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COMPARISON OF THREE DIFFERENT TECHNIQUES OF GENE TRANSFER IN HUMPBACK GROUPER (*CROMILEPTES ALTIVELIS*)

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ABSTRACT

Humpback grouper is one of the most cultured fishes in Asia, including Indonesia. The main problem faced by humpback culture is its slow growth rate. One of the methods that will be more effective and efficient to solve the problem is using transgenic technique. This study was conducted to determine the effectiveness of transfection, microinjection and electroporation techniques on gene transfer in humpback grouper. *In vitro* transfection was performed by incubating sperm to the foreign DNA (pktBP-ktGH gene construct)-transfectant complex solution, while *in vivo* was by injecting those complex solution into testis of mature males. Microinjection was conducted in 2-4 cell stage embryos using 25 µg/ml of foreign DNA solution, and duration of injection was 1, 2 and 3 seconds. Electroporation by 50 V, 30 ms of pulse length, 5 of pulse number and 0.1 of pulse interval was performed to sperm using three DNA concentration of 5, 10 and 20 µg/ml. The incorporation of foreign DNA in sperm and embryos were analyzed using PCR method. Based on PCR analysis, an optimum DNA concentration for electroporation was 10 µg/ml. Limited number of embryos could be microinjected during 20-30 min to reach 2-4 cell stage. Microinjection for 1 second showed higher survival rate of embryos, although none or very low number of larvae was hatched. Transfast was an effective DNA delivery reagent for humpback grouper sperm. Foreign DNA could be detected in sperm from two out of ten *in vivo* transfected fish at least 36 hours post transfection (hpt). By *in vitro* transfection, foreign DNA was detected in sperm at 48 hpt 25°C incubation temperature. Our study revealed that transfection, microinjection as well as electroporation could be used as transgenesis methods in humpback grouper. By means of simplicity and efficacy, however, electroporation was an appropriate gene transfer method.

Key words: Transfection, microinjection, electroporation, transgenesis, humpback grouper

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INTRODUCTION

In the last two decades, a variety of transgenic aquatic organisms have been successfully produced by researchers around the world. These transgenic organisms have been produced by introducing gene/transgene encoding protein using various transgenesis methods, that is microinjection, electroporation, transfection and particle gun bombardment (Chen *et al.* 1998; Sarmasik *et al.* 2001). Transgene can be integrated and expressed in descendants of transgenic fish. Furthermore, transgenic organisms have produced the expected phenotypes. Until now, most of the generated transgenic aquatic organisms are freshwater fish because these fish are relatively easy to maintain and spawn under laboratory condition. In contrast, marine fish receive very little attention despite the fact that marine fish have a high potential economic value in aquaculture.

Humpback grouper is one of the most cultured fishes in Asia, including Indonesia. The main problem faced in humpback culture is its slow growth rate. For instance, to reach consumption size of about 0.5 kg - 1.0 kg, this species needs 8 up to 24 months (Tucker 1991; Teitelbaum 2007). Its slow growth makes the operational cost of this fish aquaculture increases drastically. Therefore, developing a proper method that will be more effective and efficient to solve the problem is urgently needed. Application of transgenesis technique is expected to solve the problem for humpback grouper and also other marine fish, though the use of transgenic organisms in aquaculture remains controversial.

Several methods of transgenesis have been successfully applied to create transgenic fish. Devlin *et al.* (1994) utilized microinjection method to generate transgenic fish. Pacific salmon, Alimuddin *et al.* (2005) succeeded in zebrafish. Electroporation method has also been successfully used by Sin *et al.* (1993) and Symons *et al.* (1994) on production of transgenic salmon, while Patil and Khoo (1996) and Rambubu *et al.* (2005) succeeded in zebrafish. The transfection method was successfully applied for gene transfer in silver snapper (Lu *et al.* 2002) and white shrimp (Sun *et al.* 2005). This study was conducted to determine the effectiveness of transfection, microinjection and electroporation methods in gene transfer for humpback grouper.

MATERIALS AND METHOD

Transfection

Transfection method is one of gene transfer methods using a specific reagent that is capable of binding to and delivering foreign DNA to enter the cell. Foreign DNA used in this study was all-humpback grouper gene construct, pktBP-ktGH, which has been constructed by RUSNAS (National Strategic Prime Research) Program of Ministry of Research and Technology, Republic Indonesia) for genetic improvement of humpback grouper research team in 2007. pktBP-ktGH contains growth hormone gene (ktGH) and β-actin promoter (ktBP) from humpback grouper.

a. *In vitro* transfection

Preliminary experiment was conducted by two transfectanst, i.e. Transfast (Promega) and JetPei (PolyPlus) to determine their efficacy to deliver pktBP-ktGH into sperm. In this experiment, 100 µl of grouper sperm was exposed to 360 µl of physiological solution containing 10 µl pktBP-ktGH and either 30 µl Transfast or JetPei, at 17ºC and 25ºC incubation temperature for 24, 48, and 72 hours transfection.

b. *In vivo* transfection

Plasmid pktBP-ktGH was injected into humpback grouper testes prior to spawning according to the method of Lu *et al.* (2002) with slight modifications. Foreign DNA-Transfast complex solution at ratio of 1:2 (1 µg of DNA: 6 µl transfast) was dissolved in physiological solution to final volume of 500 µl, and then half of the solution was injected into the right and left testes of mature broodstock, respectively. At 12, 36, 48, 60 and 72 hours post injection, DNA from the transfected sperm was extracted and used as template of PCR amplification to determine the success of transfection method.

Microinjection

Microinjection was conducted as described previously (Alimuddin *et al.* 2005) Microinjection was carried out to fertilized grouper eggs at 2-4 cell stage. A microinjection plate that consists of ten grooves of 3% agarose gel was used to support embryos when penetrated by microinjection needle. Humpback grouper embryos were gently transferred onto the groove of microinjection plate and blastodisc should face the direction of the microinjection needle. DNA solution at concentration of 25 µg/ml was slowly injected into blastodisc. The volume of injected DNA solution was about one-fifth of the blastodisc volume (Ath-Thar 2007). Number of embryos that can be injected by one microinjector in each spawning time was 60-100 embryos. Three durations of injection (1, 2 and 3 seconds) into each embryo were examined to obtain length of injection time resulting to higher survival number of injected embryos.

Electroporation

Electroporation is a gene transfer method that utilizes a series of electric shock to help foreign DNA to enter the cell. Electroporation was carried out using Gene Pulser Xcell Electroporation System (BioRad) by Square wave method with parameters of 50 Volt, 30 ms of pulse length, 5 pulse numbers, 0.1 s of pulse interval, and using cuvettes of 0.2 and 0.4 cm gap size. These electroporation parameters were obtained from the preliminary study. Concentrations of DNA used in this study were 5, 10 and 20 µg/ml, respectively. Each DNA concentration was mixed with 25 µl grouper sperm and sodium chloride solution to final volume of 260 µl. An amount of 100 microliters of electroporated sperm was used for DNA analysis using PCR method.

DNA extraction

Transfected and electroporated sperm solution was subjected to centrifugation for 1 min at 3000 rpm to remove remaining DNA in solvent and transfection reagent. Sperm was washed two times with 200 µl physiological salt solution before performing

DNA extraction. Sperm and the injected embryos were lyzed with 500 µl of lysis buffer for 10 minutes at 95ºC. DNA was precipitated using 400 µl of ethanol 96% and then diluted with 50 µl DEPC water. DNA solution was stored at -20ºC.

PCR amplification

PCR analysis was performed in 10 µl of 1 microgram DNA extracted from sperm and embryos, 1 µl primer forward and reverse (10 pmol), 1 µl dNTPs mix, 1 µl Ex *Taq* buffer, 0.05 µl Ex *Taq* polymerase (TAKARA Bio) and SDW to reach the final volume of 10 µl. Forward and reverse primers designed and used in this study were located at 3' terminus of ktBP promoter (FBP2) and ktGH (RGH1) sequences, respectively. The sequence of those primers was 5' TTCATCCAGCTGATGATT GCCAGATGTAAC-3' and 5'-AGTTGGCTTCA-GGAGAGAGTCGACATTT AG-3'. A total of 35 cycles of denaturation for 30 s at 94ºC, annealing at 62ºC for 30 s, and extension at 72ºC for 1 min were conducted. Two microliters of PCR product was electrophoretically separated using 1.5% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light.

RESULTS AND DISCUSSION

***In vitro* transfection**

Sperms survived when transfection was conducted using Transfast reagent in both incubation temperatures, while contrarily JetPEI-transfected sperm died (Table 1). Furthermore, the results of PCR analysis showed that transfection using transfast reagent for 48 hours at 25°C incubation temperature allowed foreign DNA to incorporate into sperm (Fig. 1 lane 2, Table 1). The forward primer located at 5' terminus of ktBP (FBP1) and AP2 reverse (5'-CTATAGGGCACGCGTGGT-3') was used to ensure that the amplified DNA product was the foreign DNA. PCR product using this set primer was about 2 kb in size (Fig. 1 lane 1). The result suggested that foreign DNA was incorporated in sperm.

Table 1. Survival and incorporation of foreign DNA into *in vitro* transfected sperms using transfast and JetPei reagents at 17°C and 25°C incubation temperatures.

Treatments		Duration of transfection (hours)		
		24	48	72
Incubation temp. 25 °C	Transfast	Sperm survived, did not carry the foreign gene	Sperm survived and carry the foreign gene	Sperm survived, did not carry the foreign gene
	JetPei	Sperm died and did not carry the foreign gene	Sperm died and did not carry the foreign gene	Sperm died and did not carry the foreign gene
	Control	Sperm survived, no carry the foreign gene		
Incubation temp. 17 °C	Transfast	Sperm survived, did not carry the foreign gene	Sperm survived, did not carry the foreign gene	Sperm survived, did not carry the foreign gene
	JetPei	Sperm died and did not carry the foreign gene	Sperm died and did not carry the foreign gene	Sperm died and did not carry the foreign gene
	Control	Sperm survived,did not carry the foreign gene		

In vivo transfection

In vivo transfection was carried out using ten mature male fish by injecting DNA-transfast complex to testis through a canulation tube. This technique was developed, since fish could die when directly injected by syringe into testis through urogenital pore was conducted. Two out of ten broodstocks contained sperm carrying foreign gene at 36 hours and 60 hours after transfection, respectively. The size of DNA band of PCR product of DNA from sperm containing foreign gene was similar with that of pktBA-ktGH as template (Fig. 2, indicated by arrow head).

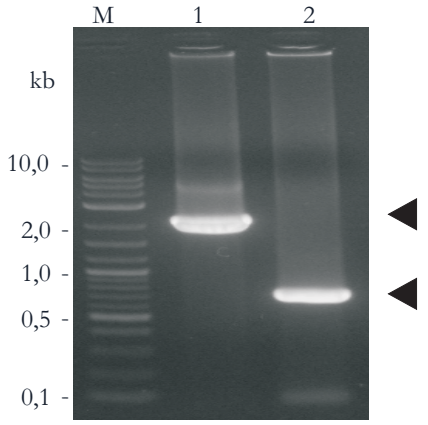

Figure 1. Results of PCR analysis with DNA template extracted from transfected sperm using Transfast. Lane 1: PCR amplification product using primers FBP-1 (5'-GTGWGTGACGCYGGACCAATC-3') and AP2 (5'-CTATAGGGCACGCGTGGT-3'), lane 2: PCR amplification product using primers as described in Materials and Methods. Arrow head indicates PCR product of foreign GH gene. Transfection was carried out for 48 hours at 25ºC. M is 2-log ladder DNA marker (BioLabs, Inc., New England). The amplified fragment is ~700 bp in size.

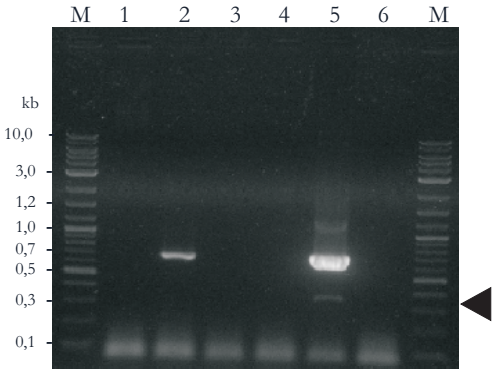

Figure 2. Results of PCR amplification with DNA that have been extracted from *in vivo* transfected sperm at 24 and 60 hours post transfection (hpt). Lane 1 and 2: PCR product of DNA from broodstock no. 2919 at 36 hpt and 60 hpt, respectively. Lane 3 and 4: PCR product of DNA from broodstock no. 2935 at 36 hpt and 60 hpt, respectively. Lane 5: PCR product using pktBA-ktGH as template, lane 6: product of PCR with no DNA template. M is 2-log ladder DNA marker (BioLabs, Inc., New England).

Microinjection

High survival rate of uninjected embryos (92%) indicated that a high quality of fertilized eggs was used for microinjection (Table 2). Percentage of survived microinjected embryos 14 hours after injection decreased with the increase in duration of DNA injection into blastodisc (Table 2). Increasing the duration of injection into blastodisc increased DNA volume entering the cytoplasm. Thus, high DNA volume in cytoplasm of blastodisc probably caused embryos to die. Similar result has been obtained for catfish (At-Thar 2007). In addition, embryo may also die when injection is too deep into yolksac (At-Thar 2007). This might also contribute to the decrease in survival rate of injected humpback grouper embryos. As blastodisc of humpback grouper is very thin, it is highly possible that the microinjection needle enters the yolksac.

Furthermore, twenty microinjected embryos for each treatment at 14 hours post injection were pooled into a tube for DNA analysis. PCR amplification product showed that all microinjected embryos group contained foreign DNA (Fig. 3). This suggested that foreign DNA was transferred into blastodisc.

Table 2. Number and percentage of developed embryos after injection of 25 μg/mL at 1, 2 and 3 seconds.

Treatment	Repetition	No. developed embryos	Undeveloped embryos	Percentage developed embryos
1 second	1	42	2	95,5
	2	41	14	74,5
	Mean			85.0±14.8
2 second	1	1	18	5,3
	2	3	42	6,7
	Mean			6.1±1.0
3 second	1	0	31	0,0
	2	0	105	0,0
	Mean			0.0±0.0
Control		508	39	92,9

Electroporation

In this study, application of electroporation method for transferring the gene revealed that high percentage of electroporated sperm was motile (Table 3). There was no difference in sperm motility of treated and untreated DNA i.e. during 5-10 minutes in water. This result suggested that electroporated sperm could fertilize the eggs as in untreated sperm. Percentage of motile electroporated spermatozoa using DNA concentration of 5 μg/ml was slightly lower (90%) compared to that of the two other treatments (100%). Slight difference of water quantity in DNA solution might take account to reduce percentage of motile sperm in 5 μg/ml DNA concentration. Furthermore, droop value when using 0.2 cm cuvette was slightly higher (6-12%) compared with 0.4 cm cuvette (0-6%). Thus, droop seems to be affected by the size of cuvette used, although no effect on electroporated sperm motility (Table 1). Droop

is a function of voltage reduction at the end of electric shock (V_0V_t) from a starting voltage V_0 (Anonymous 2006).

Table 3. Actual voltage, droop and percentage of motile sperm in electroporation using different DNA concentration and cuvette gap size

DNA concentration (µg/ml)	Repetition	Setting Voltage (volt)	Cuvette gap (cm)	Actual voltage (volt)	Droop (%)	Sperm motility (%)
	1	50	0.4	37	6	90
5	2	50	0.4	37	0	90
	3	50	0.2	37	12	90
	1	50	0.4	37	6	100
10	2	50	0.4	37	6	100
	3	50	0.4	37	0	100
	1	50	0.2	37	12	100
20	2	50	0.2	37	12	100
	3	50	0.2	37	6	100

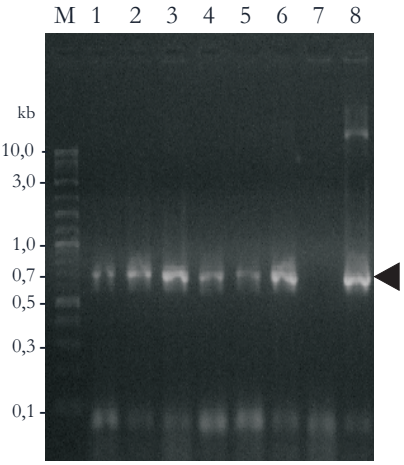

Figure 3. PCR amplification product of DNA extracted from microinjected embryos at 14 hours post injection. Lanes 1-2: 1 second injection, lane 3-4: 2 seconds injection, lane 5-6: 3 seconds injection, lane 7: PCR product with no DNA template, and lane 8 is PCR product with plasmid pktBP-ktGH as template. M is 2-log ladder DNA marker (BioLabs, Inc., New England).

PCR analysis showed that electroporated sperm in all treatments contained foreign DNA (Fig. 4). Thus, parameters of electroporation used in this study could deliver foreign DNA to enter spermatozoa. In addition, semi-quantitative PCR was applied to determine whether increase of DNA concentration used in electroporation could improve number of sperm carrying foreign gene. As shown in Figure 4, the thickness of DNA band increased by increasing concentration of foreign DNA used in this electroporation method. Volume of semen (25 µl, equal to about 175 million spermatozoa) utilized in electroporation and concentrations of the extracted

DNA from electroporated spermatozoa used as template in PCR amplification were similar among treatments. Thus, most likely increasing of DNA concentra-tion in this study increased copy number of foreign DNA in spermatozoa. Furthermore, higher copy number of foreign gene entering spermatozoa may raise the possibility of foreign gene to integrate to the host genome. Through fertilization, those spermatozoa would then contribute to enhance the number of embryo carrying foreign gene.

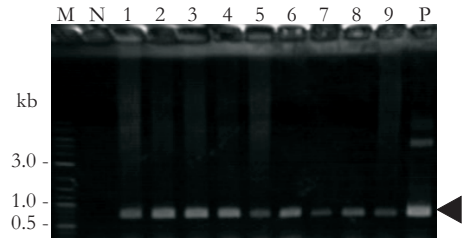

Figure 4. PCR amplification product of DNA extracted from electroporated sperm. Lanes 1-3: 20 µg/ml plasmid DNA concentration, lanes 4-6: 10 µg/ml plasmid DNA concentration, lanes 7-9: 5 µg/ml plasmid DNA concentration. M is 2-log ladder DNA marker (BioLabs, Inc., New England). N is PCR product without DNA template. P is PCR product with plasmid pktBP-ktGH as template.

Efficacy of sperm-mediated gene transfer (SMGT) technique to produce a transgenic marine species has been proved by Lu *et al.* (2002) and Sun *et al.* (2004). SMGT method has also been applied to introduce foreign gene into humpback grouper sperm. Three SMGT methods (electroporation, *in vitro* and *in vivo* transfection) were examined in this study. *In vitro* transfection using JetPei reagent succeeds to be used in white shrimp (Sun *et al.* 2005). In this study, humpback grouper sperm died when transfection was performed using JetPei reagent. Any compounds of JetPei may be toxic for humpback grouper sperm. In contrast, *in vitro* transfection using transfast reagent allowed humpback grouper sperm to survive. Transfast transfected-sperm also carried foreign gene, although this was only found when transfection was conducted for 48 hours at 25°C incubation. Furthermore, *in vivo* transfection by injecting DNA-transfast complex into testis could also deliver foreign gene to sperm. Foreign gene could be detected in transfected sperm at least 36 hours post injection. Transgenic sea bream could be obtained by *in vivo* transfection for at least 48 hous prior to spawning (Lu *et al.* 2002). In the same way with sea bream, transgenic humpback grouper may also be produced. In this study, however, only two out of ten injected broodstocks carried foreign gene in their sperm. This might be due to the difference in testis maturity. In addition, by applying the spawning system for humpback grouper, screening of founder transgenic fish generated by *in vivo* transfection method is costly, labor-extensive and time-consuming.

Microinjection is generally applied to produce transgenic fish. This method has also been used to introduce foreign gene through fertilized eggs towards generation of transgenic humpback groper. As shown in Figure 3, injected embryos carried the foreign gene at least until 14 hours post injection. However, in this study, none or very low number of injected embryos hatched. As in other marine finfish, eggs of

humpback grouper float in water, small in size and has unclear blastodisc. These conditions hamper microinjection precisely. In addition, the time to reach 2-cell stage of humpback grouper embryos is about 20-30 min post fertilization. So the number of embryos which could be injected by one microinjector is very limited (60-100 embryos). Hatching rate of uninjected embryos is high, but the survival rate of larvae is lower i.e. 5-10% in average. Based on the results in zebrafish transgenic research, the number of germline transgenic F0 is 2-4% of survived fish (Alimuddin *et al.* 2005; Alimuddin *et al.* 2008). If we assume that similar number of germline transmitted F0 in zebrafish can be achieved in humpback grouper, the number of embryos injected will be at least 1000 embryos to obtain 2 transgenic F0. This means that microinjection should be conducted 10 times, or we need more microinjectors and technicians. Thus, the use of microinjection technique to produce humpback grouper requires facilities, and is labor-extensive as well as time-consuming.

Electroporation has been reported to be a simple and mass transgenic production method. This technique also has similar efficiency with *in vivo* transfection method to produce transgenic sea bream (Lu *et al.* 2002). As shown in Figure 4, all treatments allowed the sperm to carry the foreign gene. Based on the sperm motility and results of PCR analysis, 10 μg/ml was considered as the optimum DNA concentration for electroporation of humpback grouper. This DNA concentration was lower than that used for sea bream, 25 μg/ml (Lu *et al.* 2002). Voltage (50 V), pulse length (30 μs) and number of pulse (5 pulses) were also lower compared to those of sea bream. Electroporation in sea bream is conducted using 600-2000 V, 40 μs pulse length, and up to 8 pulses. This suggests that optimum level of electroporation parameters may be species specific.

Finally, compared to the three other methods examined for humpback grouper as discussed above, electroporation to sperm is a fast, simple, and efficient transgenic method for humpback grouper. Production of transgenic humpback grouper using electroporation technique is in progress in our laboratory.

CONCLUSIONS

Electroporation to sperm was considered as an appropriate approach by means of efficacy and simplicity, to generate transgenic humpback grouper. Optimum DNA concentration for electroporation was 10 μg/ml. Production of transgenic grouper carrying genes regulating the important traits for aquaculture holds exciting possibilities for the future, though the cultivation of transgenic organisms remains controversial.

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