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CLONING, SEQUENCING AND EXPRESSION OF GOONCH *Bagarius bagarius* (Hamilton, 1822) GROWTH HORMONE

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ABSTRACT

Goonch *Bagarius bagarius* (Hamilton, 1822) is one of the largest and fastest-growing catfish of the Indus River found at Taunsa Barrage, Pakistan. The full-length cDNA of the growth hormone gene (600 bp) was amplified by reverse transcription of mRNA isolated from the pituitary gland of *B. bagarius*. The full-length growth hormone gene encodes a putative polypeptide of 200 amino acids with a molecular mass of 22.58 kDa. The precursor of *B. bagarius* growth hormone (GH) is composed of 22 amino acids as a signal peptide and 178 amino acids of a mature peptide. There were six conserved Cys residues in GH protein (20, 71, 135, 173, 190 and 198) that maintain the structural integrity of this protein. One putative N-glycosylation site was present at the 197th amino acid. The total number of positively charged (Arg and Lys) and negatively charged (Asp and Glu) residues is 42 and 31, respectively. The *B. bagarius* GH gene shows more than 90% sequence homology with other catfishes. The mature protein GH gene was expressed in *Escherichia coli* using pET-28 expression vector, and the recombinant protein of 19.5 kDa was detected through SDS-PAGE analysis. This study suggests that cloning and expression of *B. bagarius* GH gene would provide basic information for transgenic studies aimed at a faster growth rate. This recombinant GH may be produced on a large scale to exploit its growth-promoting function in other cultured fishes.

How to Cite

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INTRODUCTION

Goonch *Bagarius bagarius* (Hamilton 1822) belongs to the order Siluriformes (catfishes), family Sisoridae. It is a natural inhabitant of medium and large rivers, and is restricted to Indian subcontinent countries like Pakistan, Bangladesh, India, Nepal, Bhutan, Sri Lanka and the Maldives. It is one of the largest freshwater fish in Pakistan (Mirza, 2004). It thrives very well in rocky habitats and rapid pools of medium and large-sized rivers. It is the devastating predator of fish, frogs, shrimps and aquatic insects. It is also known as a freshwater shark that can grow more than 2 meters in length with a maximum weight of up to 120 kg, which makes it one of the heaviest fish in South Asia (Mirza, 2004). It is also known as the living fossil because through millions of years there were no evolutionary changes in the body structure of this fish species. It is recognized as a Near Threatened species in the IUCN Red List of Threatened Species (Ng, 2010).

The anterior lobe of the pituitary gland secretes growth hormone (GH) in all vertebrates. It is a protein in nature, consisting of a single polypeptide with 21-23 kDa and approximately 200 amino acids. It controls the process of normal development and linear growth in all vertebrates (Sekar et al., 2014). It is widely used for commercial purposes like animal husbandry, medicine, feed formulation for animals and farming of fish (Venugopal et al., 2002). GH controls and regulates the process of growth directly by binding with its receptor on the target tissues, called GH receptor, which is a single trans-membrane receptor and indirectly mediated by insulin-like factors I and II (Waters et al., 2006).

In many groups of fishes such as cyprinids, salmonids, silurids and sparids, various aspects of the physiology of GH have been an intense research topic for the last two decades. GH plays a pivotal role in almost all major physiological processes in fish, including the growth of skeletal and soft tissues, immune function, metabolism of carbohydrates, lipids and proteins, ionic and osmotic balance regulation and reproduction. Recently, many studies have determined that GH also plays an important role in the regulation of many behavioral aspects of the body like foraging behavior, appetite, aggression and avoidance of the predator (Bjornsson, 2002; Petro-Sakuma et al., 2020).

Venugopal et al. (2004) generated fast-growing transgenic *Labeo rohita* by electroporated sperm-mediated transfer of the vectors harboring CMV promoter fused to endogenous rohu *Labeo rohita* GH (rGH) cDNA. The gene transfer efficiency was 25%. They revealed that transgenic rohu *L. rohita* grew fast and ate less food than its control siblings. Sekar et al. (2014) cloned, characterized and reported the expression pattern of the GH gene of sutchi catfish. They reported that the gene is composed of five exons and four introns. They also reported some basic information for the development of allotransgenic and autotransgenic fish with higher growth rates. They reported that the recombinant fish growth hormone had the potential to enhance the fish growth. Nasr et al. (2014) described phylogenetic variation on the basis of the growth hormone gene of Persian sturgeon *Acipenser persicus* with other vertebrates. They reported that apart from a few species, growth hormone is a highly conserved protein in all vertebrates. Hoga et al. (2018) described the potential use of hormones in fish farming, artificial reproduction and sex reversal to improve fish industry profitability. Kamenskya and Brykov (2020) reviewed the structure of fish growth hormone genes. They reported that in most fish species, the growth hormone gene is represented by a single copy with conserved coding sequences. The variations in gene length among different species are due to the length of intron sand flanking regions. Moreover, GH is attracting the focus of many researchers throughout the world due to its potential use to evaluate evolutionary relationships, rearing and production of transgenic fish with enhanced growth rates. The administration of GH in many animal species increases their growth rate as reported for common carp *Cyprinus carpio*, rohu *L. rohita*, Nile tilapia *Oreochromis niloticus*, rainbow trout *Oncorhynchus mykiss*, coho salmon *Oncorynchus kisutch* and Atlantic salmon *Salmo salar* (Nasr et al., 2014; Celia et al., 2018). In the light of above-mentioned details, the current study was designed for the isolation, sequencing and expression of the growth hormone gene of *B. bagarius*. This study reports the details of the molecular physiology of the growth hormone gene and its protein, its phylogenetic relationship with other fish species and potential use in the future to enhance fish growth rate and generation of transgenic fish at the industrial scale.

MATERIALS AND METHODS

Fish sampling, RNA extraction and primer designing

A total of 10 live specimens of *B. bagarius* were collected by dragnet in June 2015 from the Indus River at Taunsa Barrage, South Punjab, Pakistan. All specimens were anesthetized and total RNA was extracted by the TRIzol method from their pituitary gland obtained by dissecting their skulls. The RNA pellet was dissolved in injection water and stored at -70°C. Two sets of primers (forward and reverse) were designed to amplify the full-length and mature protein growth hormone gene of *B. bagarius* given in Table 1.

Table 1. Primers used for amplification of the growth hormone of *B. bagarius*

S. No	Primer	Sequence	Tm(C)
1	FGH-R	5'-CTCGAGGGTGCAGTTGGAATCCAGGGATCTCC-3'	77.6
2	dT	5'-CGGAATTCTAGATTTTTTTTTTTTTTTTT-3'	58.4
3	FGH-F1	5'-CCATGGCTAGAGTGTTGGTGGTGCTCTCTGTGG-3'	77.7
4	FGH-F2	5'-CCATGGAGAACCAGCGGCTCTTCAACAACG -3'	76.3

The amplification of full-length growth hormone (FGH) gene from cDNA

Full-length growth hormone (FGH) cDNA was synthesized by reverse transcription through RT-PCR by FGH-R primer (Table 1). There was no cDNA synthesis by dT primer. The PCR amplification for the FGH gene of *B. bagarius* was carried out in a total volume of 25 µl containing 1X PCR buffer; 0.2 mM mixture of dNTPs; 2 mM $MgCl_2$; 50 pmol FGH-F1 primer; 50 pmol FGH-R primer; 1.5 U Taq polymerase; 50 ng template DNA and nucleus-free water. PCR profile was followed as an initial denaturation of 5 min at 94°C, followed by denaturation of 30 sec at 94°C; annealing for 30 sec at 500C; extension for 45 sec at 72°C with 30 cycles and final extension for 5 min at 72°C. The PCR amplification of the FGH gene (600 bp) was checked through gel electrophoresis and visualized on GelDoc UV trans-illuminator. The FGH gene was purified by Gel Extraction Kit (Gene JET=K0691) and confirmed by sequencing.

Cloning of FGH in pTZ57R/T and transformation to Escherichia coli DH5α cells

The purified FGH gene was ligated in the pTZ57/RT vector. The prepared competent cells of DH5α (*E. coli*) containing recombinant plasmid were cultured on LB agar media. Only white colonies were subjected to the confirmation of the FGH gene through colony PCR amplification with FGH-F1 primer and FGH-R primer (Table 1). After successful colony PCR amplification, the recombinant plasmid was again isolated from the secondary culture of transformed DH5α cells.

Sequence analysis and restriction

The plasmid containing the FGH gene was purified by Gel Extraction Kit (Gene JET=K0691) and confirmed after its sequencing from 1st Base Company, Singapore. The FGH gene was restricted by EcoR1 and HindIII, as well as Xba1 and HindIII with 1X tango buffer. The restriction process was confirmed through gel electrophoresis (Figure 1). Moreover, on the basis of FGH sequence of *B. bagarius*, a UPGMA phylogenetic tree was constructed and compared with twenty other fish species' growth hormone gene sequences to understand the phylogenetic relationship.

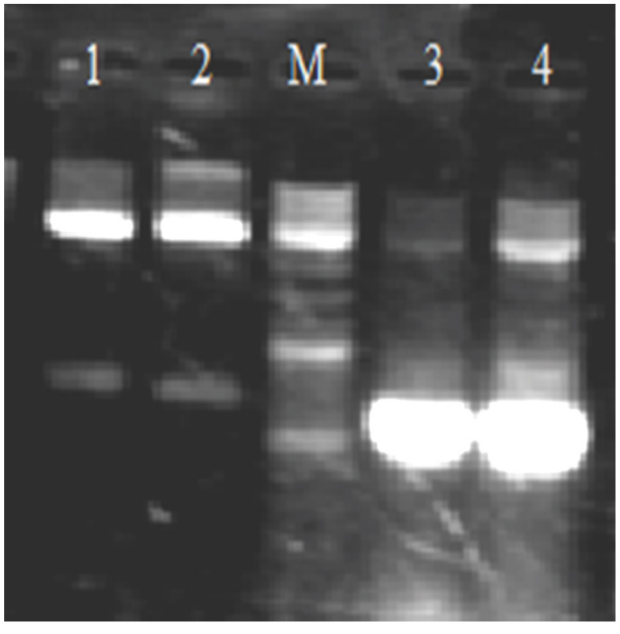

Fig 1. Lane 1-2, double restriction of full-length GH gene of *B. bagarius*, M DNA ladder mix and 3-4, plasmid PCR of full-length GH gene of *B. bagarius*

Amplification of mature protein growth hormone (MGH) gene, ligation in pET-28 vector and expression

The MGH gene was amplified through PCR from the FGH gene with FGH-F1 primer and FGH-R primer (Table 1). Both the MGH gene and pET-28a vectors were subjected to double restriction with Nco1 and Xho1 in 2X tango and 1X orange buffer. The restricted MGH gene was ligated into the double digested pET-28a vector. The ligation was confirmed by PCR. Nco1 and Xho1 in 2X tango buffer were again utilized for restriction analysis of recombinant pET-28a plasmid under the same conditions as described earlier (Figure 2). The expression of the MGH gene was carried out in *E. coli* BL21 cells. The expression was confirmed on 15% SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 3).

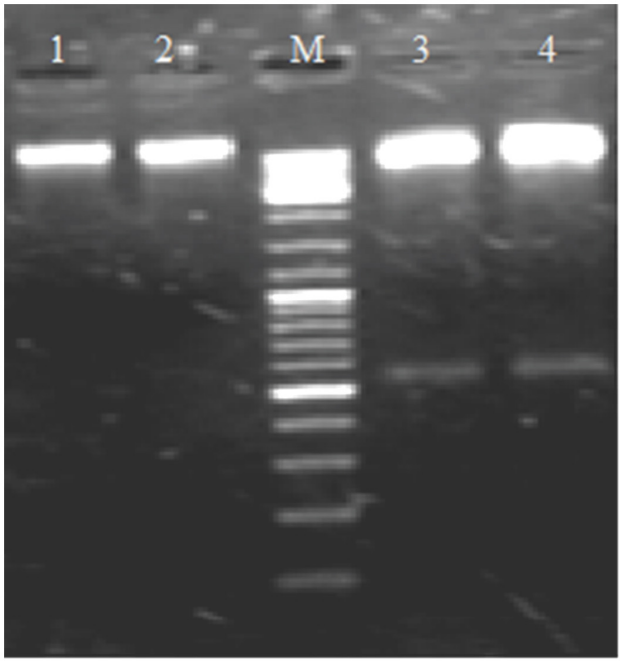

Fig 2. Lane 1-2, recombinant pET-28 plasmid with the MGH gene of *B. bagarius*, M DNA ladder mix and 3-4, restriction of the MGH gene of *B. bagarius* from pET-28 plasmid

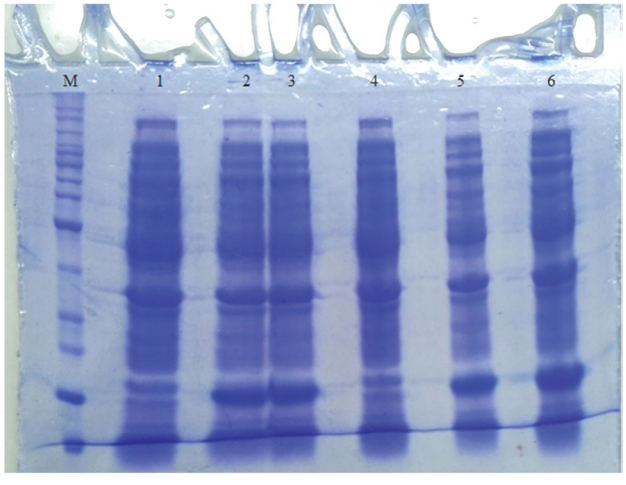

Fig 3. M protein ladder, 1 and 4, control; 2, 3, 5 and 6 expressions of MGH protein of *B. bagarius* after 2 hr and 4 hr of induction in BL21 cells of *E. coli*

RESULTS

Molecular cloning of the FGH gene of *B. bagarius* was initiated by isolation and conversion of its mRNA into cDNA by reverse transcription. In the first stage, the FGH gene for a growth hormone was amplified through PCR and analyzed on the agarose gel. The FGH was observed at the expected size of 600 bp. After cloning the cDNA fragment of the FGH gene, the sequence was submitted to NCBI GenBank with accession no. MW314139. Moreover, the FGH gene sequence was compared through BLAST for its homology with the growth hormone gene of other fishes of the order Siluriformes. The FGH gene showed more than 90% homology with other catfish species. Then the MGH gene fragment of 534 bp was amplified and confirmed in BLAST searches against NCBI database sequences with designed primer FGH-F2 from the FGH gene of *B. bagarius*. The FGH gene encodes a polypeptide chain of 200 amino acids with a molecular weight of 22.58 kD. The initial 22 amino acids represent a signal peptide that is removed from the mature protein, while the MGH gene encodes a protein of 178 amino acids of 19.5 kDa, as represented in Figure 4. The detailed complete nucleotide and amino acid sequences of the FGH gene of *B. bagarius* are given below in Table 2.

Table 2. Nucleotide and deduced amino acid sequence of full-length growth hormone gene of *B. bagarius*. The putative regions (signal peptide) are marked by bold letters, potential N-glcosylation sites are marked by underline, conserved six residues of Cys are shaded and the stop codon is represented by an asterisk.

atggctagagtgttggtggtgctctctgtggtggtggcgagtttgtacttcagtcaatgt
M A R V L V V L S V V V A S L Y F S Q **C**
gcgacattcgagaaccagcggctctttaacaacgccgtcatccgcgtgcaacacctccac
A T F E N Q R L F N N A V I R V Q H L H
cagctggctgccaagatgatggatgattttgaagaggctctgctaccagaagaacgcaaa
Q L A A K M M D D F E E A L L P E E R K
cagctgagcaagattttccccctgagtttctgcaactccgactccatcgaggctccggca
Q L S K I F P L S F **C** N S D S I E A P A
ggcaaagacgagacccagaaaagctctgtgctgaaacttttacacacatcctaccgtctg
G K D E T Q K S S V L K L L H T S Y R L
atcgagtcctgggaatttcccagcaaaaacttgggcaaccccaaccatatctccgagaag
I E S W E F P S K N L G N P N H I S E K
ctggcggacctgaaaatgggcatcggcgtgcttatagagggatgcctggacggacaaaca
L A D L K M G I G V L I E G **C** L D G Q T
agcctggatgagaacgacgctctggctccgcccttcgaggatttctaccagaccctgagt
S L D E N D A L A P P F E D F Y Q T L S
gaaggcaacctgaggaagagcttccgcctgctgtcctgctttaagaaggacatgcacaaa
E G N L R K S F R L L S **C** F K K D M H K
gtggggacttatctaagcgtggccaagtgcaggagatccctggattccaactgcaccctctag
V G T Y L S V A K **C** R R S L D S N **C** T L *

The cysteine amino acids were located at six different conserved positions in the GH protein of *B. bagarius* (20, 71, 135, 173, 190 and 198) (Table 2). Putative N-glycosylation sites were present at the 197th amino acid position of the protein. The total number of positively charged (Arg and Lys) and negatively charged (Asp and Glu) residues is 42 and 31, respectively. It is evident from the deduced amino acid sequence that *B. bagarius* growth hormone protein required 10 essential amino acids: leucine (14%), valine (6%), arginine (4.5%), isoleucine (3.5%), lysine (7%), threonine (3.5%), phenylalanine (5.5%), histidine (2.5%), methionine (2.5%), and tryptophan (0.5%). Non-essential amino acids in protein are serine (10%), glutamine (4%), alanine (6.5%), glutamic acid (7%), aspartic acid (5.5%), proline (3.5%), glycine (4%), tyrosine (2%), asparagine (5%) and cysteine (3%). To understand the genetic relationship, a phylogenetic tree was constructed from the deduced amino acid sequence of FGH of *B. bagarius* and aligned with 20 other fish species GH protein sequences of the orders Siluriformes and Cypriniformes (Table 3). It revealed that *B. bagarius* has a maximum genetic distance (65%) with catla *Catla catla* and minimum genetic distance (76%) with silver carp *Hypophthalmichthys molitrix* and grass carp *Ctenopharyngodon idella* in the order Cypriniformes, while maximum (98%) and minimum (86%) genetic difference was with giant catfish *Pangasius gigas*, pangas catfish *Pangasius pangasius*, and silver catfish *Rhamdia quelen* of the Siluriformes, respectively (Table 3).

Table 3. Genetic distance of *B. bagarius* with 19 different fish species of the orders Siluriformes and Cypriniformes

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
S. asotus	-																			
S. meridionalis	99																			
C. batrachus	93	93.5																		
P. ful-vidraco	95	96	94.5																	
H. fossilis	95	95.5	95.5	97.5																
P. gigas	96.5	97.5	95	98.5	98															
P. pangasius	96.5	97.5	95	98.5	98	100														
P. hypothalamus	96	97	94.5	98	97.5	99.5	99.5													
C. gariepinus	95	95.5	96.5	96.5	98	97	97	96.5												
C. dussumieri	94.5	95	96	96	97.5	96.5	96.5	96	99.5											
R. quelen	85.5	86	86.5	85.5	86.5	87	87	86.5	86.5	86										
I. punctatus	94.5	95	93.5	95	95.5	96.5	96.5	96	96	95.5	88.5									
C. carpio	77.5	78	76.5	77	75.5	77.5	77.5	77	76.5	76.5	72	77								
H. molitrix	77.5	78	76.5	77	75.5	77.5	77.5	78	76.5	76	72.5	77	96.2							
C. idella	77	77.5	76	76.5	75	77	77	77.5	76	75.5	72	76.5	95.7	99.5						
L. rohita	68	68.5	67	67.5	66.5	68	68	68	67	67	64.5	68.5	86.4	84	83.6					
L. gonius	69.5	70	68.5	69	68	69.5	69.5	69.5	68.5	68.5	65.5	70	89	86.6	86.2	96.6				
C. mrigala	70	70.5	69	69.5	68.5	70	70	70	69	69	66	70.5	90	86.6	86.2	96.6	99			
C. catla	66	66.5	65	65.5	64.5	66	66	66	65	65	62.5	66.5	85.2	82.8	82.3	94.7	94.7	94.7		
B. bagarius	94.5	95.5	94	97.5	97	98	98	97.5	96	95.5	86	95	76	76	75.5	66.5	68	68.5	64.5	-

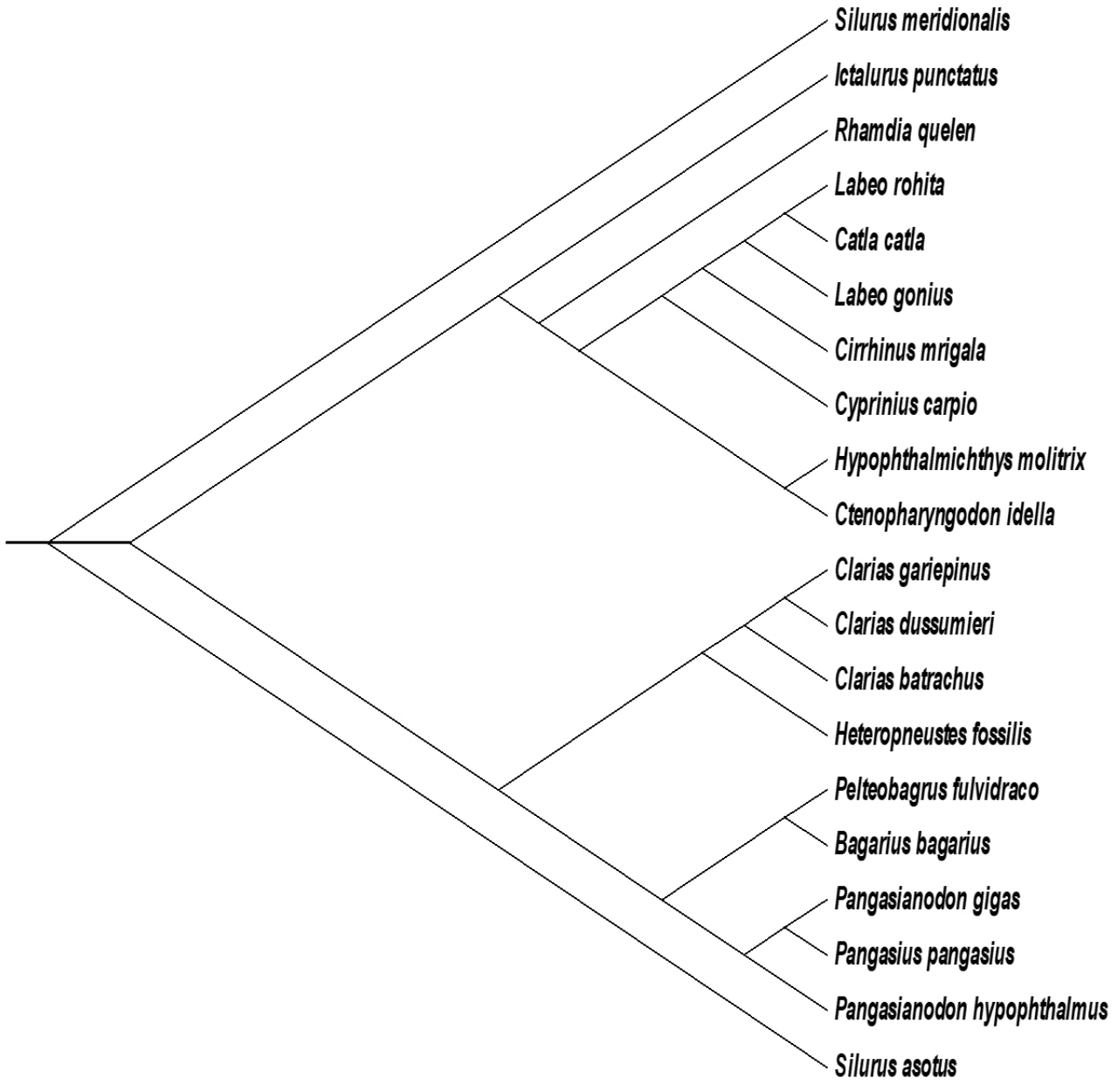

Fig4. UPGMA phylogenetic tree of twenty different fish species of the orders Siluriformes and Cypriniformes

The expected MGH protein (19.5 kDa molecular mass) was co-expressed in *E. coli* BL21 cells. The protein showed considerable expression as evident in SDS-PAGE analysis (Figure 4). The expected size confirmed that the expressed protein is the MGH protein of *B. bagarius* catfish.

DISCUSSION

This study reports the cloning and expression of GH of *B. bagarius* in the prokaryotic system. *B. bagarius* is the largest catfish found in the natural water resources of Pakistan, especially in the Indus River. The cDNA sequence of the identified GH shares more than 90% homology with other catfish species. The coding gene of the GH protein of this catfish was identified to consist of 603 bp. However, earlier studies reported that the length of fish GH gene varies across the species. The growth hormone of Cyprinidae consists of 624 bp in common carp *C. carpio* (Chiou et al., 1990), 636 bp in catla *C. catla*, 633 bp in mori *Cirrhinus mrigala*, 624 bp in rohu *L. rohita* (Venugopal et al., 2002) and 630 bp in Milkfish *Chanos chanos* (Jesus et al., 2002).

On the other hand, the nucleotide length of the GH gene is lower (short) in some species, as reported by Venktesh and Brenner (1997) in puffer (588 bp) and Tanaka (1995) in *flounder* (570 bp) fish species. However, the GH of *B. bagarius* shares the highest similarity with *Pangasius gigas* (Lemaire, 1994), *P. pangasius*, *P. hypophthalamus* (Sekar et al., 2014) and *heteropneust fossilis* (Anathy et al., 2000) catfish species of the order Siluriformes in terms

of sequence homology and the number of nucleotides. The structural organization of the GH gene consists of five exons and four introns in the Cypriniformes, Siluriformes and mammals, respectively (Chiou, 1990; Rajesh and Majumdar, 2008; Tang et al., 1993 and Barta et al., 1981). However, it consists of six exons and five introns in the Tetradontiformes (Venktesh and Brenner, 1997), Perciformes (Almuly et al., 2000) and Salmoniformes (Devlin, 1993). These reports clearly suggest that fish possess mammalian-like structural organization of GH, i.e. it is not a conserved structure in various fish species.

Kamenskaya and Brykov (2020) reported that coding sequences of growth hormone genes in various species of fish are conserved. Variations in gene length are due to introns, noncoding and flanking regions. Only regulatory elements of flanking regions are conserved. Özkan et al. (2020) reported twenty single nucleotide polymorphisms (SNP) and one deletion/insertion in the growth hormone of the buffalo population in Anatolian waters. They also suggested that these SNP may have an effect on carcass traits, reproduction, milk yield and improving growth traits. The FGH gene of *B. bagarius* encodes 200 amino acid proteins in which 178 amino acids constitute the mature GH protein. The remaining 22 amino acid residues of the N terminal region formed the signal peptide. Generally, signal peptide sequence varies from species to species compared to the mature GH protein polypeptide. In fish, as in other eukaryotes, the signal peptide is cleaved upon hormonal secretion for the production of mature protein. The mature MGH protein of carps appears to be larger (188 amino acids) compared to *B. bagarius* MGH protein (178 amino acids). The appropriate folding and functional tertiary structure of GH is due to the presence of Cys residues in the paired form that participate in the formation of a disulphide bond (54). Some fish species contain four residues of Cys in their growth hormone protein located in 49, 161, 178 and 186 amino acids, as highly conserved positions. Many studies report the presence of five cysteine residues in many fish species like Indian major carps (Venugopal, 2001), striped mullet *Mugil cephalus* (Meier et al., 2006), giant catfish *P. gigas* (Lemaire et al., 1994), milkfish *C. chanos* (Jesus et al., 2002), channel catfish *Ictalurus punctatus* (Tang et al., 1993), goldfish *Carassius auratus* (Law et al., 1996), grass carp *C. idella*, bighead carp *Hypophthalmichthys nobilis* and common carp *C. carpio* (Chang et al., 1992). Interestingly, *B. bagarius* possess six cysteine residues in its growth hormone protein polypeptide at position 20, 71, 135, 173, 190 and 198. Earlier, it was suggested that the fifth Cys residue might play an important role in the proper folding of GH protein but later studies did not support this idea (Fine et al., 1993). The occurrence of the fifth extra Cys residue in *B. bagarius* in a similar position as in *P. hypophthalmus* and carps may behave the same in GH protein. The occurrence of one putative glycosylation in *B. bagarius* GH is a common feature, as observed in many teleost growth hormones (Degani et al., 2003).

CONCLUSION

The GH gene of *B. bagarius* consists of 600 bp and encodes a protein of 200 amino acids with 23 kDa estimated molecular mass, while the mature protein gene consists of 534 bp and encodes a protein of 178 amino acids with an estimated molecular mass of 19.5 kDa. GH sequence can be used as a natural marker to understand the evolutionary relationships in fishes. The successful expression of the GH gene in *E. coli* suggested that the gene is functionally viable. Presently there is an increasing trend for the use of recombinant fish growth hormone in aquaculture. This study provides basic information for the development of allotransgenic and autotransgenic fish with a higher growth rate in aquaculture. In addition, this methodology will be helpful in heterologous GH expression for large-scale production that may be used to investigate the physiology and enhancement of fish growth in aquaculture.

KLONIRANJE, SEKVENCIRANJE I EKSPRESIJA HORMONA RASTA SOMA *Bagarius bagarius* (Hamilton, 1822.)

SAŽETAK

Som *Bagarius bagarius* (Hamilton, 1822) jedan je od najvećih i najbrže rastućih somova rijeke Ind koji obitava u Taunsa Barrage, Pakistan. Čitava dužina cDNA gena hormona rasta (600 bp) umnožena je reverznom transkripcijom mRNA izolirane iz hipofize *B. bagarius*. Gen hormona rasta pune duljine kodira polipeptid od 200 aminokiselina s molekularnom masom od 22,58 kDa. Prekursor *B. bagarius* hormona rasta (GH) sastoji se od 22 aminokiseline kao signalni peptid i 178 aminokiselina peptida. Utvrđeno je šest konzerviranih Cys djelova u GH proteinu (20, 71, 135, 173, 190 i 198) koji održavaju strukturni integritet ovog proteina. Jedno navodno mjesto N-glikozilacije bilo je prisutno na 197. aminokiselini. Ukupan broj pozitivno nabijenih (Arg i Lys) i negativno nabijenih (Asp i Glu) ostataka je 42 odnosno 31. Gen *B. bagarius* GH pokazuje više od 90% homologije sekvence s drugim somovima. Gen proteina GH istražen je u *Escherichia coli* korištenjem pET-28 ekspresijskog vektora, a rekombinantni protein od 19,5 kDa detektiran je SDS-PAGE analizom. Ova studija sugerira da bi kloniranje i ekspresija *B. bagarius* GH gena pružilo osnovne informacije za transgenetske studije usmjerene na bržu stopu rasta. Rekombinantni GH može se proizvoditi u velikim količinama kako bi se iskoristila njegova funkcija poticanja rasta kod drugih uzgojenih riba.

Ključne riječi: Hormon rasta, rijeka Ind, Taunsa Barrage, Pakistan

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