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Research Article

Characterization and phylogenetic analysisof a g-type lysozyme gene variant from the skin mucus of grass carp (*Ctenopharyngodon idella*)

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Abstract

The g-type lysozyme is one of the three major diverse lysozyme types recognized in the animal kingdom including fish. Using the RT-PCR technique, a 555 bp cDNA fragment encoding a g-type lysozymewas isolated from the skin mucus of *Ctenopharyngodon idella* using homolog primes. The cDNA named Ci-Kh, codes for 185 amino acids with a predicted molecular weight of 20.49 kDa and theoretical pI of 9.13. The sequence consists of one cysteine residue with no predicted signal peptide. Domain analysis showed e-value of 3.74e-107 with the conserved domain of lysozyme-like superfamily (cd01021) between amino acid residues 12 to 184. Multiple alignment with the lysozyme genes from other fish species revealed that this protein have a goose egg white lysozyme (GEWL) domain containing two conserved catalytic residues (Glu73 and Asp97) and N-acetyl-D-glucosamine binding site (Glu73, Asp85, Asp97, Tyr100, His101, His102, Ile119, Tyr147, Asn148). Protein structure prediction software revealed a prediction of 58% and 42% of *a*-helical and random coils for the coding sequence of Ci-Kh, respectively.The 3D model of Ci-Kh revealed that this protein was mainly composed of five main helices and random coils. Phylogenetic analysis indicated that Ci-Kh matched the group of five grass carp g-type lysozyme transcript variants with 86-99% similarity. Among the five variants of this gene, Ci-Kh sequence had the highest and lowest genetic distance with *C. idella* variant X2 (8.6%) and X3 (1.1%) sequences, respectively. We conclude that Ci-Kh as a different variant of g-type lysozyme cannot be ruled out.

Introduction

Lysozymes are important proteins that act against the invasion of bacterial pathogens. The family of lysozymes (EC 3.2.1.17) is a very old group of antimicrobial enzymes that play a key role in innate immunity, with the capability to hydrolysis of1,4-beta-linkages between N-acetyl-D-glucosamine (NAG) and N-acetylmuramic acid (NAM) in peptidoglycan, heteropolymers found in Metazoan ranging from bacteriophages up to plants and animals (Callewaert and Michiels, 2010).

Based on their differences in primary structure, catalytic feature and original source, lysozymes have been named chicken-type (c-type) (Hikima *et al.*, 1997), goose-type (g-type) (Jimenez-Cantizano*et al.*, 2008), invertebrate-type (i-type) (Zhao *et al.*, 2007), plant-type (Beintema and Terwisscha van Scheltinga, 1996), bacterial type (Holtje, 1996) and phage-type (Weaver *et al.*, 1984). Usually, g-type lysozymes are bigger (about 20-22 kDa) than c- and i-type (about 11-15 kDa), and have no signal peptide (Callewaert and Michiels, 2010). In the fish immune system, innate immunity plays a main role in protection against pathogens (Magnadottir, 2006). They are broadly distributed enzymes that are found in serum, mucus and many other tissues. Fish lysozymes have been reported in two forms c-type and g-type from the various species in different tissues, like kidneys, gills, and intestines. They are much more antibacterial than other higher vertebrate lysozymes (Saurabh and Sahoo, 2008).

Lysozyme has been well studied on a molecular level and some differences within and among fish species have been described. The first fish g-type lysozyme gene was identified in Japanese flounder (*Paralichthys olivaceus*) (Hikima *et al.*, 2001). That was the first lysozyme of g-type outside the class of birds that its functions as an antibacterial molecule in fish was considered. Since then, genes of g-type lysozymes have been reported from the number of fish species, including Senegalese sole (*Soleasenegalensis*) (Fernandez-Trujillo *et al.*, 2008), orange-spotted grouper (*Epinephelus coioides*) (Yin *et al.*, 2003), large yellow croacker (*Pseudosciaena crocea*) (Zheng *et al.*, 2007), mandarin fish (*Siniperca chuatsi*) (Sun *et al.*, 2006), Atlantic salmon (*Salmo salar*) (Kyomuhendo *et al.*, 2007), large yellow croaker (*Larimichthys crocea*), brill (Jimenez-Cantizano *et al.*, 2008), Atlantic cod (*Gadus morhua*) (Larsen *et al.*, 2009), grass carp (*C. idella*) (Ye *et al.*, 2010) and catfish (*Ictalurus punctatus*) (Wang *et al.*, 2014), turbot (*Scophthalmus maximus*) (Yu *et al.*, 2013), rainbow trout (*Oncorhynchus mykiss*) (Dautigny *etal.*, 1991) and common carp (*Cyprinus carpio*) (Savan *etal.*, 2003). Here, we focused on the primary structure and phylogeny of a g-type lysozyme from the skin mucus of *C. idella*.

Materials and methods

Animals

Grass carp were obtained from the Shahid Maleki Fish Culture Ponds located in the Khuzestan province of Iran and maintained in an indoor aquarium tank with running river water. The fish had a mean weight of 800-1200 ×g, and were adapted at 20±2°C

for at least three weeks before they were killed.

RNA isolation

After removing most of the epidermal mucus by blotting the fish skin with tissue paper, epidermal cells were collected by scraping the scales along the cephalo-caudal axis of the freshly killed fish with a sterileglass microscope slide. Total RNA from the skin was extracted using the RNX plus solution (CinnaGen, Iran) according to the manufacturer's instructions, RNA was quantified by absorbance at 260 nm.

Synthesis of cDNA

Briefly, 12 µL (2 µg each) of skin total RNA was incubated with 0.5 µg of Oligo(dT)18 primers at 70°C, for 10 min followed by a brief centrifugation. The reaction was chilled on ice for a few minutes and then 1 µL RNasin (CinnaGen, Iran), 1 µL dNTPs mixture (120 mM of each nucleotide), 2.5 µL of 5 × enzyme buffer and 1 µL (200 U) of Moloney Murine Leukemia Virus (MMulV) reverse transcriptase (CinnaGen, Iran) were added. The reaction was incubated at 42°C for 1 h.

RT-PCR

Homologous primer sets lysgF 5'-ATGGCATACATTTATGGAGACACC lysgR 5'-GTAACCCTTGCTTCTGAACCACT was prepared based on the sequence data of common carp (AB084624). The coding region amplified by these primers corresponds to 185 amino acids. RT-PCR was performed in a volume of 25 µL containing 10 ng of cDNA, 1×PCR buffer with 1.5 mM MgCl2, 0.2 µM of each primer, 250 µM of each dNTPs and 1 unit of *Taq* DNA Polymerase. The PCR program consisted of the following steps: 94°C for 2 min followed by 38 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 40 s, then a final step of 72°C for 5 min. The PCR products were electrophoresed on a 1.0% (w/v) agarose gel. DNA fragments were then extracted from the gel using the Gel Extraction Kit (CinnaGen, Iran) according to the manufacturer's instructions.

DNA sequencing and sequence analysis

The amplified cDNA fragments were subjected to sequencing in both strands using a dideoxy termination method and run on an Applied Biosystems 373 DNA sequencer. The complete cDNA was determined by using overlapping fragments. The primer was designed using Primer3 program (biotools.umassmed.edu/bioapps/primer3_www.cgi). The sequence was compared with sequences in the database using the BLAST algorithm from the NCBI site (ncbi.nlm.nih.gov). To evaluate the evolutionary relationships between lysozyme genes, we retrieved cDNA sequences of lysozyme genes of the other species from the NCBI GenBank. The putative signal peptides were analyzed using SignalP software (Nielsen *et al.*,1997) (cbs.dtu.dk/services/SignalP) and for N- (with the NetNGlyc 1.0 Server) and O-linked (NetOGlyc 3.1 Server) glycosylation sites (Julenius *et al.*, 2005). The multiple alignments were made using the CLUSTAL_W program (Thompson *et al.*, 1994) and edited with the

BOXSHADE software (ch.embnet.org/software/BOX_form.html). The CDD-Search software from the NCBI site was used to determine the conserved domains (Marchler-Bauer *et al.*, 2017). The molecular weight (MW) and theoretical isoelectric point (pI) of the deduced amino acid sequences were analyzed using the export protein analysis program (ca.expasy.org/tools). The secondary structure of the protein was predicted using the PSIPRED Protein Sequence Analysis Workbench (bioinf.cs.ucl.ac.uk/psipred). The 3D structure prediction was performed by the Phyre2 program (Kelley andSternberg, 2009).Phylogenetic analysis and genetic distance were carried out by the "neighbor-joining" method bootstrap tests 1000 using MEGA7 software (Tamura *et al.*, 2007).

Results

PCR amplification and sequence characterization

The results obtained by RT-PCR analysis confirmed the presence of lysozyme g-type in grass carp skin since the expected 555 bp cDNA fragment was amplified from the skin (Fig. 1).

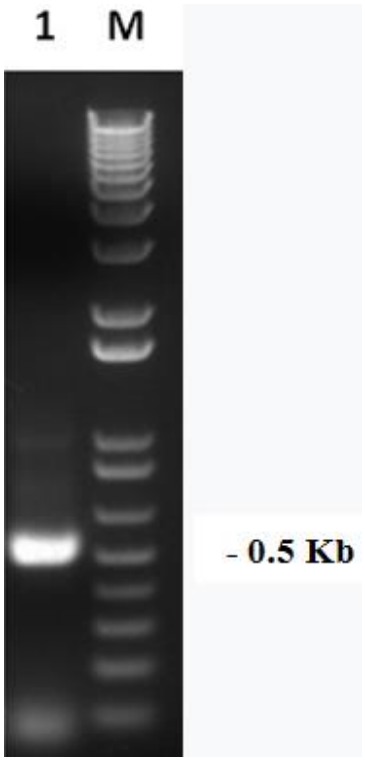

Figure 1: Agarose gel electrophoresis of g-type lysozyme PCR amplification. Lane M: DNA size marker. Lane 1: PCR amplification products.

It was called Ci-Kh. Only 5 μL of the PCR products were taken for agarose gel electrophoresis, indicating the efficient amplification of the target gene and the optimal conditions of the reaction.

A single band corresponding to the target sequence was identified with a putative translated protein composed of 185 amino acids with a predicted molecular mass of 20.49 kDa and theoretical pI of 9.13. Typical signal peptide has not been detected, in agreement with most other fish g-type lysozymes, neither possible N- or O-glycosylation sites.In order to compare the nucleotide sequence of Ci-Kh with the sequences available in the GenBank database, the BLASTn program available on the NCBI website was applied using the "Highly similar sequences (megablast)" program. This sequence showed 92-99%

identity with the sequences of *C. idella* lysozyme g-type transcript variant mRNA genes. The nucleotide sequence of Ci-Kh showed similarity with 96 hits from the order *Cypriniformes*, and a total of 99 hits belonged to the family *Xenocypridinae* (Table 1). It was revealed that *Carassius gibelio* with 13 hits has the most similarity in this family, while only seven hits were related to*C. idella*species. The sequence with accession number EU835653.1 which has already been submitted to the GenBank (Ye *etal.,* 2010) was identical to the sequence of *C. idella* variant X4 (XM_051859671.1). Five different sequences X1 (XP_051715628.1), X2 (XM_051859669.1), X3 (XM_051859670.1), X4 (XM_051859671.1) and X5 (XM_051859672.1) were included in drawing the phylogeny tree. At the protein level, the amino acid alignment of Ci-Kh sequence with *C. idella* lysozyme g variant X1 (XP_051715628.1) and *C. idella* lysozyme g variant X3 (XP_051715630.1) was 99.41% identical. The degree of Ci-Kh amino acid identity with the sequence of *C. idella* lysozyme g-type variant X5 (XP_051715632.1) was 90.53% with the value of 1e-109. This comparative analysis confirmed our sequence as g-type lysozyme.

Secondary structure and three dimensional-structure model

It was revealed that the amino acids critical for the fundamental structure and function of the g-type lysozyme were highly conserved in the fish lysozymes. Domain analysis (Marchler-Bauer *et al.,* 2017) of Ci-Khshowed e-value of 2.59e-111 with a conserved domain of lysozyme-like superfamily (cd01021) between amino acid residues 12 to184 (Fig. 2). It was revealed that this protein has a goose egg white lysozyme (GEWL) domain containing three typical conserved catalytic residues (Glu73 and Asp97) and sugar binding site (Glu73, Asp86, Gln95, Val96, Asp97, Tyr100, His101, Glu119, Tyr147, Asn148, and Gly150) (Hikima *et al.,* 2001).

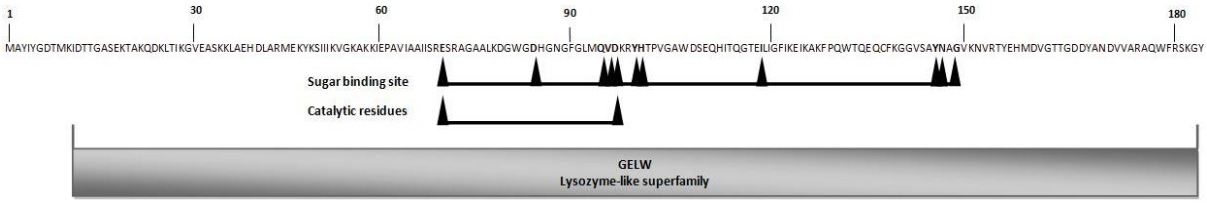

Figure 2: A schematic representation of g-type lysozyme domain of Ci-Kh.

Using the PSIPRED Secondary structure and disorder prediction program we obtained a prediction of mostly *a*-helical (44.86%), Strand (1.08%) and random coils (54.05%) for the coding sequence of Ci-Kh (Fig. 3A). The three-dimensional (3D) structure of Ci-Kh protein (Fig. 3B) was modeled using 181 amino acids (98% of the entire coding sequence) with 100% confidence the single highest-scoring template (Kelley and Sternberg, 2009). The predicted 3D structure of Ci-Kh was

related to a protein that was mostly made by five main helices and random coils.

Multiple sequence alignment of the g-type lysozyme with other fish counterparts illustrates the most conserved nucleotide sequence scattered among the entire sequence. However, the result of this alignment revealed that it contained several region sites with high homology (Fig. 4). In the multiple alignments performed in the coding region of the gene, the sequence of *C. idella* (FN428856.1) was identical to the sequence of *C. idella* variant X4. Therefore, sequence *C. idella* variant X4 was used in the multiple alignments. This sequence differed from the *C. idella* variant X2 sequence by two nucleotides (98% homology). The sequence difference between *C. idella* variant X4 and *C. idella* variant X5 was determined in 18 nucleotides (96% homology). The difference between sequence *C. idella* variant X3 and *C. idella* variant X1 was one nucleotide (99% homology).

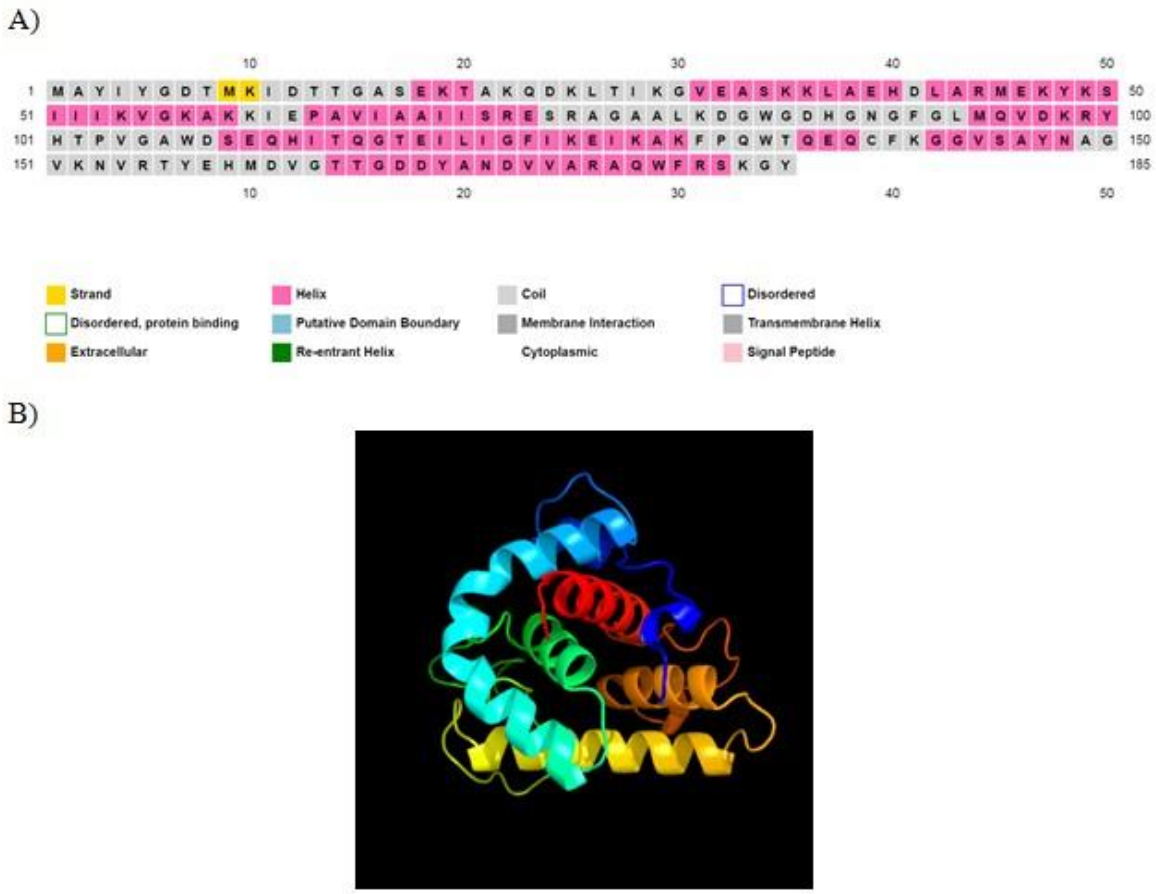

Figure 3: A schematic representation of the secondary structure (A) and three-dimensional model (B) of g-type lysozyme Ci-Kh.

When the 5 variant sequences were compared with Ci-Kh, it was found that this sequence showed the highest and lowest similarity with sequences *C. idella*

variant X3 and *C. idella* variant X1 (98%) and *C. idella* variant X4 and *C. idella* variant X2 (92%), respectively. Comparison between Ci-Kh with sequence *C. idella* variant X5 showed lower similarity (94%).

Phylogenetic analysis

The phylogenic tree was constructed for the nucleotide sequence of the g-type lysozyme gene obtained from *C. idella* in Khuzestan (Ci-Kh) and other variant sequences from *C. idella* species. The nucleotide sequence of Ci-Kh with the retrieved variant sequences was subjected to multiple sequence alignment and phylogenetic analysis using MEGA7 software using the neighbor-joining method. Among the 5 variant sequences given in Figure 5, two sequences *C. idella* variant X1and *C. idella* variant X3 with a high similarity of 99 are in the same cluster. However, Ci-Kh with bootstrap value 91 is separated from both of them.The other three sequences (*C. idella* variant X5, *C. idella* variant X4 and *C. idella* variant X2) were placed in a separate cluster. *Cyprinus carpio (*AB084624.1) sequence was used as an out-group.

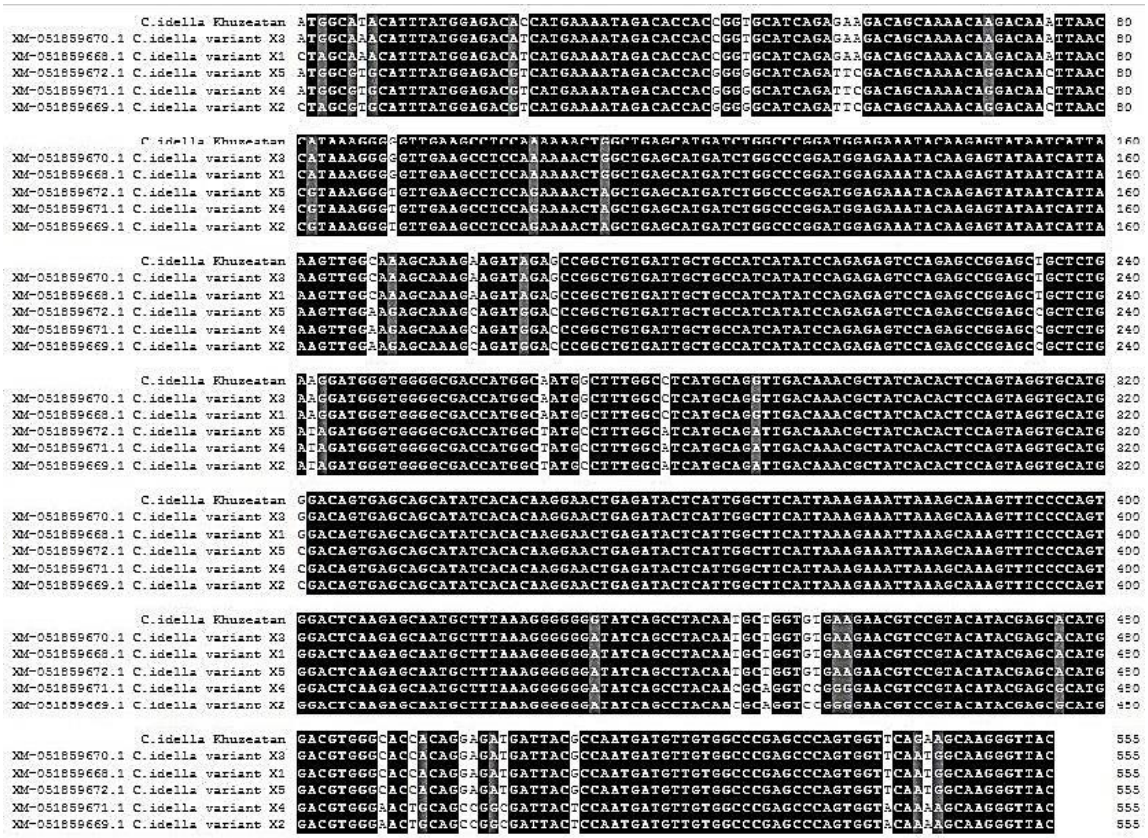

Figure 4: Comparison of the nucleotide sequences of g-type lysozyme Ci-Kh with other *C. idella* variants sequences. Shading indicates identity (black) or conservative substitutions (grey) relative to Ci-Kh.

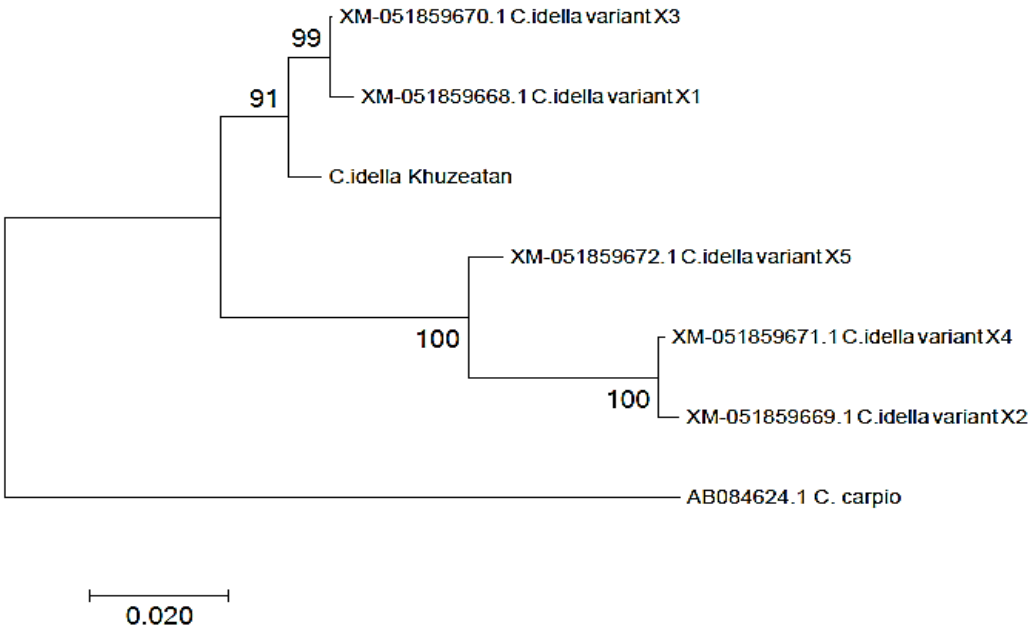

Figure 5: Phylogenetic tree constructed from the nucleotide sequences of g-type lysozyme *C. idella*-Khuzestan with other variant sequences using Neighbor-*joining* analysis. Bootstrap numbers are based on 1000 replicates. The numbers in front of the species are the accession numbers of the related gene variants in the GenBank. The numbers above the lines indicate the relationship between the groups.

In order to analyze Ci-Kh gene in the larger context of fish g-type lysozyme genes, phylogenetic analysis was conducted based on the deduced amino acid sequences from 4 *C. idella* gene variants with 9 sequences from *Cyprinidae* family, as shown in Figure 6. The protein sequence of Ci-Kh with the related sequence data was subjected to multiple sequence alignment and phylogenetic analysis using MEGA7 software using the neighbor-joining method. The constructed phylogram showed two distinct clusters (A and B). The major cluster A was further subdivided into two sub-cluster. In cluster A, the gene variants of *C. idella* variant X3 (XP-051715630.1) and *C. idella* variant X1 (XP-051715628.1)were grouped together with a strong bootstrap score of 85.Within this cluster, Ci-Kh formed a separate branch (59%) that shows moderate support for *C. idella* variant X3 and X1.The similarity of *C. idella* variant X5 (XP-051715632.1)and *C. idella* variant X4 (XP051715631.1)formed a separate branch in cluster A with a score of 99. The phylogenetic tree shows that all species belonging to Cluster B are joined together with a bootstrap score of 46. As it was expected Ci-Kh used in this study was perfectly arranged in cluster A showing higher genetic similarity with different *C. idella* isoforms and was showing higher genetic distance particularly with species in Cluster B. *Cyprinus carpio (*ANC28107.1) used as an out-group.

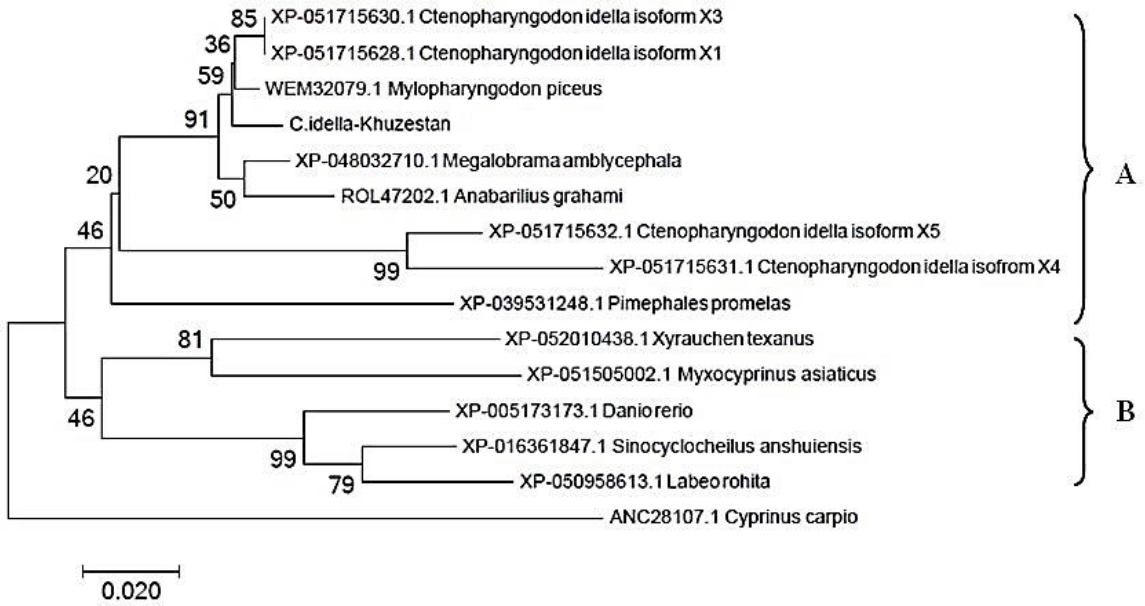

Figure 6: Phylogenetic tree constructed from the amino acid sequences of g-type lysozyme *C. idella*-Khuzestan with the related sequences using Neighbor-*joining* analysis. Bootstrap numbers are based on 1000 replicates. The numbers in front of the species are the accession numbers of the related genes in the GenBank. The numbers above the lines indicate the relationship between the groups.

Genetic distances

The genetic distance of g-type lysozyme Ci-Kh was calculated with *C. idella* nucleotide variant sequences using MEGA7 software. According to Table 1, among the five variants of this gene available in GenBank, Ci-Kh sequence had the highest and lowest genetic distance with *C. idella* variant X2 (XM 051859669.1) (8.6%) and *C. idella* variant X3 (XM-051859670.1) (1.1%) sequences, respectively.However, when amino acid was used in phylogeny, these values were 14.2% and 1.7%, respectively (Table 2).

Table 1: Taxonomy of Ci-Kh from Khuzestan based on BLASTn g-type lysozyme gene.

Taxonomy	No. of Organism	Score	No. of Hits
Bilateria	24		103
. Otophysi	23		102
. . Cypriniformes	21		96
. . . Cyprinoidei	19		89
. . . . Xenocypridinae	3		12
. . . . . Ctenopharyngodonidella	1	933	7
. . . . . Megalobramaamblycephala	1	872	4
. . . . . Hypophthalmichthys molitrix	1	307	1
. . . . Carassiusgibelio	1	712	13
. . . . Pimephalespromelas	1	712	4
. . . . Carassius auratus	1	706	11
. . . . Sinocyclocheilusrhinocerous	1	676	7
. . . . Sinocyclocheilusanshuiensis	1	676	4
. . . . Labeorohita	1	662	4

Table 1 continued:			
. . . . Sinocyclocheilusgrahami	1	660	6
. . . . Puntigrustetrazona	1	601	3
. . . . Danio rerio	1	568	11
. . . . Cyprinuscarpio	1	553	7
. . . . Gobiogobio	1	318	1
. . . . Phoxinusphoxinus	1	307	1
. . . . Squaliuscephalus	1	291	1
. . . . Barbusbarbus	1	276	2
. . . . Daniokyathit	1	274	1
. . . . Danioaesculapii	1	261	1

Table 2: The genetic pairwise distances of g-type lysozyme gene Ci-Kh compared to the related *C. idella* variant nucleotide sequences.

Specimens	**1**	**2**	**3**	**4**	**5**	**6**
1- C. *idella* Khuzestan						
2-XM-051859670.1 *C. idella* variant X3	0.011					
3-XM-051859668.1 *C. idella* variant X1	0.014	0.004				
4-XM-051859672.1 *C. idella* variant X5	0.058	0.050	0.054			
5-XM-051859671.1 *C. idella* variant X4	0.083	0.083	0.086	0.032		
6-XM-051859669.1 *C. idella* variant X2	0.086	0.086	0.083	0.036	0.004	
7-AB084624.1 *C. carpio*	0.139	0.146	0.150	0.178	0.182	0.186

Discussion

As indicated by the name, the first g-type lysozymes were recognized in the egg whites of different bird species (Canfield and McMurry, 1967). It is also broadly reported in fish (Hikima *et al.,* 2001) and mammals (Irwin and Gong, 2003). In this paper, we report the identification of a g-type lysozyme gene variant in grass carp.

Multiple alignment and phylogenetic analysis of Ci-Kh at nucleotide and amino acid sequences revealed that the gene was the g-type lysozyme. It consists of 185 amino acids, with no predicted signal peptide. In birds and mammals, the signal peptide sequence was found at the N-terminus of g-type lysozymes, but the reported g-type lysozyme (Ci-Kh) possessed no signal peptide, suggesting that the enzyme as an intracellular protein perhaps not be secreted from cells (Irwin and Gong, 2003). Although most fish g-type lysozymes lack signal peptides, some of them, such as salmon (Kyomuhendo *et al.,* 2007), have signal peptides. Some features of grass carp lysozyme (Ci-Kh), like the number of amino acids (185 aa) and the molecular weight (20.490 kDa) are close to what is estimated for a typical g-type lysozyme (Callewaert and Michiels, 2010). Sequencing results showed that Ci-Kh has one cysteine which is lower with compared to mammalian and bird lysozymes.The presence of one cysteine, as in common carp (Savan *et al.,* 2003), and S. salar (Kyomuhendo *et al.,* 2007), or two, as in *D. rerio*and *S. maximus*(Yu et *al.,* 2013), with no capability to shape a disulfide bond (Irwin and Gong, 2003), suggesting that disulfide bonds formed in g-type lysozyme were not essential for the proper folding of the catalytically active conformation (Kawamura *et al.,* 2006).

It was shown that the expression of g-type lysozyme gene showed different patterns in various species. They were expressed in different tested tissues such as the spleen, kidney, gill, skin, heart, intestine and blood of fish including Japanese flounder (Hikima *et al.,* 2001), orange-spotted grouper (Yin *et al.,* 2003) and Atlantic cod (Larsen *et al.,* 2009). Isolation of Ci-Kh transcript from the skin mucus of fish suggested that g-type lysozyme may have involvement in creating the first barrier against external pathogens and skin mainly plays key roles in innate immunity.

The g-type lysozyme of Ci-Kh containing 185 deduced amino acid residues, is similar to those in common carp (Savan *et al.,* 2003) and grass carp (Ye *et al.,* 2010) which is shorter than other fish species. For instance, the number of deduced amino acid residues of g-type lysozyme cDNA from some fish species such as Japanese flounder(Hikima *et al.,* 2001), orange-spotted grouper (Yin *et al.,* 2003), larger yellow croaker(Zheng *et al.,* 2007), mandarin fish(Sun *et al.,* 2006), brill (Jimenez-Cantizano *et al.,* 2008) and turbot (Yu *et al.,* 2013) was 193-195. The Atlantic cod has a g-type lysozyme with a longer deduced lysozyme protein having 217 aa (Kyomuhendo *et al.,* 2007). Although different fish species contain different open reading frame (ORF) lengths of g-type lysozyme cDNA, the conserved domain of g-type lysozyme protein was much similar. The deduced Ci-Kh protein contains typical GEWL domain and two conserved catalytic residues (Glu73 and Asp97) which are present in most fish species as well as in birds and mammals create strong catalytic activity (Hikima *et al.,* 2001). In Ci-Kh, the third catalytic residue at the 86th position, the aspartate, is also observed. However, studies show that the presence of the two original residues is sufficient for catalytic activity (Kuchinke, 1989). Nevertheless, as typical of fish g-type lysozyme, neighboring amino acids were also conserved. Moreover, the presence of five conserved repeats predicted to form amphipathic α-helical secondary structures is quite conserved.

Phylogenetic analysis revealed that Ci-Kh is divergent from the five other *C. idella* variant members which are retrieved from the database. Only a few nucleotide differences were found between variant X1 (XM-051859668.1) and variant X3 (XM-051859670.1) as well as variant X2 (XM-051859669.1) and variant X4 (XM-051859671.1). It is very probable that these nucleotide differences were due to "usual" genetic variations found in any population. Although, Ci-Kh showed a moderate difference with those two variants (X1 and X3 variants), its differences with other three (X2, X4, and X5 variants) both in terms of genetic distance and phylogenetic analyses are distinct.

Conclusions

It is concluded that Ci-Kh as a different variant of g-type lysozyme cannot be ruled out. Further analysis of the structure and function is necessary to understand the evolutionary pattern of g-type lysozymes in this species.

Acknowledgments

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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