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PRODUCTION OF NATURAL "XY" FEMALE OF NILE TILAPIA "*OREOCHROMIS NILOTICUS*" BY THERMAL MANIPULATION IN EGYPT

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Abstract

Nile tilapia (*Oreochromis niloticus*), a crucial freshwater fish for aquaculture, requires mono-sex culturing to prevent unwanted reproduction during grow-out, making sex control a crucial issue in aquaculture. The hormone 17-α-methyltestosterone is typically employed to generate phenotypic males in the mass production of all-male fingerlings. The extensive use of substantial amounts of sex reversal hormones in hatcheries may present health hazards to workers and ecological risks to adjacent areas. This study illustrates breeding strategies to produce genetically all-male tilapia without the use of sex hormones. This study examines the intricate sex determination system of Nile tilapia, influenced by genetic factors and temperature interactions in domestic populations. Sex reversal can occur due to feminizing temperatures experienced by fry during their sex differentiation period in shallow waters, and/or due to the impact of minor genetic variables. The 3-day post-fertilization Nile tilapia fry was reared in Agromar farm at 17-20 °C for 60 days to induce XY female sex reversal, after which the tail fin (n=21) was harvested for genotyping and cytogenetic investigation. Results indicated that there 30% of Tilapia were

reversed to XY females. The present study may provide an important unique foundation for developing genetic male tilapia XY females by natural sex reversal in Egypt.

Keywords: Tilapia, Natural production, Thermal manipulation, Female XY.

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Introduction

Fish display a wide range of sex determination and differentiation processes, which include genetic sex determination (GSD), environmental sex determination (ESD), and a combination of GSD and ESD (**Capel, 2017; Ospina-lvarez and Piferrer, 2008**). Subjecting fish to environmental stresses during the sensitive period of gonad differentiation can lead to sex-reversal, when Environmental Sex Determination (ESD) overrides Genetic Sex Determination (GSD).

Temperature is a common environmental stressor that can cause sex-reversal in fish species. This occurs when the hormones involved in gonad differentiation pathways are either up-regulated or down-regulated, leading to a change from female to male or vice versa. This phenomenon has been documented in studies by **Poonlaphdecha *et al.* (2013)** and **de Alba *et al.* (2023)**. Instances of total sex-reversal, where a female organism transforms into a male, have been documented in Nile tilapia. Conversely, male to female sex reversal has been reported in Channel catfish (*Ictalurus punctatus*) and Sockeye salmon (*Oncorhynchus nerka*) **(Baroiller *et al.*, 2009a)**. Research has discovered that the sensitivity to temperature in a particular species of fish is influenced by its family and can differ among different populations, even when they are in their natural habitat **(Bezault *et al.*, 2007)**. Sex reversal in fish, whether to male or female, can be accomplished by administering androgenic or estrogenic steroid hormones during the early stages of gonad development. Steroid hormones function

by increasing the activity of the genes responsible for the development of testes (*Dmrt1* and *Amh*) and ovaries (*Foxl2* and *Cyp19a1a*) **(Piferrer, 2001)**.

Sex control is crucial in aquaculture for certain species due to the presence of sexual dimorphism, which results in one sex being more attractive than the other for cultivation. This preference is based on differences in growth rates, age at sexual maturity, lack of development after maturation, and deterioration in flesh quality **(Tenugu & Senthilkumaran, 2022)**. According to **Toguyeni *et al.* (1997)**, male Nile tilapia have a 20% higher growth rate compared to females and also have a more efficient feed conversion ratio. Mixed sex tilapia production techniques promote undesired reproduction, resulting in issues related to overcrowding, which subsequently diminishes the value of the end product **(Budd *et al.,* 2015)**. In aquaculture, exogenous hormones or high temperature are commonly employed strategies to control sex-ratio and maturation. These methods are used to produce mono sex populations with the desired sexual genotype and to study the genetic and endocrine factors that play a role in sex determination and differentiation **(Singh, 2013)**. Some nations, such as India, and countries within the EU, Ecuador, and Costa Rica, have imposed restrictions on the use of hormone-treated fish for human consumption. This procedure is commonly used to establish mono sex tilapia populations. Nevertheless, by employing both sex-reversal and genetic manipulation techniques in fish, it is possible to generate genetically mono sex offspring suitable for commercial purposes. As an illustration, initiating hormone treatment can serve as the initial stage, resulting in the creation of XX or YY populations. These populations can then be bred together to generate offspring of a single sex. The populations of XX and YY have been effectively cultivated for tilapia and salmonids **(Taslima *et al.,* 2023)**.

Tilapia have diverse sex-determining systems, including male and female heterogametic systems and ESD (**Palaiokostas *et al.*, 2015).** The Nile tilapia species has a complicated mechanism for determining male sex

based on different sex chromosomes **(Penman and Piferrer, 2008)**. Thermo-sensitivity is also observed in Nile tilapia, with phenotypic sex linked to LG3 and LG20 **(Sissao *et al.,* 2019; Taslima *et al.,* 2021)**. Sex steroids are commonly administered during the sensitive phase of sex differentiation **(Baroiller and D'Cotta, 2019)**, typically between 10- and 30 days post fertilization. Sex-reversing genetic females to phenotypic males is more feasible in some fish species **(Meriwether and Shelton, 1981; Donaldson & Hunter, 1982)**. In species that are sensitive to temperature, such as Nile tilapia, temperatures above 32-34^{o}C can cause XX female offspring to completely change sex and become functional males **(Baroiller *et al.,* 2009b)**. Alternatively, it might lead to an imbalanced ratio of males and females in groups that have both sexes **(Kwon *et al.,* 2002)**.

It is worth noting that a higher percentage of females were found in YY and XY Nile tilapia offspring (YY offspring were produced by mating YY males with YY females) when they were exposed to higher temperatures **(Abucay *et al.,* 1999)**. **Kwon *et al.* (2002)** observed a notable feminization impact in genetically YY Nile tilapia offspring, with a female population comprising 35.5%. The survival percentage of these offspring was 32%. Additionally, when genetically XY fish were subjected to high temperatures (36^{o}C), only a tiny fraction of females was present in the group. The study conducted by **Karayücel *et al.* (2003)** found that when YY Nile tilapia juveniles were exposed to a temperature of 36 ^{o}C, they exhibited a feminization rate of 32%. Additionally, the survival rate of these progeny was observed to be 63%. The feminization techniques reported for Nile tilapia exhibit significant variability in feminization rates and often result in lower survival in practical applications. While elevated temperature treatments typically result in the masculinization of genetic females, there is evidence suggesting that genetic males can be feminized under some circumstances. The aim of this study was to evaluate the impact of thermal manipulation on the process of feminizing male Tilapia in order to produce XY females.

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Materials and Methods

Broodstock selection

Originating from the Kafr El-Sheikh private farm in Egypt, the Nile tilapia (*Oreochromis niloticus*) stock was kept at the Agromar farm located at Kilo 45 in Alexandria, Egypt. Using the methods outlined in **Taslima *et al.* (2017)**, the broodstock were chosen based on the sex-linked marker analysis.

Experimental design

After the yolk sac resorption stage (10 days after fertilization), the offspring from each family were separated into equal batches. The fry batches received a 60-day treatment at a temperature range of 17-20°C in a stationary thermal treatment facility equipped with glass holding tanks measuring 30 x 19 x 17 cm. Prior to the beginning of the experiment, the system was filled with aerated clean tap water. A Gallenkamp Thermo stirrer 85, 220-240V, EEC, with a temperature range of 0-100ºC, was used to maintain the proper temperature in the glass tanks. The heater also had a stirrer pump that facilitated the circulation of water within the plastic holding tanks.

Tilapia fry was fed three times daily at 15% of their body weight. 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 (60 days), the fries were fed with untreated feed.

Phenotypic sex differentiation by Ultrasonic sonar

About 48 hours to the time of ultrasound examination feeding was stopped to enhance scanning. During ultrasonic imaging, the fish were immobilized to expose the ventral region. Each fish was subjected to transverse ultrasound imaging and the images were recorded. The sex determination of *O. niloticus* and imaging of ovaries in females were conducted using a SIUI CTS-500 digital ultrasound machine equipped with a 6.5 MHz transducer frequency sector probe. Gender determination was done and the males were released back into the water. However, certain females were sacrificed in order to verify the accuracy and reliability of the ultrasound images.

Collection of sample and Fixation for Cytogenetics

The experiment was conducted in the Laboratory of Fish Breeding and Genetics, Agromar Farm, Alexandria, Egypt. Nile tilapia, aged 9 months and produced via artificial fertilization, were utilized for chromosome preparations in the present study. One hundred fish were collected for chromosome preparation without the application of colchicine. The solid tissue method, as described by **Kligerman and Bloom (1977)**, and the staining method established by **Howell and Black (1980)**, were employed as standard protocols for chromosome preparation and staining, respectively. Following anesthesia, the caudal fin tissues were swiftly sliced into 0.25 cm^2 pieces, deposited right away into 5–10 ml of hypotonic solution (0.56% KCl), and then incubated for 30, 40, 50, and 60 minutes (twice, respectively) at 29–30°C. The tissues were then preserved for 30 minutes (twice) in 5–10 ml of fresh, cool (7–10°C) Carnoy's solution, which was made up of 100% ethanol and acetic acid (3:1). Following the fixation procedure, tissues' epithelial cells were separated by adding three to four drops of 50% acetic acid. They were then carefully cut with a sharp scalpel on a concave glass object for 30 to 60 seconds, creating a murky cell suspension. When working with a lot of samples, the tissues were fixated, then put in Carnoy's solution and kept in a refrigerator at 4°C for no more than two weeks before proceeding to the next step. The cell suspension was carefully pipetted and squished on a very clean and warm

slide glass that was set on a hot plate (50–55°C) using a 100 µl chip micropipette. Squashing entailed the subsequent procedures: following pipetting, the entire cell suspension was aspirated from the concave glass object and deposited onto a warm glass slide from a height of 4–5 cm, then promptly re-aspirated to create rings with a diameter of 1–1.5 cm (three rings per glass slide). Following a 2–3 min air-drying period, the preparations were dyed. Preparations were stained with Giemsa stain (**Kligerman and Bloom, 1977**). Giemsa staining was performed by submerging the specimen in a freshly produced 10% Giemsa solution in a phosphate buffer solution (PBS) at pH 6.9 for 30 minutes. Giemsa-stained preparations were meticulously cleansed with distilled water and air-dried. The fully desiccated preparation was examined under 400× and 1000× magnifications with a Leica DM2000 microscope.

Genotyping

DNA extraction

Tail fins were harvested and cryopreserved in liquid nitrogen, and subsequently stored at −80°C until required. Genomic DNA was extracted using proteinase K digestion followed by phenol/chloroform extraction, as previously outlined (**Samoluk *et al.*, 2019**). The purity and content of DNA were evaluated using agarose gel electrophoresis and quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The DNA was ultimately diluted to a concentration of 20 ng/µl and preserved at −20°C for subsequent utilization. The existence of Y-linked and X-linked sequences was validated as outlined by **Sun *et al.* (2014)**. DNA was isolated from caudal fins using normal techniques and subsequently underwent PCR with the following primers in **Table 1**.

Table 1: Primers used in the present study

Primer name	Primer sequence (5' to 3')	Purpose
SCAR-5F-Y	TAAATTAATGACATTTCAGTTATG	Y-specific amplification
SCAR-5R-Y	TTACAGCAGCACCCAGAGTCAT	Y-specific amplification
SCAR-5F-X	CTGGTTTGCAATAGTTAGGGTGCT	X-specific amplification
SCAR -5R-X	CAGAAATGTAGACGCCCAGGTATC	X-specific amplification

Statistical analysis

Mean values comparisons were performed using t student test with a significance level of $\alpha = 0.05$.

Results and Discussion

Ultrasonic

Reversed "XY" female *O. niloticus* were examined using ultrasound imaging. The obtained ultrasound image is shown in **Fig. 1** and also **Fig. 2** shows the picture of sacrificed fish to validate the position and identity of the ultrasound images. Gender of female *O. niloticus* was verified using ultrasound imaging. Females of *O. niloticus* showed paired masses of ovary-like tissue which extends interiorly from the anal fins to the abdomen, **Fig. 1**. showed prominent images of paired ovaries. Visual methods were not very accurate but ultrasonic imaging is an efficient and accurate method to determine gender of mature females of *O. niloticus*. Gender determination using ultrasound was successfully performed in *O. niloticus*. Similar results were obtained by **Jenning *et al.* (2005)** and **Nzeh and Jimoh (2010)**. Sector probe with transducer frequency of 6.5 MHz was effective in determining *O. niloticus* gender. Jenning (2005) made

similar observation in stripped bass *Morone sexafilis* in Savannah River estuary in Georgia Atlanta USA. **Nzeh and Jimoh (2010)** also made similar observation in *C. gariepinus*. This technique is more accurate than visual sex determination. Early detection of eggs may be done with the help of ultrasound and manage rutting in *O. niloticus*. Ultrasound may be a useful method of separation of sexes in *O. niloticus* (**Makinde and Jimoh, 2012)**.

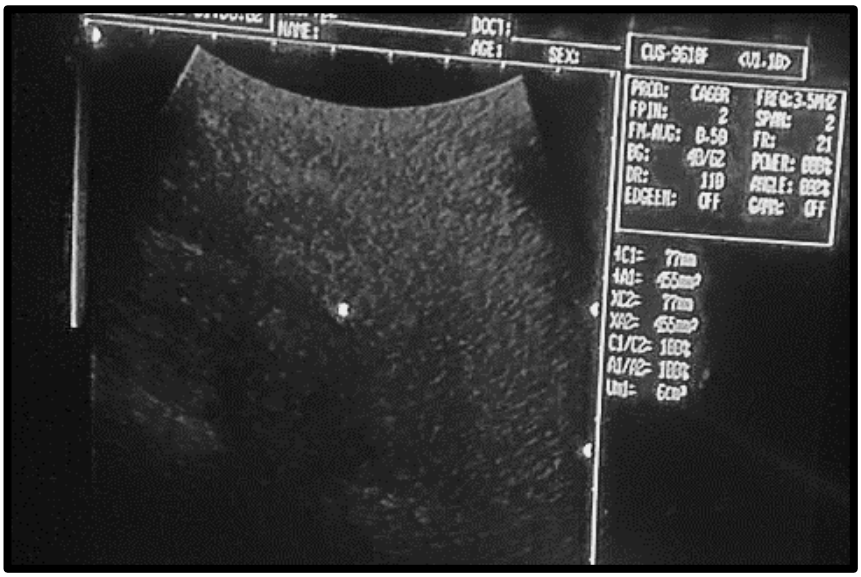

Figure 1: Ultrasonic images showing ovaries of female XY *O. niloticus*

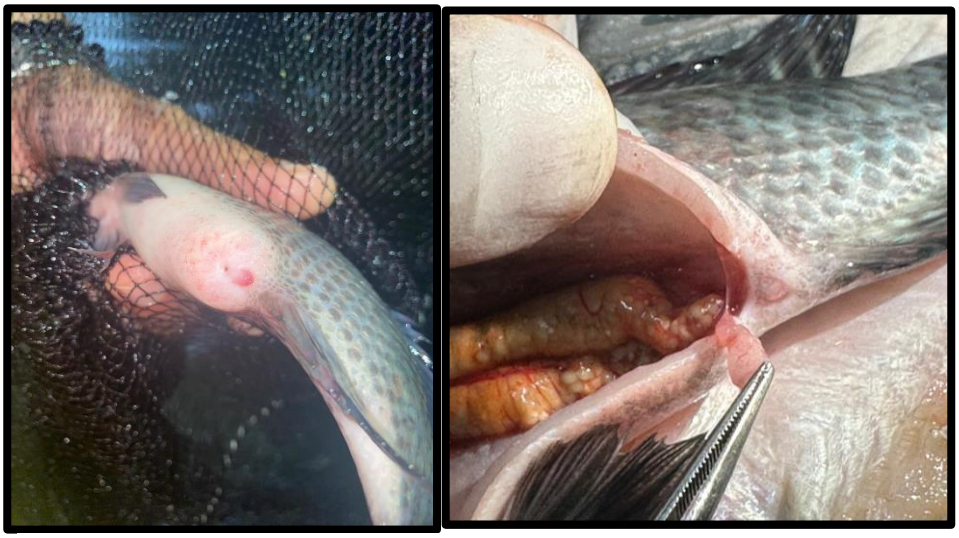

Figure 2: Photograph showing external features and Ovaries of female

Chromosomal karyotyping

Somatic chromosome number of *O. niloticus* was detected 2n = 44 in the present studies. No deviation in chromosome number was detected in each plate. The chromosomal karyotypes showed highly similar in chromosomal number as 2n= 44 in all samples were study. The structure of chromosomes was observed some abnormal in centromere position, this abnormality could be a pericentric inversion in chromosomes No. **1, 2, 7, 13, and 14** (**Fig. 3 & 4**)

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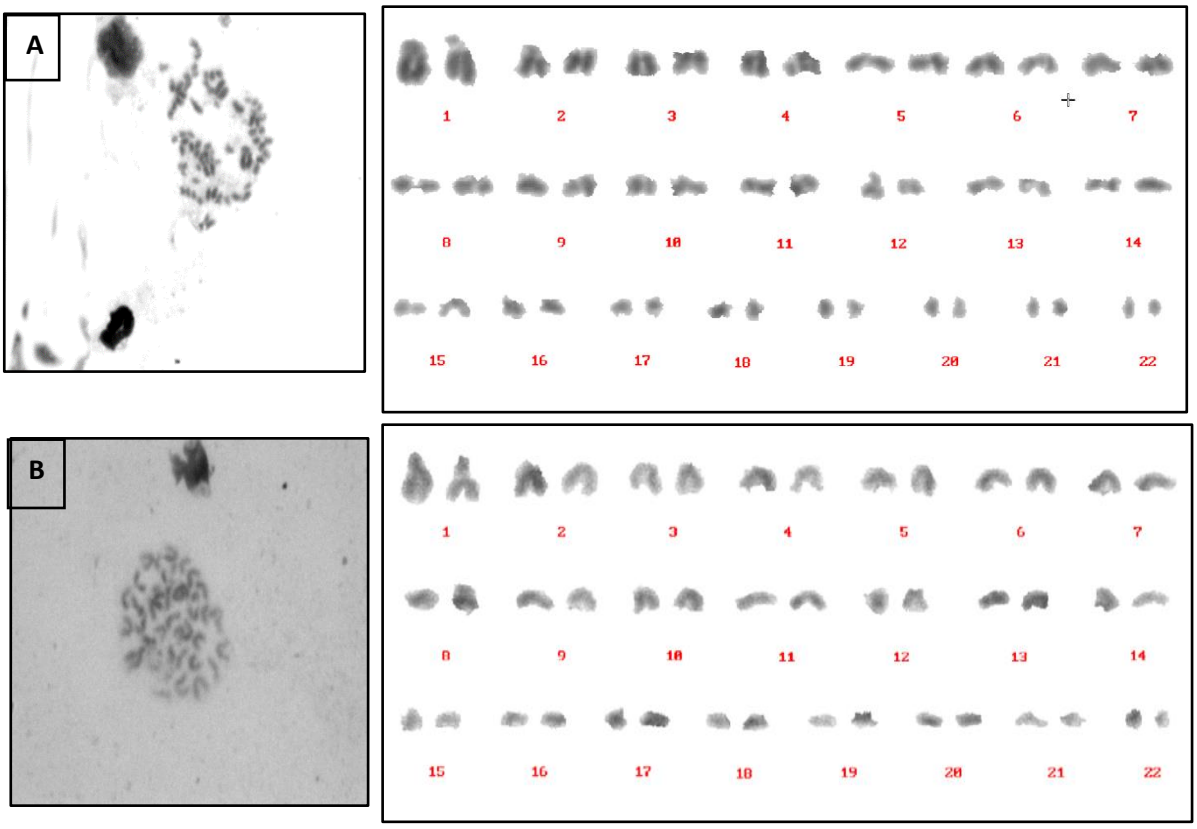

Figure 3: (A & B) showing no chromosomal aberrations with normal number of chromosomes 2n= 44

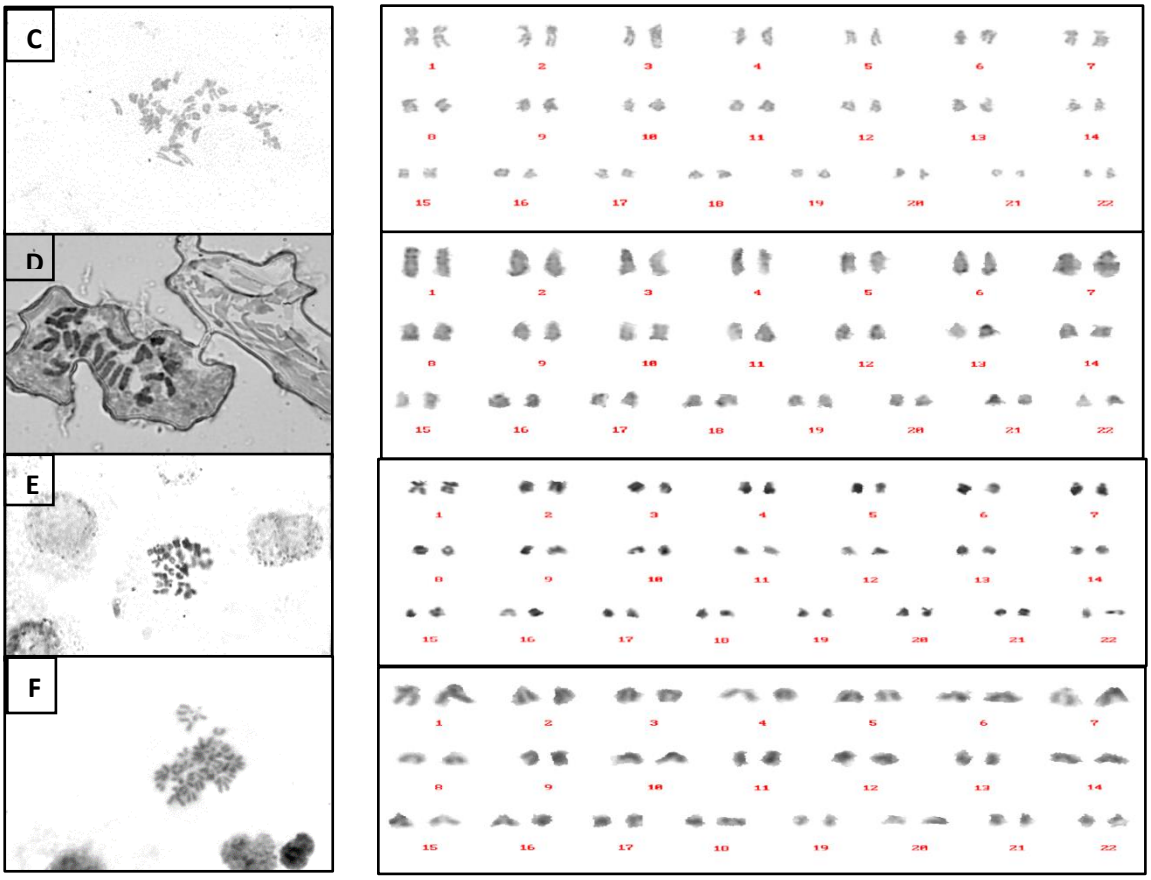

Figure 4: (C, D & E) showing a pericentric inversion in chromosomes (No. 1, 2, and 7) but chromosomal number was 2n= 44.

There may be a linkage between the chromosomal structure changes, especially pericentric inversion, and changes of X and Y genes. The examination of chromosomes is essential for species taxonomy and the comprehension of evolution. Despite extensive utilization in taxonomic

research of invertebrates and vertebrates, there is a notable deficiency of cytological data regarding fishes from this group (**Alves, 2000; Artoni and Bertollo, 2001**). Consequently, the current research is highly valuable for elucidating the cytogenetic and karyotypic organization of *O. niloticus*. The karyological analysis of teleost fish has technological challenges not faced in the examination of vertebrates, attributed to the diminutive size and elevated quantity of chromosomes (**Cucchi and Baruffaldi, 1990**). The current study on the chromosomal makeup of *O. niloticus* faced challenges in staining and slide production utilizing suitable fish tail fin tissues. Cells are prone to breakage, resulting in a higher incidence of incomplete chromosomal complements. The primary challenge faced is the morphological variance present even among homologous chromosomes inside the same nucleus (**Al-Sabti, 1991; Levan *et al.,* 1964**). Fish karyotypes are not uniform as in humans or other animal species; yet, polymorphism frequently arises within the same fish species (**Al-Sabti, 1991**). Karyotype analysis is essential for stock enhancement through polyploidy management, hybridization detection, and associated genetic engineering (**Tan *et al.,* 2004**).

In the tilapia tribe, there are two different sex chromosome systems: an XX/XY sex-determination system (e.g., in *O. mossambicus* and *O. niloticus*) (**Zhu *et al.,* 2009**). But such differences do not reflect significant genomic variation in the distribution of repeated DNAs among these species, as visualized here by FISH, at least in relation to the Cot-1 DNA. These species have a typical karyotype pattern composed of one pair (the largest) of telocentric chromosomes(t), a few sub-metacentric chromosomes (sm) and several sub teloacrocentric (st/a) chromosomes (Chen & Chen 1983; Majumdar & McAndrew 1986; Zhu *et al.,* 2009). These results suggest genomic conservation in all tilapiine species studied (Zhu *et al.,* 2011).

Genotyping

Data in **Table 2**. illustrates the thermal manipulation resulted in 30% sex reversal providing female (XY), while the control groups remained as 100% (XX) female and (XY) male (**Fig. 5**). In tilapia like in various reptiles (**Valenzuela, 2008**), genetic x environment interactions have been demonstrated in the sex-determining response to temperature modulation. In all of these species, treatments that mimic the natural thermal regimes have demonstrated to be efficient for sex inversion. Additionally, the recent data of **Bezault *et al.* (2007)** strongly suggest that in tilapias XX males and XY females can be encountered in nature. As XX males and YY males have clearly been demonstrated to be viable and fertile in tilapias, the existence of XX males and YY males in the wild is not a drawback for the population and species (**Baroiller *et al.*, 2009a**)

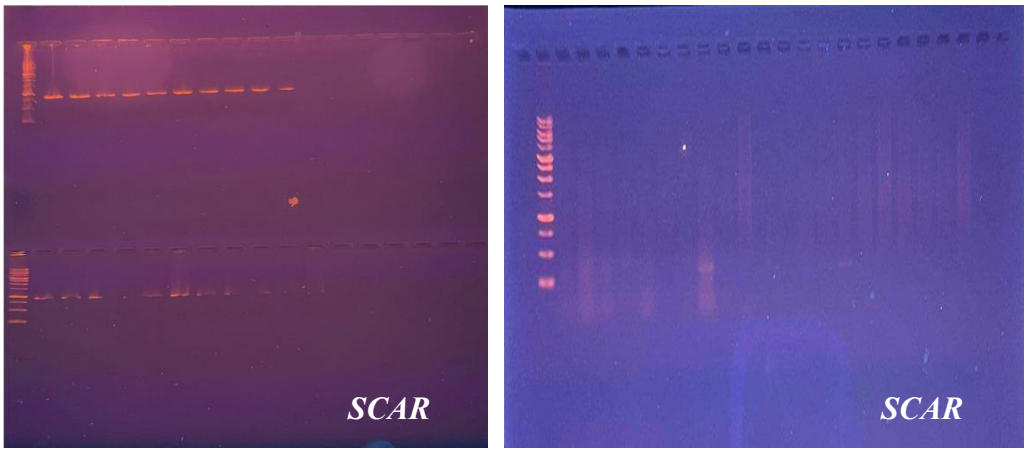

Figure 5: A. PCR markers SCAR-5F/5R-Y and SCAR-5F-X/5R

Table 2: Showing PCR results of normal XX and reversed female XY

Conclusion

No	*SCAR Y*	*SCAR X*	*Genotype*
1	+	+	**FXY**
2	+	+	**XY**
3	+	+	**XY**
4	+	+	**FXY**
5	+	+	**FXY**
6	-	+	**XX**
7	-	+	**XX**
8	+	+	**XY**
9	-	+	**XX**
10	+	+	**XY**
11	+	+	**FXY**
12	+	+	**FXY**
13	-	+	**XX**
14	+	+	**FXY**
15	+	+	**FXY**
16	+	+	**FXY**
17	-	+	**XX**
18	+	+	**FXY**
19	-	+	**XX**
20	-	+	**XX**
21	-	+	**XX**

Conclusions

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 SCAR 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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إنتاج إناث "XY" الطبيعية من سمكة البلطي النيلي *OreoChromis niloticus* عن طريق التلاعب الحراري في مصر"

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الملخص العربي

اسماك البلطي النيلي، هي اسماك تعيش في المياه العذبه، تتطلب انتاج زريعه أحادية الجنس للتربيه على مستوى تجاري و ذلك لمنع التكاثر غير المرغوب فيه خلال فترة النمو، مما يجعل التحكم في الجنس قضية حاسمة في تربية هذا النوع من الاسماك. يتم عادةً استخدام هرمون 17-ألفا-ميثيل تستوستيرون لإنتاج ذكور ظاهريا (مع عدم المساس بالمحتوى الجيني) في الإنتاج الضخم لزريعة الاسماك وحيده الجنس ذكور بالكامل. الاستخدام المكثف لكميات كبيرة من هرمونات تحويل الجنس في مفرخات الأسماك قد يشكل مخاطر صحية على الاسماك و العمال و ايضا مخاطر بيئية تنتقل للمناطق المجاورة. توضح هذه الدراسة استراتيجيات انتاج زريعة اسماك البلطي وحيد الجنس ذكور بالكامل دون استخدام هرمونات تحويل الجنس. تدرس هذه الدراسة النظام المعقد لتحديد الجنس في اسماك البلطي النيلي و مدى تأثر المحتوى الجيني للاسماك بتغير الحرارة في المراحل الاولى للزريعه. ووجد ان التلاعب بدرجات الحراره في هذه المرحله يكون له تأثير واضح في عملية التحكم في جنس الزريعه كما وجد ان درجات الحرارة المنخفضه قد تؤدي الى اتجاه معظم الزريعه الى الجنس الانثوى. تم تربية صغار اسماك البلطي النيلي في مزرعة أجرومار عند درجة حرارة 17-20 درجة مئوية لمدة 60 يومًا لتحفيز اتجاه الجنس ناحية الإناث مع احتفاظها بالمحتوى الوراثي ثابتا (XX,XY)، و تم تحديد ذلك عن طريق جمع عينات من الزعنفة الذيليه (n=21) لإجراء تحاليل لكشف المحتوى الجيني والدراسات السيتوجينية. و أشارت النتائج إلى أن 30% من اسماك البلطي قيد الدراسة قد تتجه جنسيا إلى إناث تحمل محتوى وراثي XY. و بذلك قد توفر هذه الدراسة أساسًا فريدًا ومهمًا للحصول على ذكور يكون محتواها الوراثي YY و الذي يعد الامل الوحيد لانتاج اسماك ذكور وحيدة الجنس بالكامل بدون استخدام اي كيماويات او هرمونات.