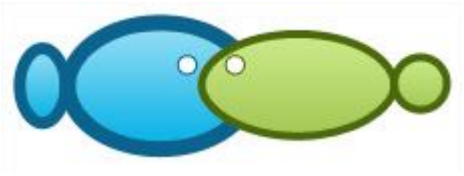

Analysis of promoter activity on tiger shrimp *Penaeus monodon* using EGFP (enhanced green fluorescent protein) as a marker gene

[1]Andi Parenrengi, [1]Rachman Syah, [1]Andi Tenriulo, [2]Alimuddin

[1] Research Institute for Brackishwater Aquaculture and Fisheries Extension, Maros, South Sulawesi 90511, Indonesia; [2] Faculty of Fisheries and Marine Science, Bogor Agricultural University, Bogor 16680, Indonesia. Corresponding author: A. Parenrengi, andi_parenrengi@hotmail.com

Abstract. One of the most critical factors in transgenic research is analysis of promoter activity for regulating the gene expression. To study promoter activity, a reporter gene should be combined with the promoter on a gene construct, so that a suitable promoter is indicated by the transient expression of the reporter gene. The present study aimed to examine the activity of an antiviral promoter (ProAV) on tiger shrimp *Penaeus monodon* using enhanced green fluorescent protein (EGFP) as a reporter gene. Promoter ProAV linked to an EGFP gene was cloned into pEGFP-N1 to obtain the pProA*V*-EGFP gene construct. A transfection technique was used to transfer the promoter regulatory constructs into fertilized tiger shrimp eggs. The insertion of the EGFP gene and its transient expression were confirmed in the shrimp embryos and larvae. The results showed that the hatching rate of tiger shrimp with transfection was 39.3% and was not significantly different ($p > 0.05$) from the rate with transfection without plasmid gene construct (43.1%) and without transfection (49.0%). This indicated that the transfection reagent and EGFP gene did not show toxicity to the shrimp embryo. The ProAV exhibited activity in regulating the transient expression of the EGFP gene in tiger shrimp embryos and larvae. EGFP expression began to show at 12 hours after transfection (hat) and peaked at 24 hours after transfection (hat), followed by a decrease at 30 hat. The results implied that the utilization of ProAV promoter could be useful in the development of transgenic tiger shrimp using the appropriate target gene.
Key Words: fluorescent gene, promoter, tiger shrimp, transfection, transgene expression.

Introduction. Tiger shrimp, *Penaeus monodon* culture is widely known and accepted as a very important aquacultural practice in Indonesia. Tiger shrimp has contributed significantly to the development of the aquaculture sector and Indonesian growing economy. However, since 2000, the culture of tiger shrimp has been devastated by white spot disease. Development of biotechnology in shrimp aquaculture provides an alternative solution for controlling shrimp diseases. Genetic engineering is a powerful tool for transfer the foreign genes to improve the genetic resources of an organism (Glick & Pasternak 2003). Some fish transgenic studies in aiming to commercial product from important traits in aquaculture, in particularly on fast growing and diseases resistance were conducted. Promoter is an important factor for the successful genetic transformation and gene expression. Antiviral promoter (ProAV) has been isolated from tiger shrimp and cloned into the pGEM-T easy vector (Parenrengi et al 2009a). In order to establish the transgenic technology for diseases resistance in tiger shrimp, the promoter activity has to be performed by *in-vivo* on fertilized egg (embryo) for analysis of transgene expression. Alimuddin et al (2003) reported that promoter activity from fish gene constructs (all-fish construct) showed higher on regulating gene expression than promoters isolated from mammals and viruses. All-fish gene constructs were successfully applied to mud loach, *Misgurnus mizolepis* (Nam et al 2001), salmon, *Salmo salar* (Yaskowiak et al 2006) and black tilapia, *Oreochromis niloticus* (Kobayashi et al 2007).

Most promoter activity studies were conducted by injection of gene construct into the muscle of organism and transfection into the cell culture (Kato et al 2007). Both methods may be difficult regarding the observation of gene expression. The recent

progress in transgene expression studies of promoter activity *in-vivo* was the transfection of the gene into embryos using a reported gene as an indicator (Muller et al 1993, 1997; Takagi et al 1994; Hamada et al 1998; Sheela et al 1998; Maclean et al 2002; Alimuddin 2003; Kobolak & Muller 2003; Her et al 2004; Kato et al 2007; Shi et al 2015; Delhove et al 2017; Wang et al 2018).

Alimuddin et al (2003) reported that transgene expression was measured by gene mRNA level and protein. The mRNA of exogenous gene was detected using a probe of labeling DNA fragment with a radioactive isotope, while protein was determined by immunological detection by antibody. However, these methods were complicated and time consuming. To develop the promoter, it was necessary to design a simple and faster method to study the transgene expression, for example, a gene encoding green fluorescent protein (GFP) isolated from luminous organ of the jelly fish. The GFP gene as a marker was easy to detect by fluorescent microscope or gene expression analysis by RT-PCR (Takeuchi et al 1999; Yazawa et al 2005b; Alimuddin et al 2007; Ath-thar et al 2008; Dewi et al 2010; Hadie et al 2010; Dewi et al 2013). The application of GFP gene was more popular to study promoter activity in transgenesis technology. The advantages of GFP gene were low level of cytotoxicity, without substrate and co-factor to illuminate, and showing transient expression under ultra violet light (Felts et al 2001; Gong et al 2003).

Activity of promoter ProAV has been proven by in-vitro technique using the GFP gene. Luo et al (2007) reported an increase of ProAV activity after its sequence of microsatellite was deleted. Therefore, ProAV used in this present study has been specially constructed using ProAV without microsatellite sequence (Parenrengi et al 2009a) isolated from tiger shrimp. The establishment of promoter activity has to done before implementing the transfection of antivirus gene as a target gene. The study aimed to assess the promoter ProAV activity by *in-vivo* on tiger shrimp using enhanced green fluorescent protein (EGFP) gene as a marker.

Material and Method

Construction of ProAV-EGFP gene. Promoter antivirus (ProAV) has been isolated from tiger shrimp and cloned into the pGEM-T easy vector (Parenrengi et al 2009a). Construction of ProAV promoter with EGFP gene was performed in pEGFP-N1 vector (Clontech). The ProAV sequence was digested from plasmid of recombinant bacteria in pGEM-T easy vector using restriction enzyme of *Bam*HI. The DNA fragment of ProAV was isolated, purified by gel extraction, and then ligated into the pEGFP-N1 vector to construct the pProAV-EGFP. The gene construct was transformed to competent cell of bacterium *E. coli* DH5α. The cloning gene was done according the procedure developed by Parenrengi et al (2009a).

The success of inserting promoter gene to the vector was determined by cracking technique. Each white colony of bacteria was transferred to the master plate using the 1.5 mL micro tube and incubated at 37^{o}C for 8 hours. Ten µL of cracking buffer (0.2 g saccharose; 40 µL NaOH 5M; 50 µL SDS 10%; and SDW until the total volume of 1 mL), 10 uL EDTA 10 mM and approximately 2 µL loading buffer and KCl 4M in ratio 1:1 was inserted to the under cap of micro tube. After incubated in room temperature for 5 minutes, the micro tube was spinned down at 5,000 rpm (revolutions per minute) for 3 seconds and vigorously mixed. The sample was centrifuged at 12,000 rpm for 5 minutes in 4^{o}C. Ten µL of supernatant was run at 0.7% gel electrophoresis and plasmid of blue colony bacteria as a control negative. The DNA plasmid inserted gene showed the bigger molecular weight compare to the control. Based on the cracking result, the positive inserted bacteria were used as a DNA template in further analysis.

In order to determine the right position of inserted promoter in vector, the orientation test was conducted by PCR technique by using the primer forward *ProAV-F*: 5'- gtcggatccagtcccacactccatcaa -3' and reverse *EGFP-R*: 5'- acgaactccagcaggaccat -3'. PCR reaction consisted of 0.05 µL *Taq* Polymerase, 1 µL 10x buffer, 0.8 µL dNTP mix, 0.8 µL $MgCl_2$, 10 ρmol each primer, 1 µL DNA template, and 4.35 µL SDW. The PCR process was carried out at a temperature of pre-denaturation at 94ºC for 3 minutes; for 35 cycles

(denaturation at 94ºC for 30 seconds, annealing at a temperature of 58ºC for 30 seconds, extension at a temperature of 72ºC for 45 seconds); and a final extension at temperature of 72ºC for 3 minutes. The PCR products were electrophorezed by 1.0% agarose gel. The approximately 1.1 kb of amplification result indicated a right ligation position.

Shrimp maintenance and embryo collection. Tiger shrimp broodstocks were maintained in a hatchery facility of nucleus center (NC), Research Institute for Brackishwater Aquaculture and Fisheries Extension, Barru, South Sulawesi, Indonesia, and fed with squid, *Loligo* sp. and sea-worm, *Nereis* sp. of 15% per body weight twice a day. Adult female (125-237 g in body weight and 22.9-27.8 cm in total length) and male shrimps (61-112 g in body weight and 18.5-22.3 cm in total length) were transferred to mating tanks, which were supplied with a constant water flow for 300% per day. The females ready to spawn were then transferred to individual spawning tanks for continuous monitoring. Under these conditions, spawning generally occurred at the night. The fertilized eggs were immediately collected (around 5 minutes) after spawning.

Activity test of ProAV promoter. Plasmid DNA of gene construction *ProAV-EGFP* was isolated from bacteria recombinant using GF-1 Plasmid DNA Extraction Kit (Vivantis) according to its manual procedure. Concentration and purity of the plasmid DNA was measured using the GeneQuant at wavelength of 260 and 280 nm. The quality of DNA genome was also confirmed by electrophoresis at 0.5% agarose gel. The concentration and purity was calculated based on the formula of Linacero et al (1998).

The fertilized egg (embryo) was collected by egg filter collector, rinsed by sea water and then made in concentrate volume of 500 mL to obtain the large number of eggs. Two mL of concentrated egg was taken and filed into Petri dish 35x10 mm for transfection process. The egg density was observed by counting of eggs in 2 mL for 3 times. Transfer of gene construct to embryo was performed by transfection method using the transfection reagent of jetPEI (Polyplus Transfection). Six µg DNA plasmid was mixed with 100 µL NaCl 150 mM and then added with the mixed of 8 µL jetPEI with 100 µL NaCl 150 mM. The solution was mixed for 15 seconds and incubated at room temperature for 15-30 minutes. The amount of 200 µL mixture solution was mixed with the concentrated eggs, homogenized and incubated at room temperature for 50 minutes.

In order to know a toxicity of reagent, the control treatments were applied to observe the hatching rate of tiger shrimp embryo. The positive control was transfection procedure without using the plasmid of construct gene, while the negative control was without plasmid and transfection reagent. The three treatments had 5 replications (3 replicates for hatching rate observation and 2 replications for sampling gene insertion and expression). The transfected egg was rinsed with sterile sea water and stocked to the stopples filed with 2 L seawater for hatching. The containers were placed in water bath with heater to maintain the temperature. The hatching rate of embryo was counted after the incubation egg for 24 hours. The transfection of construct gene to tiger shrimp embryo was performed for two trials.

To confirm the insertion of EGFP gen and activity of ProAV promoter in larvae tiger shrimp, the pooled sample (50 nauplii) was extracted to isolate the genomic DNA and total RNA using the conventional phenol-chloroform method (Parenrengi et al 2009a) and isogen kit (Parenrengi et al 2009b), respectively. The cDNA was created from RNA using Ready-To-Go You-Pime First Strand Beads Kit (GE Healthcare) based on the manual procedure. Promoter activity indicated transgene expression was observed by UV-transilluminator and RT-PCR technique. RNA was isolated after 12 hours after transfection (hat) and following by every 6 hours (18, 24, and 30 hours after transfection - hat). Transient expression of EGFP gene was detected by PCR technique using the forward primer *EGFP-F* : 5'- ggtcgagctggacggcgacg -3' and reverse *EGFP-R* : 5'- acgaactccagcaggaccat -3', and cNDA as a template PCR with the target DNA fragment of 627 bp. DNA amplification was performed in GeneAmp PCR System 2700. PureTaq RTG PCR beads kit was used as the PCR reaction and mixed with primer for each 1 µL (50 ρmol mL^{-1}). The PCR process was carried out at pre-denaturation temperatures of 94^{o}C for 3 minutes; for 35 cycles (denaturation at 94^{o}C for 30 seconds, annealing at a

temperature of 58°C for 30 seconds, extension at a temperature of 72°C for 45 seconds); and a final extension at a temperature of 72°C for 3 minutes. The PCR result was run using 1% agarose gel and documented by Gel Documentation System (Biometra).

Data analysis. Hatching rate was measured based on the number of egg hatched to nauplii compare with the total number of eggs incubated for 24 hours. To describe the effect of transfection reagent and DNA plasmid to hatching rate, the data was analyzed by Statistix Version 3.0 (NH Analytical Software) and following LSD analysis at level of 5%. Analysis of EGFP gene insertion to the larvae and transgene expression pattern was descriptively discussed.

Results and Discussion. The construction of pProAV-EGFP gene has to carry out in step of understanding whether ProAV promoter active or not on the tiger shrimp using a marker such as EGFP gene. The successful in constructing the gene and inserting to vector pEGFP-N1 was indicated by increasing the plasmid length of bacteria by cracking analysis as shown in Figure 1A. This cracking result showed that the bacteria carrying the gene construct had the plasmid length bigger than the control bacteria (without inserting the gene construct). In order to determine the right position of ligation, Figure 9B showed that 86% of clone (18 clones from 21 clones under studied) placed the right position of gene construct in vector indicated by presence of DNA fragment in length of approximately 1.1 kb (wrong position if without the DNA fragment). Both approaches (cracking and orientation analysis) were evidently confirmed the gene construct of pProAV-EGFP in plasmid of recombinant bacteria. The clones having the gene construct were transferred to the agar medium (Figure 1C) to produce a pure clone for plasmid source of gen construct of pProAV-EGFP for further study.

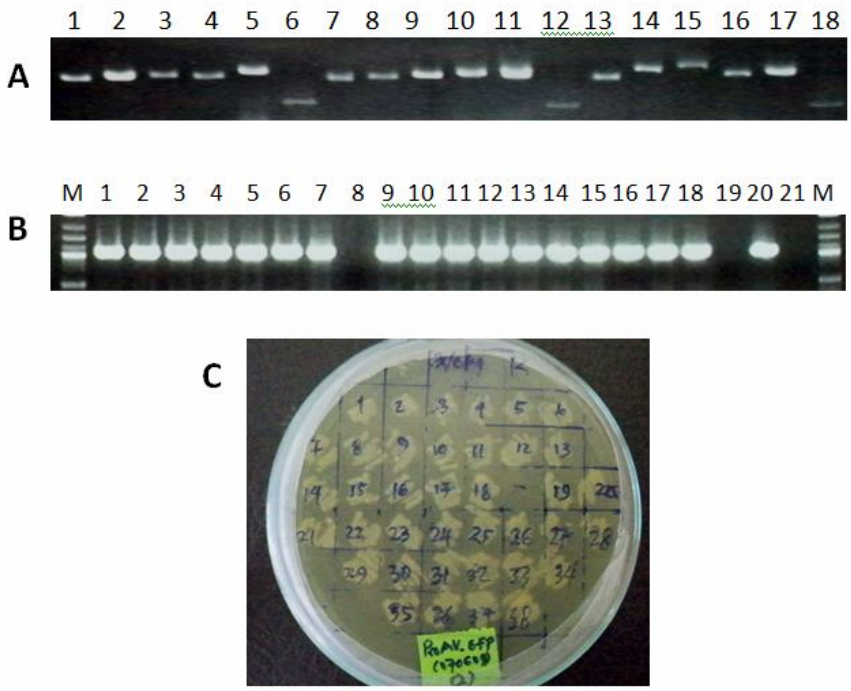

Figure 1. The results of cracking and PCR of pProAV-EGFP gene construct. A = DNA fragment from cracking of clones recombinant bacteria on agarose gel which line 1-5, 7-11, and 13-17 indicated positive marker as clones carrying the gene construct and line 6, 12 and 18 indicated negative marker as a control blue clone bacteria (without gene construct); B = the orientation ligation inserted in vector which the presence of DNA fragment which line 1-7, 9-18, and 20 indicates the right position and the absence of DNA fragment in line 8, 19, and 21 indicates the wrong position of gene construct, and M=DNA marker; and C = plating of clones bacteria carrying the pProAV-EGFP gene construct.

According to the cracking result and gene orientation analysis of promoter ProAV transformed into the vector pEGFP-N1, the plasmid has been carried a new gene construct in the vector pEGFP-N1 such as pProAV-EGFP-PolyA in size of 5.9 kb (Figure 2). The new gene construct of pProAV-EGFP-PolyA has been registered and certified to Indonesian Patent on September 26th, 2017 (No: IDP000047959) (Parenrengi et al 2017).

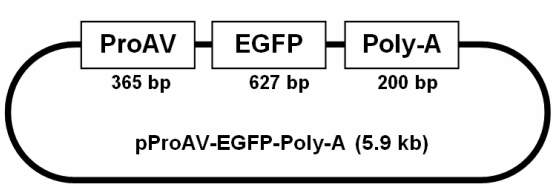

Figure 2. Map of pProAV-EGFP gene construct used for promoter activity analysis on tiger shrimp.

Verification of gene construct in the plasmid was done by presence of promoter at 368 bp and EGFP gene at 627 bp prior to be used for transfection to embryo tiger shrimp. The transfection was performed for two cycles with the embryos density was 370 pcs/2 mL and 235 pcs/2 mL, respectively. Hatching rate of embryo, number of fluorescent larvae, DNA and cDNA detection of each cycle were listed in Table 1. The average of hatching rate of embryo was 39.3% for transfection treatment, 43.1% for positive control and 49.9% for negative control (without transfection). The application of transfection reagent jetPEI and bacteria plasmid was not significantly different ($p > 0.05$) on the hatching rate of embryo. This result indicated that the transfection reagent and plasmid gene construct used this study did not show toxicity to the embryo of tiger shrimp.

The transfection reagent of jetPEI has been assessed in the previous studies on the effect of its toxicity. The transfection using jetPEI has a lower cellular toxicity (Horbinski et al 2001) and it can be naturally degradated (Ahn et al 2002). The other studies also reported that the use of gene construct plasmid did not show the negative impact to the embryo post transfection. Introduction of gene construct (promoter and β-galactosidase gene) using electroporation technique to the zebra fish was not negatively effect to the embryo with the hatching rate of 72% compared with 85% of control treatment (Sheela et al 1998). The high rate of gene transfer by transfection of gen construct of pβactP2-TSV-CP expression vector on white shrimp *Litopenaeus vannamei* could be conducted before formation of jelly layer to egg surface (72%) and post of jelly layer formation (50%). However, the low hatching rate (17.6-20.1%) of tiger shrimp embryo using the micro-injection of gene construct pJEF-GFP was reported by Yazawa et al (2005b).

Table 1

Hatching rate of embryo, number of illuminated egg, DNA and cDNA detection on tiger shrimp after transfection ProAV-EGFP gene construct

Trial	*Hatching rate (%)*			*Illuminated egg *)*	*Detection**)*	
	Transfection	*Positive control*	*Negative control*	*(embryo)*	*DNA*	*cDNA*
1	44.7±13.0[a]	60.0±3.0[a]	66.8±12.1[a]	+	+	+
2	33.9±19.7[a]	26.2±6.9[a]	31.2±14.4[a]	+	+	+

Notes: Means of hatching rate of on the same raw with similar letter are not significantly different ($p > 0.05$);
*) - illuminated egg was detected by UV-transilluminator apparatus in wavelength of 365 nm;
**) - analysis was performed on 50 embryos (pooled samples) which (+) indicated the EGFP gene expression on DNA genome and cDNA of tiger shrimp.

Using the transfectian reagent of jetPEI, the hatching rate of larvae in this present study was lower than the hatching rate of *L. vannamei* around 50-60% (Sun et al 2005). This may be a different crustacean species and quality of broodstock as well as the spawning technique. However, the result indicated that the use of jePEI and gene construct plasmid did not give the negatively effect to the hatching rate of larvae. This implied that the introduction of exogenous gene to the fertilized tiger shrimp egg (embryo) by

transfection as a potential technique in producing the transgenic shrimps as their egg size is very small (around 250-270 µm) to do micro-injection. The implementing of transfection technology have been reported to animal cell and others vertebrate organisms (Boussif et al 1995; Abe et al 1998; Demeneix et al 1998; Carballada et al 2000; Wall 2002; Sun et al 2005).

Observation of illuminated embryo/larvae was carried out using UV-transilluminator (Biometra) at wave length of 365 nm which was showing the fluorescent embryo, even the maximum emission of EGFP gene was at 507 nm (Sun et al 2005). The result showed that that illuminated embryo was 1-3 embryos (Table 1). Due to the limitation of this tool, this observation would not be addressed to describe the fluorescent in detail part or organ of embryo, but it was just to verify the insertion of EGFP gene into the embryo. In order to provide evidence the fluorescent embryo, the DNA and RNA (cDNA) analysis had to perform to the larvae for observation of EGFP gene insertion and transgene expression. The presence of DNA fragment in size of 627 bp both of DNA and cDNA analysis was a positive indicator of insertion of EGFP gene to tiger shrimp embryo, but it did not obtain from control embryo (Table 1; Figure 3A). The insertion of target gene into the embryo using the jetPEI reagent has been reported on the previous studies. The others transfection reagents was successfully used for introducing the gene into embryo such as Effectene and SuperFect from Qiagen, and Lipofectamine 2000 from Gibco BRL. Sun et al (2005) reported that rate efficiency of gene transfer to embryo white shrimp *L. vannamei* using transfection method was 40-60%, but using micro-injection and electroporation method was 10-20% and10-15%, respectively.RT-PCR analysis was used to assess the transient gene expression of EGFP gene at several interval time observations after transfection. The electrophoresis result of the transient expression of EGFP gene at 12, 18, 24, and 30 hat was shown in Figure 3B and internal control of β-actin gene expression in Figure 3C.

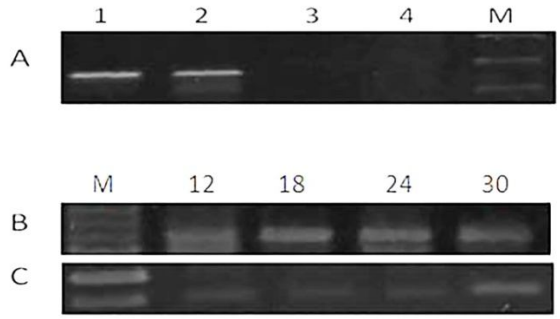

Figure 3. Analysis of DNA genome and expression of EGFP gene in tiger shrimp. A = DNA of tiger shrimp embryo carrying EGFP gene (1-2), control embryo without transfection (3-4), and DNA marker (M); B = transgene EGFP expression on observation of 12, 18, 24, and 30 hat; and C = internal control of β-actin gene expression in tiger shrimp on the same time observation.

Transient expression of EFGP gene in embryo was started to show at observation of 12 hat, however its expression was still relatively low. The EGFP gene expression then increased at observation of 18 and 24 hat which the embryo was expected in late stage and ready to hatch (nauplii), and continue to decrease expression at observation of 30 hat. Transient expression pattern of EGFP gene was reached the maximum expression at 24 hat where the embryo was hatching, while the expression of β-actin showed the relatively similar pattern along time of observations. Some studies showed the comparable expression pattern of fluorescent gene. Hamada et al (1998) reported that transient expression of wild-type GFP (wtGFP) with β-actin promoter from medaka fish started from mid-blastula stage and maximum expression occurred at the end of gastrula stage. The transient expression of humanized *Renilla reniformis* green fluorescent protein (hrGFP) on catfish embryo occurred at beginning of gastrula stage or initial epibody formed (Ath-thar et al 2008) and at blastula stage (12 hours after injection) of *Cyprinus carpio* (Alimuddin et al 2008), while the maximum transient expression was at 18 hours

after injection or the end of gastrula stage and then the transient expression slowly turned down.

Transient expression of gene was one of the replication forms in extra-chromosomal foreign DNA. Expression level would be decrease by degradation of extra-chromosome, so the foreign gene integrated into chromosome was not as much as its transient expression. The highest transient expression level of EGFP gene at 24 hat (nauplius stage) in this present study was expected to occur with DNA plasmid expression that transfected, but during embryo development stage, the DNA plasmid would be degradated by nuclease enzyme causing the lower transient expression level at observation of 30 hat. The peak of transient gene expression at same of this present study (24 hat) was also obtained on African catfish *Clarias gariepinus* using the CMV promoter with luciferase and lacZ gene as a marker (Volckaert et al 1994). As reported by Winkler et al (1991), exogenous gene replication was generally occurred only up to gastrula, in which this stadium, exogenous gene was remained in limited number, due to the degradation by restriction nuclease enzyme so the transient expression decreased. Yoshizaki (2001) stated that gene expression on the fish started from mid-blastula stage and increased to embryogenesis stage and after that the expression degreased after hatching.

The presence of illuminated gene was an indicator of promoter activity which a role of promoter as a gene regulatory. Gene expression pattern of GFP was detected not only by fluorescent microscope and quantitative PCR (real-time), but also by conventional PCR as conducted in this present study. Iyengar et al (1996) stated that the high level of transient expression that generally occurred at mid-blastula stage until gastrula stage was presumed as being result of exogenous DNA accumulation and increasing DNA replication during cleavage stage as well as accumulating from RNA polymerase-II enzyme that caused to start a transcription process in mid-blastula-transition (MBT) stage. The difference of GFP expression pattern has been observed in several tissues of transgenic zebra fish using the some promoters. Yazawa et al (2005a) reported the promoter of C3 (isolated from Japanese flounder) showed the highest activity of GFP expression in the liver, gelatinase B promoter in the pectoral fin and gills, keratin promoter in skin and liver, and TNF (tumor necrosis factor) promoter in the pharynx and heart.

The presence of transient expression of EGFP gene in tiger shrimp embryo and larvae indicated the activity of promoter proAV. The active of promoter was suspected by the presence of important transcription factors in regulating of target gene. The results implied the potential use of proAV promoter to regulate the gene target such diseases resistance in transgenic tiger shrimp. So, the gene construct of ProaAV was a useful promoter to combine with the antivirus gene (PmAV) in order to produce the resistant tiger shrimp in future.

Conclusions. ProAV promoter with EGFP gene was successfully constructed into the pEGFP-N1 vector and transformed to the *E.coli* DH5α. The promoter ProAV showed the activity to regulate the transgene expression of EGFP gene in embryo and larvae tiger shrimp. Based on the hatching rate, the transfection reagent and plasmid gene construct did not show toxicity to the embryo of tiger shrimp. The results indicated that the promoter PmAV could be used for regulating the gene target on development of transgenic tiger shrimp.

Acknowledgements. We are grateful to the Ministry of Marine Affairs and Fisheries, Republic of Indonesia for funding this study through the DIPA of Research Institute for Brackishwater Aquaculture and Fisheries Extension. We also thank to researchers and technicians at Biotechnology Laboratory of Research Institute for Brackishwater Aquaculture and Fisheries Extension, technicians of Animal Aquatic Laboratory of Bogor Agricultural University and to all those who gave assistance to support this present study.

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Received: 05 July 2018. Accepted: 25 September 2018. Published online: 17 December 2018.
Authors:
Andi Parenrengi, Research Institute for Brackishwater Aquaculture and Fisheries Extension, Jalan Makmur Daeng Sitakka No. 129, Maros, South Sulawesu 90511, Indonesia, e-mail: andi_parenrengi@hotmail.com
Rachman Syah, Research Institute for Brackishwater Aquaculture and Fisheries Extension, Jalan Makmur Daeng Sitakka No. 129, Maros, South Sulawesu 90511, Indonesia, e-mail: rachman222000@yahoo.com
Andi Tenriulo, Research Institute for Brackishwater Aquaculture and Fisheries Extension, Jalan Makmur Daeng Sitakka No. 129, Maros, South Sulawesu 90511, Indonesia, e-mail: anditenriulo@yahoo.com
Alimuddin, Faculty of Fisheries and Marine Science, Bogor Agricultural University, Jl. Rasamala, Kampus IPB Darmaga Bogor 16680, Indonesia, e-mail: alimuddin_alsani@yahoo.com

How to cite this article:
Parenrengi A., Syah R., Tenriulo A., Alimuddin, 2018 Analysis of promoter activity on tiger shrimp *Penaeus monodon* using EGFP (enhanced green fluorescent protein) as a marker gene. AACL Bioflux 11(6):1937-1946.