FOREIGN GROWTH HORMONE GENE TRANSMISSION AND EXPRESSION IN F1 TRANSGENIC BETTA FISH (Betta imbellis)

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ABSTRACT
The economical value of betta fish (Betta imbellis) is mostly determined by its body color and size. The present study was conducted to evaluate PhGH transmission, mRNA expression level and production performance of the first generation of transgenic betta (F1). Three males and three females of transgenic F0 were mated with non-transgenic to produce F1 generation, and three pairs of non-transgenic betta as control were used. PhGH transgene transmissions were analyzed using PCR method, mRNA expression was analyzed by RT-PCR and production performance was evaluated based on the fertilization level, hatching level, body length and weight. The percentage of F1 transgenic fish carrying PhGH gene was 62.5±5.89%, and mRNA expression varied amongst F1 transgenic fish. Fertilization (FR: 91.7±7.5%) and hatching rates (HR: 91.3±9.4%) of transgenic fish were higher than those of non-transgenic (FR: 70.0±10.0%; HR: 70.8±4.5%). At five-months-old, the mean body weight of males and females of transgenic F1 were 1.47 and 1.76 times higher, whereas body length were 1.32 and 1.25 times higher than those of non-transgenic fish, respectively.

Keywords: Betta imbellis, transmission, expression, gene encoding growth hormone

INTRODUCTION
The purpose of gene transfer technology in aquaculture is to obtain higher yield product with some expected characteristics. Today, the most successful transgene, which is also considered as the first one used in aquaculture. Transgene contains the gene construct encoding growth hormone (GH). Growth hormone over-expression has been proven to drastically increase growth of various fish species including common carp (Cyprinus carpio), goldfish (Carassius auratus gibelio) (Zhu and Chen, 1992, Hinit and Moav 1999), channel catfish (Ictalurus punctatus) (Dunham et al., 2002), tilapia (Oreochromis niloticus) (Martinez et al., 1996, Kobayashi et al., 2007), Atlantic salmon (Salmo salar) (Da et al., 1992), coho salmon (Oncorhyncus kisutch) (Devlin et al., 1994), Arctic char (Salvelinus alpinus) (Pitten and Moav 1999), rainbow trout (Oncorhyncus mykiss) (Devlin et al., 2001), mud loach (Misgurnus mizolepis) (Nam et al., 2001), catfish (Clarias batrachus) (Gusrina et al., 2009) and pangasius catfish (Pangasianodon hypophthalmus) (Dewi et al., 2014).

At present, the research of GH gene transfer is still limited to fish for consumption, including seaweed (Rajamuddin et al., 2016; Triana et al., 2016). The success of gene transfer in ornamental fish has been limited to only a few species including zebra and medaka (Lamason et al., 2001), mostly used as model fish in the introduction of the gene encoding green fluorescent protein in studies related to the fish developmental biology and improvement of ornamental quality (Lamason et al., 2005).

Betta fish (Betta imbellis) is one of freshwater ornamental fish that has high economical value in the export market (IBC, 2013). One of the efforts to increase the commercial value of betta fish is by producing large sized betta fish, or called as “giant betta”. The utilization of gene transfer technology could be expected to contribute on the production of fast-growing betta fish. Common methods used in the generation of transgenic fish are microinjection, electroporation and transfection. Considering the size of the betta egg is small, the transfection method is potentially applied as described by Szlelei et al. (2005). In our previous study, we have generated F0 transgenic betta fish which carried GH gene of pangasius (PhGH) (Kusrini et al., 2016). The result of this study showed that seven-month F0-generation betta fish could reach an average body weight of 5.1g, which was higher than that of the control (3.7g). In this regard, the present study aimed at evaluating the transmission and the expression of PhGH gene in F1 generation of transgenic betta fish.

MATERIALS AND METHODS
Production of Transgenic F1: F0 transgenic betta fish carrying PhGH gene is produced according to the method described by Kusrini et al. (2016). The broodstock was reared individually in 4.5-L glass aquarium in indoor hatchery of the Research Institute for Ornamental Fish, Depok, Indonesia. The fish were fed with blood-worm, twice a day
ad libitum. Matured brood-stock, indicated by the presence of foam in male and round, reddish and bulging belly in female, was paired in the spawning aquarium. Three cross-sets were made, i.e. 1) a cross between transgenic male and non-transgenic female, 2) non-transgenic male with transgenic female, and 3) non-transgenic fish pair as a control. Three different pairs of broods were prepared in each cross as the replication.

After spawning, male and female broodstocks were removed, and the eggs were hatched in the spawning aquarium. Fertilization level was calculated 4 hours after fertilization using the formula: [(number of eggs fertilized/total eggs) × 100]. White color eggs indicated unfertilized eggs. Furthermore, hatching level was calculated 24 hours after fertilization using the formula: [(number of eggs hatched/number of eggs fertilized) ×100].

Water exchange was carried out 30% of the total volume, starting from day 3 post hatching (dph). At 4 dph, the larvae were transferred into a container of 50x60x90cm³. During larvae rearing, siphoning was performed daily and water exchange at 30% of the total volume was conducted once a week.

Fish Maintenance: Larvae were reared in the styrofoam box of 34x25x30cm³. From 4 to 14 dph, the larvae were fed with Artemia nauplii and followed by moina until 30 dph and bloodworm until the end of experiment, two times a day. After 2 months old, the males were reared individually in an aquarium of 20x20x15cm³, while the females were communally reared at a density of 10 fish/L until PCR analysis was performed. The fish were fed on the frozen bloodworm twice a day to satiation. Water exchange at a level of 30% was performed once a week to keep water quality in good condition. The fish were reared for five months post-hatching. Individual weight of betta fish was measured monthly, and the biomass was calculated at the end of experiment. Survival level was calculated at the end of experiment.

Statistical analyses: Growth and survival of fish were analyzed by analyses of variance (ANOVA). Significant difference was subsequently assessed using post hoc Duncan test at a significant level of 0.05. Statistical analyses were performed using a statistical program SPSS vr.17.

Analysis of PhGH gene transmission: Identification of F1 transgenic fish that carries PhGH gene was done using PCR. After the fish was 3 months old, genomic DNA was extracted from the caudal fin using GeneJET Genomic DNA Purification Kit (Thermo Scientific Lithuania, EU) according to manufacturer instruction. The extracted DNA was dissolved in 100 mL of elution buffer.

PCR amplification was performed using maxima hot start green PCR master mix 2x (Thermo Scientific dreamtaq). The composition of PCR reaction was 12.5μL of master mix, 1 μL of PhGH primer at a concentration of 20 pmol (F3-PhGH: 5’TCT TTA GTA AAG GCG CCA CAT TCG AGA-3’ and R3-PhGH: 5’-CGA TAA GCA CGC CGA TGC CCA TTT TCA-3’ (Dewi et al., 2010), 2μL of genomic DNA (200 ng/μL), and nuclease-free water until a total volume of 25μL. PCR amplification of 35 cycles was conducted with pre-denaturation at 94°C for 5min; denaturation at 94°C for 30s, annealing at 58°C for 30s, extension at 94°C for 30s, and final extension at 72°C for 3 min.

β-actin gene was used as an internal control of loading DNA. Primers used were F: 5’- TAT GAA GGT TAT GCT CTG CCC -3’ and R: 5’- CAT ACC CAG GAA AGA TGG CTG-3’ (Alimuddin, unpublished). PCR amplification of 35 cycles was performed with the following sequences: pre-denaturation at 94°C for 3 min, denaturation at 94°C for 30s, annealing at 62°C for 30s, extension at 72°C for 30s and final extension at 72°C for 3 min.

Subsequently PCR product was separated by electrophoresis using 1% agarose gel. Documentation was done using UV transilluminator gel doc. Amplification target of PhGH gene and β-actin were 334 bp and 300 bp, respectively. Analysis of PhGH mRNA expression: Analysis of PhGH mRNA expression level in F1 transgenic was done using semi-quantitative RT-PCR method. Extraction of total RNA from caudal fin was performed using Tri Reagent Kit (Molecular Research Center, Inc. Cincinnati, OH) following the manufacturer’s instruction. cDNA was synthesized using Transcriptor First Strand cDNA Synthesis Kit (Roche) with a primer dT3’RACE-VECT (5’GTA ATA CGA ATA ACT ATA GGG CAG GCG TGG TCG ACG GCC CGG GCT GGT TTT TTT TTT TTT-3’). Amplification process was done in 30 cycles using the same primer utilized in the identification of transgenic individual. PCR product was separated using electrophoresis, documented with gel documentation system (Biorad) and evaluated descriptively. Quantification of expression was done by calculating the ratio of PhGH/β-actin.

RESULTS

Fertilization and hatching rates: Fertilization (FR) and hatching levels (HR) of F1 transgenic fish (A and B) were 30.5% and 28.9% higher (P< 0.05) than those of non-transgenic control (C), respectively (Figure 1). The fertilization and hatching levels among transgenic crosses (A and B) were not significantly different (p>0.05).
Growth performance: At 3 and 5 months after hatching, the total body length (BL) and body weight (BW) of male and female transgenic fish from crosses A and B were higher (P<0.05) than those of non-transgenic control (C) (Figure 2). Five months after hatching, the BL and BW of transgenic cross A were 1.36 and 1.65 times higher than those of non-transgenic control (C), while BL and BW of transgenic cross B were 1.25 and 1.76 times higher compared to those of non-transgenic fish cross (C) respectively. Furthermore, BL of male and female transgenic fish was 1.31 and 1.25 times higher than non-transgenic fish, while BW of male and female transgenic fish was 1.45 and 1.72 times higher than non-transgenic fish, respectively.

Figure 1: Fertilization (FR) and hatching rates (HR) of F1 transgenic fish eggs from crossovers between male F0 transgenic and female non-transgenic broodstock (A), male non-transgenic and female transgenic (B), and non-transgenic broodstock (C).

Figure 2: Average body weight of male and female transgenic betta fish Betta imbellis 5 months of rearing period. A= F1 transgenic fish from crossbreeding of male transgenic F0 and female non-transgenic; B= F1 transgenic fish from crossbreeding of male non-transgenic and female transgenic; C= non-transgenic control. Different letters above the bars with the same color indicate significant differences (p<0.05).

Figure 3: Average body length of male and female transgenic betta fish Betta imbellis after 5 months of rearing period. A= F1 transgenic fish from crossbreeding of male transgenic F0 and female non-transgenic; B= F1 transgenic fish from crossbreeding of male non-transgenic and female transgenic; C= non-transgenic control. Different letters above the bars with the same color indicate significant differences (p<0.05).
Survival: In this study, the survival of F1 transgenic betta fish and non-transgenic control after maintained for 5 months were similar (P>0.05) with the level for transgenic cross A, transgenic cross B and non-transgenic control C were 58.90±2.18%, 59.14±0.99% and 54.21±3.58%, respectively (Figure 4).

Figure 4: Survival of transgenic betta fish Betta imbellis after 5 months of rearing period. A= F1 transgenic fish from crossbreeding of male transgenic F0 and female non-transgenic; B= F1 transgenic fish from crossbreeding of male non-transgenic and female transgenic; C= non-transgenic control. The same letters above the bars with the same color indicate no significant difference (p<0.05)

Transmission of PhGH gene in transgenic F1 generation: A representative results of F1 transgenic identification by PCR method is shown in Figure 5. By using PhGH specific primer, transgenic individuals showed a 334 bp of amplification product, which was the same amplification size compared to pCcBA-PhGH plasmid as the template (Fig. 5A dan 5B). No PCR product of 334 bp was detected in non-transgenic control (Fig. 5C), indicating the specificity of the primers. In total, there were 58.89% and 66.67% of F1 transgenic progenies derived from crosses A and B, respectively.

Figure 5: Detection of PhGH gene in transgenic betta fish F1 generation produced from a cross between male non-transgenic and female non-transgenic (A), male non-transgenic and male transgenic (B), and non-transgenic control (C). M: DNA marker of 100-bp, no 1-8: individual fish number, P: PCR product with pCcBA-PhGH plasmid as template, N: without DNA template.
Expression of PhGH mRNA in transgenic F1 generation: Based on the result of RT-PCR (Fig. 6), all 53 individuals of cross A and 60 individuals of cross B showed PhGH mRNA expression. However, their expression levels were varied (Fig. 6 bottom). Furthermore, the mean PhGH mRNA expression level in the 5-month-old fish in cross B group was higher compared to fish in cross A group, both in male and female fish.

![Figure 6](image)

**DISCUSSION**

The results of FR (91.5%) and HR (91.25%) of transgenic betta fish which were higher than non-transgenic control (FR 70%, HR 70.8%) may be affected by environmental factors considering that male broodstocks were maintained in solitary, although feed was given at the same amount. Differences in environmental conditions are expected to influence spermatogenesis and oogenesis. Another factor that may consider is the total sperm count of transgenic fish which might be higher thus resulted in increase of egg fertilization, and this remains to be proved in further research. Dadras et al., (2011) reported that the density and pH of the sperm affected Persian sturgeon (*Acipenser persicus*) fertilization (79.1%) and hatching (62.5%) levels. In contrast, Kurdianto (2014) reported similar hatchability of second generation transgenic carp (94.41%) and non-transgenic control (95.09%), whereas Muir and Howard (1999) noted that there was no difference in fertilization levels between transgenic and non-transgenic carp.

The hatching levels obtained in the present study was higher than result of study on white shrimp *Litopenaeus vannamei* of which the transgenic hatchability was 50-60% (Sun et al., 2005), and on black tiger shrimp which obtained hatchability of 20.3-22.3% (Parenrengi et al., 2011). The eggs hatching levels in this study was also higher than those recorded in medaka of 70% (transgenic) and 78% (non-transgenic) (Winkler et al., 1991), in catfish of 55-93.3% (Ath-Than 2007), and in sea bream *Pagrus major* of 53-63% (Kato et al., 2007). Moreover, by applying electroporation method, FR of white shrimp in research conducted by Sun et al., (2005) was 25-35% (transgenic) and 50-60% (non-transgenic) and FR of pangasius fish (*Pangasius sp.*) was 57.11% (transgenic) and 84.11 (non-transgenic) (Dewi et
al., 2013). This difference may be due to the use of different species, the quality of broodstocks and eggs obtained, and the method of spawning or hatching used.

Exogenous PhGH gene expressed in betta fish did not affect the survival of both larvae and juvenile (Fig. 4). However, several studies found that the addition of growth hormone can increase the stamina of fish since GH is able to increase the cells that are competent in the immune system (Gala, 1991, Sakai et al., 1997, Harris and Bird, 2000).

This study used pCcBA-PhGH gene construct. The same gene construction has also been used to produce transgenic pangasius. Dewi et al. (2014) reported that the growth of F1 PhGH gene transgenic pangasius maintained for five months in the pond increased by 47.5%. This finding showed that cis-element of the common carp β-actin promoter (pCcBA) can be recognized by the trans-element of the host (Hinits and Moav 1999), resulting in higher growth stimulation (Fig. 2 and 3).

In this research, the increase in the growth of transgenic betta F1 generation was ranged 25% to 36% for body length, and 65% to 76% for body weight compared with non-transgenic fish (Fig. 2 and 3). The increased growth of transgenic F1 B. imbellis was comparable to the growth of carp transferred MMThGH gene (Wu et al., 2003). The average weight of F0 generation MThGH transgenic carp of five months old, reached 2.75 kg, while the weight of the largest control carp was 1.4 kg (Wang et al., 2001). The average growth of F1 generation MThGH transgenic carp was 42-80% faster than that of control (Wu, 2003). However, using different vector expression and host, introduction of mBA-tIGH gene to cat-fish resulted in the fish growth 7 times higher in F1 generation compared with non-transgenic control (Gusrina 2011). Moreover, research conducted by Zhang et al. (1990) on F0 transgenic carp carrying the RSVLTRtGHc gene construct resulted in various fish body sizes which were mosaic with an average body size of 22% larger than its non-transgenic sibling. In F0 Northern pike intro-duced by RSVLTR-bGHC and RSVLTR-csGHc gene construct, expression of GH gene derived from cow and Chinook salmon was detected in blood serum and was able to stimulate the growth. The average weight of F0 transgenic Northern pike increased by 30% with high mosaic level (Gross et al., 1992). These variations of growth stimulation are affected by several factors namely different types of fish, GH and different promoters used.

Integration of transgene in genome generally occurs after one-cell stage thus resulting in mosaic condition (Volckaert et al., 1994). Mosaics may occur in somatic cells and gonad cells. As shown in Fig. 5, not all F1 progenies from crosses A (58.89%) dan B (66.67%) carried the PhGH transgene. This confirmed that the transgenic F0 fish were mosaic. Transgene transmission in F1 transgenic betta fish was higher than the transmission of F1 transgenic catfish, which were 27.69% for female and 46.34% for male, respectively (Maniz et al 2014). Relatively similar transgene transmission levels have been found in GH transgenic carp, 63% (Moav et al, 1995), and in zebrafish of 53% (Sheela et al., 1998). A slightly higher result had been reported by Wu et al. (2003) of 72-88%, while Inou et al., (1990) found that all of F1 medaka fish carrying the transgene. Transgene can be integrated in one or more locations in chromosome thus resulting in variation of transgene transmission (Tewari et al. 1992).

As shown in Fig. 6, all F1 transgenic fish expressed the PhGH transgene. However, relatively high variation of expression levels within each cross was observed. Variation of transgene expression levels in F1 transgenic fish have been reported by Alimuddin, Kiron, Sato, Takeuchi, Yoshi-zaki (2008). This variation might be caused by the possibility that the transgene may have been integrated into chromosomal sites that modify its expression due to positional effects (Dobie, Lee, Fantes, Graham, Clark, Springbett, Lathe, McClennag-han, 1996). Consequently, fish strains having a high transgene expression level should be selected in order to generate F2 transgenic line in the next study.

CONCLUSION

Average transmission of PhGH transgene to the first generation of transgenic betta fish was 62.5%. The mRNA expression levels and growth stimulation were varied among F1 transgenic fish. At 5-month-old, the body length and body weight of transgenic F1 generation were 1.47 and 1.32 for male’s fish, and 1.76 and 1.25 times higher for females than non-transgenic control, respectively.

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REFERENCES

Ath-Thar, M.H.F., The effectiveness of medullary β-actin fish promoter Oryzias latipes with hr-GFP gene marker (humanized Renilla reniformis green fluorescent protein) in catfish (Clarias sp.) Descendants F0 [Thesis]. Departm-
ent of Aquaculture, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University (2007).


Kusrimi, E., E.P. Hayuningtyas, R.V. Kusumah and S. Cindelaras, The effectiveness of growth hormone gene transfer of mas on wild betta embryo (*Betta imbellis*). *Pros Forum*


