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PRODUCING SUPER MALE (ZZ) AND USING RAPD-PCR TECHNIQUE FOR SEX DIFFERENTIATION IN BLUE TILAPIA *(Oreochromis aureus)*

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ABSTRACT

A study was made to identify sex differentiation in parents and fries of *Oreochromis aureus* using RAPD- PCR and squash techniques. Fishes were collected from the experiment shared in program for producing "super males ZZ" in Egypt. The trial of sex reversal for feminization of sexually undifferentiated progeny was conducted using 17-β ethynylestradiol as a feminizing stimulating agent. Oral administration of powdered feed containing 400 and 800 mg/kg were tested for 30 days in Central Laboratory for Aquaculture Research in Abassa (CLAR). Upon termination of the experiment, average weight and length have increased using high dose. In contrast, the survival rate was increased in low dose (400 mg/kg) than high dose (800 mg/kg). Its effect was highly detectable among fry that were orally fed on 800-mg/kg hormone-treated feeds for 30 days. The mean percentage of phenotypic females was 94% ± 2.7 in the second season, females generated progeny including mean percentage of phenotypic males was 69%±4.6 while female, inter sex and non differential percentages were 26% ±3.8, 3% ± 1.5 and 2%±0.7 respectively. the sex differentiated have been using applied squash method and RAPD-PCR , Squash method illustrated that from every 10 fishes, 9 individuals were converted into super male (ZZ) . In RAPD -PCR, seven primers were used and they showed different bands patterns between male and female . The total number of DNA fragment band was 35 in male parents and 33 in female parents. While, numbers of DNA fragment bands produced by seven primers were 33 and 35 in adult converted females and fries converted females. Total numbers of DNA fragment bands were 35 and 40 in males and 33 and 35 in female. These molecular markers detected by primers (A14, B13and C05) could be used as markers associated with male or female differentiation.

Key words: *Oreochromis aureus*, sex reversal, feminization and progeny test, RAPD-PCR, squash method.

INTRODUCTION

Amongst vertebrates, fishes contain the greatest variability in sex determination mechanisms including manufactories, polyfactorial and environmental control (Bull, 1983). Plasticity in sex determination is also reported in some gonochoristic fishes species (Francis, 1992; Crews, 1996). Sex differentiation in fishes is a very flexible process with respect to evalutionary patterns observed among genera and families, and within individuals is subject to modification by external factors (*e.g.* temperature, behavioral and physiological factors). Genetic differentiation of sex in fishes can involve monogenic or polygenic systems, with factors located on the autosomes or on sex chromosomes. In the latter case, both male (XY) and female (ZW) heterogametic systems have been described (Robert and Yoshitaka, 2002). Many studies have concentrated on the genetic basis of sex determination mechanisms of *Oreochromis niloticus* and other *Oreochromis* species, as reported by Trombka and Avtalion (1993). Wohlfarth (1994) evaluated these solutions,

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especially in the developing countries, and reported that all the traditional techniques have no longer been adopted widely in aquaculture. Manual sexing is laboreus and requires skill. The major disadvantages which method are human error in sexing and the wastage of females. Interspecific and intergenic hybridization are known to produce all-male progeny. However, difficulty in maintaining pure parental stocks that consistently produces 100% male offspring, poor spawning success and incompatibility of breeders resulting in low fertility. Therefore, studies on the genetic basis of sex determination of *Oreochromis niloticus* and other *Oreochromis* species have been developed by Mair *et al*. (1991) to provide an alternative and effectual monosex breeding program for producing all- male offspring (Desprez *et al.,* 2003). Culture of monosex progeny has long been recognized as the most effective solution to the widespread problem of early sexual maturation and uncontrolled reproduction in tilapia culture. Techniques for managing recruitment have evolved from traditional means such as manual sexing and hybridization to the current practice of gynogenesis and direct steroid-induced sex reversal, which has been the industry standard for monosexing during the last couple of decades Mohamed *et al*. (2004). The monosex breeding program using YY-male broodstock might provide such a solution. The breeding program of generating YY-male comprises a number of distinct steps. The following genetic nomenclature for description of hormone - treated fishes will be used in order to skim over the steps of this program: C-XY refers to converted genotypic male into functional phenotypic female. The breeding program starts with feminization of sexually undifferentiated progeny from normal crosses (Rosenstein and Hulata 1994) .

Development of specific molecular markers associated with the sex chromosome were identified in many species of *Oreochromis* to study the sex differentiation mechanisms in many species (Nandah *et al.,* 1990; Liu and Cords 2004 and Hassanien *et al.,* 2004). Studies of the sex differentiation mechanism in tilapia are primarily based on the sex ratio of offspring obtained from inter- and intra-specific crosses, crosses between sex reversed parents and after chromosome manipulations leading to polyploid, gynogenetic and androgenetic individuals, as well as cytogenetic methods, and several hypotheses, including monofactorial, polyfactorial, autosomal and environmental sex differentiation, have been proposed (Wohlfarth and Wedekind, 1991; Trombka and Avtalion, 1993). In addition, several studies were carried out to identify heteromorphic sex chromosomes, but without success (Kornfield, 1984 and Crosetti *et al*., 1988). Hybridization with known sex-linked nucleic acid sequences and subtractive hybridization have also so far failed in the detection of sex-specific DNA markers in tilapia (McConnel, 1993).

For these reasons the present study aimed to: (1) producing supermale (ZZ) through the feminize sexually undifferentiated progeny of *Oreochromis aureu* from normal crosses using 17-β ethynylestradiol for sex reversal as a feminizing stimulating agent as well as recognizing the target converted maternal ZZ genotype. (2) use RAPD-PCR technique as a tool to differentiate parents, fingerlings and fries sex of *Oreochromis aureus.*

MATERIALS AND METHODS

Fishes and Facilities

Females spawned and eggs were extruded from the buccal cavity of the female and incubated in Macdonald jars until hatching.The first batch of hatched larvaes were collected and stocked in hapas. After the yolk sac absorption period, the so-called fry reached 7-12 mm total length and 10±3 mg body weight were used.

Hormone-treated and feed preparation

The hormone 17-β ethynylestradiol used to feminization *Oreochromis niloticus* and other *Oreochromis* species Mair and Santiago (1994) and Mohamed *et al*. (2004).

Hormone of 17-β ethynylestradiol impregnated feed weighing 1000 g each were prepared with varying dosages of hormone 400 and 800 mg/kg for feeding the two treatments. The fishes were fed on experimental diet 40% crude protein. It was finely ground and sieved to remove the particles that were too large to be ingested. An

amount of 400 mg of 17-β ethynylestradiol were weighed and dissolved in 1000 ml ethyl alcohol and also, 800 mg of 17-β ethynylestradiol were weighed and dissolved in 1000 ml ethyl alcohol 95% to prepare two treatments of diet. The two volumes of alcohol with hormone mixed thoroughly to two quantities of 1000 g feed each with even distribution of 1 cm with light mixing by hand 2-3 times and used for feeding the fry of the two treatments.

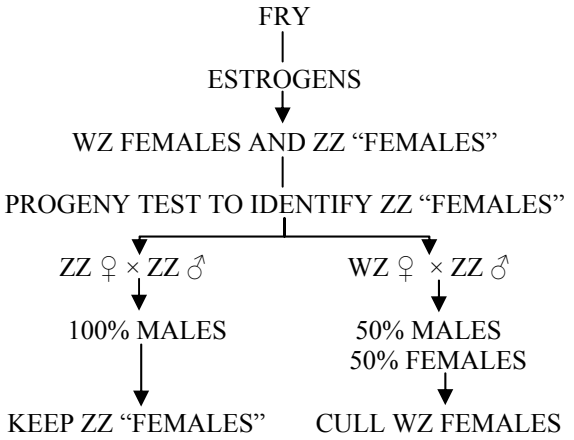
Feeding frequency was two to four times daily during the daylight. The daily feed ration schedule of Popma and Green (1985). The daily ration was divided into approximately equal weights. Feeding continued for 30 days after that fryies was fed normal diet without hormone in a hapa suspended in a concrete pond after taking the measurements of weight and length. The water temperature ranged from 22 to 28 ^{o}C, dissolved oxygen varied from 5 - 6.5 ppm while pH ranged from 7 to 8.5.

Identification of the phenotypic sex

Sexing was observed by microscopic examination of the wet squashes of gonads fix-stained in aceto-orcein fix stain according to Waindi (1994). After sex reversal, identification of the phenotypic sex of 100 fishes from each hapa was determined by microscopic examination of the gonads when the fishes reached 2 - 3 cm length. The thin gonad (thread-like structure lies along the dorsal side of the abdominal cavity) was extracted very carefully, placed on a glass slide and stained with a drop of aceto-carmine stain then it was lightly squashed with a glass cover slip and examined at 10 magnifications. The fishes were a presumptive female if densely packed oocytes were found as reported in Guerrero and Shelton (1974). The remaining fry of the treatment that had the highest percentage of mixed females (converted and normal) from the two treatments were kept to the next season in earthen pond (1000 m^2) reared to maturation. Throughout the rearing period, the water temperature ranged from 22 to 28 ^{o}C, dissolved oxygen varied from 5 - 6.5 ppm while pH ranged from 7 to 8.5. and the experiment was designed in the second season to complete the results (Tave, 1993).

Progeny test

The progeny test for the resulted "ZZ" female was designated as presented in following diagram:



Randomly Amplifid Polymorphic DNA Polymerase Chain Reaction (RAPD–PCR)

Organs sampling

The caudal fin tissue of the same individuals were cut and preserved with 70% ethanol into eppendorf tubes. Then caudal fin samples were frozen at -20˚C.

DNA extraction proceduer

Bulks of DNA were prepared for each sample. Each bulk was composed of a 5 µl of genomic DNA taken from five samples referred to the five individuals from each sample (Lukyanov *et al*., 1996). DNA was prepared from caudal fin tissue as described by (Bardakci and Skibinski, 1994).

Amplification conditions and electrophoresis

After checking genomic DNA quality, bulks of DNA samples were prepared for each sample. The Amplification conditions were conducted according to Williams *et al.* (1990).

Primers

7 primers were used and their codes and sequences are shown in Table 1.

RAPD-PCR reaction mixture

100 µM DNA, 0.4 µM Primer, 0.25 mM dNTPs, 1.5 mM $MgCl_2$, 1 X Buffer,0.5 unit Taq.

Table 1. Code and sequences of the 7 primers used for RAPD-PCR

Primer code	Primer séquence
A-03	5'AGT CAG CCA C 3'
A-14	5'TCT GTG CTG G 3'
B-8	5'CCA CAG CAG T3'
B-13	5' GTT TCG CTC C3'
C-05	5'GAT GAC CGC C 3'
C-17	5'TTC CCC CCA G 3'
D-19	5'CTG GGG ACT T 3'

RAPD-PCR program

One cycle of 94 ^{o}C for 4 min, Thirty cycles of, 94 ^{o}C for 45 sec,37 ^{o}C for 45 sec, 72 ^{o}C for 1 min and One cycle of 72 ^{o}C for 10 min.

Gel photographing

The DNA gels were immediately photographed after end of the run using UV-based gel documentation system (Bio-Rad Gel Doc 2000 apparatus).

Statistical Analysis

All gels were analyzed using Total Lab (Ver. 2.01), and SPSS (Ver. 15) software programs. The profiles were scored in a binary data (1 if the band present and 0 if absent). These results were introduced to SPSS software program in order to infer compasrision between normal and reversed males in the parents, first and second generation fries.

RESULTS AND DISCUSSION

Sex Reversal

Producing sex reversed females functional sex reversal is most easily achieved through oral application of estrogens incorporated into the feed. administered during the period of sex differentiation which is known to be 30 days according to Alvendia-Casauay and Carino (1988). The initial weight and length of *Oreochromis aureus* post-hatching fry after the yolk sac absorption period was 10 mg/fry and length was 8 mm/fry. Average length and average weight per fry in the two treatments results are shown in Table 2. It was noticed that, as the feeding dose increase, the average weight and length increased by using 17-β ethynylestradiol hormone at dose 800 mg/ kg (104 ± 0.01 mg and 23 ± 0.15 mm) than the 400 mg/kg dose (102 ± 0.004 mg and 22.5 ± 0.31 mm respectively). In contrast, the survival rate was increased in 400 mg/kg treatment (68% ± 1.53) than 800 mg/kg treatment (66% ± 4.9). This result is in agree with that of Farag *et al.* (2006) who reported that growth of fry *Oreochromis niloticus* that were fed hormone treated-feed was affected by the dosages used. Upon termination of the experiment, average weight of fry that were fed on higher doses of 17-β ethynylestradiol supplemented feed was significantly higher than those fed on lesser amounts for the same period.

Currently male monosex populations are produced mainly by androgen treatments. Due to various environmental issues related to hormone use *i.e.* possible effects of treatment residues on water quality and biodiversity with the growing concerns for food security, finding a sex control alternative based on non hazardous, consumer and environment-friendly methods represents a major challenge for aquaculture. The best way to obtain all-male populations is through genetic control (Beardmore *et al*., 2001). The possibility of mating a sex- reversed heterogametic female XY with a normal male of the same species to produce 75% male offspring. The same authors excluded some fishes species and reported that this would not be possible with *Oreochromis niloticus or O. mossambicus* but would theoretically be feasible with *Oreochromis aureus*. Researches have assured that *Oreochromis niloticus* could successfully be genetically manipulated with estrogen and the feasibility of producing “supermales YY” (Mair *et al*., 1997).

Table 2. Growth performances of first generation of *Oreochromis aureus* fry fed 17-β ethynylestradiol hormone-treated feed with two doses for 30 days

Treatment	Initial weight (mg)	Initial length (mm)	Final weight (mg)	Final length (mm)	Survival rate (%)
Control	10.00	8.00	10**0** ±0.005	2**1** ±0.10	70 ±1.30
400 mg/kg	10.00	8.00	102 ±0.004	22.5 ±0.31	68 ±1.53
800mg/kg	10.00	8.00	104 ±0.01	23 ±0.15	66 ±4.9

From the present data, 17-β ethynylestradiol proved to be an effective feminizing stimulating agent for *Oreochromis aureus*. Its effect was highly detectable among fry that were orally fed on 800-mg/kg hormone-treated feed for 30 days, mean percentage of phenotypic females (MPF) was 94% ± 2.7. It was also obvious among fry which consumed the 400-mg/kg hormone-treated feed for 30 days that MPF was 83% ± 4.3. MPF was decreased with decreasing the quantity of 17-β ethynylestradiol supplemented to the feed as indicated in Table 3. However, Popma and Green (1985) reported that fry can be effectively sex reversed in 20 days, but occasionally only 95% of the fry develop as phenotypic males using 17α-methyltestesterone for masculinization of *O. niloticus* fry. They also stated that sex reversal success is more consistent when the treatment duration is 25 to 28 days. Also, Srisakultiew (1993) reported that fries were feed for 14 days. Based on the first data on tilapia sex determination and differentiation, it has been possible to produce genetically "all-male populations" through the development of YY "supermales" (Scott *et al.,* 1989). Nevertheless, this approach is unreliable and hampered by the very long procedure of producing and identifying putative YY male individuals (Tessema *et al.,* 2006). As demonstrated in Table 3 after 30 days fry was feed with hormone 17-β ethynylestradiol treated-feed 400 mg/kg showed that male, inter sex and non differential percentages were 12% ± 4.1, 3% ± 1.1 and 2% ± 0.6, respectively. However, this percentage decreased in fry treated-feed 800 mg/kg showed that male, inter sex and non differential percentages were 4% ± 1.2, 1.3% ± 0.5 and 0.7% ± 0.1, respectively. The present observation revealed that a few genetic males might consume so little hormone during sex reversal treatment that they develop into normal functional males. Another small fraction of genetic males could be sterile individuals with Ovo-Testis as a result of insufficient amounts of hormone. The majority of the sex-reversed fry that was fed sufficient feed with high estrogen dose develop into reproductively functional females.

From the present data in the second season, when the sex reversed fry through first season was reared to sexual maturity stage, the pseudofemales (C-zz) could be differentiated form the normal ones (wz) using pair-mating of normal genotypic males to the mixed females with normal males separately in 100 isolated hapas. Progeny testing of their offspring proved that the majority of females were originally genotypic females from the moment of fertilization and their offspring had a normal sex ratio of 1:1. Females generated progeny including mean percentage of phenotypic males (MPM) was 69% ± 4.6 while female, inter sex and non differential percentages were 26% ±3.8, 3% ± 1.5 and 2% ± 0.7, respectively as reported in Table 4 exceeding the normal ratio because, hypothetically, these brooders were converted maternal C_zz genotype and acted reproductively as phonotypical functional females. Pseudofemales (C-zz) produce low number of male (69% ± 4.6) in first generation for estrogen-treated females. Also it is hypothetically supposed that one third of these males are super males of the "C-zz" genotype and two thirds should be normal males "N-zz". Further research by crossing these males after sexual maturation to normal females and testing their progeny will prove or disprove this hypothesis. The same observation was reported by Damien *et al.* (2003) who investigated a pseudofemale line in two populations of *O. aureus*, known as Egyptian Population (EP) and Israel Population (IP). In *O. aureus*, males are the homogametic sex (ZZ/ZW), and sex

Table 3. Phenotypic sex of first generation among *Oreochromis aureus* fry fed 17-β ethynylestradiol hormone-treated feed for 30 days

Treatment	Females %	Males %	Inter sex %	Non differential %
400 mg/kg	83 ±4.3	12 ±4.1	3 ±1.1	2 ±0.6
800mg/kg	94 ±2.7	4 ±1.2	1.3 ±0.5	0.7 ±0.1

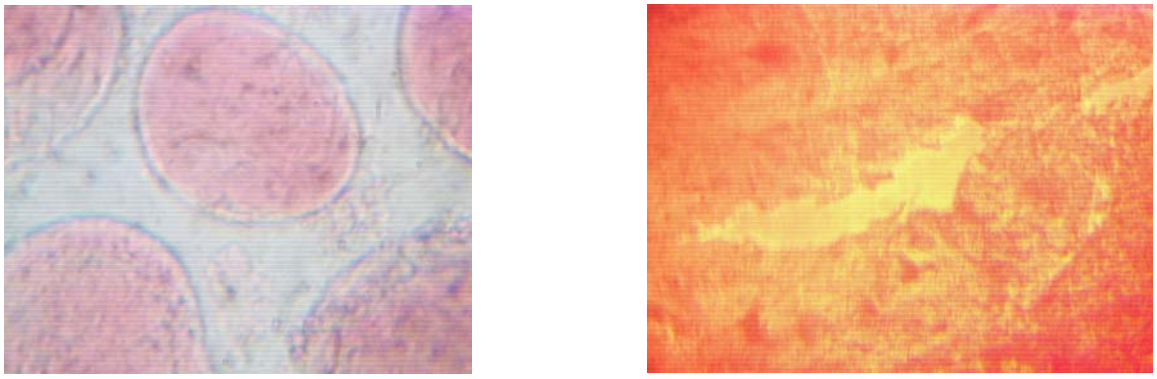
Table 4. Growth performances and phenotypic sex % (PM) of second generation of *Oreochromis aureus* fry fed 17-β ethynylestradiol hormone-treated (after 60 days from test progeny)

Growth performances	Initial weight (mg)	Initial length (mm)	Final weight (g)	Final length (cm)
	11.5±0.01	78.5±0.13	3.2± 0.28	6.1±0.41
Phenotypic Males	Females %	Males %	**Inter sex %**	**Non differentiated %**
	26±3.8	69±4.6	3±1.5	2±0.7

reversal of fry with estradiol results in the production of some functional sex-reversed fishes with a female phenotype and ZZ male genotype, known as pseudofemales. Crosses between ZZ pseudofemales and ZZ males theoretically should provide monosex ZZ male progeny only. We have studied the sex ratios of progeny from 43 IP (F_2 to F_3 generations) and 51 EP (F_1 to F_5 generations), pair-matings between normal males and pseudofemales. In IP, the male percentage in progenies ranged between 83% to 100% in F_2 and 66% to 100% in F_3. In EP, male percentage was more constant, varying from 88%to 100% in F_1, from 96% to 100% in F_3 and from 97 %to 100% in F_5. In EP, F_2 and F_4 pseudofemales produced only monosex male progeny This apparent difference in sex ratio frequency distributions between the two *O. aureus* pseudofemale lines could be due to the selection of males. The present study also shows that it is possible to fix the male sex determining factors (Z sex chromosome and genetic factors) in a line of pseudofemales, producing a high percentage of male progeny in five successive generations. While, Hackmann and Reinboth (1974) observed a feminizing effect from high levels of methyltestesterone and explained it by assuming that some of the exogenous hormone is metabolized to estrogen. This paradoxical effect probably occurs more readily at higher methyltestesterone concentrations.

Squash Method

Identification of the phenotypic sex of 100 fishes from each hapa was determined by microscopic examination of the gonads when the fishes reached 2 - 3 cm length after sex reversal and illustrated that from each 10 individuals 9 fishes were converted into male "ZZ" so 90% from population became males. Fig. 1 shows aceto-carmine squash preparation from testis and ovary of fry *Oreochromis aureua* treated with 17-β ethynylestradiol. Jalabert *et al.* (1971) studied the sex determination of *O. niloticus and O. macrochirus* by evaluating the sex ratios of hybrid progeny and concluded that *O. niloticus* had a basic XX : XY sex determination but that the sex ratios from the back cross of the male hybrid to a female *O. niloticus* did not conform to the expected ratios. Both species and their hybrid progeny had an identical karyotype (2N = 44) with no evident of sex chromosome. The authors proposed that autosomes may play a role in sex determination. Shelton *et al.* (1983) reported that sex ratios from mass spawnings of *O. niloticus* that ranged from 31% to 83% male. Also Rothbard *et al.* (1983) obtained 98 - 100% male tilapia with sex-reversal treatment of fry in outdoor concrete tanks using 60 ppm of 17a-ethynyltestosterone in the diet for 28-29 days and crosses of hormonally sex-reversed ZZ



test **ovary**

Fig. 1. Acetocarmine squash preparation from tests and ovary of fry *Oreochromis aureua* treated with 17- βethynylestradiol

phenotypic females with normal male susually produce 100% male offspring, but slight deviations have been observed (Hopkins, 1979, Mair *et al.,* 1987, Lahav, 1993, Rosenstein and Hulata, 1994).

Randomly Amplifid Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR)

The electrophoretic investigation of Randomly Amplifid Polymorphic DNA Polymerase Chain Reaction (RAPD–PCR) of parents, adult converted females, fries of converted females and mono sex super males of *Oreochromis aureus* disclosed the presence of different number fractions for DNA. Finding a polymorphism between male and female fishes samples was aimed by mixing all isolated DNA from male individuals in one tube and female samples in one tube. The seven primers were worked successfully in parents, converted adult females, first generation (converted fry females) and second generation (mono sex super males). After RAPD reaction, whole band profiles of RAPD primers were obtained. They show different band pattern between male and female individuals. Except for two RAPD primers (A14 and B13) was worked in males parents and mono sex super males and thy shared in fragment band number (5 and 4 respectively) as in Tables 5 and 6 and Fig. 2 (a and b) Where,using primer (A14) was worked in males parents and mono sex super males and thy shared in one fragment band with MS (272 bp), while primer (B13) was worked in males parents and mono sex super males and thy shared in one fragment band with MS (273 bp) Table 6 and Fig. 2(b), Also (B8) was worked in males parents and mono sex super males and thy shared in all fragment bands except the last fragment band shared in males and females parents only, and primer C5 was worked in males parents and mono sex super males and thy shared in fragment band (1 and 3) with MS(604 and 294 bp), also, A3 was worked in males parents and mono sex super males and thy shared in fragment band 6 with MS (234) Table 7 and Fig. 2(c), also one RAPD primer (D19) was worked in converated females and fries of convereted females (First generation) and thy shared in common number of fragment bands (5) as in Table 9. Also, A3 has a highest polymorphic percentage (100%) while the lowest polymorphic percentage was obtained in primers B8 and C17 (50%) in Table 9. Total numbers of DNA fragment bands produced by seven primers was 35 in male parents and 33 in female parents. While, number of bands in DNA produced by the seven primers used in this study were from 33 to 35 in adult converted females and fries converted females. So, total number of fractions in DNA were from 35 to 40 in males and from 33 to 35 in female (Table 9). Iturra *et al*. (1998) used RAPD assay and bulked segregant analysis and they found 2 markers that generate polymorphic bands amplifying preferentially in male of Mount Lassen and Scottish strains of rainbow trout. Bardakci (2001) mentioned that (RAPD) markers were successfully used in discrimination of sexes in Nile tilapia fishes *(Oreochromis niloticus*) using

Table 5. Densitometeric analysis of DNA from the caudal fin of Tilapia aurea (*Oreochromis aureus*) using primer A14

A14						
Parents				Converted female (adult)	First generation (converted fries)	Second generation (converted super male)
No.	MS (BP)	Males	Female			
1	952	1.00	1.00	0.00	0.00	0.00
2	562	1.00	1.00	0.00	0.00	0.00
3	486	1.00	1.00	0.00	0.00	0.00
4	353	100	1.00	0.00	0.00	0.00
5*	272*	1.00*	00	1.00*	1.00*	1.00*
6	234	0.00	0.00	0.00	0.00	1.00
7	151	0.00	0.00	1.00	0.00	1.00
8	126	0.00	0.00	1.00	1.00	1.00
9	91	0.00	0.00	0.00	1.00	1.00
10	75	0.00	0.00	0.00	1.00	0.00
Total band numbers		5	4	3	4	5

* Means: The band was shared between male parent and super male ZZ.

Table 6. Densitometeric analysis of DNA from the caudal finofTilapiaaurea(*Oreochromis aureus*) using primers B13, B8 and C05

B13						
Parents				Converted female (adult)	First generation (converted fries)	Second generation (converted super male)
No.	MS (BP)	Male	Female			
1	604	0.00	0.00	1.00	1.00	1.00
2	562	1.00	1.00	1.00	1.00	1.00
3	294	1.00	1.00	1.00	0.00	1.00
4*	273*	1.00*	0.00	1.00*	1.00*	1.00*
5	197	0.00	0.00	0.00	0.00	0.00
6	184	1.00	1.00	1.00	1.00	1.00
7	141	1.00	1.00	1.00	0.00	0.00
8	127	1.00	0.00	1.00	0.00	1.00
Total band numbers		6	4	7	5	6
				B8		
1	604	00	00	-0.00	1.00	1.00
2	562	1.00	1.00	1.00	1.00	1.00
3	294	1.00	1.00	1.00	1.00	1.00
4	273	1.00	1.00	1.00	0.00	1.00
5	197	1.00	1.00	1.00	1.00	1.00
6	184	1.00	1.00	1.00	0.00	0.00
7	141	1.00	1.00	1.00	1.00	1.00
8	127	1.00	1.00	0.00	0.00	0.00
Total band numbers		6	5	6	7	7
				C05		
1*	604*	1.00*	0.00	0.00	1.00*	1.00*
2	562	1.00	0.00	0.00	1.00*	00
3	294*	100*	00	1.00*	0.00	1.00*
4	273	0.00	1.00	1.00	1.00	0.00
5	197	1.00	1.00	1.00	1.00	1.00
6	184	1.00	1.00	0.00	0.00	0.00
7	141	1.00	0.00	0.00	0.00	0.00
8	127	0.00	0.00	0.00	0.00	0.00
Total band numbers		6	3	3	4	3

* Means: The band was shared between male parent and super male ZZ.

Table 7. Densitometeric analysis of DNA from the caudal fin of Tilapia aurea (*Oreochromis aureus*) using primer A03

A03						
	Parents			Converted female (adult)	First generation (converted fries)	Second generation (converted super male
No.	MS(BP)	Male	Female			
1	952	1.00	1.00	1.00	1.00	1.00
2	770	0.00	1.00	-0.00	-0.00	1.00
3	486	1.00	1.00	1.00	1.00	1.00
4	432	0.00	1.00	-0.00	1.00	1.00
5	353	0.00	1.00	1.00	1.00	1.00
6*	**234***	**1.00***	0.00	**1.00***	**1.00***	**0.00**
7	196.911	0.00	0.00	0.00	1.00	1.00
Totalband numbers		3	5	4	6	6

Table 8. Densitometeric analysis of DNA from the caudal fin of Tilapia aurea (*Oreochromis aureus*) using primers C17 and D19

C17						
No.		Parents		Converted female (adult)	First generation (converted fries)	Second generation (converted super male)
	MS(BP)	Male	Female			
1	**770**	-0.00	0.00-	0.00	-0.00	-0.00
2	**604**	-0.00	-0.00	0.00	0.00	0.00
3	**562**	0.00	0.00	0.00	1.00	1.00
4	**432**	-0.00	-0.00	0.00	1.00	0.00
5	**273**	0.00	1.00	0.00	1.00	1.00
6	**234**	1.00	1.00	1.00	1.00	1.00
7	**184**	1.00	1.00	1.00	1.00	1.00
8	**141**	00	0.00	0.00	0.00	0.00
9	**127**	1.00	1.00	1.00	1.00	1.00
10	**91**	1.00	1.00	1.00	0.00	1.00
11	**75**	1.00	1.00	1.00	0.00	1.00
Total band numbers		5	6	5	6	7
				D19		
1	**770**	0.00	0.00	0.00	1.00	1.00
2	**604**	0.00	0.00	0.00	1.00	1.00
3	**562**	0.00	0.00	0.00	1.00	1.00
4	**432**	0.00	0.00	1.00	0.00	0.00
5	**273**	1.00	1.00	1.00	1.00	1.00
6	**234**	1.00	1.00	1.00	0.00	1.00
7	**184**	0.00	1.00	1.00	0.00	0.00
8	**141**	1.00	1.00	1.00	1.00	1.00
9	**127**	0.00	0.00	0.00	0.00	1.00
10	**91.1**	0.00	0.00	0.00	0.00	0.00
11	**75.1**	0.00	0.00	0.00	0.00	0.00
Total band numbers		3	4	5	5	7

Table 9. Codes name of RAPD primers and observed band profiles of each primer

	Band profiles on agarose gels						
Primer code	**Male parents**	**Female parents**	**Converted female**	**First generation**	**Second generation**	**Marker band**	**Polymorphic %**
A-03	3	5	4	6	6	-	%100
A-14	5	4	3	4	5	1	%87.5
B-8	7	7	6	5	6	-	%50.0
B-13	6	4	7	5	6	1	%75.0
C-05	6	3	3	4	3	-	%71.4
C-17	5	6	5	6	7	1	%50.0
D-19	3	4	5	5	7	-	%75.0
Total band number	35	33	33	35	40	-	-

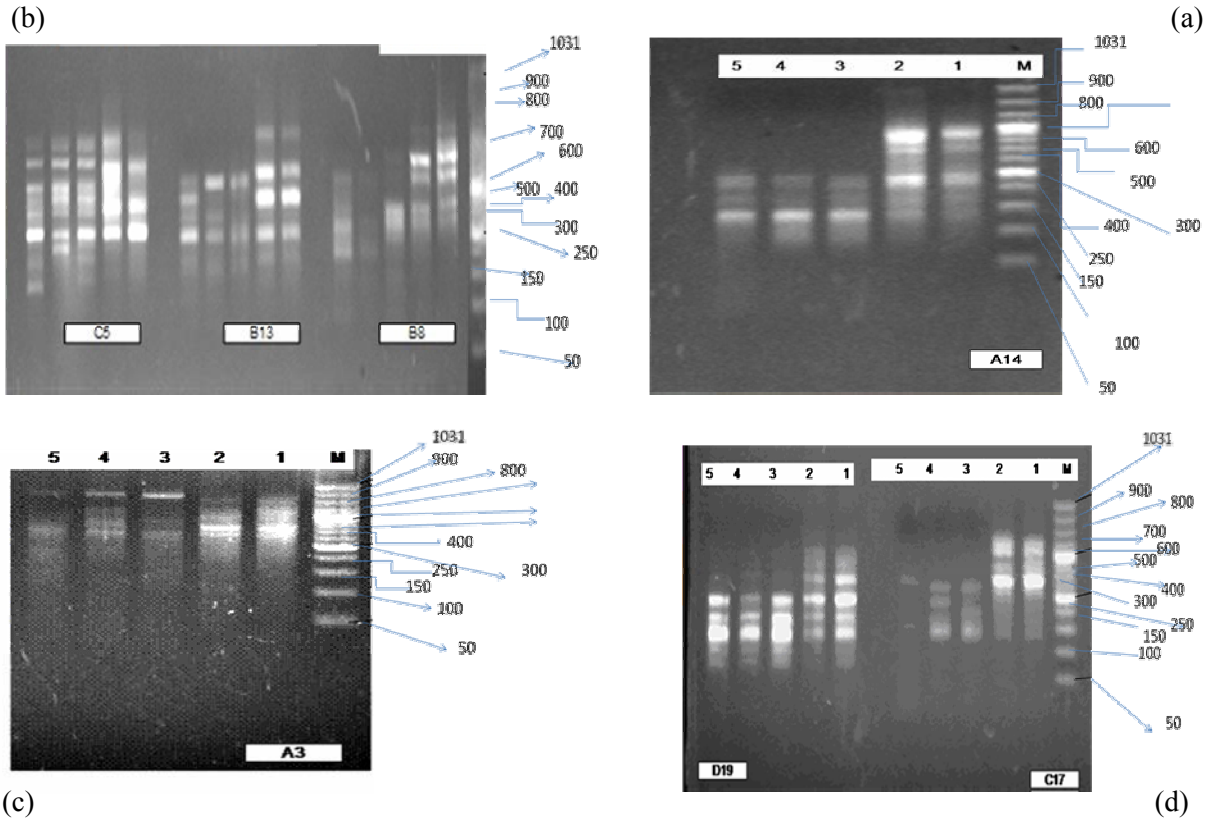


Fig. 2. Variation in electrophoretic patterns for DNA from the caudal fin of Tilapia aurea (*Oreochromis aureus*) using primers A14 ,C5, B13 , B 8, A3, C17 and D19: where lane M molecular size , lane 1and2 Parents (Males&Females), 3 Converted females and lane 4&5 First and second generation

linear discriminant functionan analysis. Also male specific RAPD markers were isolated from African catfishes, and *Clarias gariepinus.* **(**Kovacs *et al*., 2000) and Cnaani *et al.(* 2003) found two distinct QTL for sex differentiation in tilapias hybrid cross between *O. aureus* and *O.mossambinds*. Several studies have identified genetic markers linked to sex differentiation in tilapia. Durna (2009) reported that there was no sex-linked polymorphism between the male and female individuals of *A. danfordii* species using twenty RAPD primers and fifty specimens. Hormaza *et al*. **(**1994) found a single female specific RAPD marker in *Pistacia vera* using 700 primers. Moreover, the possibility of any RAPD markers being linked to a gene or a genomic region of interest is dependent on genome size, type of gene or genomic region (Bardakci, 2001).

The present data concluding that (1) hormone 17-β ethynylestradiol was effective in sex conversion and producing the ZZ super male in tilapia, (2) RAPD-PCR technique can be used to differentiate between males and females in fishes if high number of primers used.

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إنتاج ذكــور فائقة (ZZ) واســتخدام تقنية RAPD-PCR لتمييز الجنس فى البلطى الأزرق

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أجريت هذه الدراسة لتحديد الجنس فى سمكة البلطى الأوريا باستخدام تقنيات RAPD -PCR و squash method وأخذت العينات من تجربة إنتاج البلطى وحيد الجنس في برنامج يتم تنفيذه في مصر لإنتاج ذكور بلطي سوبر (ZZ) من البلطي الأزرق وذلك كحل لمشكلة التكاثر غير المرغوب فيه لسمكة البلطي في الأحواض وزيادة الإنتاج السمكي، وذلك بإنتاج ذكور البلطي الأعلى في معدلات النمو عن الإناث. لذا أجريت التجربة لتأنيث أجنة بلطي أوريا غير مميزة جنسيا باستخدام هرمون ١٧- بيتاأثينايل أستراديول عن طريق التغذية بعلف يحتوي علي نسب مختلفة من الهرمون ٤٠٠ و٨٠٠ مج/كجم ولمدة ٣٠ يوم بالمعمل المركزى لبحوث الثروة السمكية، وأكدت النتائج أن زريعة البلطي الأزرق التي تغذت علي علف يحتوي علي جرعة أكبر من الهرمون كانت متوسطات أوزانها وأطوالها (١٠٤±٠,٠١ مج و ٢٢,٥±٠,٣١ مم على التوالى) أى أنها أعلى من تلك التي تغذت علي جرعه أقل (١٠٢± ٠,٠٠٤ مج و ٢٣±٠,١٥ مم على التوالى) بعد فترة التغذية مما يؤكد فعالية هرمون ١٧- بيتاأثينايل أستراديول كعامل محفز لمعدلات النمو. وعلى العكس من ذلك لوحظ زيادة نسبة الأحياء فى التركيز الأقل ٦٨±١,٥٣% عن التركيز الأعلى ٦٦±٤,٩% خلال ٣٠ يوم من التغذية، أما بالنسبة للإنقلاب الجنسي إلي إناث فقد تم عمل أختبار أجنة للزريعة بعد انتهاء التغذية وكان تأثير الهرمون ملحوظا بدرجة كبيرة في الزريعة التي تغذت علي جرعة أكبر من الهرمون، حيث وصل متوسط نسبة الإناث إلى ٩٤± ٢,٧%، ولقد انخفضت هذه النسبة بانخفاض الجرعة في العليقة الى ٨٣±٤,٣%، بعد أن وصلت الزريعة إلي النضج الجنسي في الموسم التالي تم التخلص من الذكور ثم أجرى أختبار التزاوج بوضع الإناث المختلطة سواء المنقلبة أو الطبيعية في هابات، كل أنثي في هابة مستقلة مع ذكر طبيعى للتزاوج، تم عمل اختبار أجنة مرة أخري لزريعة الجيل الثانى الناتجة علي حدة لتمييز الإناث التي هي في الأصل ذكور C_{ZZ} وتحولت إلى إناث من الأخرى التي هي في الأصل إناث طبيعية WZ . وأظهر اختبار الأجنة للاناث المحولة نسبة عدد الذكور٦٩ ±٤,٦% والإناث وبين الجنسين والتى لا تتميز إلي إناث أو ذكور كانت٢٦±٣,٨ و٣± ١,٥و ٢±٠,٧ % على التوالى، بعد ذلك أجرى عمل الاختبارات المعملية، وقد أوضحت طريقة فحص الغدد الجنسية بعد مرور ثلاثين يوما من التغذية أن ٩٠% من العشيرة قد حدث بها التحول الجنسى حيث أنه من بين كل ١٠ سمكات كانت ٩ سمكات ذكور، وأوضحت تقنية ال RAPD-PCR والتى استخدم فيها عدد سبعة بوادىء، اختلافات ملحوظة بين كل من الآباء الذكور والإناث وزريعة وحيد الجنس، حيث أن Primer A14 قد لوحظ بة اختلافا بين حزم الاناث والذكور على حد سواء فى الآباء كما اظهر اشتراك الذكور الآباء مع زريعة وحيد الجنس فى حزمة رقم ٥ حجمها ٢٧٢,٧٦ زوج قواعد، وكذا الحال مع B13 primer و قد أوضح اشتراك الذكور الآباء مع زريعة وحيد الجنس فى حزمة رقم ٤ حجمها ٢٧٣ زوج قواعد، أيضاً Primer C5 قد أوضح اشتراك الذكور الآباء مع زريعة وحيد الجنس فى حزمة رقم ١,٣ حجمها ٦٠٤,٢٩٤ زوج قواعد. وكلا من D19 ، C17 ، A03 ، Primer B08 قد أظهروا اختلافات بين الآباء وزريعة وحيد الجنس ولكنها لم تظهر اشتراك الآباء الذكور مع زريعة وحيد الجنس فى حزمة كما فى A14,B13, C5 .

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