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# *CgGH* insertion functional domain analysis in transgenic $G_1$ and $G_2$ and $G_3$ mutiara catfish (*Clarias gariepinus*) broodstock

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**Abstract**

Catfish is one of the most important freshwater fish farming commodities in Indonesia. Higher catfish production can be achieved by cultivating transgenic catfish carrying the growth hormone (*GH*) gene of African catfish (*Clarias gariepinus* GH, *CgGH*). This research focuses on analysis of the presence of the *CgGH* gene in transgenic $G_1$, $G_2$, and $G_3$ mutiara catfish broodstock, as an indication of stable *CgGH* inheritance. *CgGH* gene was isolated using the RNeasy mini kit and RT-PCR. RT-PCR revealed amplicons measuring approximately 600 bp in transgenic $G_0$, $G_1$, $G_2$, and $G_3$ mutiara catfish. The *CgGH* consensus sequence similarities ranged from 93.76% to 97.06%, with four functional domain sites (somatotropin-1, somatotropin-2, four α-helix, N-glycosylation, four cysteine residues) of fish GH proteins. The functional domains of fish GH proteins are conserved in $G_1$, $G_2$, and $G_3$ and indicate stable exogenous GH inheritance to produce transgenic catfish strains in each generation.

**Keywords:** *CgGH*, Transgenic line, Mutiara catfish, Conserved sequence, Consensus sequence

## Introduction

The application of growth hormone (*GH*) transgenesis technology in fish has led to significant growth improvement as an effect of overexpression of the inserted *GH gene* (Hinits & Moav, 1999; Mori & Devlin, 1999; Nam et al., 2001). Transgenesis of *GH* causes excessive fish growth that is several times higher than that of non-transgenic fish, having the potential to increase fish culture yields. The growth of transgenic mutiara catfish (inserted *CgGH* sequence, GenBank accession no.MN249238.1) reported by Buwono et al. (2016; 2019b; 2021) was high (2–3 times that of non-transgenic fish) because of exogenous *GH* (*CgGH*) insertions at $G_0$, $G_1$, $G_2$, and $G_3$. This inserted transgene was successfully inherited in three generations through the reproduction of transgenic broodstock with *CgGH* transmission rates of 42.85% in $G_1$, 50% in $G_2$ and 70% in $G_3$ (Buwono et al., 2021). Transgene inserts integrated into the fish genome have been shown to be inheritance on to offspring through broodstock reproduction. Some examples include studies on trout showing the inheritance of pSV518 in $G_2$ by 49%–75% (Tewari

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et al., 1992). Transmission of the antifreeze protein gene (AFP) that was inherited in $G_2$ by the transgenic $G_1$ Atlantic salmon broodstock pair following Mendel's law by 50% showed stable inheritance in the transgenic salmon generation (Hew et al., 1992). The percentage of transgene transmission from crosses of $G_1$ transgenic and non-transgenic Nile tilapia fish (*Oreochromis niloticus*) ranged 49%–52% which is consistent with Mendelian inheritance (Rahman & Maclean, 1999). Transgene inheritance of 50% from the broodstock to the offspring of this transgenic fish possible it to be transmitted to the offspring through the reproduction of the broodstock fish.

Based on the alignment of *CgGH* sequences from transgenic mutiara catfish, $G_0$, $G_1$, $G_2$, and $G_3$ can exhibit sequence similarities among all four generations, reflecting the consistency in *CgGH* inheritance in each generation (Degani et al., 2006; Pinheiro et al., 2008; Rajesh & Majumdar, 2007). Confirmation of the presence of *CgGH* in $G_1$, $G_2$, and $G_3$ transgenic mutiara catfish broodstock to ensure transgene transmission in each reproductive offspring. It is important to evaluate the similarity of functional domains of *CgGH* sequences in the offspring of this transgenic catfish to achieve mass production of transgenic catfish lines. The similarity of functional domains in the *CgGH* sequence may indicate the similarity of the *GH* protein molecules formed between the four generations of transgenic mutiara catfish.

## Materials and Methods

### Fish used in this study

Fish used this study were kept at the Aquaculture Laboratory Universitas Padjadjaran in circular tanks containing 1,000 liters of freshwater. Fish were adapted to 12 hours' daylight photoperiod conditions. Rearing conditions were kept at optimal water quality which were 27 ± 1℃, pH 6–6.5 and optimal dissolved oxygen (continuous aeration). As much as 10% of water was replaced with freshwater while in the same time faeces and feed left over were siphoned. All fish were daily fed with commercial feed (Hiprovite 781) with the dose of 3% total biomass. Fish used for the research were the $G_0$ ♀broodstock (weighing 1,600 g, total length of 58 cm and age of 12 months), $G_1$ ♀ broodstock (weighing 1,200 g, total length 55 cm and age of 12 months), $G_2$ ♀ (weighing 950 g, total length of 50 cm and age of 11 months), $G_3$ ♀ (weighing 910 g, total length of 46 cm and age of 11 months).

### Isolation of *CgGH*

RNA was isolated from 10 mg of fish tail fin tissue of $G_0$, $G_1$, $G_2$, and $G_3$ was isolated using the RNeasy mini kit (Qiagen, Venlo, Netherlands), following the kit instructions for RNA isolation. Sampling was carried out on the tail fin of the broodstock, not taken from gonadal tissue or liver because the broodfish was used for the production of the next generation. Transgenes (including *CgGH*) can be inserted in fish tissues along the head to tail region (Rahman & Maclean, 1999; Uh et al., 2006). Synthesis of cDNA and RT-PCR (semi-quantitative PCR) of *CgGH* were performed using My Taq OneStep RT-PCR (Bioline, London, UK) with the following cycling programme: 48℃ for 20 min; 40 cycles of 95℃ for 1 min, 95℃ for 10 s, 60℃ for 30 s and 72℃ 30 s; and 72℃ for 5 min. Confirmation of transgenic catfish was achieved by detecting the presence of a PCR product of approximately 600 bp using primers GH-F (5′-ATGGCTC-GAGTTTTGGTGCTGCT-3′) and GH-R (5′-CTACAGAGT-GCAGTTGGAATCCAGGG-3′) (Buwono et al., 2021; Zhang et al., 2009).

### Sequencing of *CgGH*

The *CgGH* gene amplicon (PCR product of $G_0$, $G_1$, $G_2$ sample A and B, $G_3$ sample A, B, and C) was then sequenced using the Sanger sequencing method through service 1st BASE (Molecular Biology Company) Singapore because the amplicon size is less than 1,200 bp (Sanger et al., 1977). Nucleotide sequence similarity analysis of the *CgGH* gene in $G_0$, $G_1$, $G_2$, and $G_3$ transgenic mutiara catfish was performed using the BioEdit 7.0.5.3 software (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html) to identify the consensus sequences of the four *CgGH* inserts in the generations of transgenic catfish. To the *CgGH* nucleotide similarity analysis, we used the consensus sequence, to equate the complementary sequence from the forward direction, so that this consensus sequence could be used to *CgGH* sequence similarity analysis to $G_0$, $G_1$, $G_2$, and $G_3$ as written in Table 1 and Fig. 2 .

**Table 1. Consensus similarity of the *CgGH* sequences between transgenic mutiara catfish[1)]**

| Nucleotide (nt) | *CgGH*-$G_1$ (%) | *CgGH*-$G_2$ (%) | *CgGH*-$G_3$ (%) |
|---|---|---|---|
| *CgGH*-$G_0$ | 93.76 | 93.78 | 95.15 |
| *CgGH*-$G_1$ | | 97.06 | 96.42 |
| *CgGH*-$G_2$ | | | 96.29 |

[1)] analyzed with BioEdit 7.0.5.3 (pairwise alignment).
GH, growth hormone.


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### Functional domain analysis of the *CgGH* sequence

The presence of *CgGH* in transgenic catfish was confirmed by aligning the gene sequence with the *Clarias gariepinus GH* coding sequence (cds) in GenBank to determine the similarity of the nucleotide base sequences encoding the fish *GH* protein (Peyush et al., 2000). *CgGH* nucleotide base sequence similarity among the generation of transgenic mutiara catfish was identified using the BLAST (Basic Local Alignment Search Tools) programme (http://www.ncbi.nlm.nih.gov/BLAST/). The functional domains of *CgGH* sequences, especially amino acid residues, were analysed using SWISS-MODEL (https://swissmodel.expasy.org/) to map the molecular structure of fish *GH* protein.

## Results

### *CgGH* amplicons and *CgGH* consensus sequence

ThePCR analysis results showed that *CgGH* was amplified using the primers GH-F and GH-R in the test samples $G_0$, $G_1$, $G_2$, and $G_3$ and had a size of approximately 600 bp (Fig. 1). The size of this amplified sequence was also not markedly different from that of the *Clarias gariepinus GH* sequence in GenBank (accession nos. EF411172 and MN249238.1), with sizes of 603 and 615 bp, respectively. This indicated that the *CgGH* gene sequence present in the four generations of transgenic mutiara catfish is the *GH* gene of *C. gariepinus*. Consensus sequence analysis results of the four generations of transgenic mutiara catfish also showed high nucleotide similarity of *CgGH* consensus sequences (Table 1 and Fig. 2).

### *CgGH* functional domain

Considering that $G_0$ transgenic mutiara catfish is a germline transmitter and $G_1$ fish is produced from a transgenic × non-transgenic cross, functional domain analysis of *CgGH* (forward direction) was conducted between $G_2$ transgenic mutiara catfish resulting from crossing A (sample A coded 1st_BASE_3044995 and sample B coded 1st_BASE_3044997) (Buwono et al., 2019a) with $G_3$ transgenic mutiara catfish sample A (transgenic female-1 × transgenic male-1), B (transgenic female-2 × transgenic male-2) and C (transgenic female-3 × transgenic male-3) (Buwono et al., 2021) using the Sanger method for the sequence process while the alignment used the CLUSTALW BioEdit software. The results of the analysis showed that the *CgGH* gene sequences (forward direction) of thw $G_3$ fish (samples A, B, and C) with $G_2$ fish showed high similarity (96.21%, 96.38%, and 95.91%, respectively).

Differences in *CgGH* nucleotides between $G_2$ (code 1st_BASE_3044995 sample A) and $G_3$ fish sample A (1st_BASE_3527728_A) occurred of 16 nucleotides in the first

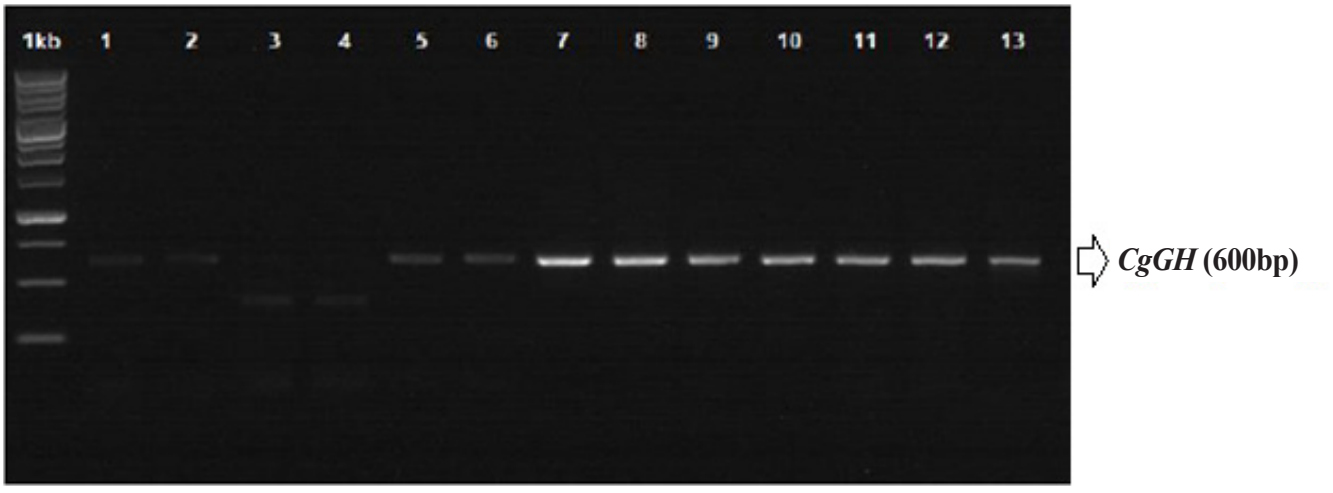


**Fig. 1. Electropherogram of the $G_0$, $G_1$, $G_2$, and $G_3$ transgenic mutiara catfish (marked by the arrow *CgGH* 600 bp).** 1kb, DNA ladder 1kb; 1, sample from fin of $G_0$ ♂ transgenic broodstock; 2, sample from fin of $G_0$ ♂ transgenic broodstock; 3, sample from fin of $G_0$ ♀ non-transgenic broodstock; 4, sample from fin of $G_0$ ♂ non-transgenic broodstock; 5, sample from fin of $G_0$ ♀ transgenic broodstock; 6, sample from fin of $G_0$ ♂ transgenic broodstock; 7, sample from fin of $G_1$ ♀ transgenic broodstock; 8, sample A from fin of $G_2$ ♀ transgenic broodstock sample B; 9, sample B from fin of $G_2$ ♂ transgenic broodstock; 10, sample A from fin of $G_3$ transgenic broodstock; 11, sample B from fin of $G_3$ transgenic broodstock; 12, sample C from fin of $G_3$ transgenic broodstock; 13, pCMV-*CgGH* plasmid; GH, growth hormone.

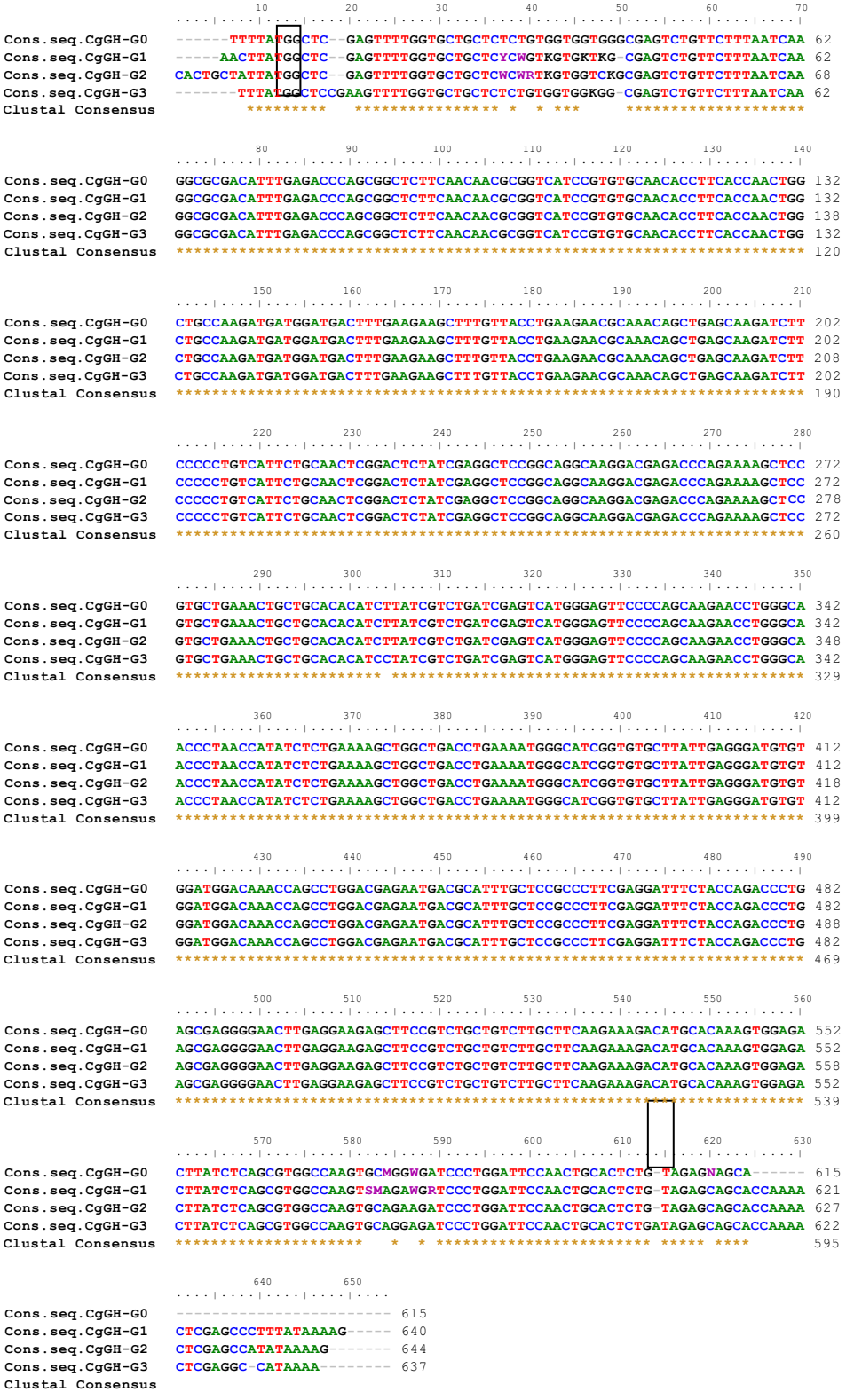


**Fig. 2. Consensus alignment of the *CgGH* sequences in $G_0$, $G_1$, $G_2$, and $G_3$ (ClustalW multiple alignment analysis BioEdit).** ATG, start codon; TAG, stop codon; GH, growth hormone.



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sequence, and in nucleotides 594, 597, 600, 606 and 610 (Fig. 3A). At nucleotide numbers 594, 597, and 600 of $G_2$ fish there was a lack of cytosine, thymine and cytosine residues, and at nucleotide 606 of $G_3$ fish, there was a lack of thymine residue. The start codon (ATG) and stop codon (TAG) were located at nucleotides 18–20 and 576–578, respectively. In $G_3$ fish of sample B (1st_BASE_3527730_B), there was a difference of 16 nucleotides in the first sequence of *CgGH* with $G_2$ fish (code

(A)


**Fig. 3.** Continued.

(B)


**Fig. 3.** Continued.



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**Fig. 3. *CgGH* functional domain in $G_2$ and $G_3$ transgenic mutiara catfish.** (A) Forward alignment of the *CgGH* in $G_2$ (1st_BASE_3044995 sample (A) with those in $G_3$ sample A (1st_BASE_3527728_A). (B) Forward alignment of the *CgGH* in $G_2$ (1st_BASE_3044997 sample (B) with those in G3 sample B (1st_BASE_3527730_B). (C) Forward alignment of *CgGH* in $G_2$ (1st_BASE_3044995 sample (A) with those in G3 sample C (1st_BASE_3527732_C). CNSDSIEAPAGKDETQKSSVLKLLHTSYRLIESW, somatotropin-1 site; CFKKDMH KVETYLSVAKC, somatotropin-2 site; LFNNAVIRVQHLHQ LAAKMMDDFEEALLP, Helix-1; TSYRLIESWEFPSKNLGNPNHIS, Helix-2; GIGVLIEGRVDGQTSLDENDAFAPPF, Helix-3; KDMHKVETYL SVAKCRRSLDSNCT, Helix-4; NCTL, N-Glicosylation; CCCC, 4-Cysteine residue; GH, growth hormone.

1st_BASE_3044997 sample B). In $G_3$ fish, nucleotides 600–602 lacked three thymine residues and nucleotide 609 lacked a guanine residue (Fig. 3B). The start codon, ATG, was located at nucleotides 17–20, and the stop codon, TAG, at nucleotides 575–577. The results of the alignment of the *CgGH* sequence of $G_2$ fish (code 1st_BASE_3044995 sample A) with that of the $G_3$ fish of sample C (1st_BASE_3527732_C) showed a difference of 14 nucleotides in the first sequence, where the start codon was located at nucleotides 18–20 and the stop codon at nucleotides 571–573 (Fig. 3C). In sample $G_3$, nucleotides 600, 601, 604, and 608 lacked adenine, thymine, adenine and guanine residues, respectively. The amino acid residues in Figs 3A–C are underlined and marked with coloured boxes after conversion using SWISS-MODEL to form a three-dimensional structure of the *GH* protein molecule.

Overall alignment of the sites of somatotropin-1 (nucleotide 186–287), somatotropin-2 (nucleotide 492–548), N-glycosylation (nucleotide 564–575), 4-residue cysteine (nucleotide 181, 493, 544, 598), helix -1 (nucleotide 56–143), helix-2 (nucleotide 261–329), helix-3 (nucleotide 354–431) and helix-4 (nucleotide 501–572) were contained in the *CgGH* sequence in both G2 of sample A (1st_Base_304495) and G3 fish sample A (1st_BASE_3527728_A) were located on the same nucleotide (nt.) (Fig. 3A). Meanwhile, the alignment of the *CgGH* functional domains between in G2 of the sample B (1st_Base_3044997) and the G3 fish sample B (1st_Base_3527730) were located on different nucleotides. In G2 of the sample B, the somatotropin-1 site at nt. 186–286, somatotropin-2 at nt. 491–547, N-glycosylation at nt. 563–574, 4-cysteine residue at nt. 186, 492, 543, 562, helix-1 at nt. 56–141, helix-2 at nt. 260–328, helix-3 at nt. 353–430 and helix-4 at nt. 500–574, while in G3 of the sample B, the somatotropin-1 site at nt. 187–287, somatotropin-2 at nt. 492–548, N-glycosylation at nt. 564–575, 4-cysteine residue at nt. 187, 493, 544, 563, helix-1 at nt. 57–142, helix-2 at nt. 261–329, helix-3 at nt. 354–431 and helix-4 at nt. 501–575 (Fig. 3B). The alignment of the position of the *CgGH* functional domain between in G2 of the sample A (1st_Base_3044995) and the G3 fish sample C (1st_Base_3527732) was also located on different nucleotides. Somatotropin-1 (nt. 187–287), somatotropin-2 (nt. 492–548), N-glycosylation site (nt. 564–575), 4-cysteine residue (nt. 187, 497, 544, 568), helix-1 (nt. 57–143), helix-2 (nt. 261–329), helix-3 (nt. 354–431) and helix-4 (nt. 501–572) in G2 of the sample A, while in G3 fish sample C is located at different nucleotides, namely somatotropin-1 (nt. 183–283), somatotropin-2 (nt. 488–544), N-glycosylation (nt. 560–571), 4-cysteine residue (nt. 183, 493, 540, 564), helix-1 (nt. 53–139), helix-2 (nt. 257–325), helix-3 (nt. 350–427) and helix-4 (nt. 493–564) (Fig. 3C).

## Discussion

The presence of *CgGH* in four generations of transgenic mutiara catfish indicates that the exogenous *GH* gene is inherited stably in each generation of *GH*-transgenic catfish. The rate of *CgGH* transmission in $G_2$ transgenic mutiara catfish was 50% and increased in $G_3$ to 70% (Buwono et al., 2021). This indicates the potential for increased *CgGH* transmission in crosses between G4 transgenic catfish as a consequence of the stability of *CgGH* inheritance in transgenic catfish offspring. Homozygous transgenic fish need to be produced to obtain stable transgene inheritance (Iwai et al., 2009). Homozygous fish were produced when crossing between heterozygous $G_2$ mud loach (*Misgurnus mizolepis*) transgenic fish (carrying CMV-H2B-GFP) to produce 50% homozygous $G_3$ progeny (Nam et al., 2000).

To confirm its stable inheritance, the stability of the *CgGH* sequence needs to be analysed for similarity as an indication that its copies in $G_0$, $G_1$, $G_2$, and $G_3$ transgenic mutiara catfish have high similarities between generations. Yang et al. (2018) also explained that the coding region in the gene sequences are generally conserved and have high similarities with those of related fish species. There was a high homology of the gene encoding the hormone oxytocin, which regulates *GH* release in ricefield eel (*Monopterus albus*), being 84.6% identical to that of Anguilliformes (*Anguilla bicolor*). High homology was shown in the gene sequences encoding *GH* in *C. gariepinus* $G_0$ fish compared with those in $G_1$ fish (93.76%), $G_2$ fish (93.78%) and $G_3$ fish (95.15%), indicating that the nucleotide sequence of *CgGH* did not change much and was conserved (Table 1). In addition, there was a tendency for an increase in the homology of *CgGH* sequences between $G_1$ and $G_2$ and $G_3$ fish by 97.06% and 96.42%, respectively. The results of another study also indicated that the *GH* sequences of blue gourami (*Trichogaster trichopterus*) and pearl gourami (*T. leeri*) showed high homology as conserved sequences, at 97% and 96%, respectively (Degani et al., 2006). The same study also showed that the Indian catfish (*Heteropneustes fossilis*) *GH* sequence had high homology (98%) with the Siluridae and Clariidae groups (Anathy et al., 2001). It was shown that the *CgGH* sequence was conserved with high homology (93.76%–97.06%) in four generations of transgenic mutiara catfish, which was required for stable exogenous *GH* inheritance in the transgenic fish generations. The consistency


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of *CgGH* consensus sequence homology in $G_0$, $G_1$, $G_2$, and $G_3$ fish, especially at the start codon (ATG) and stop codon (TAG), is shown in Fig. 2, indicating that the coding sequence of *CgGH* is conserved in the generation of transgenic mutiara catfish.

Functional domains are conserved sequences that characterise a particular gene group consisting of 40–700 amino acid residues (Xiong, 2006). Generally, five functional domains characterise fish *GH* sequences (somatotropin-1 and somatotropin-2, N-glycosylation, four α-helix structure and four cysteine residues), which are homologous (Anathy et al., 2001; Pinheiro et al., 2008). The results of SWISS-MODEL processing showed that the four characteristic sites of the *GH* molecule (somatotropin-1, somatotropin-2, α helix-1 to α helix-4, N-glycosylation and four cysteine residues) in the *CgGH* sequences of $G_2$ and $G_3$ fish were conserved and located at the same base pairs. According to Anathy et al. (2001) and Pinheiro et al. (2008), α-helix-1 is encoded by amino acid residues LFNNAVIRVQHLHQLAAKMMDDFEEALLP (underlined in blue), α helix-2 by TSYRLIESWEFPSKNLGNPNHIS (underlined in gold), α helix-3 by GIGVLIEGRVDGQTSLDENDAFAPPF (underlined in red) and α helix-4 by KDMHKVETYLSVAKCRRSLDSNCT (underlined in green). These four helix structures are bound by four cysteine residues (marked with red circles). The α-helix site is a domain that indicates the formation of a secondary structure of *GH* protein, namely, α-helix sites 1 to 4, which are important for the functional activity of these hormones (Pinheiro et al., 2008). Generally, this domain has relatively high homology among the *GH* of freshwater fish (including the catfish group). The amino acid residues CNSDSIEAPAGKDETQKSSVLKLLHTSYRLIESW (marked with purple box) are the functional domain of somatotropin-1, and the amino acid residues CFKKDMHKVETYLSVAKC (marked with yellow box) are the functional domain of somatotropin-2. The existence of these two functional domains is related to *GH* activity and synthesis of insulin-like growth factor-1and prolactin for tissue growth. The somatotropin sites (1 and 2) present in the *GH* sequences of transgenic mutiara catfish ($G_2$ and $G_3$) and in Indian catfish (Anathy et al., 2001) are both conserved. N-glycosylation site domains encoded with NCTL amino acid residues (marked with pink boxes) in *GH* protein sequences were also found to be conserved in fish (including transgenic mutiara catfish) and act as signals for protein transport to the cell surface (Degani et al., 2006). Another important site is four cysteine residues (C) in the protein-coding *GH* gene, which are involved in the formation of two disulphide bonds for the structural integrity and biological activity of the hormone (Anathy et al., 2001); the five functional domain sites were found to be conserved in *CgGH* of $G_2$ and $G_3$ transgenic mutiara catfish (Figs 3A–C). The molecular structure of *GH* protein in $G_3$ mutiara catfish (samples A–C), shown in Fig. 4A–C, was confirmed as a *GH* protein molecule (Swiss model analysis), as shown in the Siluriformes group *GH* protein molecule (Vaz et al., 2010).

These results indicate that *CgGH* from $G_s$ broodstock had been successfully inherited in up to three generations ($G_1$, $G_2$, and $G_3$) with a high degree of similarity and confirmed as fish *GH* protein. This verification was based on analysis of the functional domains of fish *GH* molecules composed of somatotropin-1 and somatotropin-2, four-helix structures, N-glycosylation and four cysteine residues that bind to the helix structure (Pinheiro et al., 2008; Vaz et al., 2010).

## Conclusion

*CgGH* (600 bp) can be inherited in $G_1$, $G_2$, and $G_3$ transgenic mutiara catfish through reproduction. The consensus sequence similarity of *CgGH* between transgenic fish in $G_1$ to $G_3$ ranged

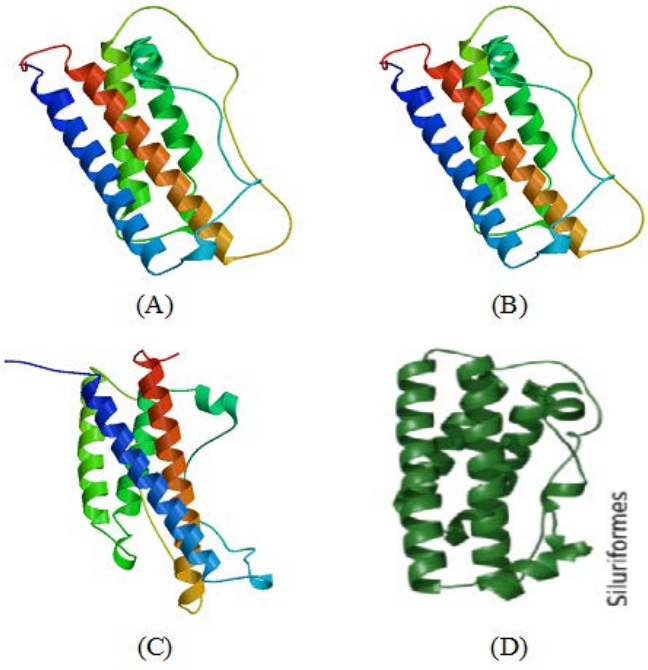


**Fig. 4. Three-dimensional model of the GH protein molecule from $G_3$.** (A) $G_3$ sample A; (B) $G_3$ sample B; (C) G3 sample C; (D) GH protein molecule from *Siluriformes*. Adapted from Vaz et al. (2010) with CC-BY -NC-SA. GH, growth hormone.

from 93.76% to 97.06%, and they had five fish *GH* protein functional domain sites (somatotropin-1, somatotropin-2, four α-helix, N-glycosylation and four cysteine residues).

### Competing interests

No potential conflict of interest relevant to this article was reported.

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### Availability of data and materials

Upon reasonable request, the datasets of this study can be available from the corresponding author.

### Ethics approval and consent to participate

This article does not require IRB/IACUC approval because there are no human and animal participants.

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