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Research Article

# MOLECULAR CHARACTERIZATION OF GROWTH HORMONE RELEASING HORMONE RECEPTOR (*GHRHR*): CLONING, TISSUE DISTRIBUTION, EXPRESSION ANALYSIS AND HOMOLOGY MODELING IN FARMED CARP, *Labeo rohita*

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**Abstract-** Growth hormone releasing hormone receptor (*GHRHR*) is a trans-membrane receptor belonging to the Class-II GPCR family. *GHRHR* activates growth-hormone production consequent upon triggering somatotroph proliferation. Unlike in mammals, information on teleosts *GHRHR* is insufficient. Here, *GHRHR* cDNA has been cloned from the brain tissue of *Labeo rohita*, a large-bodied farmed carp. It is a 1320 bp transcript consisting of an ORF (open reading frame) translatable to a 416aa long polypeptide. Relative mRNA expressions of *GHRHR* in various organs of different age groups (i.e. juvenile, young and adult) were estimated by using q-RT PCR analyses. Phylogenetically *GHRHR* was closer to Cyprinidae family. It was found to be 92-93% similar to *Carassius auratus* followed by *Danio rerio*. It was abundantly expressed in the pituitary and brain followed by male/female gonads of all age groups in rohu; demonstrating its possible functions may not be restricted to GH release only, but also in many other developmental and physiological regulations. The predicted stable 3D structure and protein-protein interactions provided the clue about its diversified networking roles. Our findings could be of great help for future studies to delineate its multidirectional neuroendocrine roles in various organs in teleosts.

**Keywords**- *GHRHR*, growth stages, *Labeo rohita,* mRNA expression, qPCR

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## Introduction

Growth in vertebrates is controlled and regulated in part by a complex cascade of hormones. Growth Hormone (GH) being the potent regulator of development and somatic growth, its synthesis is controlled by Growth Hormone Releasing Hormone (*GHRH*). *GHRH* peptide mediates its function by interacting with the N-terminal region of a trans-membrane receptor, Growth Hormone Releasing Hormone Receptor (*GHRHR*), which enhances GH production by increasing the level of cAMP, which proliferates the somatotrophs for activation [1]. However, teleosts exhibit a more complex multifactorial hypothalamic regulation of GH secretion than in mammalian vertebrates because of the unique organization of the hypothalamo-pituitary axis [2]. *GHRHR* is a Class II G protein–coupled receptor found predominantly on pituitary somatotrophs [3]. Other receptors included in this family are vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP), secretin and glucagon. These receptors share similar amino acid sequence in the seven trans-membrane domains and a N-terminal extracellular domain containing cysteine residues that are important for ligand binding [4,5]. All these receptors mediate their action by the activation of G-protein that induces an intracellular signal cascade facilitated by GTP-binding proteins, a heteromeric protein [6-8].

Several observations suggest that the *GHRH* receptor, in addition to playing a key role in normal growth, might be important in disorders involving aberrant GH secretion. Studies suggest that GH in adults acts in the regulation of various metabolic action and is proposed to act as an anabolic factor in the balance between muscle and fat, in muscle growth in response to exercise, and in muscle maintenance during starvation [9]. The *GHRH*/GH/IGF-1 system is also reported to have a variety of other actions throughout the body including stimulation of increased bone mineral mass, immune function, wound healing, heart function, skin thickness, and psychological well-being. Consistent with its mitogenic effect on somatotrophs, *GHRH* is hypothesized to act as an autocrine/paracrine factor whose local release by tumor cells may play a role in tumor growth and proliferation. Thus, the details of *GHRH* receptor function have implications in growth and development, metabolism, aging, and cancer [10].

The cDNA of *GHRH* receptors have been characterized in a number of mammals: human [9,10]; rat [9], mouse [11], sheep [12] and cattle [13]. However, in teleost, characterization of the *GHRH*/*GHRH*-R system has been limited to few species. A novel *GHRH*/*GHRHR* axis in zebrafish and goldfish, was discovered that showed a "true" *GHRH*-*GHRHR* system which is capable of stimulating GH production, while the *GHRH*-like ligand-receptor pair is in fact orthologs of vertebrate PRP-PRPR [14]. The *GHRHR* polypeptide consists of varying aa residues like 423aa in Human and Mouse [9,11], 419aa in Chicken [15], 425aa in Fugu [16], 422bp in Orange spotted grouper [17], 445aa in Zebrafish and 416aa in Goldfish [14]. In goldfish (*Carassius auratus*), both *GHRH* and *GHRH*-R mRNAs were abundantly expressed in the whole brain [14]. Functional studies for *GHRH* revealed that *GHRH* stimulates GH release from goldfish pituitary cells depending on dosage. In addition to its main function, *GHRH* was also found to play roles in the network between growth and immunity [18]. Moreover, *GHRH* may be involved in the regulation of the sexual growth dimorphism in the half-smooth tongue sole (*Cynoglossus semilaevis)*. The distribution of *GHRH* mRNA was also investigated in various tissues and developmental stages of half-smooth tongue sole [19]. cDNAs encoding *GHRH* and *GHRHR* were characterized for orange-spotted grouper (*Epinephelus coioides*) [17]. Keeping in view of its diversified participatory physiological and pathological roles played in mammals, it would be of interests its physiological functions during various stages of development. However, the expression patterns of *GHRHR* mRNA in different tissue and developmental

stages have not been investigated in any other fishes, especially economically important farmed fishes.

Among the Indian major carps, *Labeo rohita* (popularly known as rohu) is the most sought after and economically important freshwater cultured species in India and Asian Subcontinents with high nutritional value [20]. There has been diversified research work done in the species, though characterization of *GHRHR* has not yet been attempted. Therefore, for better and more systematic understanding of the *GHRH*/*GHRHR* system, we have first cloned the full-length cDNA sequences of *GHRHR* in rohu carp. The present study would facilitate a detailed understanding of the mechanism of neuroendocrine regulation of growth and reproduction in rohu.

## Materials and Methods

### Experimental animal and sample collection

The experimental animal, rohu (*Labeo rohita*) were collected from the farms of ICAR-Central Institute of freshwater aquaculture (ICAR-CIFA), Bhubaneswar, Odisha, India. For expression profiling analysis various tissues like liver, muscle, heart, kidney, brain, pituitary, intestine, testis and ovary were collected aseptically from the anesthetized fishes. Anesthesia was performed using tricaine methanesulfonate (MS222, Sigma Aldrich). For expression studies in different growth stages of rohu, tissue samples were also collected from juvenile, young and adult stages. To minimize the chances of error, each set of tissues were collected from five individuals of each stage. The collected tissue samples were snap-frozen in Liquid Nitrogen followed by storage at -80$^0$C till further use.

### RNA Isolation and cDNA Synthesis

Total RNA was isolated from different tissues such as liver, muscle, heart, kidney, brain, pituitary, testis, and ovary using TRI Reagent RNA isolation reagent (Sigma-Aldrich) as per the manufacturer's guidelines and standard protocol [21]. The possibilities of DNA contamination was eliminated from the extracted RNA by RNase free DNase I (Invitrogen, CA, USA) treatment followed by column purification (Qiagen). Quality and quantity were checked in formaldehyde agarose gel and spectrophotometer, respectively. cDNA was reverse transcribed from the total RNA (about 1µg) using SuperScript® III Reverse Transcriptase (Invitrogen, CA, USA) using the protocol provided by manufacturer. It was incubated at 65°C for 5 min followed by 50°C for 60 min and finally inactivation was carried out at 70°C for 15 min.

### Molecular cloning of full length *LrGHRHR* cDNA

Brain cDNA was amplified using primers designed from conserved region of *GHRHR* sequences of different species available in the public database, Genbank (DQ991244, DQ991247). The primer sequences are listed in [Table-S1]. PCR was performed under standard protocol with 25 µl reaction volume containing 10 picomoles of each conserved primer, 2.5mM of each dNTP and 0.25 U of *Taq* polymerase with thermal regime of 94°C (5 min), 35 cycles at 94°C (0.5 min), 62°C (0.5 min), 72°C (1 min) and final extension of 72°C (5 min). The PCR products were resolved in 1.5% agarose gel and desired band was excised and purified using Gel purification kit (Qiagen, USA) and bi-directionally sequenced in ABI 310 Genetic Analyzer. The sequence was verified as the partial cDNA sequence of *GHRHR* using BLASTn program (http://www.ncbi.nlm.nih.gov/blast). The Rapid Amplification of cDNA Ends (RACE) PCR was done using Smater RACE cDNA amplification kit (Clontech, US) as per manufacturer's protocol. An end to end PCR strategy was followed to obtain complete sequence of rohu *GHRHR* mRNA. The PCR conditions remain same as above. The amplified products were gel purified and ligated to pGEMT Easy vector (Promega, USA) and transformed to a chemically competent *E. coli* DH5α strain. The plasmids containing inserts which were screened through blue white screening were sequenced using an automated ABI 310 Genetic Analyzer.

### *In silico* analysis of *LrGHRHR* and evolutionary relationship identification

Amino Acid sequence was predicted by the online tool, ExPASy translate tool (http://expasy.org/tools/dna.html) and these predicted amino acids were verified and validated using BLASTp program of the National Center for Biotechnology Information. Prediction of the presence and location of signal peptide was done using SignalP v4.1 program (http://www.cbs.dtu.dk/services/SignalP/). Domain structure analysis was performed using the SMART program of ExPASy tools (http://smart.embl-heidelberg.de/) and the architect was achieved by Domain Graph (DOG) software version 2.0. Theoretical isoelectric point (pI) and molecular weight (Mw) of the protein were predicted using the ExPASy ProtParam Server (http://web.expasy.org/protparam/). Multiple sequence alignments were performed with ClustalW software. Evolutionary relationship was analyzed by constructing a phylogenetic tree using MEGA 6.0 [22]. Maximum likelihood tree was constructed using Kimura-2-parameter model in Mega 6.0 software and the reproducibility of the tree was evaluated with 1000 replicates using bootstrap method. The percentage of similarity and identity of *LrGHRHR* protein with that of other species was calculated by Matrix Global Alignment Tool Program [23].

### Expression profiling of *LrGHRHR* mRNA in various growth stages of rohu

Relative levels of *GHRHR* mRNA expression were determined in different tissues of rohu in triplicate for each cDNA samples (three independent experiments) against β-*actin* gene as reference using SYBR Green Real-time Master Mix II (Roche Diagnostics, Germany) in a Light Cycler 480 II RTPCR instrument (Roche Diagnostics, Germany). The reaction was conducted in 10 µl reaction containing 1 µl of cDNA (diluted to 50ng/ µl ), 0.3 µl of each forward and revere primers (10pM). Thermal cycles for the reaction were as : one cycle of pre-incubation at 94 $^o$C for 5mins followed by amplification (95 $^o$C for 10 sec, 58 $^o$C for 10 sec and 72 $^o$C for 20 sec) and melting curve analysis (95 $^o$C for 20 sec, 63 $^o$C for 1 min). The reactions were carried out in triplicate at annealing temperature 58$^o$C as per manufacturer's instructions and described elsewhere [20,21]. Expression profiling was also done in various growth stages (juvenile <6months, young<1year and adult>1year) of rohu to study the level of *GHRHR* with increasing growth. The details of the primers are provided in the [Table-S1]. Specificity of the primers was confirmed using the melting curve analysis followed by a high-resolution agarose gel electrophoresis to authenticate the presence of transcripts of exact molecular size. The relative expression level of each mRNA normalized to β-*actin* was calculated by δCT method.

### Statistical Analysis

All the experiments were performed in triplicate and the data generated were expressed as relative value to β-*actin*, which was used to normalize any difference in efficiency of reverse transcriptase. The threshold cycle (Ct) value (the number of PCR cycle at which fluorescence was and decreased linearly with increasing input target quantity) was obtained from the quantitative Real-Time PCR system software (Roche Diagnostics, Germany) These Ct value were used to calculate fold change for the gene expression using Pfaffl method [24]. All data are expressed as mean ± SEM and analyzed by one-way ANOVA.

### Secondary Structure, prediction of homology model and validation

Secondary structure prediction was done by SOPMA and the confidence level was checked in PsiPred (http://bioinf.cs.ucl.ac.uk/psipred/). Physiochemical data were generated from the ProtParam (http://web.expasy.org/protparam/) program of ExPASy server. Three-dimensional protein models of *LrGHRHR* were constructed by homology modelling approach. A suitable template was identified by PSI-BLAST [25] against the Protein Data Bank (PDB) proteins available in the NCBI web server and appropriate template was selected on the basis of sequence similarity. Multiple sequence alignments were performed using Clustal W [26]. Three-dimensional models of the proteins were constructed by using the MODELLER 9.12 program [27] based on its alignment with the template protein and visualized by Discovery Studio. The best model was chosen depending on the DOPE score obtained for each model. The generated models were validated on Structural Analysis and Verification Server (SAVES) as described. Ramachandran plot statistics [28] was used to evaluate the best model and also to inspect the favorable and unfavorable regions of the modelled structure.

**Post translational modification site prediction in *LrGHRHR* protein**

Glycation of ε amino groups of lysines in *LrGHRHR* protein was predicted using NetGlycate 1.0 (http://www.cbs.dtu.dk/services/NetGlycate/). Phosphorylation sites were predicted using a NetPhos2.0 server (www.cbs.dtu.dk/services /NetPhos/). The neural network prediction for the phosphorylation sites in serine, threonine and tyrosine in eukaryotes is predicted by NetPhos 2.0. Sumoylation sites for *LrGHRHR* protein was predicted by using SUMOplot™ analysis program (http://www.abgent.com/sumoplot). Protein-protein interaction and functional protein association network was predicted using STRING server (http://string-db.org/).

**Table-S1** *Information of the oligos used in this study*

| Information | Gene | Primer ID | 5'-3' sequence |
|---|---|---|---|
| Partial | GHRHR | F1 | GAGTGTGAGTATATCTTTCAGCT |
| | | F2 | CTCGAAACTATATCCACATGCAG |
| | | R1 | GTTCAGTTTCTGCACCAGAATCC |
| | | R2 | CCTCCTGGTTCAGGAAACAGT |
| RACE | | 5' GSP | ACAGCAGGACAGGGCAAGTGGATCGTC |
| | | 5' NGSP | CTACAGCATCCCATACTGGCAGACAA |
| | | 3' GSP | ACTGCACGCTCTCTACGGCGGCATGTA |
| | | 3' NGSP | CTTCTGGTTGCTGGTTGAAGCCA |
| | | 5'GSP2 | TCGAGCACAGTGTAATCGCCTAACGAGC |
| | | 5'NGSP2 | TCTGGGCCAACAAACTACAGCATCCCA |
| | | 3'GSP2 | TGGTCTGCATCATCCTCTGGATCTGTTCCA |
| | | 3'NGSP2 | CACCTTCATCCTGAAATCGGTGGCTGTGTT |
| Fragmented PCR (end-end PCR) | | 1F1 | CTCGACTGGAGAGATGCTGCCGTGT |
| | | 2F1 | GGCGATTACACTGTGCTCGAAACT |
| | | 3F1 | GCTGTGGACATGACTGGAGACATGCGCT |
| | | 4F1 | ACGGAGTGTCACTGCTGTCGCTCTCTGT |
| | | 1R1 | AGCAGTGATCTGTGTCGTCRCTGGAGAA |
| | | 2R1 | GAGCTGAAACTTCACATGCGC |
| | | 3R1 | TGTTCATGGTTTCTGGAGGTGTTGAGGT |
| Real time | | RTF1 | ACACCGTTGGTTACGGAGTGTC |
| | | RTR1 | AGCGTGCAGTGATCTGTGTCG |
| | Beta Actin | F1 | ATCCTGACCGAGAGAGGCTACAG |
| | | R1 | CCTTACGGATATCGACGTCAC |

**Result**

**Characterization of *LrGHRHR* mRNA sequence**

A partial sequence of 674bp of *LrGHRHR* (Accession Number: KF935239) was obtained by PCR amplification using degenerate primers [Table-S1]. Following Blast analysis, the obtained partial cDNA exhibited 91% homology with counterparts of *C. auratus*, *D. rerio*, etc. (data not shown). To obtain complete CDS of *GHRHR* from this partial sequence information, RACE-PCR was attempted towards 5'- and 3'-ends. However, we failed to generate sequence information even after repeated trials, the exact reasons remain unknown. Alternatively, a fragmented PCR strategy was opted to obtain complete sequence. Using different sets of primers [Table-SI] two different amplicons of 598 bp in 5'-end and 902 bp in 3'-end were obtained, purified and bi-directionally sequenced. By combining all the above generated sequences, we could able to get a total of 1320 bp *LrGHRHR* sequence comprising of an open reading frame (ORF) of 1251bp translatable to 416 amino acids (aa) (Accession Number: KU695553) [Fig-1] with a predicted molecular weight of 47.53 kDa and isoelectric point of 6.73. When compared with other related fish species, the *LrGHRHR* protein showed 91% sequence similarity with *C. auratus* and 88% with that of *D. rerio*. A signal peptide sequence of 23aa (positioning 1 to 23) was identified by Signal P analysis. The comparison of this protein sequences with the counterparts of other species, available in public domain, provided the clue regarding their sequence convergence [Fig-2]. From the SMART program analysis, a seven trans-membrane domain and a HormR domain (spanning 54-124 aa) were identified [Fig-2]. Rohu *GHRHR* also contained an RLTK motif, which is considered as a defining feature of the secretin receptor family [Fig-1].

The alignments of all the available vertebrate *GHRHR* aa sequences showed seven conserved cysteine (Cys) residues including another conserved glycosylation site in the N terminus [Fig-2]. Documentation of the conserved Cys residues in the second and third extracellular loops is also available [Fig-2]. A total of 8 serine, 4 threonine and 7 tyrosine phosphorylation sites were predicted by Netphos 2.0 program. A comparison of the architect of *LrGHRHR* domains with its counterparts are depicted in [Fig-3]. These results suggested that *GHRHR* gene is present and expressed in the brain of rohu carp, *L. rohita*

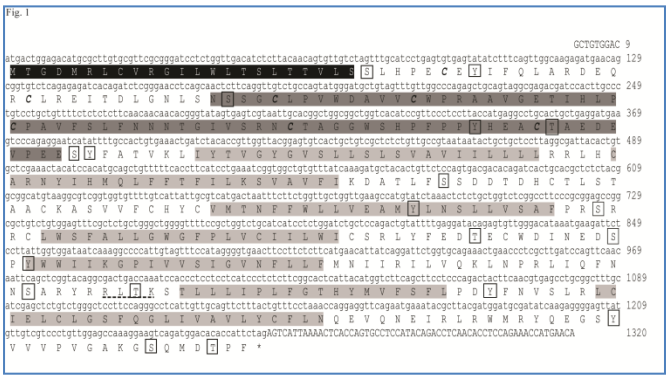

**Fig-1 Complete nucleotide and deduced amino acid sequence of *GHRHR* of *Labeo rohita* (*LrGHRHR*). The signal peptide is shaded in black, the HormR domain is shaded in dark grey and the seven Tm domains are shaded in light grey color. The phosphorylation sites are shown in boxes and the "RLTK" is underlined with broken lines.**

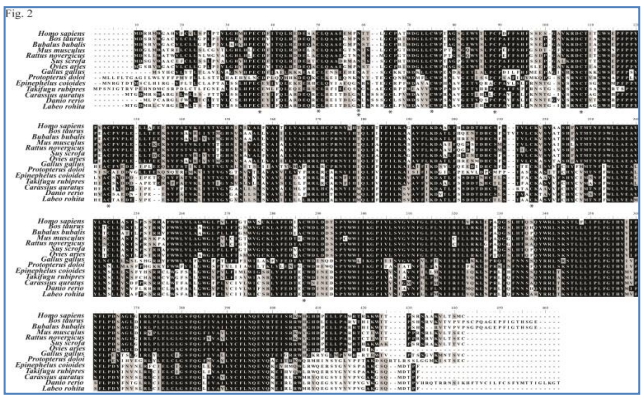

**Fig-2 Multiple sequence alignment of *GHRHR* complete CDS denoting * as conserved cysteine residues and ** denotes conserved glycosylation site at N-termini.**

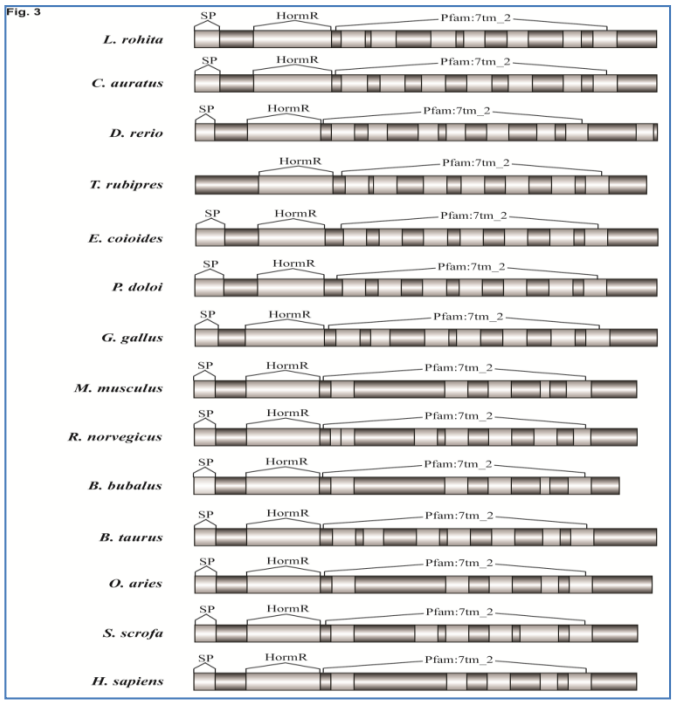


**Fig-3 Schematic representation of structural architect of *LrGHRHR* along with other species counterparts visualized by Domain Graph (DOG, V2.0) software. SP, signal peptide. The accession numbers are from the GenBank datasets.**

**Evolutionary and similarity analysis of *LrGHRHR***

To evaluate the evolutionary relationships, a rooted phylogenetic tree was constructed by the Neighbor-joining method and Maximum-Likely hood method using poison model with deduced full-length *GHRHR* protein sequence of various species available in the public databases. Members of the GPCR family showed highest degree of aa identities forming two distinct subgroups supported by high bootstrapping values, one of which consisted of teleost *GHRHR* proteins including *L. rohita, C. auratus, D rerio, E. coioides, P. dolloi*, and *T. rubripes*; whereas the other was belonging to mammalian *GHRHR* proteins, including those of *H sapiens, B. taurus, B. bubalis, S. scrofa, O. aries, M. musculus, and R. norvegicus* [Fig-4]. *GHRHR* of *G. gallus* clustered in between mammals and teleost. Thus, a clear genetic separation of teleost from mammals was obtained. In the mammalian cluster, *H sapiens, B. taurus, B. bubalis, S. scrofa, O. aries* showed tight cluster, whereas two rodents, *M. musculus* and *R. norvegicus* formed a separate cluster. Teleostean cluster was sub-divided into three sub-groups, one consisting *L. rohita, C. auratus* and *D. rerio*; *T. rubipres* and *E. coioides* forming the second group; and *P. dolloi* alone in the cluster.

Similarity and identity of *LrGHRHR* with its counterparts of other species were in agreement with the evolutionary analysis [Table-1] confirming its relation with other teleosts as well as mammals. Maximum level of similarity was with *C. auratus* (96.6%) and *D. rerio* (85.4%). Investigating similarity and identity of HormR domain and Pfam: 7tm_2 domain of *LrGHRHR* with its teleostean counterparts, it exhibited maximum similarity/identity in the tune of 94.4/91.5%, 97.6/92.7% with *C. auratus* respectively.

**Expression profiles of *LrGHRHR* in different growth stages tissues**

Hypophyseal *GHRH* is known to have growth promoting activities via binding to its receptor (*GHRHR*) present in various organs. The relative mRNA expression levels of *LrGHRHR* in various organs (e.g. liver, muscle, brain, pituitary, heart, intestine, kidney, testis and ovary) was estimated to confirmed its site of synthesis and action as reported earlier [Fig-5a]. The least expressed intestine was considered as default setting 'one' for comparative estimation of fold-changes in other organs. Most abundant transcript level was documented in the pituitary (3109-fold change) followed by brain (2733-fold change). Elevated mRNA level was also detected in male/female gonads. As expected, *LrGHRHR* mRNA expressions were also documented in other peripheral organs. To confirm its potentiating role in the growth trait, the *LrGHRHR* expression was estimated by qPCR in brain, pituitary, liver , muscle and gonads of different age groups (i.e. juvenile, young of about 1 year and adult aged more than one year) [Fig-5b]. A remarkable high expression was found in the pituitary of juveniles with a similar fashion in brain. Noticeably the expression level was also high in male and female gonads. The level of expression in liver and muscle tissue of different stages were relatively low.

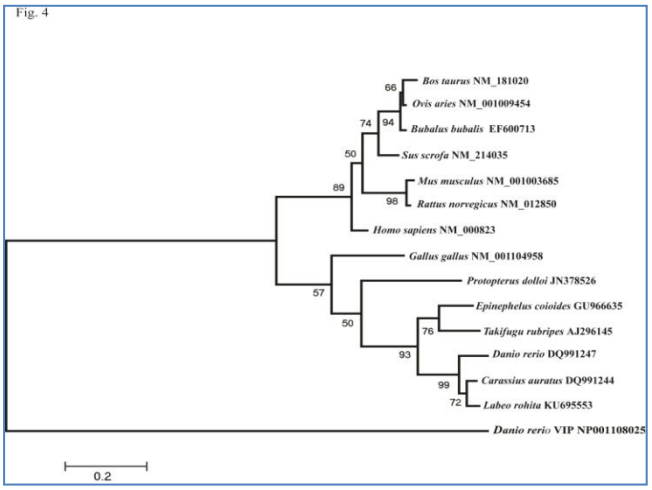


**Fig-4 Evolutionary relationship of *LrGHRHR* protein with other species counterparts by bootstrap analysis using Maximum likelyhood (1000 replicates) in MEGA 6.0 program. The bootstrap values are given next to the branches. GenBank accession numbers are indicated for each individual.**

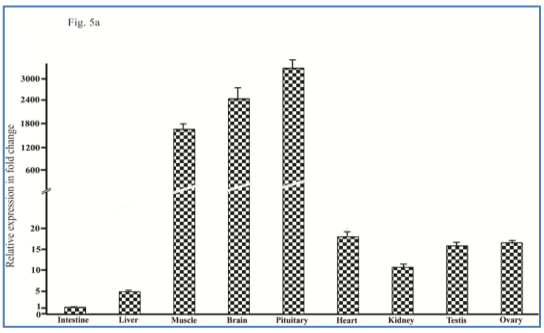


**Fig-5a Expression profiling of *LrGHRHR* (a) Tissue distribution of *GHRHR* in *Labeo rohita* assessed by qPCR analysis.**

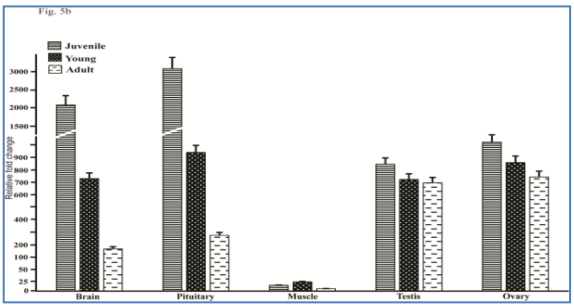


**Fig-5b Expression of *LrGHRHR* in different growth stages of rohu (juvenile, young, adult). The qPCR data in each tissue was normalized with the β-*actin*, used as reference genes. Relative expression was quantified with respect to intestine. The data represent the mean of three independent experiments (each in triplicate). All the experiments were performed under significance level P<0.05 Protein-**

**Table-1** *Percentage similarity and identity of LrGHRHR and its domain with other species counterpart*

| | Total Amino acid | | Domain region | | | |
|---|---|---|---|---|---|---|
| | | | HormR | | Pfam:7tm_2 | |
| | Similarity (%) | Identity (%) | Similarity (%) | Identity (%) | Similarity (%) | Identity (%) |
| *Danio rerio* | 85.4 | 79.3 | 84.7 | 75.0 | 94.3 | 89.1 |
| *Carassius auratus* | 96.6 | 92.5 | 94.4 | 91.5 | 97.6 | 92.7 |
| *Takifugu rubripes* | 81.6 | 70.4 | 74.6 | 62.0 | 91.9 | 82.2 |
| *Epinepheulus coioides* | 86.3 | 73.3 | 80.3 | 66.2 | 94.3 | 82.6 |
| *Protopterus dolloi* | 70.4 | 54.4 | 56.3 | 45.1 | 81.8 | 66.0 |
| *Gallus gallus* | 74.9 | 56.2 | 63.9 | 54.2 | 86.2 | 64.8 |
| *Ovis aries* | 69.2 | 52.0 | 63.4 | 50.7 | 76.5 | 58.3 |
| *Sus scrofa* | 68.6 | 51.3 | 64.8 | 50.7 | 76.1 | 58.7 |
| *Bos taurus* | 66.9 | 50.9 | 63.4 | 49.3 | 76.3 | 58.6 |
| *Bubalus bubalis* | 67.7 | 51.1 | 64.8 | 50.7 | 76.5 | 57.9 |
| *Rattus norvegicus* | 67.4 | 50.4 | 59.2 | 46.5 | 76.9 | 58.7 |
| *Mus musculus* | 67.4 | 51.3 | 60.6 | 46.5 | 76.1 | 58.7 |
| *Homo sapiens* | 68.6 | 51.4 | 60.6 | 47.9 | 77.7 | 60.3 |

Protein-protein interacting network of *GHRHR* [Fig-6] was determined by STRING server. STRING analysis revealed that *LrGHRHR* is capable of interacting with other glucagon/secretin family members like vip (153 aa), adcyap 1a (199 aa), and adcyap 1b (175 aa) with 0.730, 0.566 and 0.564 scores respectively [Table-2].

**Table-2** *Protein –Protein Interacting network as determined by STRING*

ghrhr-growth hormone releasing hormone receptor (432 aa) *(Danio rerio)*

Predicted Functional Partners:

| | | Neighborhood | Gene Fusion | Cooccurrence | Coexpression | Experiments | Databases | Textmining | [Homology] | Score |
|---|---|---|---|---|---|---|---|---|---|---|
| dip2c | DIP2 disco-interacting protein 2 homolog C (Drosophila) (1547 aa) | | | | | | | | | 0.769 |
| vip | vasoactive intestinal peptide (153 aa) | | | | | | | | | 0.730 |
| CDK5RAP1 | CDK5 regulatory subunit associated protein 1 (577 aa) | | | | | | | | | 0.601 |
| EPB41L1 | erythrocyte membrane protein band 4.1-like 1 (877 aa) | | | | | | | | | 0.596 |
| ctnnbl1 | catenin, beta like 1 (563 aa) | | | | | | | | | 0.590 |
| adcyap1a | adenylate cyclase activating polypeptide 1a (199 aa) | | | | | | | | | 0.566 |
| adcyap1b | adenylate cyclase activating polypeptide 1b (175 aa) | | | | | | | | | 0.564 |
| pou1f1 | POU domain, class 1, transcription factor 1 (350 aa) | | | | | | | | | 0.533 |
| zmynd11 | zinc finger, MYND domain containing 11 (640 aa) | | | | | | | | | 0.476 |
| spcs3 | signal peptidase complex subunit 3 homolog (S. cerevisiae) (180 aa) | | | | | | | | | 0.472 |

Fig. 6

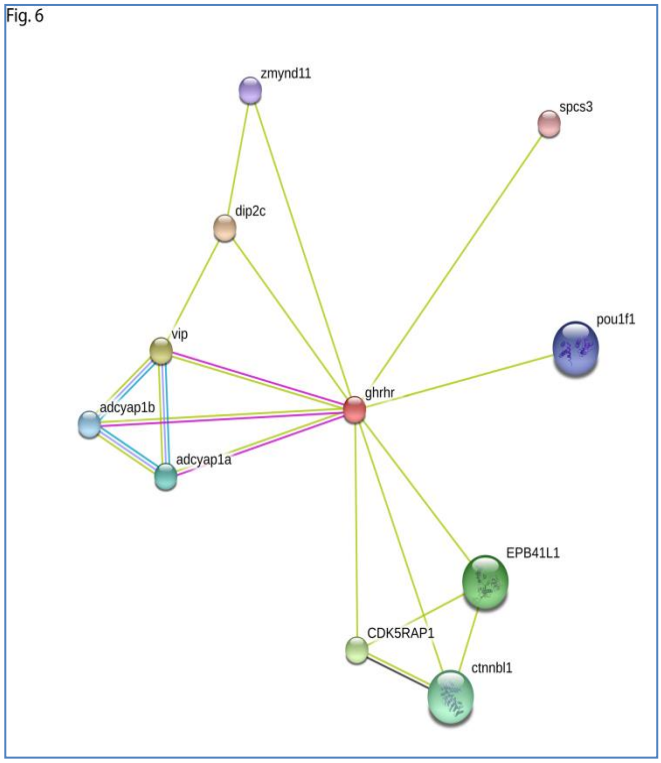


**Fig-6 Protein-protein interaction of *LrGHRHR* predicted using STRING server.**

**Secondary Structure and Homology Modeling of *LrGHRHR***

The computational structural biology approach is being applied for the prediction of protein structure. The secondary structure analysis using SOPMA showed that *GHRHR* possesses turns and helix lacking beta sheets. It has 39.90% alpha helix, 28.12% random coil, 25.24% extended strands and 6.73% beta turns. The confidence level of the predicted secondary structure was validated using PsiPred [Fig-S1]. The 3D structure of the *GHRHR* protein of fish species has not yet been determined experimentally. In the absence of crystal structure, we attempted to build a 3D model of *L. rohita* so as to assume physiochemical properties of interacting molecules [Fig-7a]. For homology modeling, PSI Blast was used to find the appropriate template for modelling. Structural analysis of the 3D model of *LrGHRHR* was performed using Procheck [29] and verified 3D program in Structural Analysis and Verification Server tools (SAVES). The Ramachandran plot analysis using Procheck showed 93.1% of the amino acid residues in most favored region, 5.8% in additional allowed region and 0.7% in the generously allowed region [Fig-7b].The number of residues in disallowed region remained 0.4% following model refinement. A good quality model would be expected to have over 90% of amino acid residues in the most favored region, and during homology modelling 98% of the amino acid residues must be present in the allowed region. These results confirmed that the quality of modelled protein prediction was a good. The ERRAT value for the predicted model was 68.881 [Fig-S2]. Further, quality of protein folds of *LrGHRHR* homology model was evaluated using ProSA-web program. In general, folding energy of proteins showed negative values and these values correspond with the stability and nativity of the molecules. We note that the ProSA web revealed a Z-score value of -2.43 and its energy remained negative for almost all amino acids, indicating

the acceptability of the homology-modelled structure. Thus, ERRAT Plot values and Z-score increased the confidence of the acceptability of the model predicted.

Fig. 7a

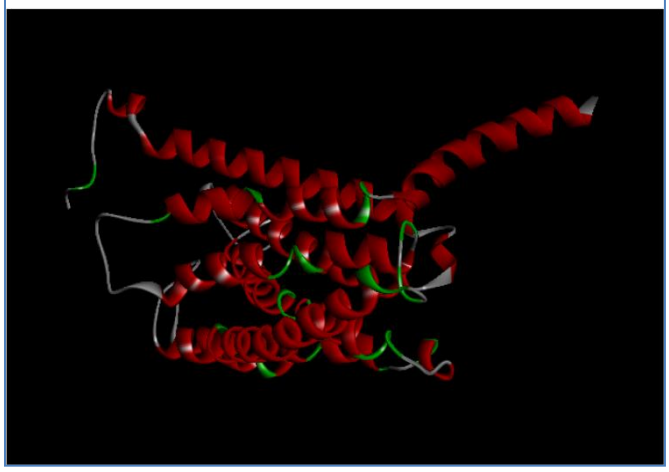

**Fig-7(a) 3D Structure of *LrGHRHR* modelled by the Modeller 9.12 structure prediction software**

Fig 7b

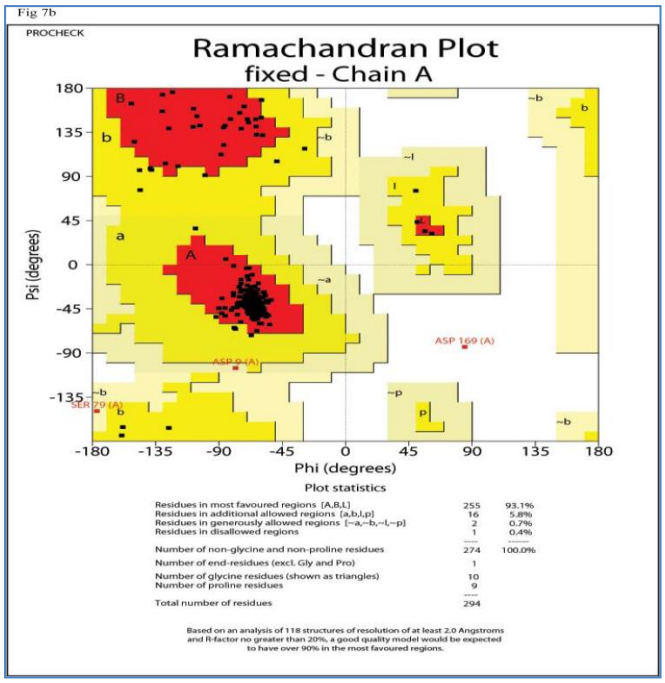


**Fig-7(b) Ramachandra Plot validation by Procheck of *LrGHRHR***

## Discussion

The present study reports the cloning, characterization, structural prediction and expression profiling of *GHRHR* from the brain of Indian major carp, *Labeo rohita*, for the first time. Analysis of mRNA sequence of *GHRHR* in rohu revealed an ORF of 416 aa residues bearing the typical features of GPCR family members. We found that the aa sequences of *LrGHRHR* were highly homologous to counterparts of other species (fish as well as higher vertebrates) in terms of conserved cysteine residues and putative glycosylation/ phosphorylation sites. Domain structure of *LrGHRHR* was found to be similar with that of other species counterparts but the number of aa and Trans-membrane (TM) region varied from species to species. Sequence similarity analysis has been a striking feature in this study, where it has shown high similarity with 95-60% with other counterparts. Such high level of conservation through evolutionary process suggests higher degree of functional convergence with regards to its functional importance. Furthermore, various other striking features, common to the secretin receptor superfamily, were also observed in the *LrGHRHR*. For example, it contained a large N-terminal extracellular domain with seven conserved Cys residues, which are considered to form a ligand-binding pocket [30] and responsible for receptor activation. Secondly, one N-linked glycosylation site and one conserved aspartate (D) positioned at 71 were found in this long N-terminal extracellular domain. This consensus glycosylation site together with conserved aspartate are reported to be essential for high-affinity ligand binding [31]. Thirdly, *LrGHRHR* also contained the basic RLTK motif in the third intracellular loop, which is known to be involved in G protein coupling [32]. In addition, like other family-A GPCRs, the *LrGHRHR* contains two conserved Cys residues between the first and second extra-cellular loop regions. These residues possibly form a disulfide bond, and thus could contribute to form a stable tertiary receptor structure as suggested earlier [31, 33].

Similarly, evolutionary analysis using phylogenetic tree showed that *LrGHRHR* is closely related to teleost and form separate subgroups depending on their habitat (marine, freshwater). *LrGHRHR* was closest to goldfish (*C. auratus*) rather than zebra fish (*D. rerio*) although both of them fall under same family, Cyprinidae. This might be because goldfish is more close to rohu in the evolutionary process. *LrGHRHR* seems to be the most conserved amongst teleosts relative to mammalian counterparts. High degree of similarity/identity value observed between goldfish and zebra fish indicates its structural and functional similarity among the teleosts.

It is generally believed that *GHRH* and *GHRHR* are synthesized in hypothalamus of brain and play critical role in somatotrophs cell growth and GH release by high affinity binding [34, 35]. In the present study, we also found elevated expression of *LrGHRHR* transcript in the brain of rohu similar to that of the previous findings reported in goldfish, zebra fish and orange-spotted grouper. *LrGHRHR* also expressed in other tissue including muscle, heart, kidney, testis and ovary correlating its possible action not only in GH release but also in many other developmental and physiological functions. Expression of *GHRHR* in other tissue predicts that it may play a role in controlling finely tuned paracrine and/or autocrine networks. The mRNA patterns in the brains and pituitary of different age groups revealed that there is a need of increased GH function in juvenile stage and young growing fishes. Similar pattern of higher documented evidences also supports its growth related function.

Investigation of protein–protein interactions is a wide-ranging essential approach to know the interacting proteins with the desired protein. For example, functional networking studies have been useful for drug discovery, understanding metabolic pathways, and predicting or developing genotype–phenotype associations [36-38]. *GHRH* belongs to the family of gut-neuropeptide hormones inclusive of glucagon, secretin and vasoactive intestinal peptide (VIP). All receptors for this peptide hormone family seem to be involved in similar kind of signaling cascades. Upon hormone binding, the interaction of these receptors with guanine nucleotide binding protein 'Gs' cause the stimulation of adenylate cyclase activating polypeptide (ADYCAP). VIP is also found in brain that plays a key role in communication between individual brain cells and also facilitates its role in pituitary development and GH release with *GHRH*. In the present study, the protein-protein interaction analysis has been an addition to the fact showing that *LrGHRHR* has a functional linkage with VIP and ADYCAP of secretin/glucagon family. Together, *LrGHRHR* plays multidirectional finely-tuned biological roles including the main function of GH binding. This is also in line with our transcriptomic results showing its most abundance in the brain and gonads. However, transcript level expression profiling requires to be validated by protein levels in future. Nevertheless, the current study provided the basis of undertaking future research studies so as to elucidate the exact networking functions, especially linked to reproduction, mediated by *GHRHR* for this commercially important farmed carps. Furthermore the prediction of secondary and tertiary structure of *GHRHR* will also facilitate to understand the mechanism behind the receptor ligand binding and their functional aspects.

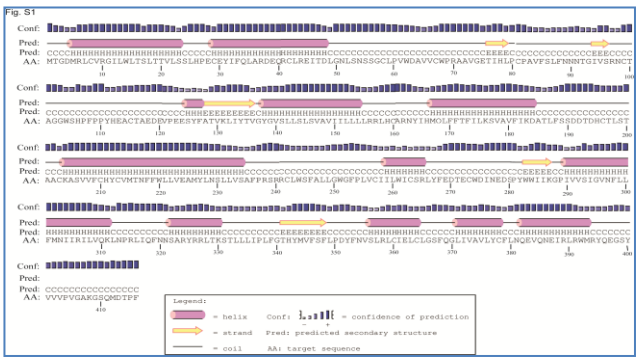

**Fig-S1 Secondary structure of LrGHRHR presenting confidence level of prediction for predicted secondary structural elements. It has 39.90% alpha helix, 28.12% random coil and 25.24% extended strands.**

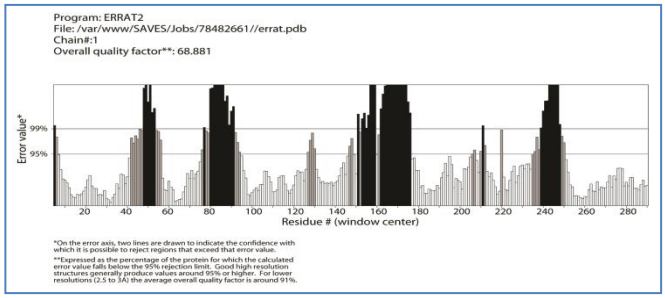


**Fig-S2 Errat plot for the stability of homology modeled structure of *LrGHRHR***

## Conclusion

In summary, we have identified and characterized a full length cDNA sequence of *GHRHR* expressed in the brain tissue of *Labeo rohita.* Abundant expression of *GHRHR* in brain provides evidence to the fact that hypothalamic *GHRH* acts on the pituitary to stimulate GH synthesis and release. Evolutionary analysis showed that *GHRHR* is conserved among other mammalian counterparts providing a clue that it might have similar diverse physiological functions in in teleost. We have also provided evidence for the presence of *GHRHR* in various growth stages of rohu, which might be involved, in promoting body growth and muscle mass. Furthermore, the findings of this study would help us to clearly understand the mechanism behind GH release for enhanced growth and also will serve as a stepping stone in finding its association with many physiological traits including its associated reproductive roles

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The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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