

Full Length Research Paper

Extraordinary mullet growth through direct injection of foreign DNA

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The present study aims to produce a genetically modified grey mullet, *Mugil cephalus*, with accelerated growth through direct injection of foreign DNA isolated from the liver of shark (*Squalus acanthias* L.) or African catfish (*Clarias gariepinus*) into muscles of fingerlings fish at the dose of 40 µg/fish. The results show a significant ($P \leq 0.05$) improvement in most of the growth performance and body composition parameters of genetically modified grey mullet fingerlings injected with shark DNA compared to both genetically modified grey mullet injected with catfish DNA and the control fish, while the results of feed conversion ratio (FCR) and protein efficiency ratio (PER) indicate that fish injected with shark DNA or catfish DNA had significant ($P \leq 0.05$) superiority compared to their control. The results of the random amplified polymorphic DNA (RAPD) fingerprinting show highly genetic polymorphic percentage among grey mullet that received foreign DNA and their control using different random primers. This may be due to some fragments of foreign DNA randomly integrated into grey mullet genome. Therefore, the result indicates a possible easy and rapid way for improving fish characteristics.

Key words: Grey mullet, growth, foreign DNA, genetically modified.

INTRODUCTION

Grey mullet, *Mugil cephalus*, is a euryhaline fish widely distributed in tropical and subtropical estuaries. Mulletts are catadromous spawning migrating fish; the young life before maturity remains predominantly in the system of rivers and lakes (Lee and Tamaru, 1988; El-Deeb et al., 1996). Natural spawning of grey mullet in captivity has not yet been demonstrated (Lee et al., 1988; El-Gharabawy and Assem, 2006). Reports on induced spawning and larval rearing in grey mullet are primarily based on experiments carried out in Taiwan (Kuo, 1995; Liao, 1997) and Hawaii (Weber and Lee, 1985; Lee et al., 1987, 1988; Tamaru et al., 1989) where fertilized mullet eggs have been obtained consistently. However, no commercial production of mullet eggs has been reported to date in the Mediterranean basin. Grey mullet commands high price and the ability of juvenile and adult to tolerate large fluctuation of salinity qualifies them as an attractive species for farming (Monbrison et al., 2003; El-Gharabawy and Assem, 2006).

Since the first batch of transgenic fish was produced in China (Zhu et al., 1985 and 1986), many laboratories all

over the world have turned to the study of transgenic fish to gain new farming strains with the traits of fast-growing, disease resistance, cold or salt tolerance, sexual maturation, food quality and preservation (Shears et al., 1991; Chen et al., 1996; Martinez et al., 1996; Hernandez et al., 1997; Martinez et al., 1999, 2000; El-Zaeem, 2001, 2004 a, b; El-Zaeem and Assem 2006; El-Zaeem et al., 2011; El-Zaeem, 2011 a, b). A commonly used method to introduce foreign DNA is by microinjection into the nucleus or cytoplasm of fertilized eggs. This method, however, requires some skill and involves some difficulties and it is time consuming (Inoue et al., 1990; Sin et al., 1993). To avoid the difficulties accompanying microinjection, much more convenient methods are required, especially if such techniques are to be applied in aquaculture for fast breeding of commercially important species. The most common potential mass methods are: 1) the use of electroporation of fertilized eggs (Inoue et al., 1990; Inoue, 1992; Xie et al., 1993), 2) electroporated sperm (Muller et al., 1992; Symonds et al., 1994), 3) the use of sperm cells as vector to introduce foreign DNA into

Table 1. The sequences, GC % and the annealing temperatures of the primers used.

Primers	Sequence 5' - 3'	GC (%)	Annealing Temperature (°C /s)
1	GGA CTG GAG TGT GAT CGC AG	60	
2	GGT GAC GCA GGG GTA ACG CC	70	30
3	CAG GCC CTT CCA GCA CCC AC	70	30
4	GTA AAA GTC CTG GTT CCC CG	55	30
5	GGC GGA GCT GGA GGG CCT GG	80	30

fish eggs (Khoo et al., 1992) and 4) direct injection of foreign DNA into fish gonads (El-Zaeem, 2001).

A quick method to introducing foreign DNA injected directly into the muscle tissue was reported (Wolff et al., 1990; Ono et al., 1990) in adult mice, (Thomson and Booth, 1990) in rat, and (Hansen et al., 1991; Rahman and Maclean, 1992; Tan and Chan 1997; Xu et al., 1999; El-Zaeem 2004a; Hemeida et al., 2004; El-Zaeem and Assem 2004; Assem and El-Zaeem 2005; El-Zaeem et al., 2012) in fish. This procedure is useful because muscle injection is much easier than the others and very rapid results are obtained (Rahman and Maclean, 1992). The foreign DNA was presented extrachromosomally up to six months following injection (Wolff et al., 1990). Moreover, Sudha et al. (2001) reported that the expression of muscular injection of DNA was evident in several non muscle tissues, such as skin epithelia, pigment cells, blood vessel cells and neuron-like cells.

Therefore, the aim of this work was to study the effect of direct injection of foreign DNA extracted from shark (*Squalus acanthias* L.) or African catfish (*Clarias gariepinus*) into skeletal muscles of grey mullet (*Mugil cephalus*) on the productive performance characteristics. Moreover, genetic polymorphism among normal and injected fish was studied using random amplified polymorphic DNA (RAPD) fingerprinting.

MATERIALS AND METHODS

Fish origin

The grey mullet, *M. cephalus*, used in this study were collected from the Mediterranean sea and transfer to the Laboratory of Breeding and Production of fish, Animal and Fish Production Department, Faculty of Agriculture, (Saba-Bacha) Alexandria University, Alexandria, Egypt.

Preparation of genomic DNA

High molecular weight DNA was extracted according to Brem et al. (1988) method. Isolation of DNA was accomplished by reducing liver sample from shark (*S. acanthias* L.) and African catfish, *C. gariepinus* to small pieces, which were then transferred to a microfuge tube and incubated overnight until the samples were digested in a buffer containing 50 mM Tris, 100 mM ethylenediaminetetraacetic acid (EDTA; pH 8.0), 100 mM NaCl, 0.1% sodium dodecyl sulphate (SDS) and 0.5 mg/ml proteinase K. After incubation, samples were extracted twice for 15 to 20 min with one volume of phenol/chloroform (1:1) and then again twice for 15

min with one volume of chloroform/isoamyl-alcohol (24:1). The aqueous phase was then precipitated with 2.5 volumes of 100% ethanol in the presence of 1/10 volume 3 M sodium acetate (pH 6.0). The pelleted DNA was washed with 70% ethanol and dissolved in 0.1X saline sodium citrate (SSC) buffer. The DNA concentrations were measured by ultraviolet (UV) spectrophotometry. The extracted DNA was restricted by *EcoR*I restriction enzyme type II. The DNA between guanine and adenine was digested according to Tsai et al. (1993).

Experimental setup

Management

Ninety fingerlings of grey mullet (*M. cephalus*) with an initial live weight (2.11 ± 0.01 g) were divided randomly into three groups and three replicates for each group. Each group was stocked separately at a rate of 1.0 fish/17.5 L in a half of rectangle fiberglass tank (total volume, 350 L, which was divided by plastic sieved connected with iron frame. Each tank was supplied with fresh water at a rate of 0.5 L/min with supplemental aeration. Fish were fed twice daily with pellet diet (28% protein) to satiation six days a week, and weighed biweekly for 63 days.

Injection of foreign DNA *in vivo*

The DNA concentration of 40 µg/0.1 ml/fish (El-Zaeem, 2004a; El-Zaeem and Assem, 2004; Hemeida et al., 2004; Assem and El-Zaeem, 2005) were prepared from each type of DNA using 0.1X SSC buffer and injected into grey mullet muscles using a hypodermic needle. The injection was applied on two groups of grey mullet fingerlings, while the third group was left without injection as a control.

Quantitative traits studied

The following traits were measured; body weight (g), weight gain (g), specific growth rate (SGR %/day), survival ratio, feed intake, Feed conversion ratio (FCR) and protein efficiency ratio (PER). Whole body composition of fish was analyzed according to the standard methods (AOAC, 1984) for moisture (oven drying), protein (micro-Kjeldahl method) and lipid (ether extract method).

Random amplified polymorphic DNA (RAPD) analysis

By the end of the experiment, genomic DNA was extracted from tissue of injected fish and their control according to the method described by Baradakci and Skibinski (1994). In this work, 20 base long oligonucleotide primers (Table 1) were used to initiate PCR amplifications. Primers were randomly selected on the basis of GC content and annealing temperature for RAPD-PCR amplification.

Table 2. Growth performance, survival and feed utilization of grey mullet injected with shark and catfish DNA.

Treatment	IBW	FBW	WG	SGR (%/day)	Survival (%)	Feed intake (g)	FCR	PER
Control	2.11 ± 0.01	3.77 ± 0.11 ^c	1.66 ± 0.13 ^c	0.92 ± 0.06 ^c	100 ± 0.00 ^a	2.87 ± 0.05 ^c	1.73 ± 0.10 ^a	2.08 ± 0.12 ^b
Shark DNA	2.11 ± 0.02	5.65 ± 0.35 ^a	3.55 ± 0.37 ^a	1.57 ± 0.12 ^a	75 ± 7.07 ^b	5.22 ± 0.04 ^a	1.48 ± 0.14 ^b	2.45 ± 0.23 ^a
Catfish DNA	2.10 ± 0.01	4.83 ± 0.18 ^b	2.73 ± 0.16 ^b	1.32 ± 0.04 ^b	70 ± 0.00 ^b	4.40 ± 0.04 ^b	1.62 ± 0.08 ^{ab}	2.24 ± 0.1 ^{ab}

Means having different superscripts within column are significantly different ($P \leq 0.05$). Initial and final body weight (IBW and FBW) = body weight at start and end of experiment; weight gain (WG) = final weight - initial weight; specific growth rate (SGR % / day) = $(\text{Log}_e \text{ final weight} - \text{Log}_e \text{ initial weight}) / 100 / \text{number of days}$; feed conversion ratio (FCR) = dry feed intake/weight gain; protein efficiency ratio (PER) = weight gain/protein intake.

The polymerase chain reaction amplifications were performed following the procedure of Williams et al. (1990, 1993). The reaction (25 μL) was carried out in a medium that consisted of 0.8 U of Taq DNA polymerase (Fanzyme), 25 pmol dNTPs and 25 pmol of random primer, 2.5 μL 10X Taq DNA polymerase buffer and 40 ng of genomic DNA. The final reaction mixture was placed in a DNA thermal cycler (Eppendorf). The PCR programme included an initial denaturation step at 94°C for 2 min followed by 45 cycles with 94°C for 30 s for DNA denaturation, annealing as mentioned with each primer, extension at 72°C for 30 s and final extension at 72°C for 10 min were carried out. The samples were cooled at 4°C. The amplified DNA fragments were separated on 1.5% agarose gel and stained with ethidium bromide. DNA marker (bp 1000, 900, 800,....., 100) was used in this study. The amplified pattern was visualized on an UV transilluminator and photographed by Gel Documentation system.

Scoring and analysis of RAPDs

RAPD patterns were analyzed and scored from photographs. For the analysis and comparison of the patterns, a set of distinct, well-separated bands were selected. The genotypes were determined by recording the presence (1) or absence (0) in the RAPD profiles. Furthermore, the genetic similarity (GS) of the three groups of injected mullet and their control, based on RAPD fingerprinting were analyzed by the index of similarity using the formula given by Nei and Li (1979): $B_{ij} = 2 N_{ij} / (N_i + N_j)$, where N_{ij} is the number of common bands observed in individuals i and j , and N_i and N_j are the total number of bands scored in individuals i and j , respectively, with regards to all assay units. Thus, GS reflects the proportion of bands shared between two individuals and ranges from zero (no common bands) to one (all bands identical). Genetic dissimilarity (GD) was calculated as: $GD = 1 - GS$ (Bartfai et al., 2003).

Statistical analysis

Data of the phenotypic traits were analyzed using the following model (CoStat, 1986):

$$Y_{ij} = \mu + T_i + B_j + E_{ij}$$

Where, Y_{ij} is the observation of the ij^{th} parameter measured; μ is the overall mean; T_i is the effect of i^{th} dose; B_j is the effect of J^{th} block; E_{ij} is the random error. Significant differences ($P \leq 0.05$) among means were tested by Duncan's multiple range test (Duncan, 1955).

RESULTS AND DISCUSSION

The data in Table 2 shows that the final body weight

(FBW), weight gain (WG), specific growth rate (SGR %/day) and feed intake of mullet injected with shark DNA were significantly ($P \leq 0.05$) increased compared with the mullet injected with catfish DNA and the control groups. The highest record of survival was achieved by the control group and differed significantly ($P \leq 0.05$) from those of the fish injected with each shark and catfish DNA. In addition, the best FCR and the highest PER were recorded by mullet injected with shark DNA, but did not differ significantly ($P \leq 0.05$) from that of fish injected with catfish DNA. The results of the previous studies (El-Zaeem and Assem 2004; El-Zaeem 2004a; Hemeida et al., 2004; Assem and El-Zaeem 2005; El-Zaeem et al., 2012) state that the optimal dose of foreign DNA isolated from different donors and injected into different fish species, was 40 $\mu\text{g} / 0.1 \text{ ml} / \text{fish}$. The injected fish had significant ($P \leq 0.05$) improvement of growth performance, body composition, feed utilization and immunity traits. The results of this work are consistent with these findings.

The results of body composition by the end of the experiment show that protein content of mullet injected with shark DNA were significantly ($P \leq 0.05$) higher than those of mullet injected with catfish DNA or the control group, while lipid content of control group was significantly ($P \leq 0.05$) lower than those of mullet injected with shark DNA or catfish DNA (Table 3). Martinez et al. (2000) and Lu et al. (2002) reported that anabolic stimulation and average protein synthesis were higher in transgenic fish than that of non-transgenic fish. The improvement of most traits may be explained according to Hemieda et al. (2004); they reported that, genetically investigation of Nile tilapia injected directly with shark DNA into skeletal muscles was carried out. The concentrations of such DNA up to 40 $\mu\text{g} / 0.1 \text{ ml} / \text{fish}$ probably activated gradually cell proliferation in modified muscle tissues. Also, the measurements of DNA content in the muscles of modified fish indicated that shark DNA may be acting as a mutagen and it had no carcinogenic effect. This is mostly responsible for the enhancement of the productive performance shown in the modified fish injected with foreign DNA.

Compared with the traditional approaches, genetically modified breeding avoids the productive isolation between two different species. Since more manipulated

Table 3. Body composition of grey mullet injected with shark and catfish DNA.

Treatment	Moisture (%)	Crude protein (%)	Crude fat (%)
At the start	73.98±0.04	13.97±0.02	8.89±0.05
At the end			
Control	72.06±0.06	15.67±0.02 ^c	9.20±0.04 ^b
Shark DNA	71.98±0.04	16.16±0.07 ^a	10.50±0.08 ^a
Catfish DNA	72.01±0.07	15.80±0.06 ^b	10.47±0.05 ^a

Means at the end of experiment having different superscripts within column are significantly different ($P \leq 0.05$).

Table 4. The percentage of polymorphic (PB%) of control (T1) versus mullet injected with shark DNA (T2), control (T1) versus mullet injected with catfish DNA (T3) and mullet injected with shark DNA (T2) versus mullet injected with catfish DNA (T3).

Primers	T1 vs. T2			T1 vs. T3			T2 vs. T3		
	NTB	NPB	PB (%)	NTB	NPB	PB (%)	NTB	NPB	PB (%)
1	4	4	100	6	4	67	4	4	100
			20	13	3	23	11	5	45
			45	6	6	100	5	5	100
			75	4	2	50	10	4	40
			0	8	2	25	8	2	25
Average			48			53			62

NTB, Number of total bands; NPB, number of polymorphic bands.

genes are available for foreign DNA transfer, it is hopeful for the investigators to shorten the breeding period through directional genetic breeding (Wang et al., 2001). Also, Sin (1997) reported that the phenotypic changes, such as increased growth rate, are usually more prominent in the transgenic fish than those obtained by artificial selection or through efficient feeding regime. Furthermore, the technique used in this work is concerned with the utilization of the whole gene, introns and exons and not only exons through mRNA and reverse transcriptase treatments (Ali 2001). Thus, there is no need to utilize any kind of virus as the total DNA facilitates the introduction of foreign genes into cells with the aid of introns which act as retro-transposons (Hickey and Benkel, 1986).

The identification of the injected fish and their control was made using RAPD technique. Five random primers (Table 1) were tested for their ability to produce DNA polymorphism in genomic DNA of each genotype selected. All the five random primers examined produced different RAPD bands patterns. The number of amplified fragments detected varied depending on primers and treatments. Moreover to ensure that the amplified DNA fragments originated from genomic DNA, not from primer artifacts, negative control was carried out for each primer/genotype combination. No amplification was detected in the control reactions. All amplification products were found to be reproducible when reactions were repeated using the same reaction conditions (Table 4 and Figure

1). Data of genetic diversity among the injected fish and their control showed that the highest genetic polymorphic percentage (62.00%) was found between mullet injected with shark DNA and catfish DNA, while the lowest percentage (48.00 %) was recorded between mullet injected with shark DNA and their control (Table 4 and Figure 1). The results of genetic polymorphic between mullet injected with catfish DNA and their control show the percentage (53.00 %). This may be due to the differences in DNA molecule among normal and injected fish as a result of direct injection of foreign DNA isolated from shark or catfish. Moreover, some fragments of foreign DNA may be randomly integrated into mullet genomes. This integration could be functional or silent integration (Yaping et al., 2001).

The results of this work are consistent with the findings obtained in previous studies (El-Zaeem, 2001; Hemeida et al., 2004; Ali, 2002; Assem and El-Zaeem, 2005, El-Zaeem and Assem, 2006; El-Zaeem et al., 2011; El-Zaeem 2011 a, b). Also, the sensitivity of the RAPD marker played an important role in the detection of these differences (Ahmed et al., 2004; Ali et al., 2004; El-Zaeem et al, 2006; El-Zaeem and Ahmed, 2006; El-Zaeem 2011 a, b). The specific characterization of the RAPD method (random, uncharacterized multiple genome loci; dominant nature of markers; and possibility of migration of no-homologous bands) result in limitations based on RAPD analysis alone. Despite these limitations, the RAPD analysis can be used effectively for initial

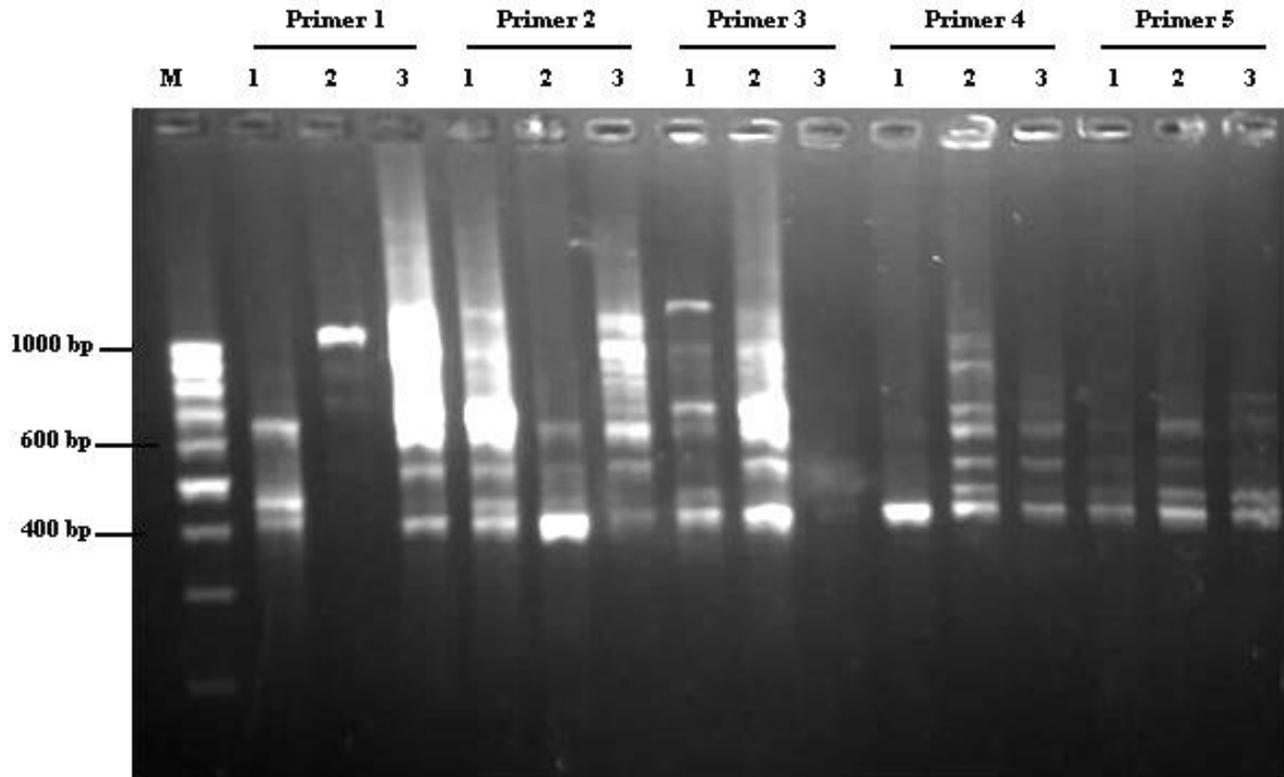


Figure 1. DNA fingerprinting illustrating polymorphism among *Mugil cephalus* muscle tissues following direct injection with different types of DNA. Lanes M, 1, 2 and 3 are DNA marker, control, mullet injected with shark and catfish DNA, respectively.

assessment of genetic variation among fish species (Barman et al., 2003). The main advantages of RAPD markers are the possibility of working with anonymous DNA and the relatively low expense, and it is fast and simple to produce RAPD marker (Hadrys et al., 1992; Elo et al., 1997; Ali et al., 2004).

On the other hand, the success of the growth enhancement in this study with injected fish is impressive and underscores their potential usefulness in aquaculture. Thus, mullet injected with shark DNA show a very good response, with more than two fold weight increase compared with non-injected control. In addition, most of the productive performance traits of injected fish were improved significantly. In this connection, several studies reported that transgenetically growth, body composition and feed utilization enhanced fish show some promise of improvement on both counts (Chatakondi et al., 1995; Rahman et al., 1998; Rahman and Maclean, 1999; Maclean and Laight, 2000; Matinez et al., 2000; Devlin et al., 2004 a, b; Kang and Devlin, 2003; Stevens and Devlin, 2000, 2005; Dunham et al., 2002; Raven et al., 2006; Hallerman et al., 2007; Oakes et al., 2007; El-Maremie, 2007; El-Zaeem et al., 2011; El-Zaeem, 2011 b).

In conclusion, the results of this study suggest that genetically modified *M. cephalus* with extraordinary growth rate can be produced using a feasible and fast

methodology.

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