of the standard was run along with the cDNA samples in the same 96-well PCR plate and served as a reference for quantification. The expression level of each gene was calculated as arbitrary units normalized to the expression of β-actin. Statistical analysis was performed using SPSS 21.0 software. One way-analysis of variance (ANOVA) followed by the LSD post hoc test was used to evaluate the transcript expression levels. A *t*-test was performed to determine whether there was a significant difference ($P < 0.05$) between sets of samples.

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