Damanhour Journal of Veterinary Sciences 8 (2), (2022) 22–25

Damanhour Journal of Veterinary Sciences

Journal homepage: https://djvs.journals.ekb.eg/

Early sexing of Tilapia nilotica (*Oreochromis niloticus)* by using short sequence repeats (SSRs) molecular markers

Medhat H. Hashem[1*]

[1]Animal Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Sadat City, Egypt

Abstract

Aquaculture is a promising of the Nile tilapia (*Oreochromis niloticus*, L.), an important issue in Egypt. The present study's objective was to use short sequence repeats markers (SSRs) in the early sexing of *O. niloticus*. Molecular markers identification in fish includes SSRs markers that were very useful for investigating sex-determination system, sex-related genes, and sex chromosomes. The fin-clip tissue of examined fish was used for DNA extraction and the amplification of SSRs and sex determination; twenty primer pairs were tested. The results showed that, from the tested 21 primers of SSRs markers, four primers had significant differences between males and females. It was observed that females were homozygous, and males may be heterozygous or homozygous for the alternative bands. The females were homozygous, males were heterozygous for two SSRs markers, the females were homozygous, and males were homozygous for the alternative alleles for the other two markers. In conclusion, the results of the present study and the previous ones confirm the possibility of using short sequence repeats markers (SSRs) in the sex determination of fish species, including Nile tilapia (*Oreochromis niloticus*, L.). SSRs markers could be used in the early sexing detection of the Nile tilapia breeding programs and for producing supermale YY without needing progeny test.

Keywords: Sex determination; *Oreochromis niloticus*; SSRs markers; breeding program.

*Correspondence: Medhat H. Hashem
Animal Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Sadat City, Egypt
Email: medhat_helmy@yahoo.com
P ISSN: 2636-3003
EISSN: 2636-2996
DOI: 10.21608/djvs.2022.141182.1074
Received: May 27, 2022; Received in revised form: June 24, 2022; accepted: June 27, 2022
Editor-in-Chief:
Prof Dr/Ali H. El-Far (ali.elfar@damanhour.edu.eg)

1. Introduction

Nile tilapia is expected to be the most imperative of all aquaculture fish species in the 21st century. Tilapia has many favorable features, such as the tolerance to adversative environmental conditions; it can survive at low dissolved oxygen conditions, relatively fast growth, and efficient food conversion. The all these characteristics make it one of the best fish for farmers (Hashem et al., 2020). The production of the world tilapia aquaculture rose from 380,000 tons in 1990 to 6 million tons in 2018. This makes it the fourth-largest fish in global aquaculture (Martínez-Cordero et al., 2021).

The major problem in tilapia breeding programs is that the growth of females is slower than that of males, the early maturation of sex, and uncontrolled reproduction (Lazaro-Velasco et al., 2019). Monosex production upon supermale YY followed by a production of all-male progeny by hybridization between supermale and normal female, known as the most effective solution to resolve these problems due to mixed-sex or females breeding (Mair et al., 1997; Gennottea et al., 2012; Hashem et al., 2019).

Several methods can be used for YY production. One method is using sex reversal hormones to obtain functional females XY followed by a progeny test to separate them from normal females (XX). Then female XY is crossed with male XY to obtain supermale YY as described by Mair et al. (1997). Production of supermale YY through hormonal treatment followed by progeny test takes a long time within an average of 8 years to produce the first generation.

On the other hand, it is very difficult to differentiate between the sex chromosomes and autosomes of nearly most fish species, including the Nile tilapia based on their size and shape (De Rosa et al., 2017). For sex differentiation and to reduce time required for novel "supermale YY breading program, microsatellite or short sequence repeats molecular markers (SSRs) can be used for early sexing of individuals without progeny test.

In the past few years, many molecular markers have been developed to study sex-specific molecular markers in different fishes (Silva et al., 2012, Adhikari et al., 2014; Al-Qurainy et al., 2018). Molecular markers identification in fishes is very useful for investigating the sex determination system, sex-related genes and sex chromosomes identifying. It can also be used to study the effect of environmental factors on sex differentiation (De Rosa et al., 2017).

Molecular markers, such as microsatellites or short sequence repeat markers (SSR), are now being used in aquaculture and fisheries (Al-Abedi, 2019). SSR is a repetitive DNA sequence which has important influence on the function and evolution of genomes and chromosomes (Li et al., 2017; Samoluk et al., 2019). There is big need for continuous development of the fish quality in

Egypt. So, this investigation aimed to use short sequence repeats markers (SSRs) in the early sexing of the Nile tilapia (*Oreochromis niloticus,* L.).

2. Material and Methods

2.1. Extraction of fish genomic DNA

Adult Nile tilapia fish were obtained from the fish breeding lab., animal biotechnology department, GEBRI, USC, Sadat city, Egypt. Fin-clip tissues of examined fish were used for DNA extraction using the genomic DNA extraction kit (G-Spin) TM (iNtRON Biotechnology, Inc. Korea) and according to the manufacturer's protocol with some modifications in tissue lysis. The concentration of extracted DNA was quantified with a NanoDrop spectrometer, and each DNA sample was diluted to a final concentration of ~50 ng/µl.

2.2. SSRs primers

Twenty-one primer pairs were tested for the amplification of SSRs and sex determination. These SSRs were identified using the tilapia genome sequence on linkage group 23 (LG23) as described by Eshel et al (2012). The twenty-one primers are shown in Table 1.

The PCR reaction conditions for SSR were optimized by using purified DNA in a total volume reaction of 25 µl containing 12.5 µl of master mix (2X-TOPsimple^{TM} PreMIX-nTaq, enzynomics, Korea), 0.5 µM of each Primer (2.5 µl of 5 µM of each primer stock solution), 2.0 µl of 50 ng of template DNA and de-ionized water up to 25 µl. PCR conditions were 5 min at 94°C followed by 40 sec at 94°C, 40 sec at 55°C, and 1 min at 72°C for 30 cycles, and the final extension step for 10 min at 72°C. The PCR reaction was conducted by using Applied Biosystems® ProFlex™ PCR System.

2.3. Detection of fragments

PCR products were tested by electrophoresis on a 4% agarose gel medium cooked at low temperature. The electrophoresis run was performed at 60 V in DNA electrophoresis unit (Bio-Rad) for 90 to 120 mins. Obtained DNA bands sizes were detected through 100 bp DNA ladder and were visualized using UV-trans-illuminator. To detect the fragment size for every SSR region before PCR, BLAST searches for every primer sequence against the unpublished tilapia genome (http://cichlid.umd.edu/blast/blast.html).

2.4. Statistical analysis:

Obtained results data were analyzed using the one-way ANOVA procedure of SPSS v.23 (SPSS Inc., Chicago, Illinois, USA). Tukey's multiple comparison tests were used to detect significant differences among the samples' SSRs bands. Data were expressed as means ± SD, with a p-value of 0.05 considered significant.

3. Results and Discussion

In tilapia, sex determination (SD) is controlled via major and minor genetic and environmental factors; in addition to genetic mapping of sex determination (SD), genes in species of purebred tilapia showed that SD should be analyzed and considered as a quantitative trait (Lee et al., 2004, Shirak et al., 2008). Molecular markers identification in fishes, including short sequence repeat (SSR) marker, is a very useful tool for investigating of sex-determination system, sex-related genes, and sex chromosome identification (De Rosa et al., 2017).

Table 1. Primers for SSRs that were used in the study.

Primer name	Forward primer	Reverse primer
SSRP1	CCTGAAACTCAGGCGCTGTA	GCTCTCACCAAGGTCAGCAA
SSRP2	GCCTTGTGCCACTGTAGGAG	AACCTGCCTCTCCTGGAATC
SSRP3	TGTGGGGTTTTTGAAGCCTA	GAAACCCCCTCTTCCTTGTG
SSRP4	TGAAGCAAACAGAGGCCATT	GCTGGGTGAGGGGTTTTGTA
SSRP5	CAGACTGTCCCCATCCTCAA	AGGGAGCTGGATCTGCCTAA
SSRP6	TTGACTACCGGCTTGCATTC	GCCCGAACATAAGATGTCCA
SSRP7	TGCTCTCACTGCTGAGCAAA	CGCAAATGTTAGGCCAGAAA
SSRP8	AAGACCCGTTCTTCGTCGTC	TTCATTCCACCTGCTCCAAA
SSRP9	GTGAGGCAAGTCCGGTTTCT	TGATCCACGGCGTATTGAGT
SSRP10	GTGGGCAAAAACAAGCCATT	TGTTTCAGTGTGAACGTGTGTG
SSRP11	GATTGTGGCCTGGTCAAGTG	TCCGTTTGTCTGCTGTGTGA
SSRP12	CAATGTGGCAATGTGTCCAA	CGGTGTCTCTGTGTCGTGTG
SSRP13	AGGCCTTTCATCGCTGTTTT	ACCCTGTAGATGAGCGCAAA
SSRP14	CCCTGCCCTGAACTACCTTC	GCTGCAAGCAAATGAAAAGC
SSRP15	CATGCTGATGGAGACCGATT	TCAAGACGCAATGGAGTGTG
SSRP16	AAGGGAAAGTGGCTCAGCTC	GTTGCTTCCCCACAGTTTCA
SSRP17	GGTGGGACTGTGGTGTATGG	GGTGGATTGCAAGCAACATT
SSRP18	AGGAGAAGTCGCAGGTGACA	GGCACAGTTGCCTGGTACAT
SSRP19	TGAATCTTCCCACAGCAACA	GTTGGTGCCAACAAAGCAAT
SSRP20	TTAATCCTGCCCACCTCTCC	AAGCAAAAGCATTTTCATGTTCA
SSRP21	CGAGCTGCTTTGTTGTCTGA	CGAACCGAAAATGAGAATGC

Through the rapid development of sequencing methods, many fish species' genomes are sequenced and unpublished or published in public databases. These sequenced genomes provide an excellent opportunity for determining SSR loci at these genomes and SSR markers development. SSR molecular markers are DNA markers based on PCR amplification. They have several advantages over

other molecular markers, such as high information, abundant quantity, and co-dominance marker (Xiao et al., 2016**;** Li and Ye, 2020).

The present study tested 21 SSRs marker primers for SD in Nile tilapia *O. niloticus*. The results of PCR amplification for SSRs are presented in Fig. 1

From the tested 21 SSRs marker primers, four primers that gave differences between males and females of Nile tilapia (SSRP1, SSRP7, SSRP13, and SSRP17) as mentioned in Table 2. But other primers showed no clear differences between males and females on the 4% agarose gel electrophoresis.

Two alleles were found for each locus that was detected by each primer. It was observed that females were homozygous, and males may be heterozygous or homozygous for the alternative alleles. The females were homozygous, and males were heterozygous for markers SSRP1 and SSRP17. As well as, the females were homozygous, and males were homozygous for the alternative ones as in SSRP7 and SSRP13. The same results were reported by Shirak et al (2008) who obtained 12 adjacent SSRs markers found in the studied Nile tilapia genome region. Al-Abedi et al (2019) studied six SSR markers loci in 38 samples of *S. rivulatus* from the Mediterranean Red Sea. They reported that the males and females from the Mediterranean and the Red Sea were homozygous for the same loci alleles. The other studied loci were homozygous in some individuals and heterozygous or homozygous in others for alternative alleles. In different chromosomes of tilapia species, different loci of sex determination have been mapped. From SSRs-based studies, two unlinked SSRs loci are linked to phenotypic sex in blue tilapia (Lee et al., 2004).

Figure 2 shows the occurrence % of SSRs fragments of *Tilapia nilotica* treated with four primers (SSR P1, SSRP7, SSR P13, and SSR.P17). The results reveal significant differences between males and females of an adult fish of *O. niloticus*. Song et al (2012) and Nie et al (2021) found that males showed more fluctuations than females. As well as, Through the creation of a high-density SSRs genetic map of *C. semilaevis* fish, several SSRs markers that are sex-linked were identified, and five of these markers were established to be related to sex.

This work used 4% agarose gel electrophoresis for PCR product detection. This may be the reasonfor the low productibility of the method compared with other techniques such as, polyacrylamide gel electrophoresis as separating methods, capillary electrophoresis, or sequencing. But these methods need a molecular biology laboratory that includes relatively expensive equipment and are laborious and time-consuming compared with an agarose gel electrophoresis, which is popular and inexpensive. So, this method could be helpful for wide use, as mentioned by Samoluk et al (2019).

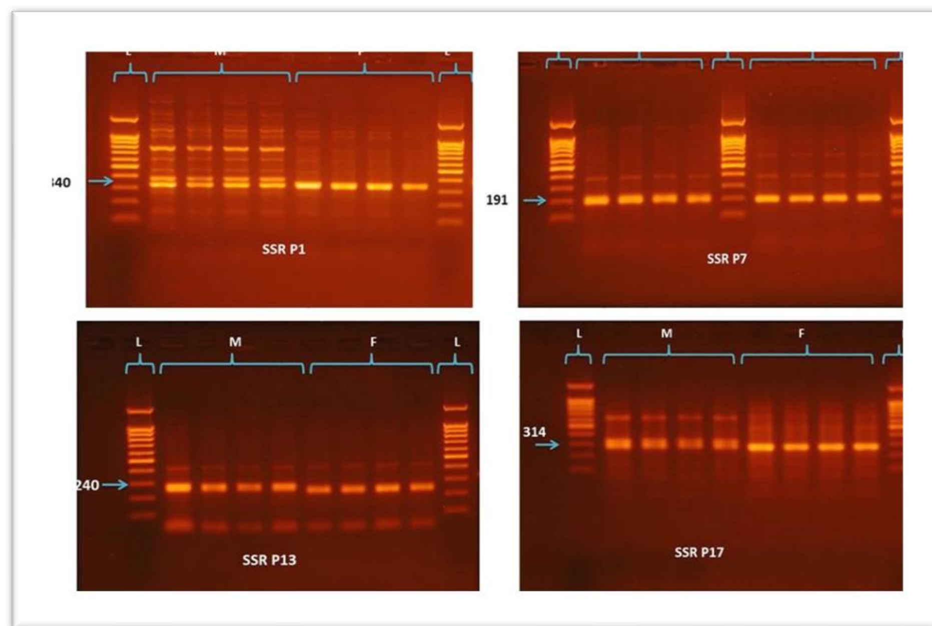

Fig. 1. Agarose gel electrophoresis of PCR products of SSR primers (SSR P1, SSRP7, SSR P13, and SSR.P17).

Table 2: SSRs' primers

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

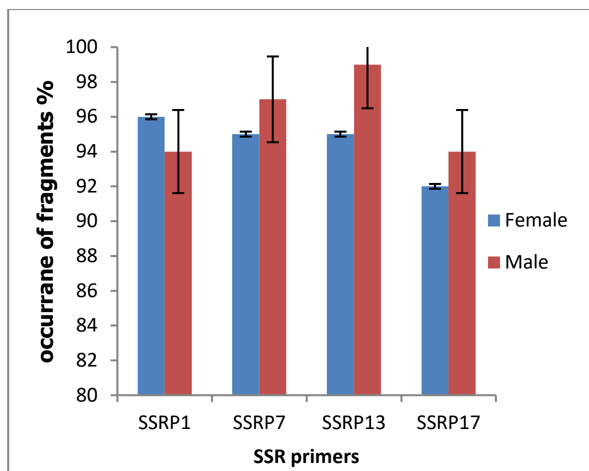

Fig. 2: Occurrence % of SSRs fragments of *Tilapia nilotica* (*Oreochromis niloticus*) tested with four primers (SSR P1, SSRP7, SSR P13, and SSR.P17) as mean ± SD

4. Conclusion

The molecular identification could confirm the possibility of using short sequence repeats markers (SSRs) in the sex determination of fish species (*Oreochromis niloticus,* L.). The results of the present study revealed that there were insignificant differences between males and females of Nile tilapia. In addition, SSRs markers detected by agarose gel electrophoresis could be used in the early sexing detection of the breeding programs for mono-sex supermale production without needing progeny test.

Acknowledgments

This study was funded by the Science and Technology Development Fund (STDF), Egypt, Grant # 5667, and the authors gratefully acknowledge STDF for its support.

5. References

Adhikari, S., Saha, S., Bandyopadhyay, T.K., Ghosh, P. 2014. Identification and validation of a new male sex specific ISSR marker in pointed gourd (*Trichosanthes dioica Roxb*.). The Scientific World Journal, 16896, doi: 10.1155/2014/216896.

Al-Abedi, A.S., Hashem, M.H., Abd El-Azeem. R.M., Khaled. A.E. Abdelsalam, R.N. 2019. Genetic variations of the Lessepsian rabbitfish populations from Red and Mediterranean Sea. Middle East Journal of Applied Sciences 9 (3): 679-685.

Al-Qurainy, F., Al-Ameri, A.A., Khan, S., Nadeem, M., Gaafar, A.Z., Tarroum, M. 2018. SCAR marker for gender identification in date palm (*Phoenix dactylifera* L.) at the seedling stage. International Journal of Genomics, doi: 10.1155/2018/3035406.

De Rosa, L.M., Cruz, V.P., Serrano, E.A., Mendes, N.J., Wasko, A.P., Senhorini, J.A. Pardo, B.G., Martínez, P., Oliveira, C., Foresti, F. 2017. Identification of a sex-specific molecular marker in *Salminus brasiliensis* (Characiformes) based on SCAR marker. Journal of Applied Ichthyology 33: 1024-1026. http://dx.doi.org/10.1111/jai.13430

Eshel, O., Shirak, A., Weller, J. I., Hulata, G., Ron, M. 2012. Linkage and Physical Mapping of Sex Region on LG23 of Nile Tilapia (*Oreochromis niloticus*). G3 (Bethesda), 2(1): 35-42.

Gennottea, V.E., Franc_oisb, C., Rougeota, J., Ponthierc, S., Deleuzec, C., 2012. Sperm quality analysis in XX, XY and YY males of the Nile tilapia (*Oreochromis niloticus*). Theriogenology , 78: 210-217.

Hashem, M.H., Abdel-Azim, R.M., Abel-Rahman M.M., Mousa I.E. 2019. Brackish water for cultivation of *Oreochromis niloticus* with sex reversed female by diethylstilbestrol. International Multidisciplinary Scientific GeoConference: SGEM 19 (3.1): 65-72.

Hashem, M.H., Tayel, S.I., Sabra, E.A., Yacoub, A.M., Heiba, A.A. 2020. Impact of the water quality of El-Rahawy Drain on some genetic and histopathological aspects of *Oreochromis niloticus*. Egyptian Journal of Aquatic Biology and Fisheries 24 (2).19-38.

Lazaro-Velasco, A., Isidro-Cristoba, H.M., Alcántar-Vázquez, J.P., Antonio-Estrada, C., Calzada-Ruiz, D., Moreno-de La Torre, R. 2019. Effect of the combination of a cold-water temperature and exogenous estrogens on feminization, growth, gonado somatic index and fat muscle content of Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758). Latin American Journal of Aquatic Research, 47(1): 52-64.

Lee, B.Y., Hulata, G., Kocher, T.D. 2004. Two unlinked loci controlling the sex of blue tilapia (*Oreochromis aureus*). Heredity 92: 543-549.

Li, J., Ye, C. 2020. Genome-wide analysis of microsatellite and sex-linked marker identification in *Gleditsia sinensis*. BMC Plant Biology 20: 338. https://doi.org/10.1186/s12870-020-02551-9.

Li, S.F., Su, T., Cheng, G.Q., Wang B.X., Li, X., Deng C.L., Gao, W.J. 2017. Chromosome evolution in connection with repetitive sequences and epigenetics in plants. Genes. 8: 290-5.

Mair, G.C., Abucay, J.S., Skibinski, D.O.F., Abella, T.A., Beardmore, J.A. 1997. Genetic manipulation of sex ratio for the large-scale production of all-male tilapia, *Oreochromis niloticus*. Can. J. Fish. Aquat. Sci. 54: 396-404.

Martínez-Cordero, F.J., Delgadillo, T.S., Sanchez-Zazueta, E., Cai, J. 2021. *Tilapia aquaculture in Mexico: assessment with a focus on social and economic performance.* FAO Fisheries and Aquaculture Circular No. 1219. Rome, FAO.

Nie, Z., Peng, L.ü., Zhang, R., Tu, Y., Liu, Z., Li, Y., Tang, C. 2021. A simple and rapid method for fish sex identification based on recombinase-aided amplification and its use in *Cynoglossus semilaevis.* Scientific Reports, 11: 10429. https://doi.org/10.1038/s41598-021-89571-z

Samoluk, S.S., Chalup, L.M.I., Chavarro, C., Robledo, G., Bertioli, D.J., Jackson, S.A., Seijo, G. 2019. Heterochromatin evolution in Arachis investigated through genome-wide analysis of repetitive DNA. Planta 249: 1405-15.

Shirak, A., Golik, M., Lee, B.Y., Howe, A.E. Kocher, T.D., Hulata1, G., Ron M.,SeroussiE. 2008. Copy number variation of lipocalin family genesfor male-specific proteins in tilapia and itsassociation with gender.Heredity, 101: 405-415.

Silva, E.M., Wong, M.S.L., Martins C., Wasko, A.P. 2012. Screening and characterization of sex-specific DNA fragments in the freshwater fish matrincha, *Bryconama zonicus* (Teleostei: Characiformes: Characidae). Fish Physiology and Biochemistry, 38: 1487-1496.

Song, W., Li, Y., Zhao, Y., Liu, Y., Niu, Y., Pang, R., Miao, G., Liao, X., Shao, C., Gao, F., Chen S. 2012. Construction of a high-density microsatellite genetic linkage map and mapping of sexual and growth-related traits in half-smooth tongue sole (*Cynoglossus semilaevis).* PLoS One 7 (12): e52097. doi: 10.1371/journal.pone.0052097.

Xiao, Y., Xia, W., Ma, J., Mason, A.S., Fan, H., Shi, P., Lei, X., Ma, Z., Peng, M. 2016. Genome wide identification and transfer ability of microsatellite markers between palmae species. Front Plant Sci. 10 (7): 1578-88.