Pak. J. Biotechnol. Vol. 15 (1) 1-9 (2018)
www.pjbt.org
ISSN Print: 1812-1837
ISSN Online: 2312-7791

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

Eni Kusrini[12], Alimuddin[2*], Muhammad Zairin Jr[2], Dinar Tri Soelistyowati[2]

[1]Research Institute for Ornamental Fish Culture, Ministry of Marine Affairs and Fisheries, [2]Department of Aquaculture, Faculty of Fisheries and Marine Science, Bogor Agricultural University, Bogor, Indonesia. E-mail*: alimuddin@ipb.ac.id; alimuddin@apps.ipb.ac.id

Article received 4.7.2017, Revised 27.12.2017, Accepted 3.1.2018

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 *Ph*GH 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. *Ph*GH 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 *Ph*GH 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 (*Carrasius 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*) (Du *et al.,* 1992), coho salmon (*Oncorhynchus kisutch*) (Devlin *et al.,* 1994), Arctic charr (*Salvelinus alpinus*) (Pitten and Moav 1999), rainbow trout (*Oncorhynchus 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 Szelei *et al.* (2005). In our previous study, we have generated F0 transgenic betta fish which carried GH gene of pangasius (*Ph*GH) (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 *Ph*GH gene in F1 generation of transgenic betta fish.

MATERIALS AND METHODS

Production of Transgenic F1: F0 transgenic betta fish carrying *Ph*GH 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 cros-ses were made, i.e. 1) a cross between transgenic male and non-transgenic female, 2) non-transge-nic 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 repli-cation.

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 50×60×90cm^3. 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 34×25×30cm^3. 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 20x20x15cm^3, 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 *Ph*GH gene transmission: Identification of F1 transgenic fish that carries *Ph*GH 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 µLof *Ph*GH primer at a concentration of 20 pmol (F3-*Ph*GH: 5'TCT TTA GTC AAG GCG CGA CAT TCG AGA-3' and R3-*Ph*GH: 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 72°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 *Ph*GH gene and β-actin were 334 bp and 300 bp, respectively.

Analysis of *Ph*GH mRNA expression: Analysis of *Ph*GH 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 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*/beta 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 ($p>0.05$).

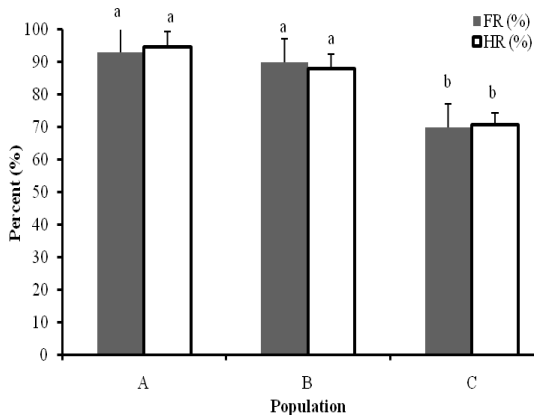
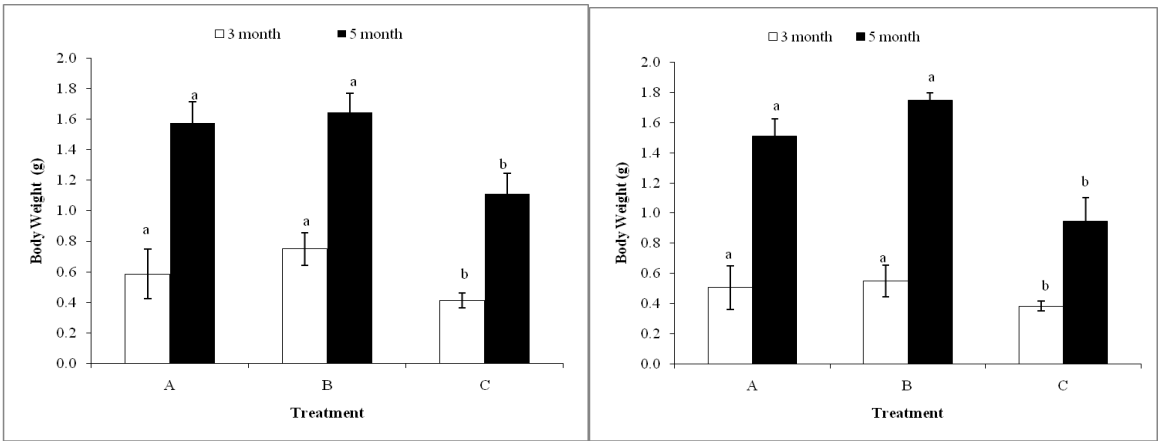


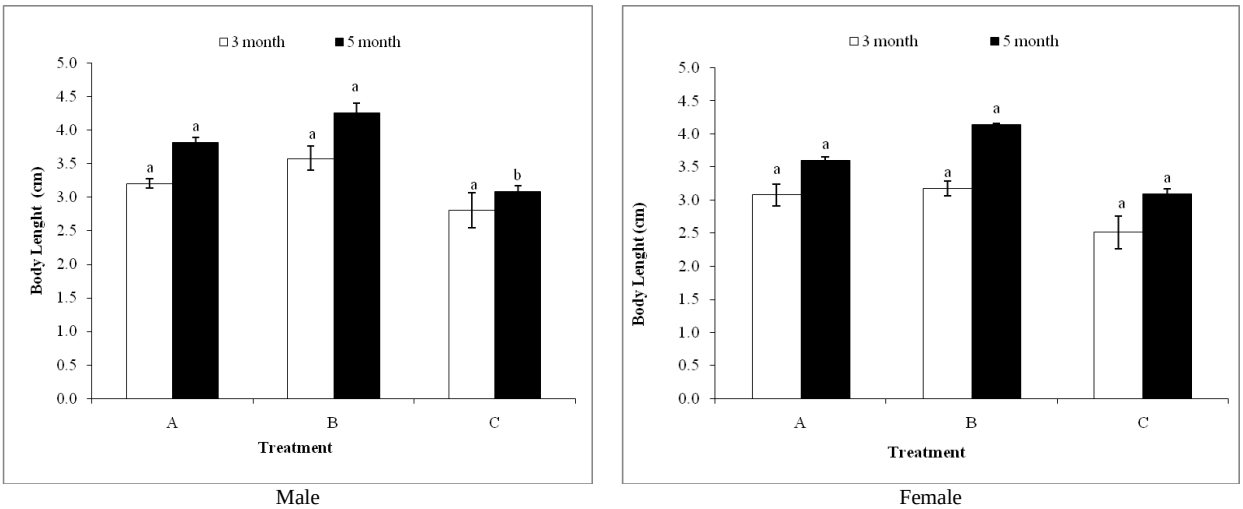
Figure 1: Fertilization (FR) and hatching rates (HR) of F1 transgenic fish eggs from crossess between male F0 transgenic and female non-transgenic broodstock (A), male non-transgenic and female transgenic (B), and non-transgenic broodstocks (C).

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 dan 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.



Male Female

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 ($p<0.05$)



Male Female

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).

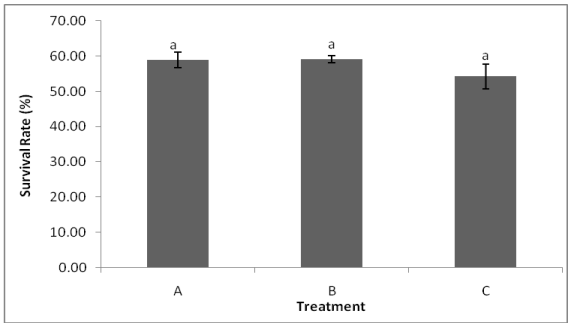


Figure 4: Survival of transgenic betta fish *Betta imbellis* after 5 months of rearing period. A= F1 transgenic fish from crossbreeding of male transgenic F0 and female non-transgenic; B= F1 transgenic fish from crossbreeding of male non-transgenic and female transgenic; C= non-transgenic control. The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

Transmission of *Ph*GH gene in transgenic F1 generation: A representative results of F1 transgenic identification by PCR method is shown in Figure 5. By using *Ph*GH specific primer, transgenic individuals showed a 334 bp of amplification product, which was the same amplification size compared to p*Cc*BA-*Ph*GH 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.

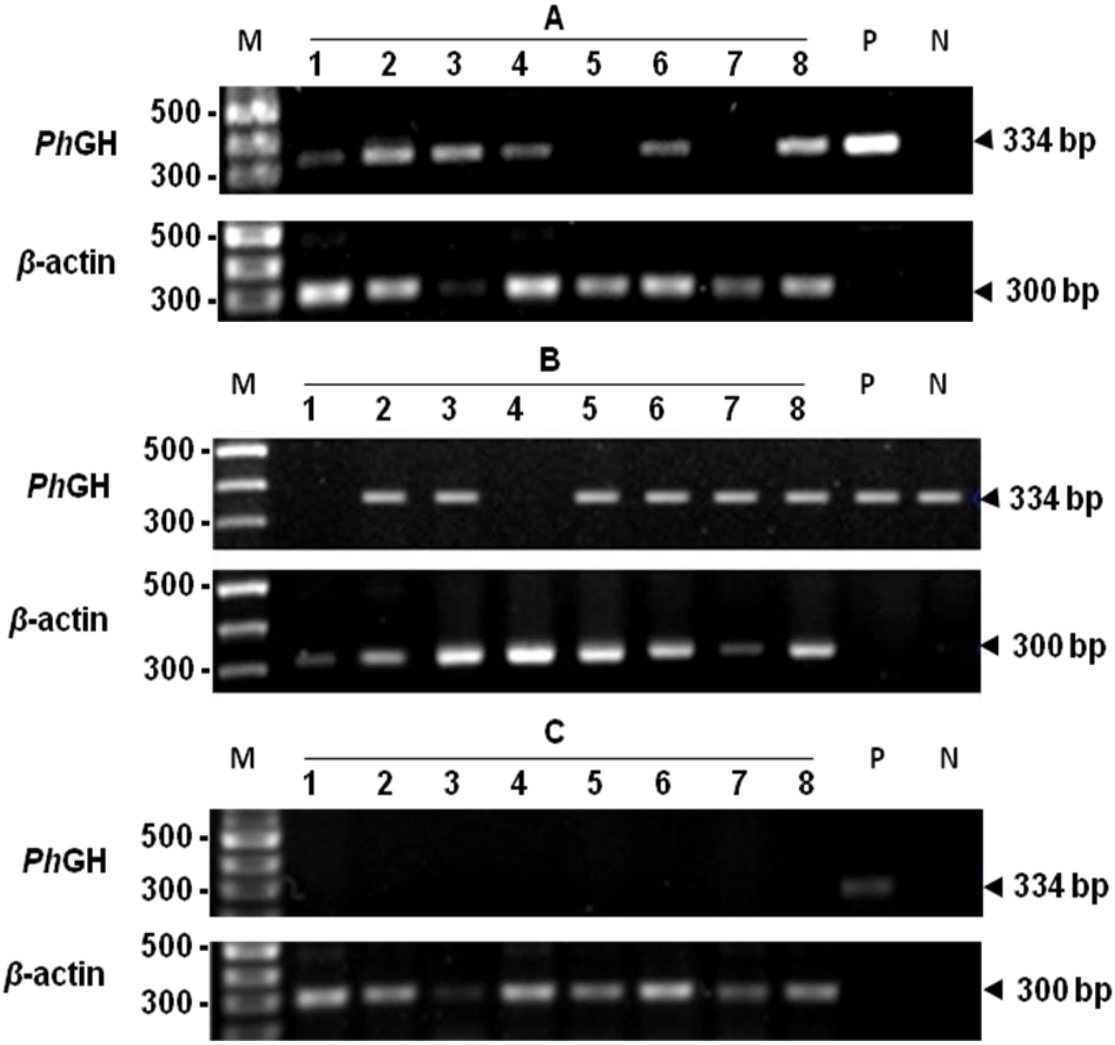


Figure 5: Detection of *Ph*GH gene in transgenic betta fish F1 generation produced from a cross between male non-transgenic and female non-transgenic (A), male non-transgenic and male transgenic (B), and non-transgenic control (C). M: DNA marker of 100-bp, no 1-8: individual fish number, P: PCR product with p*Cc*BA-*Ph*GH plasmid as template, N: without DNA template.

Expression of *Ph*GH 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 *Ph*GH mRNA expression. However, their expression levels were varied (Fig. 6 bottom). Furthermore, the mean *Ph*GH 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.

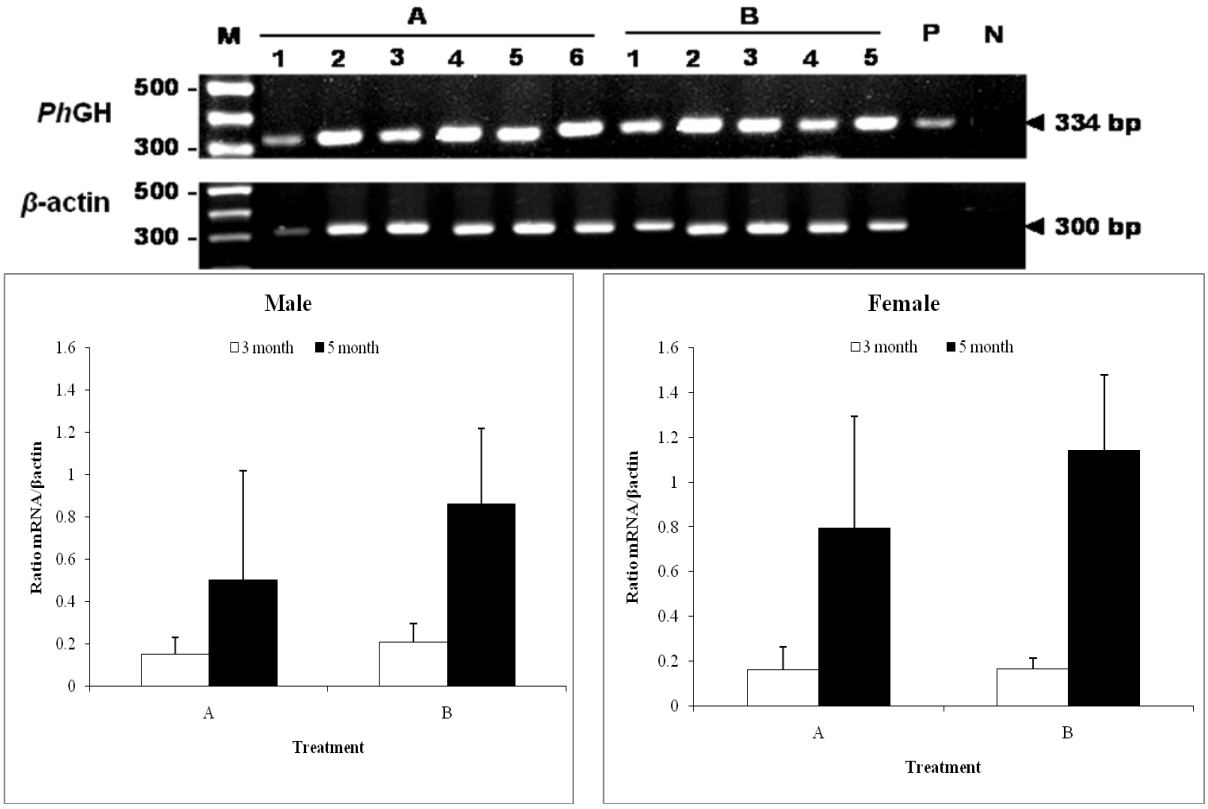


Figure 6: Electrophoregram for RT-PCR product (top) and semi-quantitative expression level of *Pangasianodon hypophthalmus* growth hormone (*Ph*GH) in transgenic betta fish F1 generation (bottom). M: DNA marker of 100-bp, A: F1 transgenic fish from crossing between male transgenic and female non-transgenic; B: male non-transgenic and femal transgenic, Number 1-6: individual F1 transgenic, P: PCR product with plasmid template of p*Cc*BA-*Ph*GH, N: PCR product without template. β-actin was used as an internal control of loading cDNA. Number on the right side is the size of DNA fragment of target PCR product.

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-Thar 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 *Ph*GH 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 p*Cc*BA-*Ph*GH gene construct. The same gene construction has also been used to produce transgenic pangasius. Dewi *et al*. (2014) reported that the growth of F1 *Ph*GH 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 (p*Cc*BA) 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 gro-wth 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 RS-VLTRrtGHc gene construct resul-ted 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 stimula-tion 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 *Ph*GH transgene. This confirmed that the transgenic F0 fish were mosaic. Transgene transmission in F1 transgenic betta fish was higher than the transmitssion 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 *Ph*GH transgene. However, relatively high variation of expression levels within each cross was observed. Variation of transgen expression levels in F1 transgenic fish have been reported by Alimuddin, Kiron, Satoh, Takeuchi, Yoshizaki (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, McClenaghan, 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 *Ph*GH 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.

ACKNOWLEDGEMENT

This research was partially funded by APBN 2016 of Research and Development Center for Ornamental Fish, Agency for Marine and Fisheries Research and Development, Ministry of Marine Affairs and Fisheries, Republic of Indonesia.

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).

Alimuddin, V. Kiron, S. Satoh, T. Takeuchi and G. Yoshizaki, Cloning and over-expression of a masu salmon (*Oncorhynchus masou*) fatty acid elongase-like gene in zebrafish. *Aquaculture* 282: 13–18 (2008).

Dadras, H., H. Khara, N. Baradaran, and E. Shahkar, Effect of sperm pH and density on fertilization and hatching rates of Persian sturgeon (*Acipenser persicus* Borodin, 1897). *International Conference on Chemical, Environmental and Biological Sciences*. Pataya, Thailand 358-361 (2011).

Devlin, R.H., C.A. Biagi, T.Y. Yesaki, D.E. Smailus and J.C. Byatt, Growth of domesticated transgenic fish: growth hormone transgene boots the size of wild but not domesticated trout. *Nature* 409: 781-782 (2001).

Devlin, R.H., T.Y. Yesaki, C.A. Biagi, E.M. Donaldson, P. Swanson and W.K. Chan, Extraordinary salmon growth. *Nature* 371: 209-210 (1994).

Dewi, R.R.S.P.S., H. Marnis, R. Suprapto and N. Syawalia, Production of fast growing catfish generation F-0 using transgenesis method. *J. Ris. Akuakultur*, 7(2): 173-180 (2013).

Dewi, R.R.S.P.S., Gene transfer through sperm mediation to produce transgenic fish, *Pros. Konf. Akuakultur Indonesia 2011*, p 372-378 (2011).

Dewi, R.R.S.P.S., J. Darmawan and I. Nurlaela, Transmission and expression of the growth hormone gene phenotype in Siamese catfish. *J. Ris. Akuakultur* 9(1): 31-37 (2014).

Dobie, K.W., M. Lee, J.A. Fantes, E. Graham, A.J. Clark, A. Springbett, R. Lathe and M. McClenaghan, Variegated transgene expression in mouse mammary gland is determined by the transgene integration locus. *Proc. Natl. Acad. Sci. U. S. A.* 93, 6659–6664 (1996).

Du, S.J., Z. Gong, G.L. Fletcher, M.A. Shears, M.J. King, D.R. Idler and C.L. Hew, Growth enhancement in transgenic Atlantic salmon by the use of an "all fish" chimeric growth hormone gene construct. *Biol Tech* 10: 176-180 (1992).

Dunhan, R.A., G. Warr, A. Nichols, P.L. Duncan, B. Argue, D. Middleton and Z. Liu, Enhanced bacterial diseases resistance of transgenic channel catfish (*Ictalurus punctatus*) possesing cecropin genes. *Mar Biotechnol* 4:338-344 (2002).

Gala, R.R., Prolactin and growth hormone in the regulation of the immune system. *Proc Sot Exp Biol Med*. 198:513-527 (1991).

Gong, Z., B. Ju, and H. Wan, Green fluorescent protein (GFP) transgenic fish and their applications. *Genetica* 111(1): 213-225 (2001).

Gross, M.L., J.F. Schneider, N. Moav, B. Moav, C. Alvarez, S.H. Myster, Z. Liu, E.M. Hallerman, P.B. Hackett, K.S. Guise, A.J. Farae and A.R. Kapiscinski, Molecular analysis and growth evaluation of northern pike (*Esox lucius*) microinjected with growth hormone genes. *Aquaculture* 103: 253-273 (1992).

Guan, B., W. Hu, T. Zhang, Y. Wang and Z. Zhu, Metabolism traits of "all-fish" growth hormone transgenic common carp (*Cyprinus carpio* L.). *Aquaculture*, 284: 217-223 (2008).

Gusrina, Alimuddin, K. Sumantadinata and U. Widyastuti, Transfer of gene encoding growth hormone of tilapia (tiGH) on catfish (*Clarias* sp.) by method of microinjection. *J. Ris. Akuakultur* 4(3):333-340 (2009).

Hinits, Y. and B. Moav, Growth performance studies in transgenic *Cyprinus carpio*. *Aquaculture*, 173: 285-296 (1999).

IBC, International Betta Congress. *A Giant Betta Journal*. 2 (2013).

Inoue, K., S. Yamashita, J. Hata, S. Kabeno, S. Asada, E. Nagahisa and T. Fujita, Electroporation as a new technique for producing transgenic fish. *Cell. Differ. Dev*., 29: 123-128 (1990).

Kato, K., M. Takagi, Y. Tamaru, S.I. Akiyama, T. Konishi, O. Murata and H. Kumai, Construction of an expression vector containing a β-actin promoter region for gene transfer by microinjection in red sea bream (*Pagrus major*). *Fish Sci* 73:440-445 (2007).

Kobayashi, S., Alimuddin, T. Morita, M. Miwa, J. Lu, M. Endo, T. Takeuchi and G. Yoshizaki, Transgenic Nile tilapia (*Oreochromis niloticus*) over expressing growth hormone show reduced ammonia excretion. *Aquaculture* 270: 427-435 (2007).

Kurdianto, Performance growth and nutrient composition of the fish seed body *Cyprinus carpio* transgenic third generation of growth hormone. Essay. Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University. 37 (2014).

Kusrini, 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*

Inovasi Teknologi Akuakultur Pp. 539-548 (2016).

Lamason, R.L., M.P.K. Mohideen, J.R. Mest, A.C. Wong, L. Heather, H.L. Norton, M.C. Aros, M.J. Jurynec, X. Mao, V.R. Humphreville, J.E. Humbert, S. Sinha, L. Jessica, J. Moore, W. Pudur, G. Zhao, G. Ning, I. Makalowska, P.M. McKeigue, D. O'Donnell, R. Kittles, J.P. Esteban, N.J. Mangini, D.J. Grunwald, M.D. Shriver, V.A. Canfield and K.C. Cheng, A putative cation exchanger, affects pigmentation in zebrafish and humans**.** *Science* 310(5755): 1782-1786 (2005).

Martinez, R., M.P. Estrada, J. Berlanga, I. Guillen, O. Hernandez, E. Cabrera, R. Pimentel, R. Morales, F. Herrera, A. Morales, J.C. Pina, Z. Abad, V. Sanchez, P. Melamed, R. Lleonart and J. de la Fuente, Growth enhancement in transgenic tilapia by ectopic expression of tilapia growth hormone. *Mol Mar Biol Biotech* 5:62-70 (1996).

Meier, K.M., C. Castano, J. Laurino, J.A. Levy, and L.F. Marins, cDNA cloning and phylogenetic analysis of growth hormone genes from the mullet (*Mugil platanus*, Mugilomorpha, Mugilidae) and the halfbeak (*Hemiramphus brasiliensis*, Atherinomorpha, Hemiramphidae). *Atlantica*, 28: 97-102 (2006).

Moav, B., Y. Hinits, Y. Groll and S. Rothbard, Inheritance of recombinant carp β-actin/GH cDNA gene in transgenic carp. *Aquaculture*, 137: 179-185 (1995).

Muir, W.M. and R.D. Howard, Possible ecological risks of transgenic organism release when transgenes affect mating success: sexual selection and the Trojan gene hypothesis. *Proc Natl. Acad. Sci. USA* 96:13853–13856 (1999).

Nam, Y.K., J.K. Noh, Y.S. Cho, H.J. Cho, K.N. Cho, G. Kim and D.S. Kim, Dramatically accelerated growth and extraordinary gigantism of transgenic mud loach (*Misgurnus mizolepis*). *Trans Res* 10:353-362 (2001).

Ohlsson, C., S. Mohan, K. Sjögren, A. Tivesten, J. Isgaard, O. Isaksson, J.O. Jansson and J. Svensson, The role of liver-derived insulin-like growth factor-I. DOI: 10.1210/er.2009-0010. *Endocr. Rev*., 30(5): 494-535 (2009).

Perez-Sanchez J., The involvement of growth hormone in growth regulation, energy homeostasis and immune function in the gilthead sea bream (*Sparus aurata*): a short review. Fish Physiol. Biochem., 22:135-144 (2000).

Parenrengi, A., A. Tenriuolo, S. Tonnek, and S. Lante, Transfer of antiviral gene with different DNA concentrations to tiger shrimp (*Penaeus monodon*) embryo. *J. Ris. Akuakultur*, 6(3): 353-361 (2011).

Pitkanen, T.I., A. Krasnov, H. Teerijoki and H. Molsa, Transfer of growth hormone (GH) transgenes into Arctic charr (*Salvelinus alpinus* L.). I. Growth response to various GH construct. *Genet. Anal. Biomol. Eng.* 15: 99-105 (1999).

Rajamuddin, M.A.L., Alimuddin, U. Widyastuti and E. Harris, Transformation of kappa (κ)-carrageenase gene from *Pseudoalteromonas* in seaweed (*Kappaphycus alvarezii* Doty). *Pak. J. Biotechnol.* 13(3): 157-163 (2016).

Ryynanen, H.J. and C.R. Primmer, Varying signals of the effects of natural selection during teleost growth hormone gene evolution. *Genome* 49: 42-53 (2006).

Sakai, M., Y. Kajita, M. Kobayashi and H. Kawauchi, Immunostimulating effect of growth hormone: in-vivo administration of growth hormone in rainbow trout enhances resistance to *vibrio anguillarum* infection. *Veterinary Immunology and Immunopathology* 57: 147-152 (1997).

Sarmasik, A., Application of gene transfer technology for genetic improvement of fish. T*urkish Journal of Zoology* 27: 1-6 (2003).

Shears M.A., G.L. Fletcher C.L, Hew, S. Gauthier and P.L. Davies, Transfer, expression, and stable inheritance of antifreeze protein genes in Atlantic salmon (*Salmo salar*). *Mol. Mar. Biol. Biotech*., 1: 58-63 (1991).

Sheela, S.G., J.D. Chen, S. Mathavan and T.J. Pandian, Construction, electroporatic transfer and expression of ZpβypGH and ZpβrtGH in zebrafish. *J. Biosci.* 23: 565-576 (1998).

Sun, P.S., N.C. Venzon, F.R.O. Calderon and D. M. Esaki, Evaluation of methods for DNA delivery into shrimp zygotes of *Penaeus* (*Litopenaeus*) *vannamei*. *Aquaculture* 243: 19-26 (2005).

Szelei, J., L. Varadi, F. Müller, F. Erdelyi, L. Orbán, L. Horvath and E. Duda, Liposome-mediated gene transfer in fish embryos. *Trans. Res. Journal* 3: 116-119 (1994).

Tewari, R., C. Michard-Vanhée, E. Perrot and D. Chourrout, Mendelian transmission, structure and expression of transgenesfollowing their injection into the cytoplasm of trout eggs. *Trans. Res.* 1: 250-260 (1992).

Triana, S.H., Alimuddin, U. Widyastuti, Suharsono, E. Suryati, and A. Parenrengi, The method of agrobacterium tumefaciens-mediated MmCu/Zn-SOD gene transformation in the red seaweed (*Kappaphycus alvarezii*). *Pak. J. Biotechnol.* 13(4):221-230 (2016).

Volckaert, F.A., B.A. Hellemans, P. Galbusera, F. Ollevier, B. Sekkali and A. Belayew, Replication, expression, and fate of foreign DNA during embryonic and larval development of the African catfish (*Clarias gariepinus*). *Mol Mar Biol Biotechnol* 3:57-69 (1994).

Wang, Y., W. Hu, G. Wu, Y. Sun, S. Chen, F, Zhang, Z. Zhu, J. Feng and X. Zhang, Genetic analysis of "all-fish" growth hormone gene transferred carp (*Cyprinus carpio* L.) and its F1 generation. *Chin. Sci. Bull*. 46: 1174–1177 (2001).

Winkler, C., J.R. Vielkind, and M. Schartl, Transient expression of foreign DNA during embryonic and larval development of the medaka fish (*Oryzias latipes*). *Mol. Gen. Genet.* 226: 129-140 (1991).

Wu, G., Y. Sun and Z. Zhu, Growth hormone gene transfer in common carp. *Aquat. Living Resour*. 16: 416-420 (2003).

Zhang, P., M. Hayat, C. Joyce, L.I. Gonzalez-Villasenor, C.M. Lin, R.A. Dunham, T.T. Chen and D.A. Powers, Gene transfer, expression and inheritance of pRSV-rainbow trout-GH in the common carp (*Cyprinus carpio* Linnaeus). *Mol. Reprod. Develop*. 25, 3-13 (1990).

Zhu, Z., G. Li, L. He and S. Chen, Novel gene transfer into the fertilized eggs of goldfish (*Carassius auratus* L. 1758). *J. Appl. Ichthyol*. 1: 31-34 (1985).