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Full Length Research Paper

In Silico characterization of growth hormone from freshwater ornamental fishes: Sequence analysis, molecular modelling and phylogeny

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The present investigation includes *in Silico* sequence analysis, three-dimensional (3D) structure prediction and evolutionary profile of growth hormone (GH) from 14 ornamental freshwater fishes. The analyses were performed using the sequence data of growth hormone gene (*gh*) and its encoded GH protein. The evolutionary analyses were performed using maximum likelihood (ML) estimate and maximum parsimony (MP) methods. Bootstrap test (1000 replicates) was performed to validate the phylogenetic tree. The tertiary structures of GH were predicted using the comparative modelling method. The suitable template for comparative modeling protein databank (PDB IDs: 1HWG A) has been selected on the basis of basic local alignment search tool (BLASTp) and fast analysis (FASTA) results. The target-template alignment, model building, loop modelling and evaluation have been performed in Modeller 9.10. The tertiary structure of GH is α-helix structure connected by loops, which forms a compressed complex maintained by two disulfide bridges. The resultant 3D models are verified by ERRAT and ProCheck programmes. After fruitful verification, the tertiary structures of GH have been deposited to protein model database (PMDB). Sequence analyses and RNA secondary structure prediction was performed by CLC genomics workbench version 4.0. The computational models of GH could be of use for further evaluation of molecular mechanism of function.

Key words: Growth hormone, *in Silico*, somatotropin, growth hormone gene (*gh*) mRNA, freshwater ornamental fish.

INTRODUCTION

The growth hormone (GH), which is a single chain polypeptide, synthesized, stored and secreted by the somatotroph cells within the lateral wings of the anterior pituitary gland, plays an essential role in the regulation of growth and development, by promoting the division, differentiation and enlargement of cells (Moore et al., 1982; Copelandet and Nair, 1994; Corin et al., 1990) as well as osmoregulation in fishes (Sakamoto et al., 1997) and many physiological activities of fish (Stacey et al., 1984; Peter et al., 1986; Sumpter et al., 1991; Trudeau, 1997; Degani et al., 2003). GH is phenotypically associated with characteristics of interest to animal breeding, such as growth, reproduction and osmo-regulation (Duan, 1998; Gomez et al., 1998; Mccormik, 2001). Genomic GH sequences and cDNAs have been used as a phylogenetic marker for different taxonomic groups including fishes (Koren et al., 1989; Chang et al., 1992; 2004).

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Abbreviations: 3D, Three dimensional; **BLAST,** Basic Local Alignment Search Tool; **EBI,** European Bioinformatics Institute; **FASTA,** fast analysis; **PDB,** protein databank; **PMDB,** protein model database; **RMSD,** root mean square deviation; **GH,** growth hormone; ***gh,*** growth hormone gene; **ML,** maximum likelihood; **MP,** maximum parsimony.

Table 1. Nucleotide sequence statistics of the *gh* cDNA sequence.

Taxon	GenBank accession numbers	Length (bp)	MW (kDa)	Melting temperature (°C) (salt)= 0.1 M	Frequency of A + T	Frequency Of C + G
C. lalia	AY873788	846	272.169	83.70	0.541	0.459
O. goramy	JF310708	828	266.763	84.90	0.512	0.488
Channa marulius	GQ214245	846	272.302	84.09	0.532	0.468
C. striata	EF447030	863	277.985	84.09	0.532	0.468
Channa gachua	GQ214244	848	273.088	84.24	0.528	0.472
C. punctata	GQ214243	848	272.936	84.38	0.525	0.475
Channa diplogramme	GQ214246	845	271.897	84.45	0.523	0.477
T. trichopterus	AF157633	881	283.163	83.28	0.552	0.448
Trichogaster leerii	AY873789	840	269.892	83.64	0.543	0.457
M. albus	AY265351	825	265.577	84.88	0.513	0.487
Oreochromis niloticus	HM565014	904	291.385	83.90	0.537	0.463
O. mossambica	AF033805	615	198.197	86.03	0.485	0.515
O. urolepis hornorum	EF371465	839	270.553	84.50	0.522	0.478
T. tinca	GU205401	1040	335.307	81.65	0.591	0.409

In addition, GH may be the most promising growth-promoting agent in aquaculture (Zohar, 1989), since it is essential for somatic growth and reproduction in bony fishes and osmoregulation in euryhaline fishes (Sciara et al., 2006). Among vertebrates, GH is essential for normal growth and is involved in the regulation of several anabolic processes (Xu et al., 2001).

The teleost growth hormone gene (*gh*) can be grouped into two types: on one hand are genes of the siluriforms and cypriniforms, which consist of five exons and four introns (5-exon type), and on the other hand are those of the salmoniforms, perciforms and tetradontiforms, which consist of six exons and five introns (6-exon type). Structurally, the latter differs from the former by the presence of an intron inserted at the 5^{th} exon (Moriyama et al., 2006). The *gh* has been shown to serve as a natural marker for studies of evolutionary genetics of various fishes because of its sequence conservation, sufficient length and minimal amount of homoplasy (Marins et al., 2003; Chen et al., 2004; Pinheiro et al., 2008). The aim of the present study was to annotate the coding sequence of the *gh* and perform a *gh*-based phylogenetic analysis among ornamental fish species. This study, for the first time focuses on prediction of *gh* mRNA structure, sequence comparison and comparative modelling for characterization of GH.

Like other native Indian freshwater fish species of economic importance, ornamental fish genetics needs greater attention for animal breeding programs. The *gh* is known to be linked to a number of molecular markers and quantitative trait loci. In this study, we have performed *in Silico* analysis with an attempt to characterize the *gh* (cDNA and mRNA) from 14 ornamental fish species and its encoded GH protein. Our results provide meaningful information for further studies on the ornamental fish breeding and fish phylogeny. On the other hand, the secondary RNA structures, which are often conserved, are essential in understanding the biological processes (Wuyts et al., 2002; Zwieb et al., 2003). Many computational methods have been developed for predicting RNA structures (Bachellerie et al., 2002; Perriquet et al., 2003; Hofacker et al., 2004).

Although, there has been availability of sequence information for GH from different fish groups, yet species-specific structural information are lacking. Therefore, the biochemistry and molecular mechanism of their functions in fishes are still not very well understood due to lack of their structural information. Thus, an attempt has been made to predict the three-dimensional (3D) folding pattern (Zemla et al., 1999) of GH from 14 ornamental fish species and their sequence analysis.

MATERIALS AND METHODS

Acquisition and alignment of sequences

The study was extended to data mining and sequence analyses of *gh* and GH protein from the sequence information extracted from GenBank (NCBI) and protein knowledgebase (UniProtKB), respectively (Boeckmann, 2003; Apweiler et al., 2004) (Tables 1 and 2). The sequences were simultaneously aligned using CLUSTAL-W (Higgins et al., 1994) and Modeller version 9v10 (Fiser et al., 2000) programs.

Sequence analysis and RNA structure prediction

The *gh* nucleotide and GH protein sequence analyses and GH mRNA structure prediction were performed in the CLC Genomics Workbench 4.0 (CLC Bio, Hyderabad). The physicochemical parameters of GH were computed using CLC Genomics Workbench and ProtParam (Gasteiger et al., 2005). The important calculations for the amino acid composition, atomic composition, theoretical pI, molecular weight, formula, extinction coefficients,

Table 2. Ornamental fish GH protein statistics.

Taxon	UniProtKB accession number	Number of amino acid	MW (Da)	pI	Negative charged residues	Positive charged residues	Formula	AI	GRAVY
C. lalia	Q5FYZ3	204	23303.6	6.43	23	22	$C_{1041}H_{1651}N_{281}O_{311}S_7$	99.85	-0.180
O. goramy	F1JZV8	204	23283.6	6.43	23	22	$C_{1037}H_{1643}N_{279}O_{313}S_8$	96.03	-0.215
Channa marulius	D6MLR5	204	23216.6	6.90	22	22	$C_{1032}H_{1658}N_{282}O_{311}S_7$	105.15	-0.168
C. striata	C0SKH7	204	23172.5	6.08	23	21	$C_{1032}H_{1654}N_{278}O_{312}S_7$	106.08	-0.129
Channa gachua	D6MLR4	204	23143.5	6.42	22	21	$C_{1030}H_{1651}N_{279}O_{311}S_7$	104.66	-0.151
C. punctata	D6MLR3	204	23172.5	6.08	23	21	$C_{1032}H_{1654}N_{278}O_{312}S_7$	106.08	-0.129
Channa diplogramme	D6MLR6	204	23120.4	6.90	22	22	$C_{1026}H_{1646}N_{280}O_{312}S_7$	101.81	-0.204
T. trichopterus	Q98UF6	204	23411.8	6.43	23	22	$C_{1049}H_{1659}N_{279}O_{311}S_8$	98.92	-0.175
Trichogaster leerii	Q5FYZ2	204	23397.8	6.43	23	22	$C_{1048}H_{1657}N_{279}O_{311}S_8$	98.92	-0.175
M. albus	Q7T231	204	22964.3	6.51	22	21	$C_{1022}H_{1642}N_{276}O_{311}S_6$	105.64	-0.117
Oreochromis niloticus	Q53WZ5	204	23110.2	5.95	22	19	$C_{1019}H_{1624}N_{280}O_{318}S_7$	97.94	-0.250
O. mossambica	Q6LAL0	204	23110.2	5.95	22	19	$C_{1019}H_{1624}N_{280}O_{318}S_7$	97.94	-0.250
O. urolepis hornorum	A3FEU9	204	23105.2	5.95	22	19	$C_{1021}H_{1625}N_{279}O_{317}S_7$	97.45	-0.257
T. tinca	D7RPP4	210	23699.2	6.32	25	24	$C_{1042}H_{1684}N_{290}O_{317}S_{11}$	97.00	-0.175

MW, Molecular weight; pI, isoelectric point; AI, aliphatic index; GRAVY, grand average of hydropathicity.

half-life,instability index, aliphatic index, hydrophobicity and charge versus pH were carried out under sequence analysis.

Molecular phylogenetic analysis

The sequences for the *gh* were separately aligned using ClustalW 1.6 (Thompson et al., 1994) integrated in software MEGA5 (Tamura et al., 2011), using default parameters. *gh* sequences were translated into amino acids (aa) of GH protein prior to analysis. Both *gh* and GH datasets were subjected to phylogenetic analyses. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). The evolutionary history was inferred by using two different methods namely the maximum parsimony (MP) (Eck and Dayhoff, 1966**)** and maximum likelihood (ML) estimate (Jones et al., 1992). Nucleotide substitution model that best fits each dataset and the model parameters were estimated using Akaike information criterion implemented in the program MODELTEST version 3.7 (Posada and Crandall, 1998) (Table 4).

Three-dimensional structure prediction

Basic local alignment search tool (BLASTp) (Altschul et al., 1997) and fast analysis (FASTA) (Pearson and Lipman, 1988, 1990; Pearson, 1991) searches were performed independently with protein databank (PDB) (Kauranov et al., 2006; Berman et al., 2007) for obtaining a suitable template. The significance of the BLAST results was assessed by expect values (e-value) generated by BLAST family of search algorithm (Altschul et al., 1991). The target-template alignment (Lassmann and Sonnhammer, 2005) was carried out using ClustalW version 2.1 (Higgins et al., 1994) and Modeller 9.10 (Fiser et al., 2000) programmes. Comparative (Homology) modelling was conducted by the Modeller version 9.10 (Marti-Renom et al., 2000; Fiser and Sali, 2003). The final 3D structures with all the coordinates for GH were obtained by optimization of a molecular probability density function (pdf) of Modeller (Eswar et al., 2006). The molecular pdf for homology modelling was optimized with the variable target function procedure in Cartesian space that employed the method of conjugate gradients and molecular dynamics with simulated annealing (Sali and Blundell, 1993).

The 3D structures for GH were evaluated (Giorgetti et al., 2005) by ERRAT (Colovos and Yeates, 1993) and ProCheck (Laskowski et al., 2003) programmes. After fruitful verification, the coordinate files were successfully deposited to protein model database (PMDB) **(**Tiziana et al., 2006**)**. All the graphic presentations of the 3D structures were prepared using Chimera (Peterson et al., 2004) and RasMol programs (Sayle and Milner-White, 1995).

RESULTS

Data mining and sequence analysis

In ornamental fishes, the *gh* ranged from 615 to 1040 nucleotide long and with molecular weights of 198.197 to 335.307 kDa. The melting temperature ranged from 81.65 to 86.03 at 0.1 M salt concentration (Table 1). The nucleotide sequence

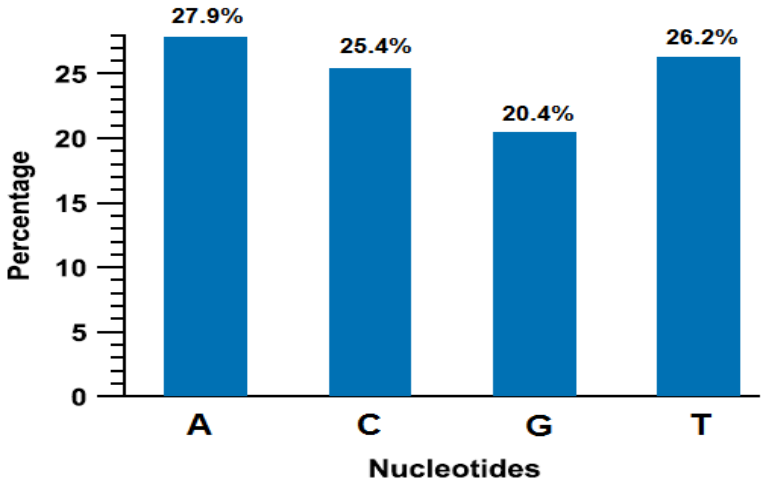

Figure 1A. Nucleotide composition (% in average) in the *gh* cDNA sequence in the ornamental fishes based on 14 *gh* sequences.

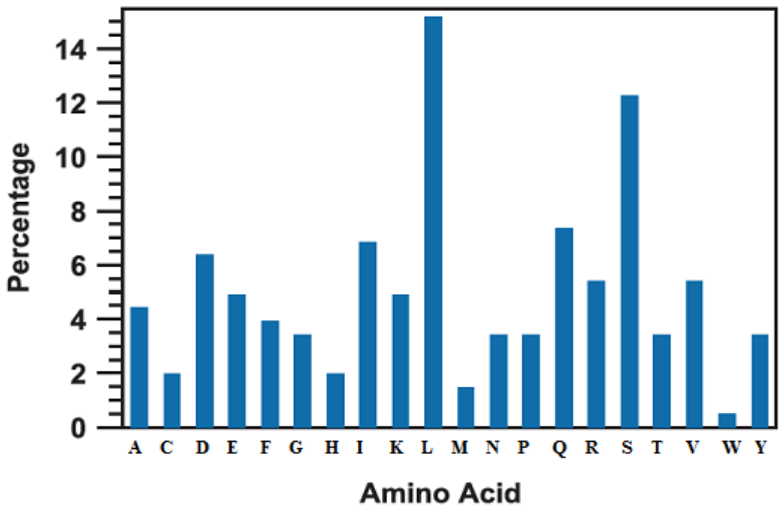

Figure 1B. Distribution of amino acids for GH protein in the ornamental fishes.

analysis based on the homologous *gh* mRNA (cDNA) sequence showed the domination of A:T in the *gh* (Figure 1A). The frequency of AT in different ornamental fish ranged from 0.485 (in *Oreochromis mossambica*) to 0.591 (in *Tinca tinca*). On the other hand, frequency of GC ranged from 0.409 (in *T. tinca*) to 0.515 (in *O. mossambica*) (Table 1).

The sequence alignment of *gh* mRNA (cDNA) sequence detected insertions of 'GTGTT' and 'TTCTA' from 105th to 109th position in *O. mossambica* and *T. tinca*, respectively. Another insertion of 'AG' has been observed from 87 to 88th positions in both the species. Again, insertion (TTTTTTC) has been observed in the genus *Oreochromis* from 800 to 806 positions in the alignment. Deletion of 'GTTT' is detected from 42 to 45 position in all the *Channa* species. There is a deletion of 'CGA' (667 to 668 positions) in all the *Channa* species. *T. tinca* has a deletion of 'CT' at 34 to 35 positions in the alignment. *Trichogaster* sp., *Colisa lalia* and *Monopterus albus* has deletion of 'A' at 33rd position. Deletion of 'CTTGC' has been observed in the genus *Oreochromis* from 773 to 777 positions. Deletion of 'ACCCCTAT' (685 to 692 position) has been observed in *T. tinca* and *Oreochromis* sp. (supplementary file).

The primary structures of GH are shown to be comprised of 204 to 210 amino acid residues. The amino acid leucine (L=16%) and serine (S=12.3%) has been found predominantly rich in the GH of these 14 ornamental fish species (Figure 1B). Sequence analysis of GH revealed negative hydropathy on the average (-0.117 to -02.57) (Table 2 and Figure 1C). The molecular weight of GH in the ornamental fishes of the present

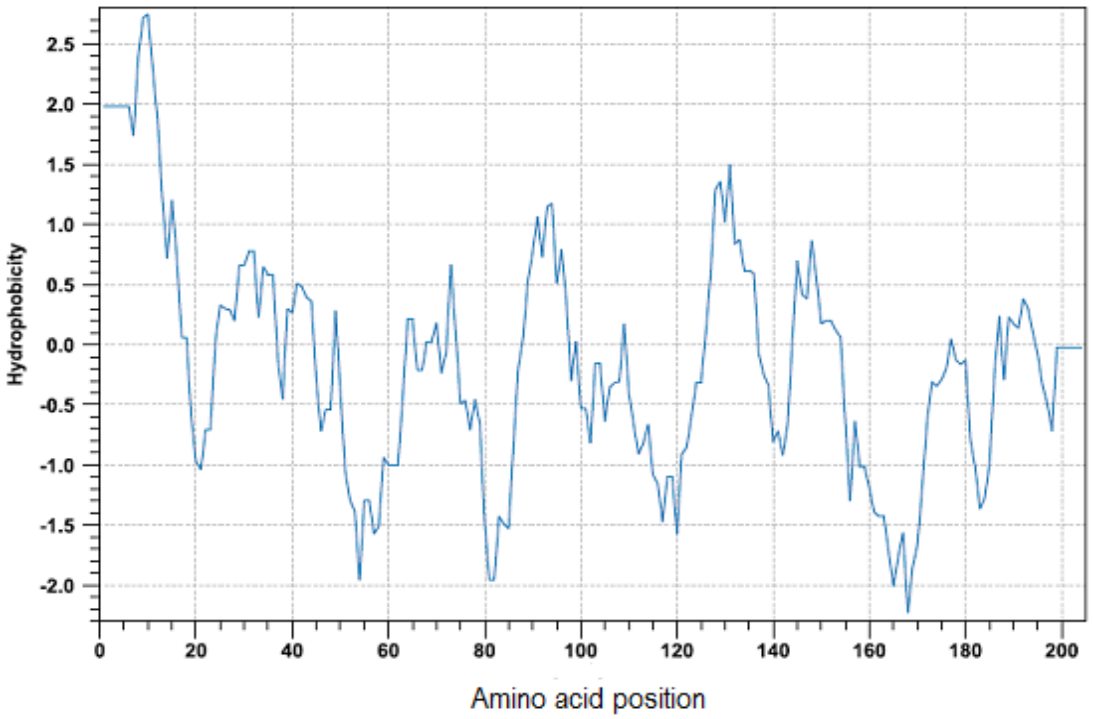

Figure 1C. Plot of local Hydropathy for GH (Kyte-Doolittle scale, Kyte and Doolittle, 1982).

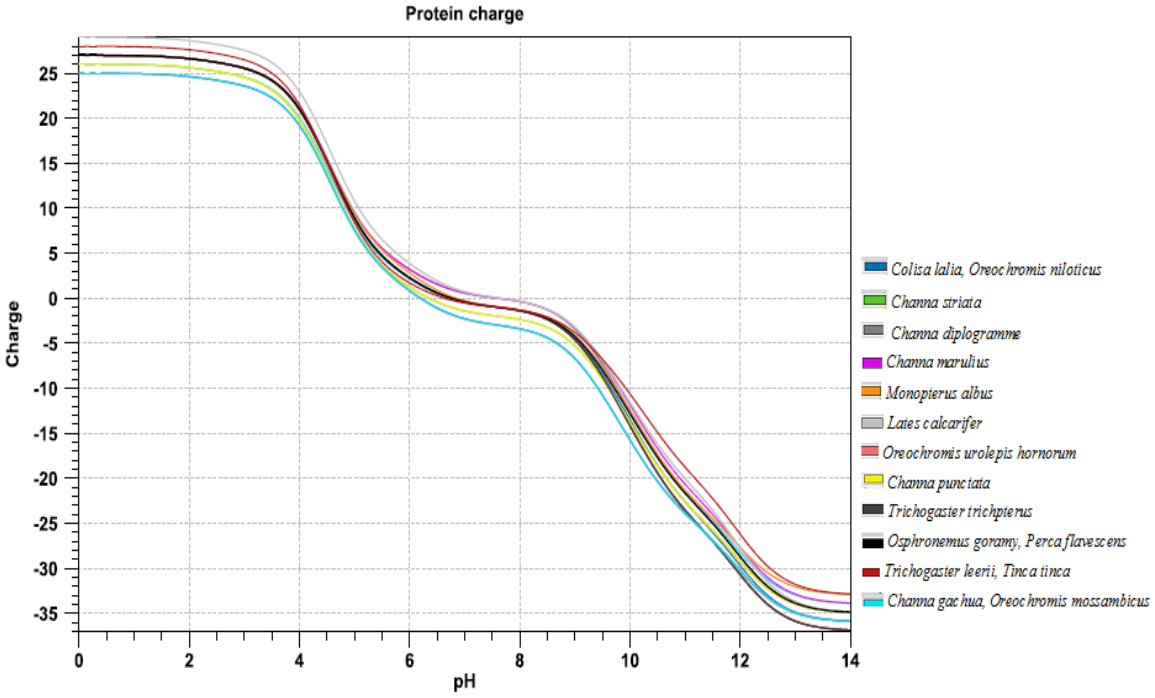

Figure 1D. Electrical charge as a function of pH for GH in the ornamental fishes.

study ranged from 22964.3 Da (in *M.* albus) to 23699.2 Da (in *T. tinca*). The isoelectric point of the GH ranged from 5.95 (in *Oreochromis* sp.) to 6.90 (in *Channa* sp.) (Table 2 and Figure 1D). Extinction coefficients for GH are 17670 [Abs 0.1% (=1 g/l) 0.758] and 317420 [Abs 0.1% (=1 g/l) 0.748]. The instability index (II) of GH was computed to be 59.46. There were 22 to 25 negatively charged and 19 to 24 positively charged amino acid resides in the GH sequence. The aliphatic index for GH was computed in the range of 96.03 to 106.08 (Table 2).

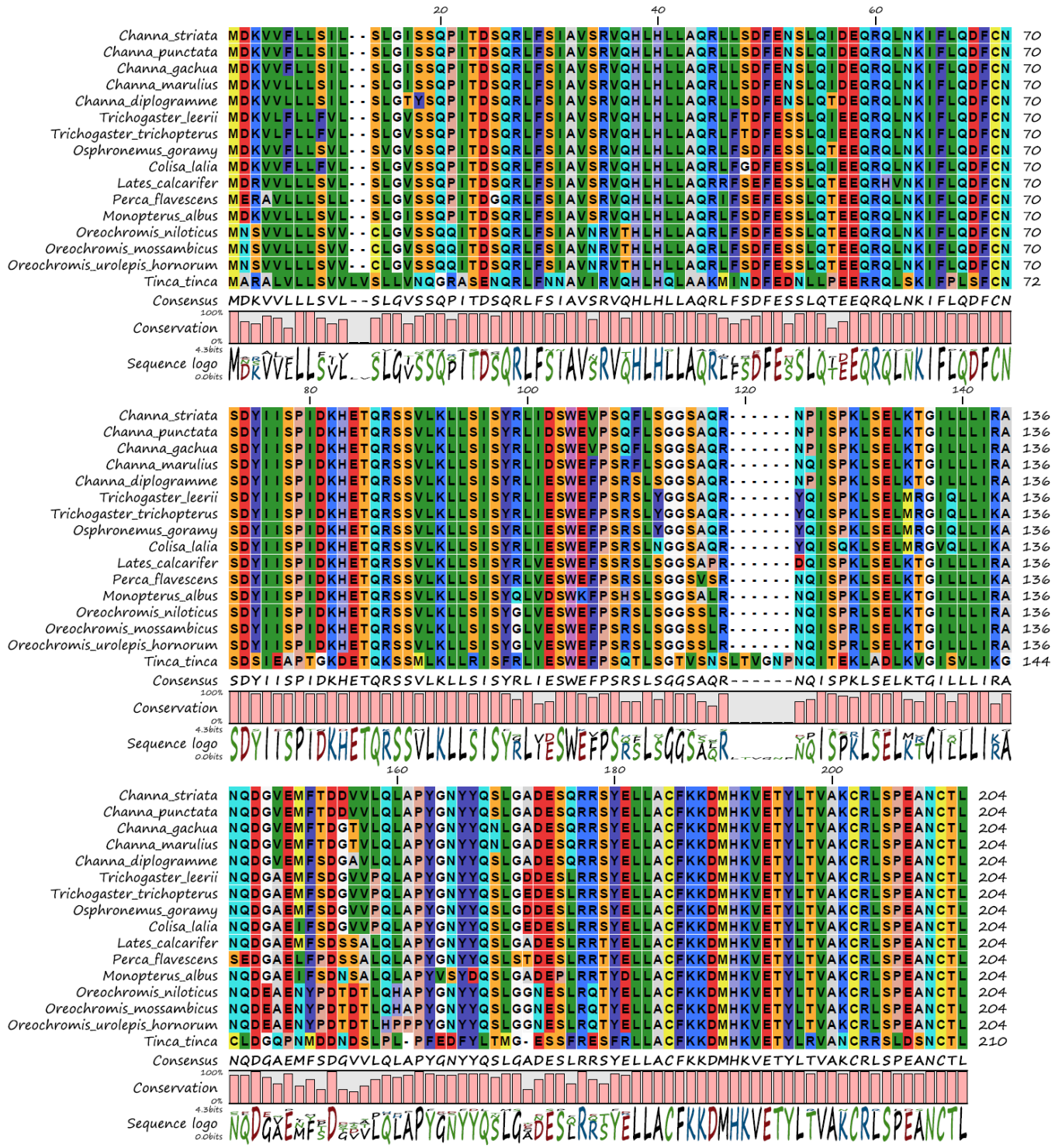

Figure 2. Multiple amino acid sequence alignment of GH protein among the 14 ornamental fish species. (-) represent sequence not conserved. The sizes of the letter in the sequence logo represent the degree of conservation of respective amino acid in each alignment position.

Multiple sequence alignment of the GH protein showed that *T. tinca* has an insertion of 'LV' and 'LTVGNP' in the 12 th to 13th and 119th to 124th positions, respectively. Similarly, deletion has been observed in *T. tinca* at 161th and 272nd positions. The genus *Oreochromis* differed from *Channa* in the position19th, 34th, 99th, 156th and 157th in the alignment (Figure 2). The homology searching demonstrated that the *T. tinca* GH shares a high

Table 3. Summary of *gh* mRNA structure.

Taxon	Minimum folding energy ΔG (kcal/mol)	Bulge	Hairpin loop	Interior loop	Multiloop	Stem
C. striata	-260.8	9	15	18	12	27
T. trichopterus	-272	14	18	16	15	33
O. goramy	-277.2	13	16	13	13	29
T. tinca	-290	16	17	28	13	29
Trichogaster leerii	-254.1	12	16	19	12	28
C. lalia	-241.5	7	17	23	14	31
Oreochromis niloticus	-282.5	6	17	23	11	28
Channa diplogramme	-264.2	14	15	16	12	27
Channa marulius	-265.5	9	16	19	13	29
Channa gachua	-262.7	6	14	20	12	26
C. punctata	-259.2	8	15	17	13	28
M. albus	-267.3	20	13	18	11	24
O. mossambica	-215.4	6	16	11	11	27
Oreochromis urolepis	-255.7	11	14	19	11	25

Table 4. Maximum likelihood model parameters for data sets as estimated in model test (Posada and Crandall, 1998).

Parameter	*gh*	GH protein
Model	T92+G	JTT+I
Bayesian information criterion (BIC) scores	9596.7	3120.7
Akaike information criterion, corrected (AICc) value	9394.5	2966.6
Maximum likelihood value (*lnL*)	-4669.2	-1457
Gamma distribution (*G*)	0.98125	n/a
invariable (*I*)	n/a	0.220026
Transition/transversion bias (*R*)	1.5677	n/a
Total positions in the final dataset	727	202

homology at the nucleotide and amino acid levels with those of grass carp (92% nt, 98% aa), of silver carp (91% nt, 98% aa) and common carp (88% nt, 98% aa).

Secondary structure *gh* mRNA

The minimum folding energy (ΔG) of the *gh m*RNA structure ranged from -215.4 kcal/mol (*O. mossambica*) to -290 kcal/mol (*T. tinca*) (Tables 3). The number of bulges ranged from 6 (*O. mossambica*) to 20 (*M. albus*), while hairpin loops ranged from 13 (*M. albus*) to 18 (*Trichogaster trichopterus*) and stems ranged from 24 (*M. albus*) to 33 (*T. trichopterus*) (Tables 3).

The sequence analysis indicated that a minor variation in bulge, hairpin loop, interior loop and in stem (Table 3) exists in the *gh* secondary mRNA structure. Minor structural changes have been observed in some parts of the mRNA secondary structure with variation in the minimum folding energy (Table 3 and Figure 3).

Molecular evolution of GH

The evolutionary tree of both *gh* and GH protein in ornamental fishes supports the fact that the families Channidae (*Channa* spp.) and Belontidae (*C. lalia*, *Trichogaster* spp.) along with Osphronemidae (*Osphronemus goramy*) are sister groups, while family Cichlidae (*Oreochromis* sp.) is their successive sister group. Family Synbranchidae (*M. albus*) was represented as an intermediate clade between Belontidae and Cichlidae. Family Cyprinidae (*T. tinca*) is represented as out group in the phylogenetic tree.

Evolution of gh

Pairwise distances of *gh* are shown in Table 5. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). There were a total of

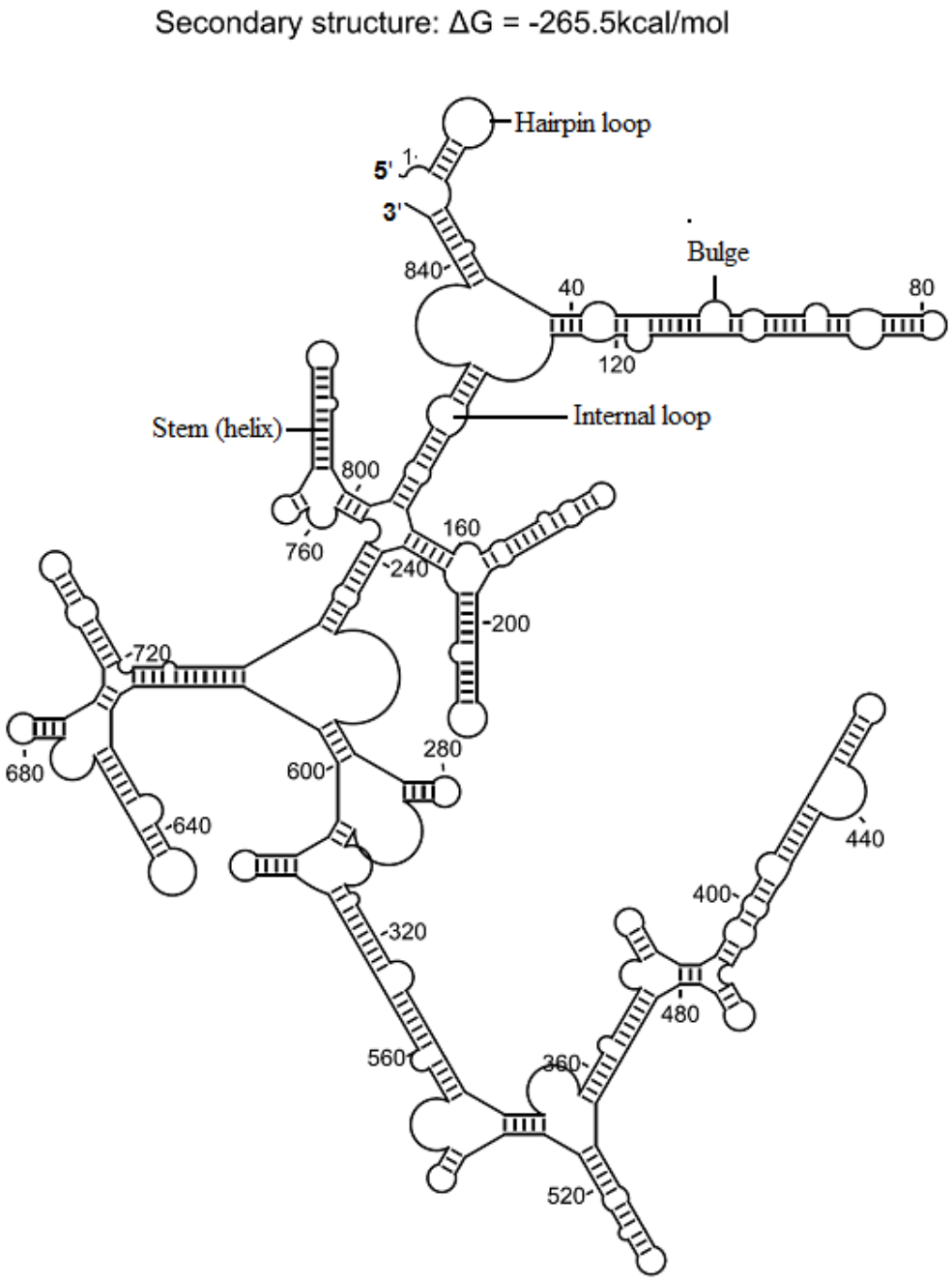

Figure 3. Lowest energy secondary structure of growth hormone (GH) mRNA (*C. marulius*). The numbers represent nucleotide positions of *gh* mRNA sequence.

727 positions in the final dataset. The pairwise distance of *gh* sequences among the 14 ornamental fish species of the present study revealed shortest genetic distance (0.007) between *Channa striata* and *Channa punctata*. The longest genetic distance (0.597) exists between *O. mossambica* and *T. tinca* (Table 5).

1) The evolutionary history was inferred using the MP method. The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Nei and Kumar, 2000) with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree was drawn to scale; with branch lengths calculated using the average pathway method (Nei and Kumar, 2000) and are in the units of the number of changes over the whole sequence (Figure 4A).

2) The evolutionary history was inferred by using the ML method based on the Tamura 3-parameter model (Tamura, 1992). The ML tree with the highest log

Table 5. Pairwise distance *gh*.

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14
C. striata	-													
T. trichopterus	0.193													
O. goramy	0.146	0.109												
T. tinca	0.557	0.569	0.558											
Trichogaster leerii	0.190	0.022	0.102	0.561										
C. lalia	0.205	0.096	0.131	0.578	0.098									
Oreochromis niloticus	0.257	0.259	0.226	0.583	0.257	0.282								
Channa diplogramme	0.100	0.179	0.146	0.549	0.179	0.190	0.268							
Channa marulius	0.063	0.172	0.140	0.539	0.169	0.193	0.248	0.084						
Channa gachua	0.045	0.180	0.143	0.538	0.177	0.193	0.253	0.094	0.021					
C. punctata	0.007	0.187	0.140	0.553	0.184	0.199	0.254	0.096	0.056	0.039				
M. albus	0.179	0.206	0.179	0.571	0.201	0.227	0.219	0.186	0.161	0.166	0.175			
O. mossambica	0.326	0.323	0.301	0.597	0.323	0.345	0.110	0.327	0.318	0.322	0.323	0.293		
O. urolepis hornorum	0.267	0.261	0.228	0.582	0.260	0.283	0.014	0.274	0.253	0.260	0.264	0.223	0.118	-

likelihood (-4725.1438) is shown. A discrete gamma distribution was used to model evolutionary rate differences among sites [(5 categories (+*G*, parameter = 3.5077)]. The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 0.0000% sites) (Figure 4B).

In the *gh* (cDNA) phylogeny, the families Channidae (*Channa* spp.) formed a clade with bootstrap support 92 and 100% in the MP and ML trees, respectively. Family Belontidae (*C. lalia*, *Trichogaster* spp.) and Osphronemidae (*O. goramy*) are clustered together at the bootstrap support 99 and 100% for MP and ML trees. In another cluster, family Cichlidae (*Oreochromis* sp.) formed a separate clade with bootstrap value 100%. The family Synbranchidae (*M. albus*) represented in an intermediate clade and along with Channidae, Belontidae and Osphronemidae is separated from Cichlidae at a bootstap percentage 73 and 97% in the MP and ML trees, respectively. Family Cyprinidae (*T. tinca*) is represented as out group in the phylogenetic tree (Figure 4).

Evolution of GH protein

Pairwise distance of GH protein is shown in Table 6. The analysis involved 14 protein sequences. All positions containing gaps and missing data were eliminated. There were a total of 202 positions in the final dataset. The pairwise distance of GH sequences among the 14 ornamental fish species of the present study revealed significant distance (0.654) between *M. albus* and *T. tinca* (Table 6).

1) The evolutionary history was inferred using the MP method. Tree #1 out of 6 most parsimonious trees (length =169) is shown. The consistency index is (0.857143), the retention index is (0.900000) and the composite index is 0.825444 (0.771429) for all sites and parsimony-informative sites (in parentheses). The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Nei and Kumar, 2000) with search level 1 in which the initial trees were obtained with the random addition of sequences (Figure 5A).

2) The evolutionary history was inferred by using the ML method based on the JTT matrix-based model (Jones et al., 1992). The tree with the highest log likelihood (-1461.4701) is shown in Figure 5B.

In the evolutionary tree GH protein, Belontidae (*C. lalia*, *Trichogaster* spp.) and Osphronemidae (*O. goramy*) formed a clade with bootstap support 98% in MP an ML trees. This clade is separated from the clade formed by Channidae (*Channa* spp.), with bootstap value 75 and 82% for the MP and ML trees, respectively. Further, family Cichlidae (*Oreochromis* sp.) is separated from family Synbranchidae (*M. albus*) with bootstrap probability 67 and 83% for MP and ML trees. All the *Oreochromis* spp. have been clubbed together with a bootstrap support of 99 and 97% for MP and ML phylogeny.

The predicted 3D structure of GH

Based on BLASTp and FASTA results, 1HWG (Chain A, Complex of Human GH; Identities 34%) was considered to be the best template for homology modelling. The model of GH has 7 to 11 helices, 10 to 17 helix-helix interactions, 5 to 13 beta turns, 0 to 5 gamma turns and 2 disulphide linkages (Table 8 and Figure 6). The overall quality factors predicted by ERRAT verification programme for the 3D structures of GH are around 95 (Figure 7). Procheck verification proved that the models

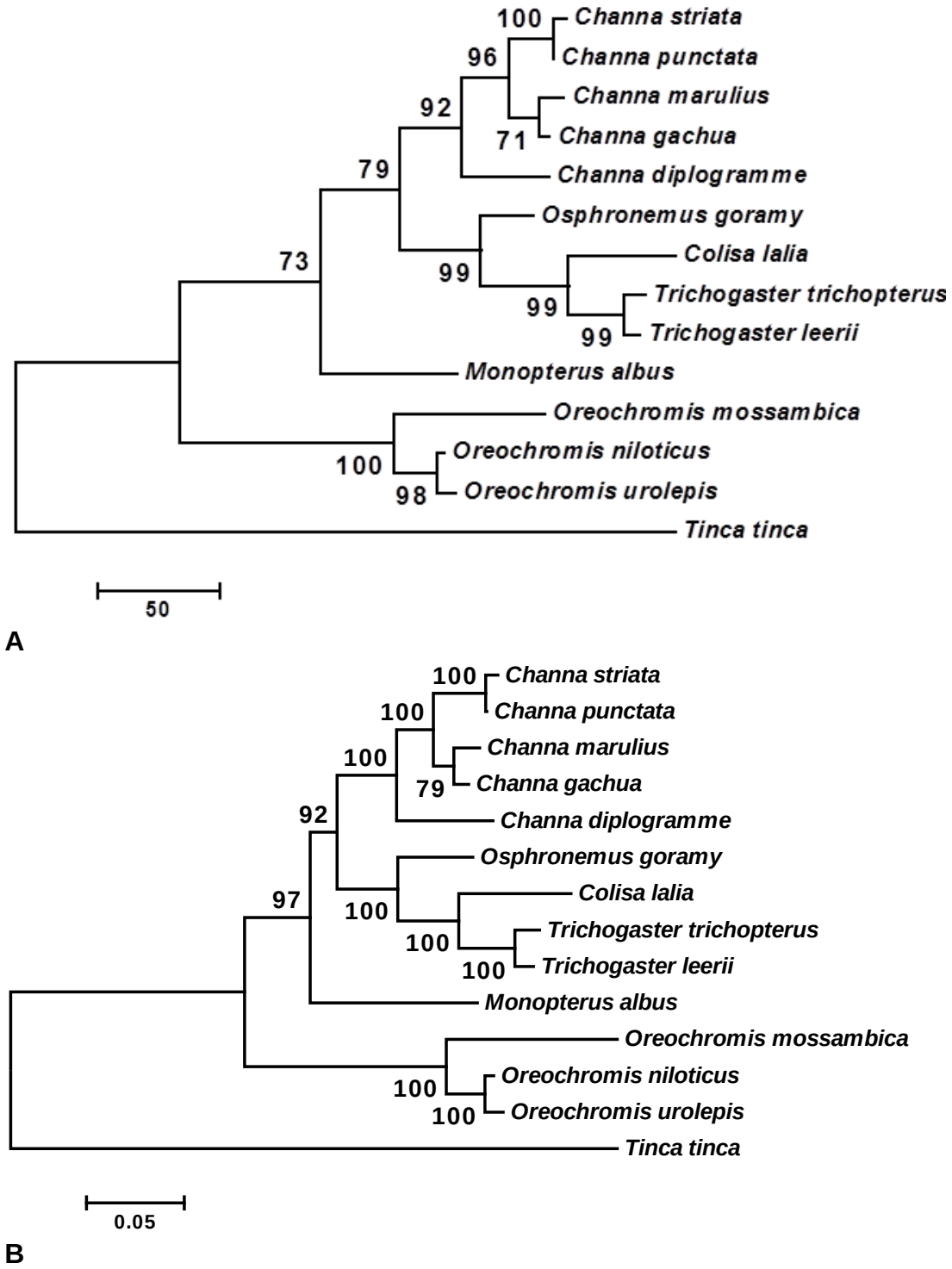

Figure 4. Molecular phylogenetic anaylsis of ornamental fish *gh*. A, MP tree; B, ML tree based on the Tamura 3-parameter model (Tamura, 1992). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The scale bars represent the branch lengths measured in the number of changes (substitutions per site) over the whole sequence.

are of good quality (88.8 to 96.3%) as judged by Ramachandran plot (Ramachandran and Sasisekharan, 1968). The number of glycine and proline resides in the plot ranged from 7 to 9 and 6 to 8, respectively as

Table 6. Pairwise distance GH protein.

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14
C. lalia	-													
O. goramy	0.040													
Trichogaster_leerii	0.030	0.025												
Channa gachua	0.143	0.126	0.132											
C. striata	0.138	0.121	0.126	0.015										
Channa diplogramme	0.126	0.099	0.115	0.056	0.056									
Channa marulius	0.132	0.115	0.121	0.020	0.035	0.046								
M. albus	0.155	0.138	0.155	0.149	0.143	0.132	0.132							
O. urolepis hornorum	0.208	0.184	0.202	0.221	0.215	0.184	0.196	0.167						
C. punctata	0.138	0.121	0.126	0.015	0.000	0.056	0.035	0.143	0.215					
T. trichopterus	0.030	0.025	0.000	0.132	0.126	0.115	0.121	0.155	0.202	0.126				
Oreochromis niloticus	0.196	0.172	0.190	0.208	0.202	0.172	0.184	0.155	0.015	0.202	0.190			
T. tinca	0.635	0.626	0.617	0.635	0.635	0.626	0.626	0.654	0.617	0.635	0.617	0.626		
O. mossambicus	0.196	0.172	0.190	0.208	0.202	0.172	0.184	0.155	0.015	0.202	0.190	0.000	0.626	-

observed in the Ramachandran plot statistics (Table 7). After fruitful verification, the tertiary structures of GH have been deposited to PMDB (Tiziana et al., 2006) (Table 8). InterPro scan for sequence motifs matched in scan against PROSITE, PRINTS, PFam-A, TIGRFAM and PRODOM motifs reveled that GH belongs to the somatotropin/prolactin family. Search of GH sequence versus superfamily HMM library revealed 11 motifs with superfamily name 4-helical cytokines (Motif 47266; residue ranges 19 to 146 and 150 to 202).

Sequence search against existing PDB entries revealed that GH in the ornamental fish has 38.6% sequence identity with the structure of human GH (Hgh) (PDB ID 3 hh and 1 hwg).

DISCUSSION

GH in ornamental fishes in the present study has 204 to 210 amino acid residues with molecular weight of 22.96 to 23.7 kDa. There are four cysteine residues (at 69^{th}, 177^{th}, 194^{th} and 202^{nd} positions) in the GH sequence containing 204 aa (Table 2). However, there are 210 amino acid residue in *T. tinca* and the five cysteine residues (at 71^{st}, 145^{th}, 183^{rd}, 100^{th} and 108^{th} positions) establishes itself as an out-group. The high Leucine content (Figure 1B) of the amino acid sequence of the GH is responsible for increased kinetics of the protein synthesis and controlling protein breakdown rates (Garlick, 2005).

Sequence analysis of GH protein revealed negative hydropathy on average (Figure 1C), which signifies the polar and hydrophilic in nature of the GH. The instability index (II) of GH in the present study (59.46) classifies GH as unstable. The formation of cysteine disulfide bonds assumes highly conservative nature of GH and all known vertebrate GH contains two disulfide bonds between cysteine-57 and cysteine-165 and between cysteine-182 and cysteine-189 (Scanes and Campbell, 1995).

The present findings demonstrated two isoforms of GH and the out-group species *T. tinca* belongs to the first isoform with 210 amino acid residues, while the other 13 species could be included in the second isoform with 204 amino acid residues, where the residues 12 to 13 and 119 to 124 are missing, compared to *T. tinca* (Figure 2). Similarly, the *gh* cDNA (mRNA sequence) of *T. tinca* contains an open reading frame of 1040 nucleotides (Table 1) encoding a pre-protein of 210 amino acid residues, while the all other species of the families Channidae (*Channa* spp.), Belontidae (*C. lalia*, *Trichogaster* spp.), Osphronemidae (*O. goramy*), Cichlidae (*Oreochromis* sp.) and Synbranchidae (*M. albus*), the GH cDNA retains an open reading frame of 615 to 904 nucleotides encoding a pre-protein of 204 amino acid residues. The GH isoform(s) and their physiological significance in different fish remains unclear, but emerging data provide suitable evidence for season and nutrition related changes in the somatototropic axis activity (Pe´rez-Sa´nchez et al., 2002).

Hong and Schart (1993) analyzed the silver carp *(Hypophthalmichthys molitrix)* g*h* with the suggestion that the arrangement of exons and introns are identical to the GH genes of common carp, grass carp and very similar to mammals and birds, but quite different from the GH genes of tilapia and salmonids. The sequence predicts a polypeptide of 210 aa including a putative signal peptide of 22 hydrophobic aa residues. Comparison of the *gh* sequence in the present study indicates that there is a high degree of homology, both at the nucleotide and amino acid level, among the fish species.

AT-rich elements (Figure 1A) involved in homeotic protein regulation of the *gh* (Rhodes and Yamada, 1995). The minimum folding energy (ΔG) of the *gh m*RNA

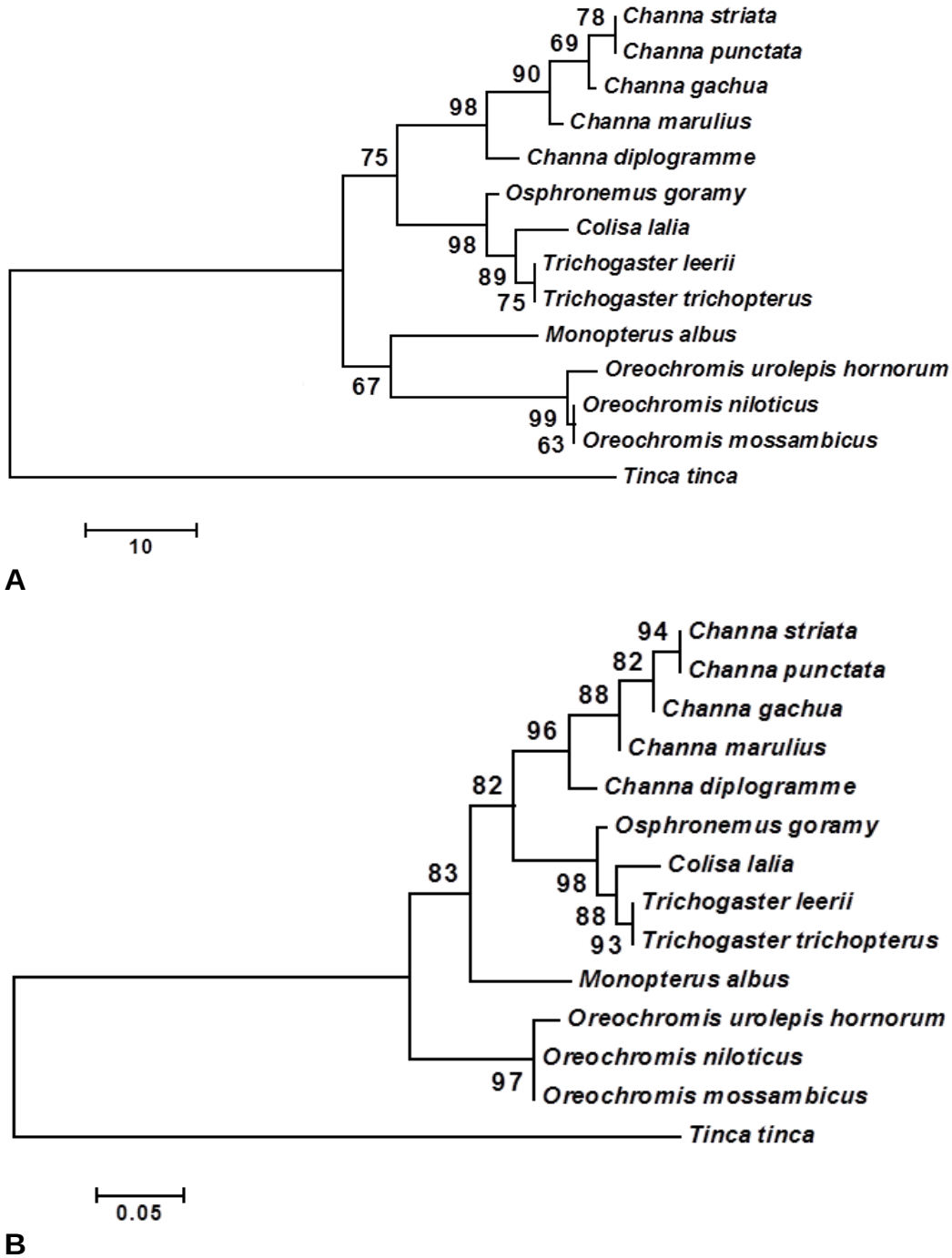

Figure 5. Molecular phylogenetic anaylsis of ornamental fish GH protein. A, MP tree; B, ML tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The scale bars represent the branch lengths measured in the number of changes (substitutions per site) over the whole sequence.

structure (-215.4 kcal/mol to -290 kcal/mol) is suggestive of highly stable structure (Table 3 and Figure 3). Timothy et al. (2004) suggested a relationship between the richness of AT/AU and mRNA stability for the regulation of the gene expression. Many of the known regulatory pathways for mRNA stability involve proteins that interact with specific AU-rich elements in the 3′-untranslated region (3′ UTR) of the transcript. In particular, rapid context-specific regulation of the stability of mRNA transcripts encoding highly active proteins, such as GH,

Table 7. Ramachandran plot statistics.

Taxon	Most favoured regions [A,B,L] (%)	Additional allowed regions [a,b,l,p] (%)	Generously allowed regions [~a,~b,~l,~p] (%)	Disallowed regions [XX] (%)	End-residues (excl. Gly and Pro)	Glycine residues	Proline residues
C. lalia	96.3	3.2	0.5	0.0	2	9	6
O. goramy	91.4	8.1	0.5	0.0	2	8	7
Channa marulius	92.6	6.9	0.5	0.0	2	8	6
C. striata	89.9	9.0	1.1	0.0	2	7	7
Channa gachua	88.8	11.2	0.0	0.0	2	8	7
C. punctata	89.3	9.6	0.5	0.5	2	7	7
Channa diplogramme	91.4	7.5	1.1	0.0	2	8	7
T. trichopterus	90.9	8.0	0.5	0.5	2	8	7
Trichogaster leerii	92.0	6.4	1.6	0.0	2	7	7
M.albus	91.0	8.5	0.0	0.5	2	6	7
Oreochromis niloticus	90.4	8.0	0.5	1.1	2	8	6
O.mossambica	90.4	8.0	0.5	1.1	2	8	6
O. urolepis hornorum	89.2	9.7	1.1	0.0	2	7	7
T. tinca	90.6	6.2	1.0	2.1	2	8	8

Table 8. The PMDB ID assigned to the submitted structures.

Taxon	Number of helices	Helix-helix interaction	Number of beta turns	Number of gamma turns	PMDB ID
C. lalia	8	10	6	1	PM0077770
O. goramy	7	10	8	5	PM0077775
Channa marulius	11	16	5	3	PM0077767
C. striata	11	14	4	2	PM0077769
Channa gachua	8	12	11	4	PM0077766
C. punctata	11	17	5	2	PM0077768
Channa diplogramme	8	14	11	4	PM0077765
T. trichopterus	11	17	5	-	PM0077777
Trichogaster leerii	7	11	12	5	PM0077778
M. albus	10	17	13	5	PM0077771
Oreochromis niloticus	11	15	5	-	PM0077772
O. mossambica	11	15	5	-	PM0077773
O. urolepis hornorum	7	11	10	1	PM0077774
T. tinca	9	15	9	3	PM0077776

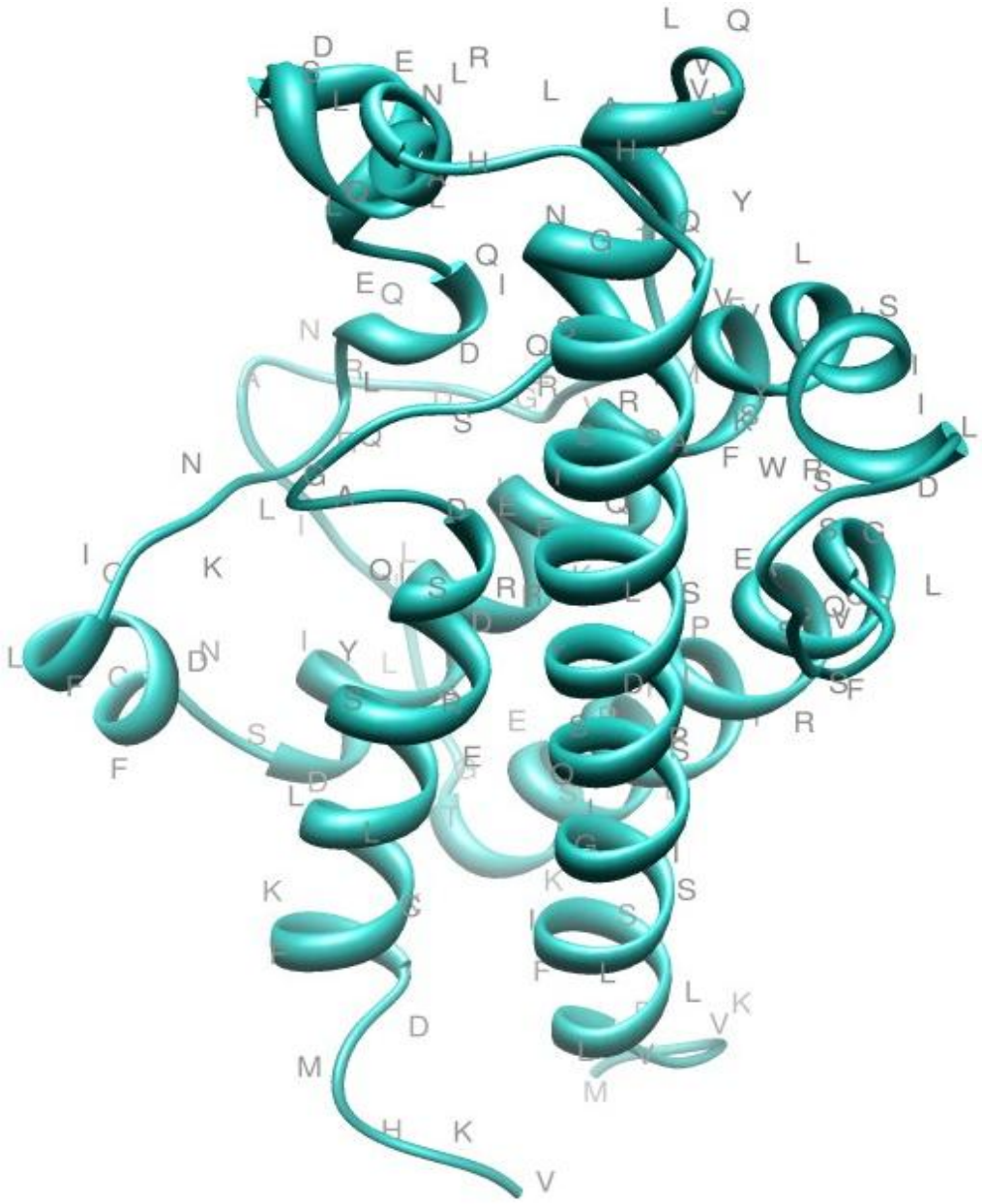

Figure 6. The predicted homology model of ornamental fish GH structure, as displayed by UCSF Chimera.

appears to play a key role in the control of these molecules and the processes they mediate (Timothy et al., 2004). Somvanshi et al. (2008) suggested that the genetic algorithm (GA) simulates natural folding pathway during RNA synthesis, which in fact enables the addition of new stems to growing RNA chain as well as allows the removal of unfavourable pairings. Their *in Silico* analysis GA on the evolutionary stability of the Influenza virus allows the prediction of tertiary interactions including RNA pseudoknots and the MFE is obtained from the secondary RNA structure. Secondary structure prediction of *gh* mRNA finds very clear evidence that the GH in 14 ornamental fish species has an evolutionary relationship, although it is not completely clear whether this was a relationship of homology or complementarities between the 5 prime and 3 prime directions of mRNA, demands further characterization.

Though the number of amino acid residues has been obtained 171 to 217 in human, bovine and in some fishes, yet the ornamental fish group of the present study presented 204 to 210 amino acid residues. However, the present *in Silico* analysis has been failed to explain the discrepancy in the amino acid residues. The structural relationship of GH is consistent with phylogeny. In a

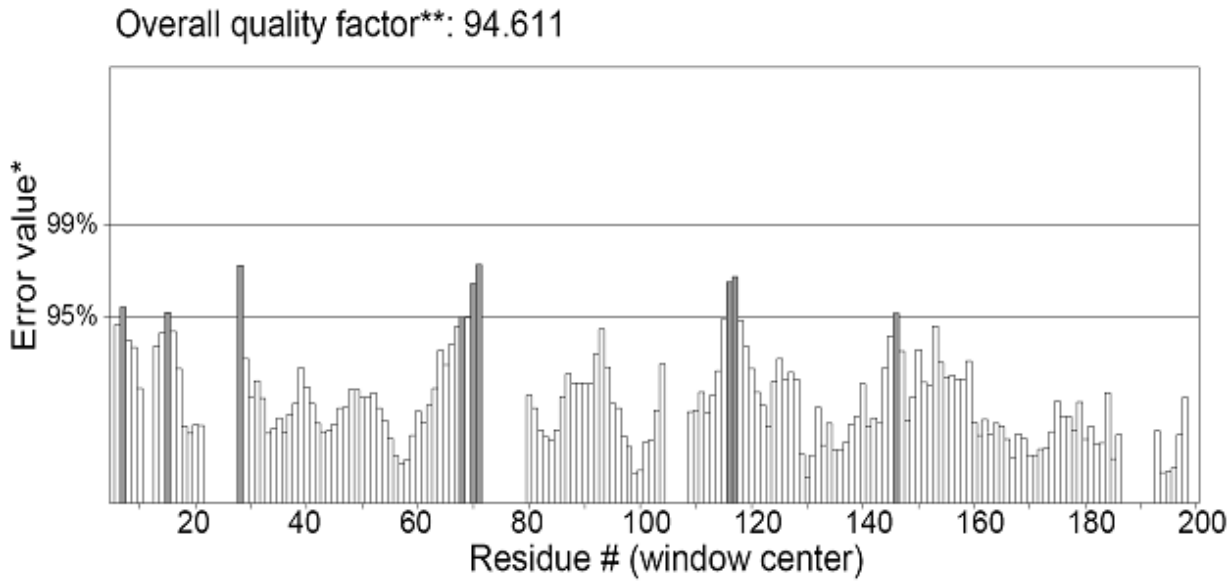

Figure 7. Structure validation results showing overall quality of 3D structure of GH (ERRAT2 Verification). The two lines at 95 and 99% represent error axis, to indicate the confidence with which it is possible to reject regions that exceed that error value. The dark grey bars indicate residues under the 95% confidence limit.

recent study, Kocour and Kohlmann (2011) studied *gh* polymorphisms in *T. tinca,* comprising 1758 to 1763 bp in length. Polymorphisms in the *T. tinca gh*, a representative of the five-exon type and are not as extensive as in fishes with the six-exon GH gene.

Both the tree building methods (MP and ML) used in the present study revealed almost similar tree topology in the final *gh* and GH protein phylogeny. However, *M. albus* (family Synbranchidae) was found to be the successive sister taxa of family Belontidae (*C. lalia*, *Trichogaster* spp.), Osphronemidae (*O. goramy*) and Channidae (*Channa* spp.), but an intermediate of family Cichlidae (*Oreochromis* sp.) in the ML and MP tree of *gh* and ML tree of GH protein. On the other hand, in the MP tree of GH protein, *M. albus* (family Synbranchidae) has formed a direct clade with Cichlidae (*Oreochromis* sp.). *T. tinca* (family Cyprinidae) is a distinct out-group of all the other taxa, as depicted by ML and MP trees of *gh* and GH protein phylogeny. The present study revealed independent evolution of GH at nucleotide and protein level in the ornamental fish families that is, one in Belontidae, Osphronemidae and Channidae group and the other Cichlidae and Synbranchidae, where Cyprinidae is a out group (Figures 4 and 5).

Pairwise genetic distance analysis of this investigation demonstrated close relationship between *C. striata* and *C. punctata* (Table 5) and *T. tinca* is definite outgroup. Also, major distance has been putforwarded between *T. tinca* and *O. mossambica* (Tables 5 and 6).

GH is structurally and apparently evolutionarily homologous to prolactin and chorionic somatomammotropin. Putative conserved domains of somatrophin-like GH superfamily were also detected in the BLAST result of the present study.

Growth rates of many fish species used in aquaculture are naturally slow, but are currently being enhanced by traditional methods of domestication and selection (Hershberger et al., 1990). The efficiency of growth and feed-conversion can also be increased in finfish by creating transgenic fish that incorporate a gene construct encoding GH, giving 3–11-fold gains in weight (Rahman et al., 1998). The number of residues varies slightly with GH from different fish species. Chen et al. (1995) studied the growth rates in transgenic mice and demonstrated that aa residues in the third alpha-helix of GH involved in growth promoting activity. GH has two disulfide bridges which are conserved in fish species (Harvey et al., 1995).

In hGH, four major isoforms were identified with the number of aa residues 217 (MW 24,847 Da), 202 (MW 22,992 Da), 179 (MW 20,561 Da) and 171 (MW 19,802 Da), respectively (Zhan et al., 2005). The largest isoform (217 aa, 24.85 kDa) is typically referred to hGH, and is most predominant. However, further wet lab analysis is required in order to study the GH isoforms in fish.

The models presented here were deposited in the public domain database and could serve as a guide for the allocation of aa residues in each fold which is important for further investigations on molecular mechanism of functions. The study was performed for sequence analyses and prediction of 3D structure of GH using the homology modeling. A series of molecular modeling and computational methods were combined in order to gain insight into the 3D structure. Further study, investigating the role of other factors in GH biosynthesis in wet lab is in progress. Much is still to be learned about how the GH can manipulate a sequence of base pairs in such a

peculiar way that results in a fully functional organism.

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