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Development of the Gene Transfer Systems for Cultured Fish

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ABSTRACT

In order to find out whether the tyrosinase gene can be expressed in fish *in vivo* as a natural marker, we introduced mouse tyrosinase gene (a 14.1 kb ptrTYR5 with *Xba*I digestion) into an albino mutant of catfish (*Clarias fuscus*). Results showed that around 33% of treated catfish appeared a black stain at the injected site after the 5th day following the third injection. The color spots became increasingly dark, with some dense black pigments appearing on the skin surface after the 7th to 8th day. Onset of this foreign gene expression was postition- and/or individual-dependent and showed a transient pattern.

A foreign gene was introduced into fertilized black porgy (*Acanthopagrus schlegeli*) eggs via electroporation. Results showed that embryos treated at 3 kV, 2^{11} pulses and 8 cycles at the one-cell stage led to the highest survival and hatching rates. However, the survival and hatching rate ranges for the untreated and electroporated groups decreased from 44-80% to 30-50% and from 38-54% to 24-37%, reapectively. Blottings and PCR proved that 15% of the examined seven-day-old fry persisted the transgene.

Loach (*Misgurnus anguillicaudatus*) sperm was used to study the sperm-mediated transfer system. Results showed that the most effective parameter for sperm mobility was amplitude. Compared with the untreated sperm, the fertility of electroporated-sperm in the absenec of DNA showed a slight decrease, i.e., 88 vs 76%; but no difference of hatchability, i.e., 72 vs 68%. But the fertility and hatchability of elcetroporated-sperm in the presence of DNA was significantly lower, i.e., 72% and 61%, respectively. A exogeneous growth hormone cDNA was introduced into loach embryos via electroporated sperm. The genomic DNA was extracted from two-week-old loach fry and analyzed by dot blot hybridization. The success rate of gene transfer was 52.2% (94 positive out of 180 fry examined). Furthermore, Southern blotting analysis of the genomic DNA isolated transgenic loach showed that the positive band with the same molecular size as the transferred DNA (4 kb), with higher size (>23 and 8 kb) and with smaller size (2.4, 0.7 and 0.4 kb).

Key words: *Electroporation, Fish, Gene transfer, Microinjection, Skin.*

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INTRODUCTION

Transgenic animals procide a powerful system for *in vivo* study of gene regulation, expression and function. Many approaches have been developed to introduce different types of DNA molecules into zygotes with varying degrees of success. In fish, the most popular methods are (1) microinjection of foreign DNA into oocyte nuclei (Ozato et al., 1986; Tsai et al., 1995), cytoplasm of the developing embryos (Chourrout et al., 1986; Dunham et al., 1987; Zhang et al., 1990) and fertilized eggs (Fletcher et al., 1988; Dunham et al., 1992; Hew et al., 1992; Lu et al., 1992). In order to find out whether the tyrosinase gene can be expressed in fish *in vivo* and served as a screening marker,we microinjected mouse tyrosinase gene into an albino mutant of catfish *(Clarias fuscus).* (2) Electroporation of DNA fragments into eggs (Inoue et al., 1990; Powers et al., 1992). Electroporation of fertilized eggs could overcome some gene transfer problems in cultured fish, i.e., the invisible pronuclei and tough chorion. The efficiency of gene transfer by electroporation for fertilized eggs is ten to hundred times greater than that of microinjection (Powers et al., 1992) transfer efficiency. The black porgy (*Acanthopagrus schlegeli*) is one of the most common marine aquaculture fish species in Asia. Taxonomically, it is classified as part of the order Perciformes, suborder Percodei, and family Sparidae. Physiologically, the black porgy belongs to the group of protandrous hermaphroditic species. In this paper, we will describe the optimal electroporation conditions for the transferring of a gene into black porgy embryos. (3) Use of sperm cells as a vector for introducing foreign DNA into oocyte. This method has been attempted resently because it is considered as a "mass" gene transfer. Fish sperm is apparently tougher and easier to handle than mammalian sperm. Simply adding water is enough to activate fish sperm. These advantages make fish sperm a suitable material for studying sperm-mediated gene transfer. We evaluate the possibility of using fish sperm treated with electroporation as a "mass" gene transfer carrier for loach (*Misgurnus anguillicaudatus*) oocytes.

MATERIAL AND MEEHODS

Experimental animals

Albino mutants of catfish were obtained from a local hatchery. And so did black porgy. These specimens were induced to spawn gametes via muscle injection of 100 U LH-RH per kg of body weight. *In vitro* fertilization was conducted in order to control the embryonic development stages for the transferring of foreign genetic material.

Loach gametes for *in vitro* fertilization were obtained from 20 to 50 g in body weight, purchased locally, and maintained on a natural light and dark cycle.

Egg collection

Mature female loach (over 30 g) were induced to ovulate by intramuscular injection of chorionic gonadotropin (CG) : one third dosage of 15 IU CG per gram of body weight was injected after 2 h. Eggs were spawned 10-13 h after CG injection. The eggs from a total of 16 female loach were collected, mixed, weighed and divided into groups for one trial.

Sperm preparations

Testes were obtained from mature male loach (over 25 g) or loach injected with 10 IU CG/g body weight for 10-13 h. Testes were mixed with 1 ml phospate buffer saline (PBS) and homogenized in a glass homogenizer for 1 min at room temperature. The homogenates without tissue debris were pooled together. The sperm suspensions were prepared by resuspending pellets in a final concentration of 1 to 2 x 10^8 cells per ml.

Transferred DNA

Plasmid ptrTYR5 (kindly provided by Prof. Schutz, German) of size 14.1 kb was consisting of 5' 270 bp upstream region (including promoter), exon 1 (900 bp), intron A (8.2 kb), fused exon 2-5 (900 bp). (Beermann et al., 1991). Purified plasmid was linearized by *Xba*I digestion and resuspended in 10 mM Tris · HCl and 1 mM EDTA (pH 8.0) buffer at a concentration of 400 ng/ml.

The opAFPGHc fragment, which consists of the antifreeze protein promoter of ocean pout fused with the 5'-untranslated region, coding region and 3'-untranslated region of chinook salmon GH cDNA (Du et al., 1992), was recovered by *Eco*RI digestion of the plasmid, opAFP-GHc (kindly provided by Prof. Hew, Canada) .

Electroporation

A noncontact mode instrument (Baekon 2000, CA) was used to carry out electroporation. The conditions for electric field strength were varied from 1 to 12 kV, burst time from 0.6 to 2.2 s, number of pulese per cycle from 2^4 to 2^{13} , and number of cycles from 2 to 14. Their resultant influences on sperm mobility of loach were examined microscopically and categorized by following the description of Guest et al. (1976). For black porgy, 90 different combinations of amplitude, cell and four-cell embryos in order to examine rates of survuval (12 h after fertilization) and hatching (3 days after fertilization).

DNA analysis of sperm treated with DNase

The genomic DNA of sperm were extracted and analyzed by dot blot using a digoxigenin labelled probe to optimize the concentration of DNase capable of completely digesting opAFPGHc persisting outside of the loach sperm. After complete digestion by DNase, the electroporated-sperm were washed several times with buffer and their genomic DNA analyzed by Southern blot using a digoxigenin-labelled opAFPGHc probe. The genomic DNA of the incubated-sperm was assayed after DNase digestion and PBS washing.

Dot blot and Southern analysis

Genomic DNA of sperm, embryo and fry were extracted (Inoue et al., 1990), and 1 μ g was spotted on a nylon membrane and hybridized to a ^{32}p-labeled opAFPGHc. For Southern blot analysis, genomic DNA was digested with *Eco*RI, electrophoresed on a 0.8% agarose gel, transferred to a nylon membrane, and then hybridized to the probe. Hybridization was carried out in a solution of 50% formamide, 5 X Denhart's solution 0.1% SDS, 6 X SSC and a denatured probe at 42°C overnight. Membranes were washed

twice in a solution containing 1 X SSC and 0.1% SDS for 15 min at 42°C, and twice in a solution of 0.1 X SSC and 0.1% SDS for 30 min at 68°C before autoradiography.

PCR analysis

Four oligonucleotide primers were synthesized for PCR analysis according to Du et al. (1992). PCR was carried out by 20-30 ng templates, 10 *p*mol of each primer, 200 μ M of each dNTP, 10 μ g of bovine serum albumin, 1.75 mM $MgCl_2$ and 1 U Taq DNA polymerase. PCR consisted of 25 denaturing cycles at 92°C for 1 min, annealing at 50°C for 2 min, and extension at 72°C for 3 min with a 10 min extension at 72°C.

RESULTS

【 I 】 Microinjection of tyrosinase gene for catfish (Tseng et al., 1995)

A black stain appeared at the injected site between the 5th and 6th day following the third injection. This spot gradually spread to form a black pattern in the positive reaction area 7- to 8- days later (right side of Fig. 1; p.193). The color became increasingly dark, with some dense black pigments appearing on the skin surface. Two weeks later, the black stains began to fade, and after the third week only a few faint spots remained. No black spots or patterns appeared on the left side - - the side injection with the solution minus the DNA fragments (left side of Fig. 1; p.193). Of the 6 catfish we injected, we found that 2 of them (33%) were positive in right area.

The dorsal side close to the anal fin was another area where spots appeared; hwever, we observed that not all of the injected area on the dorsal side showed positive reactions, even following a sequence of four injections.

【 II 】 Electroporation for black porgy's embryos (Tsai and Tseng, 1994)

Amplitude effect. For black porgy embryos, electroporation amplitude should not be too strong. The survival rates of embryos treated at either 6 kV or 9 kV were extremely low, i.e., 0-7% and 0-4% for groups of 6 kV and 9 kV, respectively; whereas, that of the untreated control groups averaged 47%. Under microscopic observation, we found some embrys treated at 6 kV fused together. Furthermore, if embryos were treated at 9 kV, some embryos showed serious damage.

Four-cell stage of treated embryos. The developmental stage of fertilized eggs is one of the most important factors related to higher survival and hatching rates for electroporated black porgy embryos. After treatment at 3kV,2^{11} pulses, and 8 cycles, the survival and hatching rates of electroporated four-cell stage embryos in the presence of opAFPGHc decreased dramatically to 2.8% and 0.9% , respectively. Similar results were also observed for group of four-cell embryos treated at 2.5kV, 2^{11} pulses ,and 4 cycles; in this case,the survival and hatching rates were 1.7% and 0.3% , respectively.

Optimal electroporation conditions. After examining the 90 conbinations of amplitude, number of pulses, and cycles, we found that embryos treated at 3kV, 2^{11} pulses, and 8 cycles at the one-cell stage showed the highest survival and hatching rates.

The average survival rates following three different experiments were 35% (3 groups) 30% (10 groups) and 50% (20 groups); whereas the untreated control group avarages 47%, 44% and 80%, respectively. The average hatching rates for those groups previously described versus the control groups were 32 vs 48%, 24 vs 38%, and 37 vs 54%, respectively. Embryos treated at 2.5 kV, 2^{11} pulse, and 4 cycles at the one-cell stage showed the second highest rates: 28-30% survival and 19-22% hatching, or average. However, compared to the untreated control groups, the survival and hatching rates of electroporated fertilized black porgy eggs under our experimental conditions revealed small decreases.

Dot blot and Southern blot analyses. Genomic DNA was extracted, from seven-day-old fry and analyzed by dot blot hybridization with a digoxigenin-labelled plasmic (pAFP-GHc). Results showed that the transferred opAFPGHc was detected in some o the experimental fry; whereas, none of the fry from the untreared control group showec positive transference (Fig. 2; p.194).

PCR analysis. As Du et al. (1992) observation, three PCR products with molecula weights of 855, 333, and 199 bp were generated from three oligonucleotide pair (primers A/B, A/D, and C/D) chosen for the PCR amplification of a Southern blot-positive genome, respectively (Fig. 4; p.195). On the other hand, when the primer set were chosen from either non-transgenic black porgy in the experimental group or from the control black porgy, no PCR products were produced.

【III】 Sperm-mediated gene transfer for loach (Tsai et al., 1995)

DNA uptaken by sperm. After electroporation. after digestion with 10 μ g DNase a 37°C for 1 h, no positive reaction was found on dot or Southern blot analysis of sperm suspension (100 μ L) mixed with opAFPGHc (0.1 μ g). However, positive reaction were found for groups treated with 0 (control), 0.1 and 1 μ g of DNase.

After the diluted sperm srspensions were electroporated in the presence o opAFPGHc, the suspension was treated with 10 μ g DNase at 37 °C for 1 h. The genomic DNA of pooled sperm (10^7 cells) was extracted and analyzed by dot blo hybridization. Results showed that DNA isolated from the electroporated sperm witl opAFPGHc was positive whereas DNA isolated from the electroporated sperm in th absence of opAFPGHc was negative.

Southern blot analysis of genomic DNA isolated from the electroporated sperm i the presence of opAFPGHc showed a positive band with a molecular weight of 4 kl which was the same size as that of opAFPGHc, 50 ng of transgene was electroporate into 10^7 sperm. However, the genomic DNA obtained from the electroporated sperm i the absence of opAFPGHc (mock-treated grorp) was negative. Moreover, sperm incubated with opAFPGHc without electroporation treatment were also negative (Fig. p.195).

Effect on fertility and hatchability of electroporated sperm. Compared with th untreated sperm (control group), the fertility of electroporated-sperm in the absence o DNA fragments (mock-treated group) showed a slight decrease, i.e., 88 vs 76%; but no

much difference of hatchability, i.e., or 72 vs 68% (column C vs C1 under II in Table 1; p.193). Compared to the control group, the fertility of electroporated-sperm in the presence of opAFPGHc (experimental group) was also lower, i.e., 90 vs 62% (trial I) and 88 vs 72% (trial II). Hatchability appeared slightly significant difference between the control and experimental groups, i.e., 71 vs 67% (trial I) and 72 vs 61% (trial II).

Analysis of transgenic loach by dot and Southern blottings. Genomic DNA was extracted from two-week-old loach fry and analyzed by dot blot hybridization. The success rate for opAFPGHc gene transfer was 52.2% (94 positive out of 180 fry examined). When 26 genomic DNA randomly chosen from 94 dot-positive ones were digested with *Eco*RI, run on the agarose gel and analyzed by Southern blotting using a probe, 92.3% (24 out of 26) of samples were identified as positive reaction. A 4 kb positive band (same size as the transgene) was shown on 58% (14 out of 24) of the transgenic fry loach. Nevertheless, different size band(s) were observed: >23, 8, 2.4, 0.7, 0.4 (Fig. 6; p.196).

DISCUSSION

Mouse tyrosinase gene was expressed in the skin of catfish albino mutant following direct injection of the gene into the skin. Onset of this foreign gene expression was position- and/or individual-dependent and showed a transient pattern.

Although we have not yet fully analyzed all of the experimental black porgy (approximately 2000 fry), the ovseved rate of successful gene transfer in seven-day-old fry was approximately 15% (18 out of 120 fry examined); this is close to the rate reported for eletroporation in medaka eggs, i.e., 4-20% (Inoue et al., 1990; Lu et al., 1992), and for microinjection in four species, including medaka, i.e., 16-27% (Ozato et al., 1989; Inoue et al., 1990; Lu et al., 1992), zebrafish, common carp and channel catfish, i.e., 0-35% (Powers et al., 1992). However, the observed success rate in the present study was not as high as that reported for electroporation in zebrafish (35-75%) (Powers et al., 1992). These inconsistent results, including survival rate of black porgy embryos, may be due to electroporation conditions, instruments, fish species' characeristics, developmental embryo stages, and the quality of fertilized eggs used.

The fertility of the electroporated sperm (mock-treated group and experimental groups) was slightly less than that of untreated sperm. We observed that some sperm fused together occasionally after electroporation, although the motility of sperm did not show any difference under microscopic observation after electroporation. We propose that those fused sperm might cause lower fertility.

Similar to the control group, cumulative mortality of the mock-treated group was auound 20-30%. However, the mortality of the group hatched using electroporated-sperm treated with opAFPGHc was comparatively high, i.e., 40-50%. Because the mortality of the mock-treated group was close to that of the control group, the higher mortality which occurred in the experimental group may gave been due to the random integration of transferred gene or/and the expression of the exogenous GH gene.

Since 0.1 μ g opAFPGHc in sperm suspension prior to electroporation could be completely digested by 100 μ g/ml DNase, the positive hybridization found in the dot

and Southern analysis suggest that foreign DNA was introduced into sperm using electroporation conditions outlined in this study. Our findings are in agreement with the results of Gagne et al. (1991), who demonstrated that foreign plasmid could be introduced into bovine oocytes by electroporated spermatozoa. However, if sperm was incubated with opAFPGc, the exogenous DNA was not transferred into sperm. This result was consistent with that of Gavora et al. (1991), who failed to transfer DNA into eggs of mice and chicken by using spermatozoa incubated with bacterial plasmid DNA. The result was also in agreement with Chourrout and Perrot (1992), who failed to detect transgenesis in rainbow trout by incubated sperm. These observations strongly support Muller et al. (1992), who demonstrated that a foreign gene was introduced into fish with electroporated sperm, but not with incubated sperm. However, it was contrary to the report of Khoo et al. (1992), who suggested that foreign plasmid could be introduced into zebrafish by simply using sperm incubated with DNA. Therefore, the exact mechanism for sperm-mediated DNA transfer needs further study.

Although we have not yet analyzed all the experimental loach, the rate of successful gene transfer that we found in two-week-old fry was approximately 50%. It was close to that reported for electroporation in zebrafish embryos, i.e., 35-75% (Powers et al., 1992). Nevertheless, the success rate of this study was not only higher than that reported for microinjection in medaka, i.e., 16-27% (Ozato et al., 1986; Inoue et al., 1990; Lu et al., 1992), zebrafish, common carp and channel catfish, i.e., 0-35% (Powers et al., 1992), but also higher than that reported for electroporation in medaka eggs, i.e., 4-20% (Inoue et al., 1990; Lu et al., 1992) and for electroporation in sperm of carp, tilapia and catfish, i.e., 2.6-4.2% (Muller et al., 1992). The reasons why our success rate was superior to that of Muller's report (1992) might be due to the difference of electroporation condition, equipment and fish employed.

Electroporation bas often been used for gene transfer into culture cells and embryos. In this report we have clearly demonstrated that foreign genes can be transferred into fish embryos by using electroporated-sperm as a vector. Compared to conventional microinjection and electroporation for fish eggs, electroporation of fish sperm is a relatively simple mass gene transfer procedure.

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Table 1. The fertilization and hatching rate of loach. C: the control group (sperm without treatment); C1: the mock-treated group (sperm electroporated without foreign DNA); E: the experimental group (sperm electroporated with opAFPGHc). One gram of eggs contains approximately 3000 eggs (Tsai et al., 1995).

Trial	I		II		
	C	E	C	C1	E
Egg weight (g)	**8.2**	**9.6**	**14.8**	**8.4**	**12.9**
Fertilized rate (%)	**90**	**62**	**88**	**76**	**72**
Hatching rate (%)	**71**	**67**	**72**	**68**	**61**

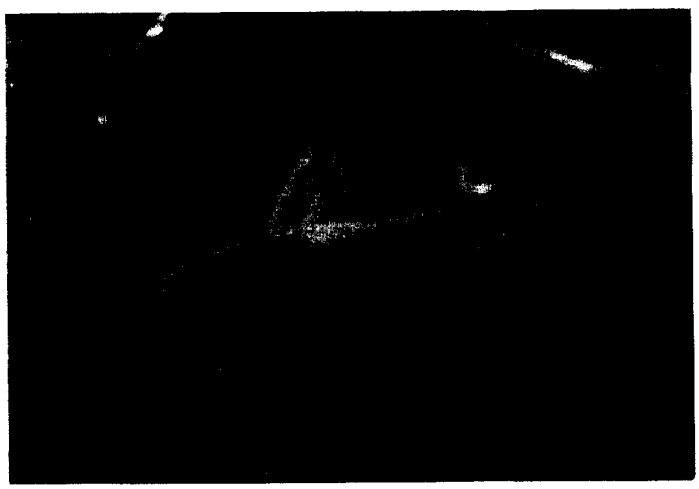

Fig. 1. Black staining in albino walking catfish observed at the dorsal side of the front right trunk (arrow tip) where a mouse tyrosinase gene was injected subcutaneously. No black pattern was observed on the left side where the solution for injection was without DNA (Tseng et al., 1995).

Fig. 2. Dot blot analysis of genomic DNA from seven-day-old fry. Row Al, positive control (opAFPGHc); Rows A2 to A7, untreated control group; Row B, experimental group (Tsai and Tseng, 1994).

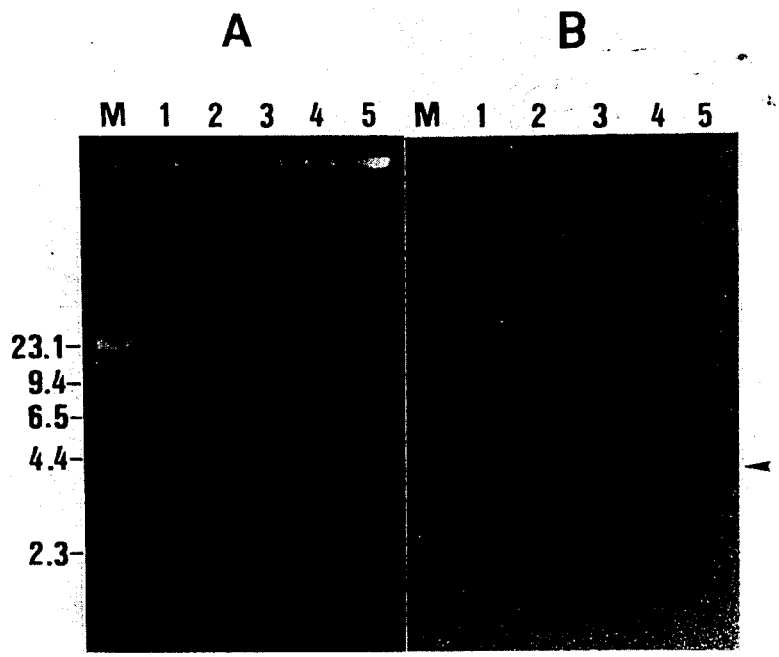

Fig. 3. (A) Gel electrophoresis and (B) Southern blot analysis of genomic DNA from seven-day-old fry. Lane 1, opAFPGHc used for transferring; lane 2, plasmid pAFP-GHc (pUC with opAFPGHc) digested with *Eco*RI; lane 3, untreated control genome without digestion; lane 4, dot-negative genome digested with *Eco*RI; lane 5, dot-positive genome digested with *Eco*RI; and lane M, molecular marker. Arrowhead indicates the transferred DNA (4 kb) (Tsai and Tseng, 1994)

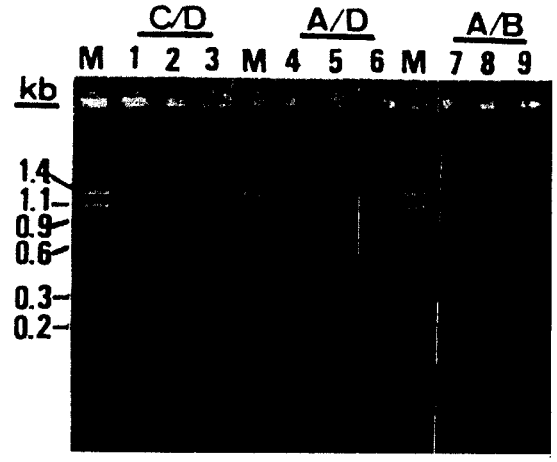

Fig. 4. PCR analysis of genomic DNA from seven-day-old fry. Primer sets used are listed in the first row. Lanes 1,2,4,5,7 and 9: dot-positive samples from the experimental group; lanes 3,6 and 8: samples from the control group; and lane M, molecular marker (Tsai and Tseng, 1994).

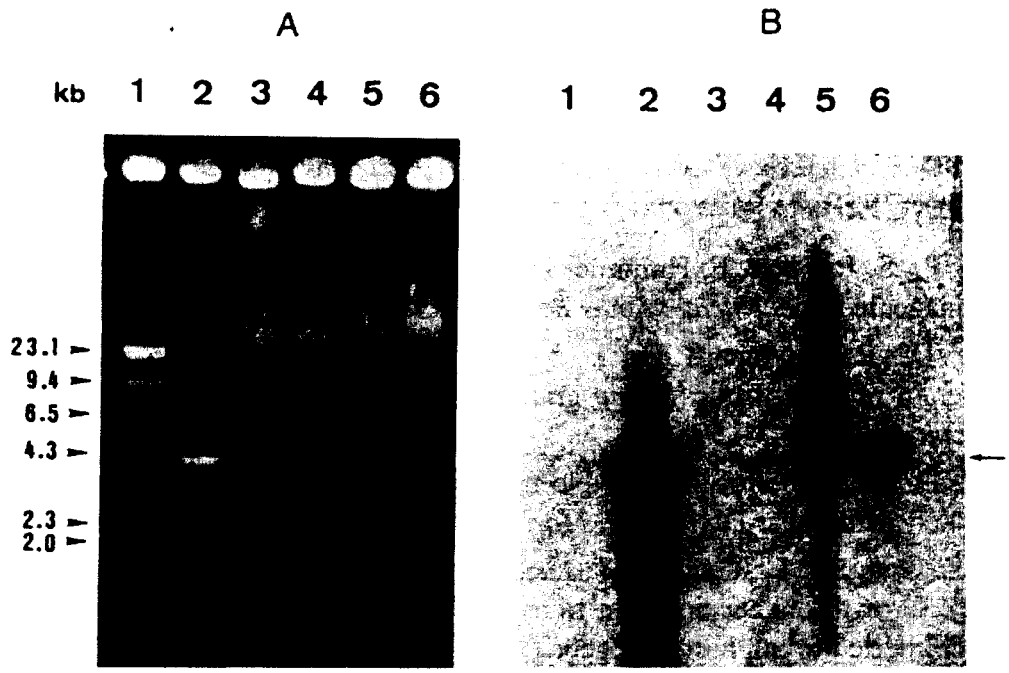

Fig. 5. Southern blot analysis of genomic DNA isolated from sperm. (A), genomic DNA run on the gel; (B), transferred to a membrane and hybridized to opAFPGHc probe. Lane 1, molecular marker, *Hin*dIII-digested lambda; lane 2, 500 ng of opAFPGHc (positive control); lane 3, sperm incubated with water; lane 4, sperm incubated with opAFPGHc solution for 30 min; lanes 5 and 6, sperm electroporated with opAFPGHc. Arrow indicates the molecular size of electroporated DNA (4 kb) (Tsai et al., 1995).

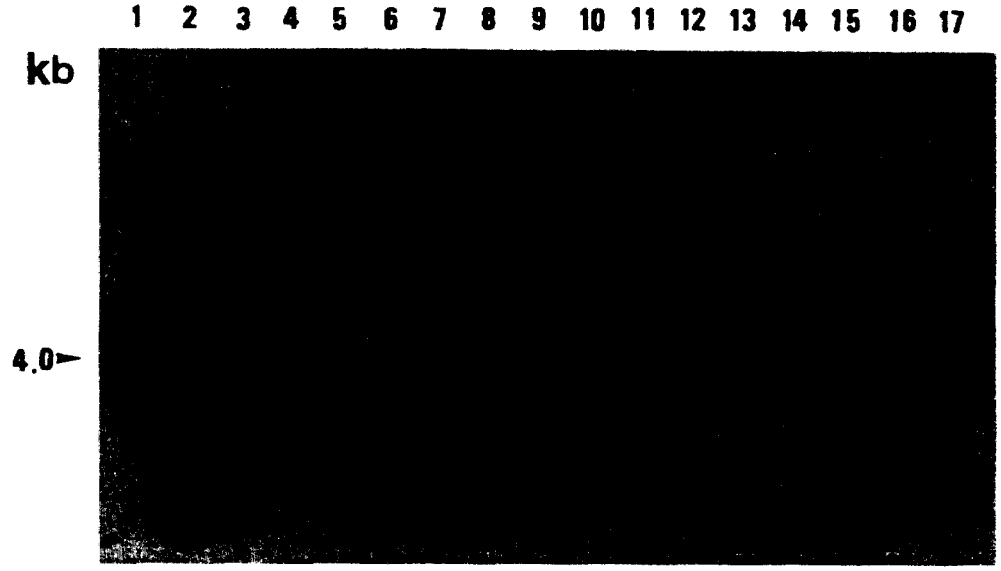

Fig. 6. Southern blot analysis of presumptive transgenic 2-week-old loach developed from electroporated-sperm carrying opAFPGHc. *EcoRI*-digested genomic DNA was electrophoresed on a gel, and carried out Southern blot hybridization. Lane 1, molecular marker in kb; lane 2, opAFPGHc (positive control) used for transferring; lane 3, blank; lanes 4 to 17, different presumptive transgenic individuals. Arrow indicates molecular size of the transgene (Tsai et al., 1995).

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經濟魚類各種基因轉殖技術的開發

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利用顯微注射的方法將帶有黑色素基因的質體ptrTYR5 (14.1 kb,用*Xba*I切成線型)打到白色變種鯰魚(Albino walking catfish *Clarias fuscus*)肌肉，結果發現33%處理的白色鯰魚在注射的背部區域約在第5天左右有黑色斑出現，在第7天及第8天達到最高表現量。但兩個星期後則逐漸消失。而在打不含DNA溶液的背部區域(控制組)就沒有黑斑顯出。而實驗組對外來基因有正反應的程度則因個體和(或)部位而有所明顯差異。

外來的生長激素基因經電破方式進入黑鯛(*Acanthopagrus sehlegeli*)的授精卵內。在90種不同的電破條件配合下(電壓、脈衝數及週期等)，我們發現3kv電壓，2^{11}脈衝數及8個週期的處理可以得到最高的活存率及孵化率。但是和控制組相比，仍顯示活存率的降低，如從44-80%降至30-50%；同時孵化率也會從38-54% 降至24-37%。用點墨、南方氏浸漬法和PCR的方式都可以偵測到七天大的小魚含有轉殖進來的基因。其轉殖率約15%。

先在不加外源性異質基因下，對泥鰍(*Misgurnus anguillicaudatus*)精子懸浮液進行電破，分別觀察不同係數組合如電壓從2至12kv, 穿孔時間從0.8到1.6 sec，脈衝數從2^6至2" 週期數從2至12，對精子的影響。結果發現對精子運動能力的影響最明顯的是電壓。電壓到8 kv以上在顯微鏡觀察處理過的精子其的運動能力下降；若10 kv以上時即看不出有運動能力。而電破後精子的運動能力又與雄魚的成熟度有關。對電破處理的精子做人工受精結果證明：電擊沒有含DNA的精子其受精率為76% 和孵化率68% (卵數為72312±276個)；與不處理的精子的88% 受精率和72% 的孵化率相比稍微下降。而對電擊含DNA的實驗組而言(卵數為73740±270個)，則降低較顯著：如授精率降至72% 和孵化率降至61% 。處理外源基因的精子受精後所發育的魚苗，經點墨雜合分析其genomic DNA，證明含有外源基因的魚苗有52% (180隻中有94隻)。若再用南方氏雜合分析其*Eco*RI所切的genomic DNA發現轉殖魚除了含有與轉殖DNA一樣大小(4kb)之外，也有大段(>23及8kb)以及小段(如2.4,0.7及0.4kb)的正反應DNA條紋存在。

從這些證據充份顯示無論是皮下顯微注射、授精卵電破或者精子電破法都能成功地將外來基因片斷轉殖到不同的經濟魚類。因此，這些技術的開發和建立對本省經濟魚類的基因轉殖研究是項重要的基礎。

關鍵詞：電破法、魚、基因轉殖、顯微注射、皮膚。

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