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Tissue expression profiling, molecular and functional characterization of the antimicrobial peptide β-defensin, *Mc*–BD, from Grey Mullet, *Mugil cephalus*

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ABSTRACT

β-defensins, an important class of antimicrobial peptides, believed to be synthesized in the gill region of the fishes and which forms a part of its innate immune mechanism, help them tide over the highly challenging conditions they live in. The present study isolated a partial transcript, *Mc*–BD, of 117 bp coding for 38 amino acids, which was confirmed to be of β -defensin family by BLASTp online tool. *Mc*–BD was predicted to be 4.6 kDa, with the amino acid arginine making up 18.4% of its composition. Phylogenetic analysis showed an evolutionary relationship of the defensins with the defensins of *Channa striata, Planiliza haematocheila* and *Epinephelus coioides*. The tissue-wise expression profiling of the gene showed a maximum level of expression in the gill, which ascertains the fact that the expression is significant in tissues involved in host defense. Functional characterization revealed its antimicrobial properties, which could be tapped for the overall development of aquaculture, which is the most happening event in this 21st century. The constraints hampering the smooth functioning of the aquaculture industry could be aptly addressed with the help of careful manipulation of the properties of β-defensins.

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1. Introduction

Fish are poikilothermic aquatic vertebrates that are the most affordable source of animal protein for many people around the world. This is being made available year-round to consumers from low to high-income nations via the mode of aquaculture (Naylor et al., 2021). Aquaculture is a collective effort of breeding, raising, and harvesting fish, shellfish, and aquatic plants. Maintaining sustainable, productive fisheries and aquaculture is primary in safeguarding nutritional security, increasing income, improving livelihoods, promoting economic growth, and protecting the environment and natural resources. According to the Food and Agricultural Organization (FAO) 2016, about 40 % of total fish production in 2014 was the contribution of the aquaculture sector. This amounted to 74 million tons of fish worth 160 billion dollars. World aquaculture production is expected to increase by 32 percent over 2018 by 2030 (FAO The State of World Fisheries and Aquaculture, 2020).

Grey mullet (*Mugil cephalus*), belonging to the Mugilidae family, is an important food fish species found in tropical and subtropical waters worldwide. It is an important and widely distributed fishery and aquaculture species and meets the protein requirements of people of the Pacific basin, Southeast Asia, India, the Mediterranean and Eastern European countries, Central and South America. Mullet has been farmed in India since ancient times and has been extensively cultured in Bengal, Madras, and Kerala since 1947.

The intensive culture practices in the aquaculture industry have triggered the use of antibiotics for tackling fish diseases, leading to a grave situation of antibiotic resistance. Antimicrobial peptides (AMPs) are host defense molecules that constitute the innate immune mechanism of fishes. The specific mode of action of AMPs has made it a potential alternative to antibiotics in combating pathogens (Paria et al., 2018). A broad spectrum of action against bacterial, viral, fungal, and protozoan pathogens also characterizes them.

β-defensins are a group of small cationic AMPs produced by tissues involved in host defense (Ganz, 2003). In addition to the antimicrobial activity, immunomodulatory, fertility, and developmental properties are also attributed to it. Three pairs of disulphide-linked cysteines (C1-C5, C2-C4, C3-C6) act to stabilize the α-helical β-sheet folds. The tissue distribution of β-defensins is extensive and diverse and is reported to be expressed in most tissues of Channel catfish (*Ictalurus punctatus*) (Zhu et al., 2017), Gilthead seabream (*Sparus aurata*) (Cuesta et al., 2011), Nile tilapia (*Oreochromis niloticus*) (Dong et al., 2015), and Blunt snout bream (*Megalobrama amblycephala*) (Liang et al., 2013).

As fish live in an environment with a rich load of microbial community, it has developed an efficient mechanism to counteract possible infections (Shabir et al., 2018). The target of the present study is the isolation and characterization of β-defensins in the grey mullet (*Mugil cephalus*), as they are one of the prominent classes of AMPs found in fishes. Also, not much study on the AMPs from mullet has been conducted so far except for a study on β-defensin from soiny mullet (Qi et al., 2016). An attempt has also been made to find the tissue-wise expression exhibited by the identified β-defensin, *in silico*. As there has been prolific research in the area of AMPs, the identification and characterization of yet another host defense molecule from an important food fish species opens up possibilities of exploring its potential and manipulating it for possible applications in aquaculture.

2. Materials and Methods

2.1 Sample collection and RNA isolation

Samples of grey mullet, *Mugil cephalus*, were collected from the Fisheries Station, Kerala University of Fisheries and Ocean Studies (KUFOS), Puthuvyppu, Kochi, Kerala.

The sampled fish was transported live to the laboratory, providing proper aeration. Tissues such as heart, liver, muscle, gall bladder, skin, stomach, intestine, brain, gill, and blood from the lamellar artery were extracted from the fish under sterile and RNase-free conditions. The tissues were preserved in TRI reagent (Sigma) and stored at -80°C till further use. The preserved tissues, which retain the pink colour of the TRI reagent, were subjected to RNA isolation following the TRI reagent protocol (Sigma). The quality and quantity of the isolated RNA were checked spectrophotometrically at 260 and 280 nm and also by 0.8 % agarose gel electrophoresis. The RNA samples which gave a value of 1.8 - 1.9 for the 260/280 absorbance ratio were selected for cDNA synthesis.

2.2 cDNA synthesis and screening for β-defensin gene by polymerase chain reaction (PCR)

cDNA synthesis of the samples with 5 µg total RNA was carried out by reverse transcription using 2 µM Oligo $d(T)_{20}$, 20 U RNase inhibitor, 100 U M-MLV Reverse Transcriptase, 3.5 mM $MgCl_2$, 2 µM dNTPs, and 1x RT buffer. The synthesis was carried out at an incubation step at 42 °C for 1 h followed by an enzyme inactivation step at 85 °C for 15 min. The cDNA thus synthesized was diluted five times to get an effective concentration of 1 µg cDNA/µL for further investigations. The efficiency of conversion of mRNA into cDNA was checked by screening for the presence of the β-actin housekeeping gene. The PCR of 1µg of cDNA was carried out in a 20µL reaction volume using 1x standard Taq buffer (10 mM Tris-HCl, 50 mM KCl at pH 8.3), 200 µM dNTPs, 0.4 µM each of the β-actin forward and reverse primers and 1U Taq DNA polymerase. Once the presence of β-actin gene was confirmed, yet another PCR amplification was done to screen for the presence of the gene of interest, β-defensin, using the specific forward and reverse primers (F: 5′-cgatgaagggactgagcttggttc-3′; R: 5′- gtgctaagaccgcatagcacagc-3′) (Anooja et al., 2020). The screening was carried out following a thermal profile with an initial denaturation step at 95 °C for 2 min followed by 35 cycles of 94 °C for 15 s, 60 °C for 30 s (annealing) and 72 °C for 30 s (extension) and a final extension step at 72 °C for 10 min. Agarose gel electrophoresis (1.0 % agarose mixed with 1X TBE (Tris-Boric acid-EDTA) buffer and Ethidium Bromide (EtBr, SRL, India) as the staining agent) was used to confirm the presence of the gene of interest in the amplicons. The gel was visualized in a UV Trans-illuminator Gel Doc XR system.

The positive samples that gave an amplification for the gene were treated with Exo-CIP rapid PCR cleanup kit. The sequencing of the amplicon was done using ABI Prism 377 DNA sequencer (Applied Biosystem, USA) at Agrigenome sequencing facility, India.

2.3 Sequencing and *in silico* analysis

The nucleotide sequences obtained were processed using Gene Tool and BioEdit software, and homology search was done in the BLAST online analysis tool of NCBI (http://www.ncbi.nlm.nih.gov/blast). The sequence was translated to its corresponding amino acid sequence using Expert Protein Analysis System (ExPASy - http://au.expasy.org/). *In silico* analyses were done using online tools and databases to decipher the physicochemical, functional, and antimicrobial properties of the peptide. The peptide sequence obtained from the present investigation was compared to the selected pre-deposited sequences of the like from the NCBI database to deduce the Multiple Sequence Alignment by the ClustalW and the phylogenetic tree by the neighbour-joining method using the MEGA 6.0 and GeneDoc software. The phylogenetic tree was constructed to understand the evolutionary bifurcations that happened to the gene of interest. The signal peptide prediction software SignalP 5.0 was used to identify the peptidase cleavage site in the peptide. The hydropathicity of the peptide was computed by the ProtScale using the Kyte & Doolittle amino acid scale (https://web.expasy.org/protscale/). The PSIPRED online server (http://bioinf.cs.ucl.ac.uk/psipred/) was used to predict the secondary structure of the peptide. The transmembrane prediction program DAS (Dense Alignment Surface) was used to find the transmembrane segments within the peptide. The ProtParam tool of ExPASy (https://web.expasy.org/peptide_cutter/) was used to predict the molecular weight, theoretical pI, percentage of amino acids present, GRAVY (Grand Average of Hydropathy) score, and the estimated half-life. The peptide cutter tool predicted the possible cleavage sites in the peptide for the selected enzymes. The Kyte-Doolittle graph of hydropathy was plotted to identify hydrophobic amino acids in the peptide, thereby predicting its possible structure. AGGRESCAN software was used to predict the hot spots in the protein. The peptide which is anticipated to have antimicrobial properties was validated using the antimicrobial peptide database (APD3) and confirmed using the Database of Antimicrobial Activity and Structure of Peptides (DBAASP v3.0). AVPpred (http://crdd.osdd.net/servers/avppred/index.html) online tool was used to predict the antiviral property of the peptide, and the anticancer property was predicted using ACPred (http://codes.bio/acpred/) online tool.

2.4 Tissue level expression profiling & ImageJ analysis

The target gene expression in the grey mullet, *M. cephalus* tissues was studied using semi-quantitative RT-PCR. The internal control gene employed was the β-actin, which showed a consistent constitutive expression in the tissues analysed. The tissues analyzed were heart, liver, muscle, gall bladder, skin, stomach, intestine, blood, brain, and gill. The reagents used and the conditions followed for the amplification reaction were the same as that used for the PCR performed to screen AMPs. For the semi-quantitative analysis, PCR for each sample was done in triplicates. The PCR cycle had been optimized to amplify the housekeeping gene and the target gene at the logarithmic phase. The amplification of the gene of interest was confirmed by 1.5 % agarose gel electrophoresis and visualized in the UV transilluminator. The intensity of the gel bands obtained was measured using ImageJ analysis software (https://imagej.nih.gov/).

3. Results

A partial β-defensin gene of 117 bp could be isolated by RT-PCR from the gill tissue of grey mullet, *Mugil cephalus*, which is hereafter designated as *Mc*-BD. BLASTn analysis of the nucleotide sequence confirmed it to be a member of the β-defensin family. The ExPASy translate online tool could deduce a 38 amino acid peptide from the 117 bp sequence (Fig. 1). Subsequent BLASTp analysis further confirmed the identity of the peptide. The obtained β-defensin sequence was deposited in GenBank under the accession number MT344089.

BLASTn analysis of the *Mc*-BD nucleotide sequence revealed 98.29 % identity to the β-defensin isoform from *Channa striata* for a query coverage of 100 %. Similarities were also found to the β-defensin isoforms from *Liza haematocheila* (95.73 %), *Siniperca chuatsi* (94.02 %), *Lates calcarifer*, *Sebasticus marmoratus*, *Epinephelus coioides* (93.16 % each) and *Trachinotus ovatus* (92.31 %). Sequence comparison of the amino acids by the BLASTp algorithm also showed 100 % similarity with the β-defensin AMP of *C. striata*. Similarities were also obtained for the β-defensins from *Planiliza haematocheilus*, *E. coioides*, *S. chuatsi*, *Gadus morhua* (97.37 % each), *Nothobranchius guentheri* (94.74 %) and *Oryzias latipes* (94.59 %).

The results of BLAST analysis were confirmed by the multiple sequence alignment results of both nucleotide and amino acids. A high degree of similarity with other reported β-defensins and the presence of conserved sequences were evident in the results and, the maximum similarity was found to *C. striata*, *P. haematocheilus* and *E. coioides* in multiple sequence analysis (Fig. 2).

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cag tac tgg acc tgt ggg tat aga gga ctc tgc aga cgg
 Q   Y   W   T   C   G   Y   R   G   L   C   R   R
ttc tgc cat gct cag gaa tac atc gtc ggt cat cat ggt
 F   C   H   A   Q   E   Y   I   V   G   H   H   G
tgc cct cgg cga tac aga tgc tgt gct atg cgg tct tag
 C   P   R   R   Y   R   C   C   A   M   R   S   *
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Fig. 1. The nucleotide and the ExPASy translated amino acid sequence of *Mc*-BD (MT344089), the stop codon is denoted by the red fond

The bootstrap neighbour-joining tree was constructed by retrieving the pre-deposited β-defensin sequences from the GenBank. The phylogenetic tree constructed for the amino acid also showed *Mc*-BD clustered in a clade along with *C. striata*, *P. haematocheilus,* and *E. coioides* (Fig. 3).

The ProtParam tool of ExPASy predicted a molecular weight of 4.6 kDa for the peptide with a theoretical isoelectric point (pI) of 9.38. The peptide had seven Arg (R) residues which amount to about 18.4 % of the total amino acids. Cys (C) (15.8 %), Gly (G), and Tyr (Y) (10.5 % each) were also found in significant amounts. The peptide with the N-terminal Gln (Q) residue is estimated to have a half-life of 0.8 h in mammalian reticulocytes, *in vitro*, 10 min in yeast *in vivo,* and 10 h in *E. coli in vivo*. This protein with a computed instability index of 34.98 was classified as stable. The aliphatic index was calculated to be 33.42 and the GRAVY score was -0.7. The PeptideCutter tool of ExPASy predicted one cleavage site for pepsin (pH 1.3), whereas pepsin (pH>2) was found to have eight possible cleavage sites in the peptide. Thermolysin and trypsin had seven possible cleavage sites in the peptide. The enzymes enterokinase and thrombin were predicted not to cut the peptide. The antimicrobial peptide database (APD3)

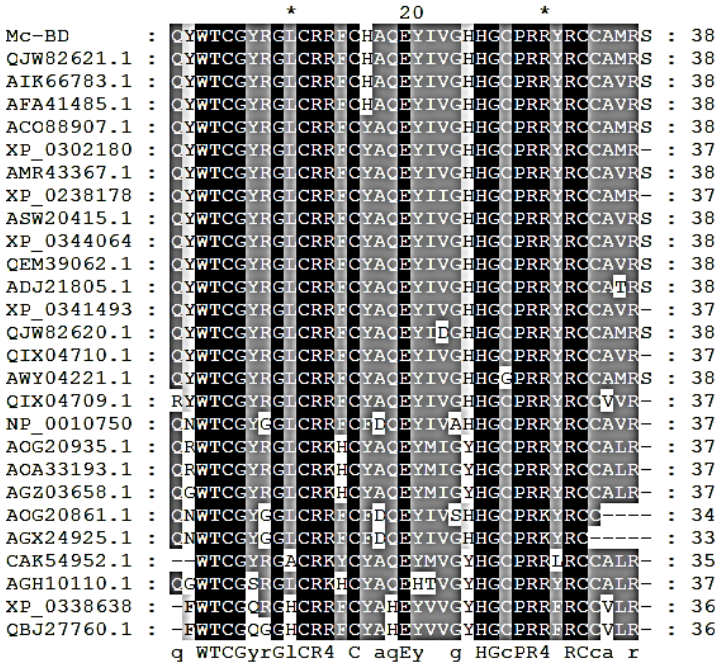

Fig. 2. Multiple sequence alignments of the amino acid sequence of *Mc*-BD (MT344089) with previously reported β-defensins from fishes showing sequence similarity obtained using GeneDoc program 2.7.0. Black shaded area shows completely conserved residues and grey shaded regions indicate partially conserved sequences

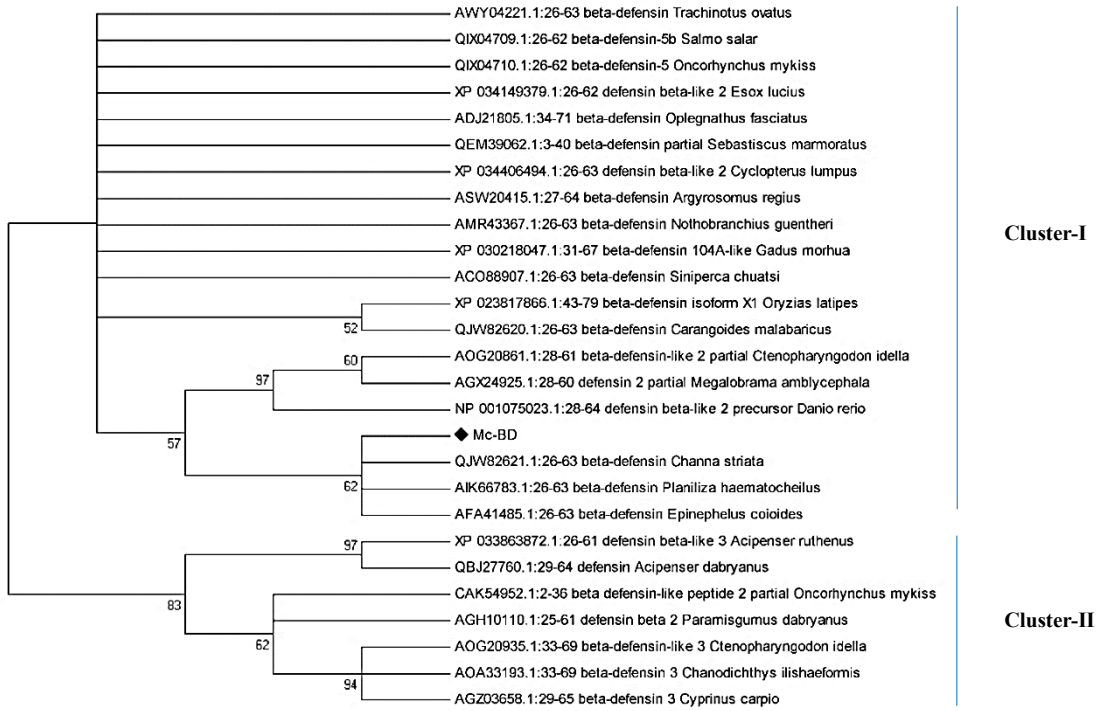

Fig. 3. Bootstrapped neighbour-joining tree obtained using MEGA 6.0 illustrating relationships between the amino acid sequence of Mc-BD (MT344089) with other reported β-defensins from fishes. Values at the node indicate the percentage of times that particular node occurred in 1000 trees generated by bootstrapping the deduced amino acid sequence

confirmed the above findings and predicted the hydrophobic ratio of *Mc*-BD to be 36 % and the total net charge +6.75. The Boman index (protein-binding potential) of the peptide predicted is 2.79 kcal/mol. The sequence alignment of *Mc*-BD using the database gave 65.11 % similarity to Chinese loach, *Paramisgurnus dabryanus* (AP02225), which showed specific activity against *Aeromonas hydrophila* and *Bacillus subtilis.* DBAASPv3.0 predicted the peptide to be in the category of AMP and, when used to check the antimicrobial activity of *Mc*-BD against specific microbes, revealed that the peptide is active against *Pseudomonas aeruginosa* ATCC 27853, and *Klebsiella pneumonia* with a MIC value less than 25 µg/ml. And the peptide was found to be not active against human erythrocytes, i.e., hemolytic activity was not detected. The SignalP 5.0 tool estimated the sequence obtained in the present study to be a partial one that lacks the signal peptide region. The hydropathicity of *Mc*-BD as illustrated by the Kyte-Doolittle plot shows positive peaks (hydrophobic regions) at positions 7, 13, 18, 19, and 24 with the maximum value at position 18 (0.478) (Fig. 4). AGGRESCAN software predicted the presence of one hotspot with the sequence EYIVG with the normalized hotspot area of 0.367. PSIPRED online tool predicted the secondary structure of the peptide with an N-terminal helix (Fig. 5). The Phyre2 server predicted the three-dimensional structure of the *Mc*-BD with 91.4 % confidence based on the template c2rngA (Fig. 6a). A secondary structure model was also predicted by the server, which confirmed an N-terminal α-helix followed by a beta-strand (Fig. 6b). The peptide was predicted to have antiviral and anticancer activity by the AVPpred and ACPred online tools, respectively.

Examination of 1.0 % agarose gel for tissue-wise expression profiling of the *Mc*-BD showed the presence of gene transcripts in all the tissues examined, with the expression in gills being the most and that in the brain meagre or nil at all. The β-actin housekeeping gene was found to have a consistent expression in all the tissues studied (Fig. 7).

4. Discussion

Defensin, a prominent family of AMP in vertebrates, is believed to have evolved early and is widely distributed in plants, animals, and fungi (Casadei et al., 2009). Among the α-, β-, and θ-defensins, β-defensins are found to be the most ancient ones and are found in fishes and characterized

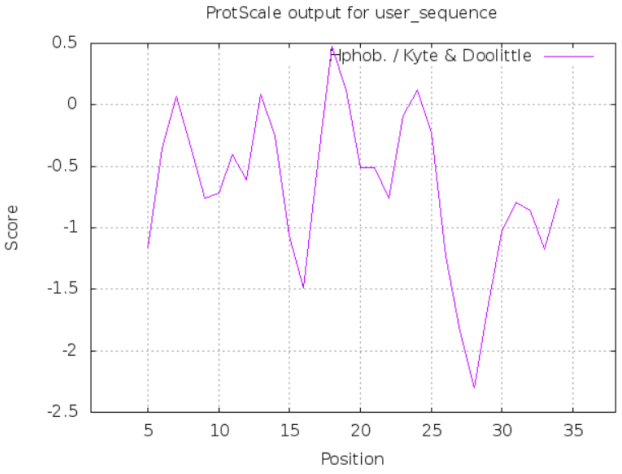

Fig. 4. The hydropathicity of *Mc*-BD plotted by Kyte-Doolittle plot with positive peaks (above 0) indicating potential hydrophobic residues in the peptide

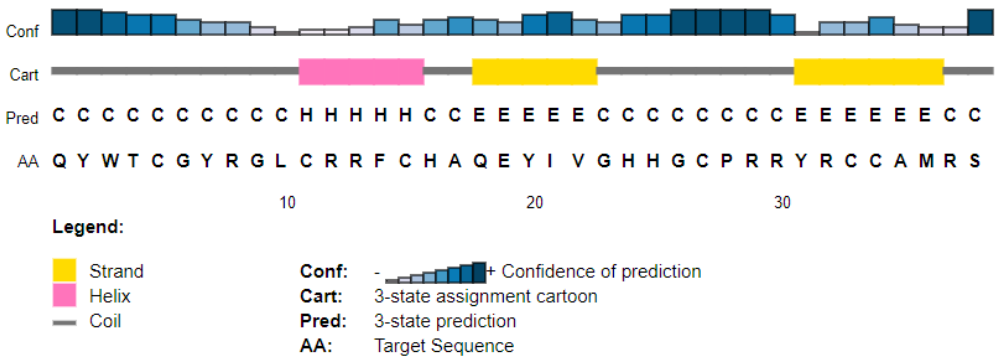

Fig. 5. The PSIPRED secondary structure prediction software predicting the helix, strand, and coiled regions of *Mc*-BD

Fig. 6a. The tertiary structure model of the Mc-BD built by the Phyre[2] server using c2rngA as a template showing the 3 beta-sheets in the structure

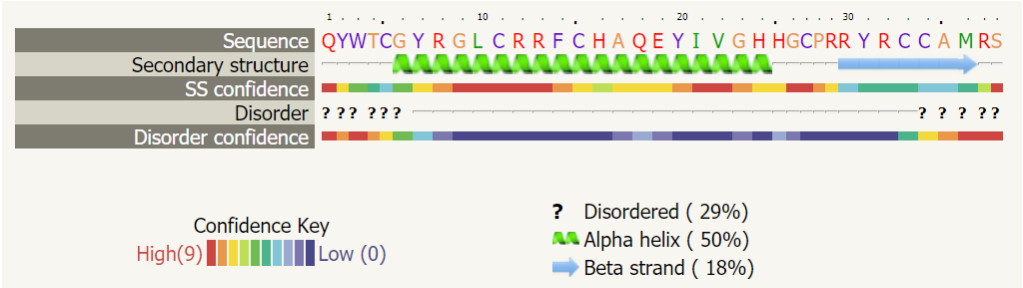

Fig. 6b. The secondary structure of Mc-BD as predicted by the Phyre[2] server showing an N-terminal α-helical region followed by the beta-strand

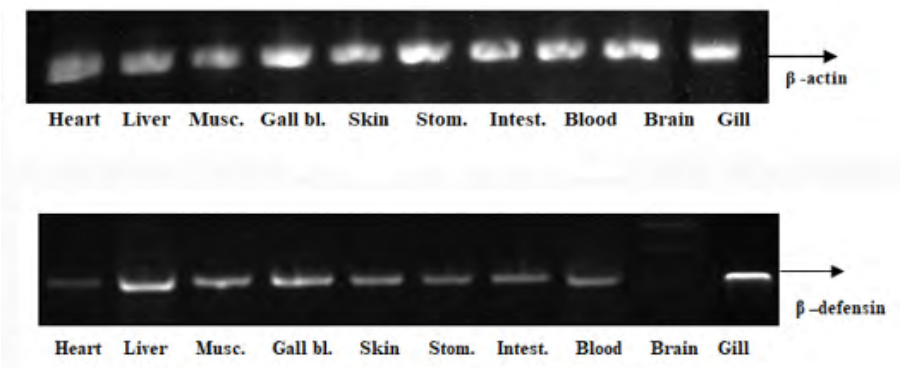

Fig. 7. The tissue-wise expression profiling of *Mc*-BD using semi-quantitative Real-Time PCR. The agarose gel images were analyzed by the ImageJ software

by the presence of six conserved cysteine residues and three disulphide bonds stabilizing their structures (Zou et al., 2007). They also have a varied expression pattern compared to the α- and θ-forms (Casadei et al., 2009). Earlier β-defensins had been characterized in fugu, zebrafish, and tetraodon (Zou et al., 2007). In general, various studies on isolation, molecular and functional characterization, antibacterial and antiviral properties, expression pattern, cell function modulation have also been conducted on β-defensins from fishes like medaka (Zhao et al., 2009), olive flounder (Nam et al., 2010), blunt snout bream (Liang et al., 2013), zebrafish using the database mining approach (Zou et al., 2007), common carp (van der Marel et al., 2012; Li et al., 2014), soiny mullet (Qi et al., 2016), channel catfish (Zhu et al., 2017), golden pompano (Zhou et al., 2019), rainbow trout (Harte et al., 2020), and turbot (Zhuang et al., 2021).

The sequence analysis of *Mc*-BD shows a resemblance to other identified vertebrate β-defensin sequences and shares the common characteristic features of defensins like cationic charge and low molecular weight. The peptide also revealed the presence of six conserved cysteine residues that may form three disulphide bonds that stabilize the structure and aid in their functional properties (Kluver et al., 2006). *Mc*-BD isolated in the present study is found to have the five amino acid residues between C1 and C2 and a conserved PRRYR motif between the C4 and C5 characteristic of fish β-defensins (Casadei et al., 2009). Multiple sequence alignment of *Mc*-BD with other fish defensins showed seventeen completely aligned residues and also the presence of G residues two places upstream of C2 and one place upstream of C4, which can be considered characteristic of fish defensins (Casadei et al., 2009). The phylogenetic tree constructed based on these sequences showed that the tree had two clades, one with beta-defensin 2, 3 and another bigger one with beta-defensin 2 and beta-defensin 5 clustered together and *Mc*-BD placed well within this clade along with *C. striata*, *P. haematocheilus* and, *E. coioides*.

The tissue-wise expression profiling of the *Mc*-BD showed expression of the gene in all the tissues examined, with the expression in gills being the most and that in the brain giving very low or no expression at all. The maximum level of β-defensin gene expression in the gill ascertains the fact that the expression is significant in tissues involved in host defense. Previous studies on the gene expression of β-defensin in fish reported high level of expression in the mucosal and immune tissues with a differential expression pattern which is found to be species-specific (Qi et al., 2016). Studies on rainbow trout showed an increased level of β-defensin in the gills, gut, and skin on challenging with *Yersinia ruckeri* (Casdei et al., 2009). This inference is substantiated by the findings in golden pompano showing upregulated β-defensin levels in the head kidney and spleen when challenged with *V. harveyi* (Zhou et al., 2019) and the yellow croaker showed constitutive expression in all the tissues with an increased level in head kidney and blood (Li et al., 2020). A high level of expression of β-defensin was noticed in the skin of turbot (*Scophthalmus maximus*), and an up-regulated expression was noticed in the mucosal tissues like skin, gill, and intestine upon bacterial challenge (Zhuang et al., 2021).

5. Conclusion

In conclusion, a partial sequence of 38 amino acid was identified from the grey mullet *Mugil cephalus* which was confirmed to belong to the β-defensin family on *in silico* analysis using the BLAST algorithm. The physicochemical properties also confirmed its identity. The peptide was also predicted to have antimicrobial activity against certain microbes. Since not much work has been done in fishes from the family Mugilidae, the present study will help understand the function of β-defensin in mullet and develop strategies for economically and environmentally viable aquaculture.

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