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Feminization and Molecular Sexing of the Nile Tilapia (*Oreochromis niloticus*, L) Through Sex Reversal Hormone Diethylstilbestrol and Molecular Markers

Reham M. Abd El-Azeem
Environmental Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Sadat City, Egypt
Corresponding Author: reham.abdelazeem@gebri.usc.edu.eg

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ABSTRACT
This study aimed to determine the optimum dosage and duration of diethylstilbestrol hormone (DES) required to produce the highest percentage of females and the use of short sequence repeats (SSRs) markers for the sexing of the Nile tilapia (*Oreochromis niloticus*). Fries with a total length of 9-11mm were used. DES hormone was added to the fries' feed in two concentrations (100 and 200mg/ kg of feed) for 28 and 40 days, excluding the control feed (without DES). To differentiate between normal females (XX) and sex-reversed females (XY), progeny test and SSRs markers were used. The hormonal treatment of 200mg/kg feed for 28 and 40 days gave the highest females percentage, with a slight difference between them (85.51 and 86.54, respectively). While, the treatment for 28 days gave a higher percentage of survival than 40 days (23,00 and 17.33, respectively). From 22 tested females, three XY females were obtained by progeny test and SSRs markers. Production of sex-reversed females can be used as a first step for the production of supper male YY of the Nil tilapia, and SSRs markers can be considered as a promising molecular marker for sex determination that can be used to reduce the duration of the Nile tilapia breeding program.

INTRODUCTION

Fish and its products represent an important source of proteins and essential micronutrients for balanced nutrition (**Srinivasan *et al*., 2015, Hashem *et al*., 2020; Abdel-Hamid *et al*., 2023**). Among cultivated fish, tilapia nilotica (*Oreochromis niloticus*) has many advantages, for example, the tolerance to adverse conditions of the environment; it can live at low oxygen conditions and somewhat gain fast growth. All of these characteristics make tilapia the fourth-largest fish in worldwide aquaculture (**Hieba *et al*., 2021; Martínez-Cordero *et al*., 2021; Hashem, 2022**). The world tilapia aquaculture production increased from 380,000 tons in 1990 to six million tons in 2018. This makes tilapia the fourth-largest fish in global aquaculture (**Martínez-Cordero *et al*., 2021**).

The major problem in the breeding programs of tilapia nilotica is that the females grow slower than the males, in addition to the uncontrolled reproduction and the early sexual maturation.

To avoid these problems, the production of all-male tilapia is the most commonly used method. All-male tilapia culture removes sex behavior and consequently uncontrolled reproduction, allowing the production of marketable-sized individuals. Therefore, it has been known as the most effective method to increase the production of the Nile tilapia under commercial cultural conditions. The production of monosex, all-male tilapia, is based on crosses between YY males and XX females (**Alcántar-Vázquez *et al*., 2014; Alcántar-Vázquez, 2018**).

For the production of super males YY, The estrogen hormones are used to induce sex reversal of males (XY) to females (XY), followed by progeny testing and breeding program to generate 'YY' male genotypes (**Mair *et al*., 1997; Gennottea *et al*., 2012; Hashem *et al*., 2022**).

The previous studies used three important estrogens hormones (estradiol-17β, diethylstilbestrol and 17α-ethinylestradiol) to induce sex reversal of males (XY) to females (XY) (**Hamdoon *et al*., 2013; Marín-Ramírez *et al*., 2016; Juárez-Juárez 2018; Lázaro-Velasco *et al*., 2019; Hashem, 2019**). These studies used different concentrations of hormone (50, 100 and 200 mg/kg diet) with different durations (10, 20, 25 and 40 days).

For most fish species, it is very difficult to differentiate between the sex chromosomes and autosomes based on their shape and size (**De Rosa *et al*., 2017**). For novel "supermale YY breading program and sex differentiation and reducing the time required, short sequence repeats markers (SSRs) can be used for fish early sexing without progeny test (**Al-Abedi, 2019; Hashem, 2022**). SSR has an important impact on the function and evolution of chromosomes and genomes (**Li *et al*., 2017; Samoluk *et al*., 2019**).

Therefore, this study aimed to determine the optimal dosage and duration of diethylstilbestrol hormone (DES) required to produce the highest percentage of females and the use of short sequence repeats (SSRs) markers for the sexing of the Nile tilapia (*Oreochromis niloticus*).

MATERIALS AND METHODS

Hormonal treatment of Fry

Fry specimens with a total length of 9-11mm were obtained from females' mouths from the fish breeding laboratory, Animal Biotechnology Department, GEBRI, University of Sadat City. Fries were stocked in groups in 48L glass aquaria for adaptation for one day to reduce the mortality before treatment started. They were divided into 15 groups, 100 individuals for each group. Groups were served as a control and the rest for

different DES concentrations (100 mg/kg and 200 mg/kg of feed) with different feeding durations of 28 and 40 days (three groups for each treatment).

All aquaria were filled with tap water, which was stored for one day before use. Supplemental aeration was provided through air stones by using air pumps. Temperature, dissolved oxygen and pH were kept at normal levels throughout the treatment period.

The diethylstilbestrol (DES) hormone (Sigma chemicals) was applied to the feed of the fries (30% protein), while the control feed was without DES. Feeds were prepared as described by **Guerrero (1975)**.

Fry samples were fed three times daily at 15% of their body weight for 28 or 40 days. The fecal matter and other waste materials were daily siphoned off, with 10 to 15% of water to control ammonia content in water.

To allow growth, the amount of feed was weekly adjusted through evaluating the sample's weight considering each aquarium. After the end of the treatment duration (28 or 40 days), the fries were fed with untreated feed.

Evaluation of treatment efficacy

For evaluating the treatment efficacy at the sexing time (90 days), all experimental individuals were sexed and the survival percentage was calculated. The gonadal tissue was removed with a pair of fine forceps and placed on a glass slide and stained with aceto-carmine using the standard squash technique (**Guerrero & Sheilton, 1974**). Gonads were classified as either ovaries or testes via a light microscope; the sex was determined and the sex ratio was calculated to evaluate the hormonal treatments.

Progeny test

To differentiate between normal females (XX) and the sex-reversed females (XY), progeny test and SSRs were used. The produced fry samples from 22 treated females (The remaining individuals from the DES-treated fish, 200mg/kg feed) were separately stocked in glass aquaria. Fry samples were reared until they attained a mean mass of about 10g. The sex of each female progeny was detected using the gonad squash technique as previously described**.**

Genomic DNA extraction

Total DNA was extracted from the fin-clip tissue of examined fish. DNA was extracted through the salting out procedure (Ma et al., 1996). A NanoDrop spectrometer was used for the quantification of DNA concentration, and each sample of DNA was diluted to ~50 ng/µl final concentration.

SSRs primers and PCR amplification

Four pairs of primers were used for SSRs amplification, sex determination and differentiation between normal females (XX) and sex-reversed females (XY). These SSRs were recognized using the genome sequence of tilapia as reported by **Eshel *et al* (2012)** and **Hashem (2022)**. The four primers are revealed in Table (1).

Table 1. The four primers were used to differentiate between normal females (XX) and sex-reversed females (XY).

Primer name	Forward primer	Reverse primer
SSRP1	CCTGAAACTCAGGCGCTGTA	GCTCTCACCAAGGTCAGCAA
SSRP7	TGCTCTCACTGCTGAGCAAA	CGCAAATGTTAGGCCAGAAA
SSRP13	AGGCCTTTCATCGCTGTTTT	ACCCTGTAGATGAGCGCAAA
SSRP17	GGTGGGACTGTGGTGTATGG	GGTGGATTGCAAGCAACATT

The conditions of the PCR reaction were optimized by using 10µl of master mix (Enzynomics, Korea), 0.4µM of each Primer, 2.0µl of 50 ng of DNA and de-ionized water up to 20µl. PCR conditions were 94^{o}C for 5 min, followed by 50 sec at 94^{o}C, 45 sec at 55^{o}C and 60 sec at 72^{o}C for 30 cycles, and a final step for 10 min at 72^{o}C. Applied Biosystems® ProFlex™ System was used for conducting the PCR reaction. The normal female was used as a control female (CF) to confirm the reversed females. 4% Agarose gel electrophoresis was used for PCR product detection. Obtained DNA fragments sizes were identified through a 100 bp DNA ladder.

RESULTS AND DISCUSSION

The variances between male and female stained gonads using standard squash technique are illustrated in Fig. (1).

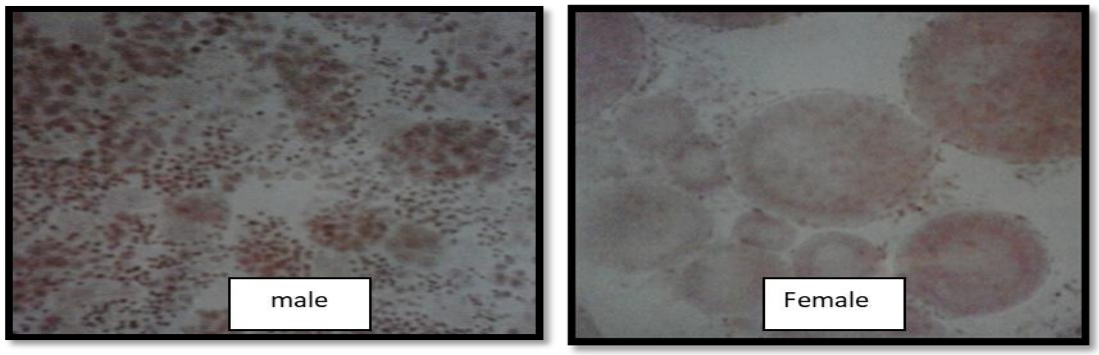

Fig. 1. The variances between male and female stained gonads using standard squash technique

At the sexing time (90 days), 50 individuals from each treatment were separated and reared until sexual maturation for progeny test and molecular sexing to detect females XY. Then, the rest individuals were sexed and the survival percentage was calculated. The evaluation results of the hormonal treatment efficacy are presented in Table 2. From Table 2, the hormonal treatment of 200mg/kg feed for 28 and 40 days gave the highest females percentage with slight difference between them (85.51 and 86.54, respectively), but the treatment for 28 days gave a higher percentage of survival than 40 days (23,00 and 17.33, respectively). **Hamdoon *et al.*, (2013)** achieved the highest percentage of females by using 100mg DES/kg feed for a 40- day feeding. The obtained results showed that feeding fries at 50mg or 100mg concentrations for both 25 and 40 days improved the percentage of females significantly than that of the control. **Lázaro-**

Velasco *et al*. (2019) obtained 100% feminization by using the estrogen hormones estradiol-17$_{\beta}$, and 17α-ethinylestradiol in combination with cold-water temperature. **Shved *et al*. (2009)** and **Lázaro-Velasco (2014)** used 120 mg/kg and 125 mg/kg feed from EE2 hormone for the Nile tilapia, and they achieved higher feminization rates of 83% and 86.5%, respectively.

Table 2. The effects of different levels of DES hormone (100 and 200mg/kg feed) for different feeding durations (28 and 40 days) on the percentage of survivals and percentage of females of *O. niloticus*

treatments		No. of recovered individuals	Mean percentage of survivals	No. of males and females		Mean Percentage of females
				Males	Females	
Control		188	31.33	90	98	52.13
100mg/kg	**28 days**	146	24.33	48	98	67.12
	40 days	154	25.67	40	114	74.03
200mg/kg	**28 days**	138	23,00	20	118	85.51
	40 days	104	17.33	14	90	86.54

On the other hand, in the commercial breeding of the Nile tilapia, obtaining all-male individuals through exogenous hormones decreases the time to produce tilapia of commercial size and eliminates the problems associated with the early sexual maturation of female's maturation (**Varadaraj & Pandian, 1989; Lázaro-Velasco *et al*., 2019**). However, the organizations concerned with human's health disallow the use of hormones in the food that will be consumed by humans. It was mentioned that, using hormones should be established by the government for human and environmental safety (**Hashem, 2022**). One of the most essential unconventional techniques in commercial tilapia production is the production of supper males YY. While, the initial stage of YY production involves the feminization of XY individuals to produce XY females; hormones will not be found in the final product that will be consumed and will significantly reduce the level of hormones in the environment (**Marín-Ramírez *et al*., 2016**).

In the present study, XY females were determined through progeny test and SSRs markers. Progeny of the sex ratio of 3:1 (males: females) or near that of some females were obtained and indicated that females are XY females. From the 22 tested females, three XY females were obtained, as presented in Table (3).

Sex determination in tilapia is controlled by major and minor inherited and environmental causes. Genes in tilapia showed that sex determination should be studied as a quantitative trait (**Lee *et al*., 2004; Shirak *et al*., 2008**). In fishes, molecular markers including SSRs markers are a very beneficial tool for studying sex determination, sex-related genetic factors and sex chromosome recognition (**De Rosa *et al*., 2017**). In the

different chromosomes of tilapia, different loci of sex detection have been detected. From SSRs-based studies, two unlinked SSRs loci were linked to the phenotypic sex in blue tilapia as reported in the study of **Lee *et al*. (2004**).

Table 3: The obtained XY females from DES hormonal treatment and the percentage of males in their progeny from progeny test.

Obtained females	No. of recovered individuals	No. of males	No. of females	Percentage of males (%)
1	106	77	28	72.6
2	94	70	24	74.5
3	91	68	23	74.4

The results of the progeny test of the following study indicated that there are three XY females. The obtained XY females were confirmed through SSRs markers (Fig. 2). The normal XX female was used as a control female (CF) to confirm the reversed XY females (RF).

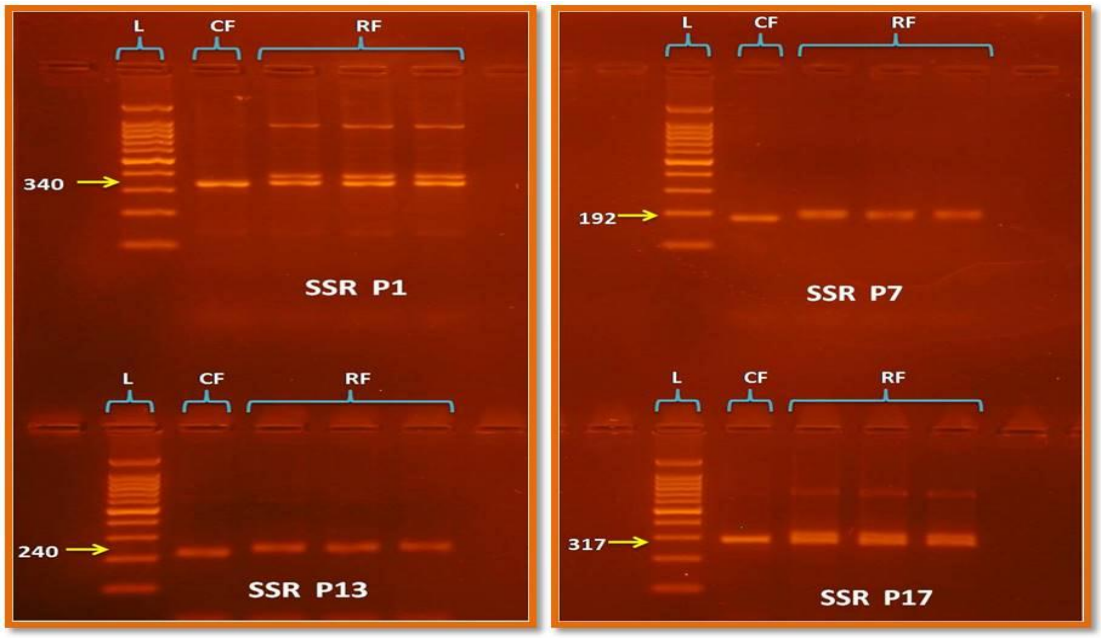

Fig. 2. Agarose gel electrophoresis of SSR P1, SSR P7, SSR P13 and SSR P17 of control female (CF) and reversed females (RF)

It was found that females were homozygous, and females XY may be heterozygous or homozygous for the other alleles. The females were homozygous, and reversed females (XY) were heterozygous for markers SSRP1 and SSRP17. Whereas, the females were homozygous, and reversed females were homozygous for the other ones as

in SSRP7 and SSRP13. **Hashem (2022)** reported the same results when he used the same primers for the early sexing of the Nil tilapia. He postulated that, SSRS markers can be used to distinguish between normal females (XX) and reversed females (XY). Females XY is a functional female but still genetically male and will give the same alleles as males. The normal female is always homozygous for alleles; however, the male is heterozygous or homozygous for the alternative alleles of SSRs.

SSRs markers were studied by **Shirak *et al* (2008)** and the authors obtained 12 adjacent markers in the studied genome region of the, Nile tilapia. While 38 samples of *S. rivulatus* were examined by **Al-Abedi *et al* (2019)**. They studied six SSR markers loci and reported that, the females and males were homozygous for the same loci alleles. Whereas, the other loci were homozygous in some samples and heterozygous or homozygous in others for the alternative alleles.

In conclusion, the production of sex-reversed females can be used as a first step for the production of supper male YY of the Nil tilapia, and SSRs markers can be considered as a promising molecular marker for sex determination that can be used to reduce the duration of the Nile tilapia breeding program. This program includes the feminization of the fry for the production of XY females that are confirmed by SSRs markers. The progeny of XY females with normal males will contain 25% of YY males that will be separated from XY after the sexual maturation stage by crossing each with normal female (XX).

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