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EXCLUSION OF TWO MICROSATELLITE MARKERS IN THE SEX-LINKAGE STUDY OF NILE TILAPIA (*Oreochromis niloticus* L.)

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ABSTRACT

Monosex Nile tilapia (*Oreochromis niloticus)* is highly preferred in semi-intensive and intensive culture systems to prevent uncontrolled reproduction and to obtain fast growing male. Production of all male tilapia is being practiced by the hatcheries of Bangladesh mainly by administering androgen hormones (particularly 17-α-methyl-testosterone) with feed in a mixture of undifferentiated fry for about a month. The direct application of hormone to such food chain often arises question in respect to public health and safety. The alternative to this is the production of putative supermales, a rather safe but longer procedure to obtain all male progeny. However, sex determination system in tilapia is fairly complex. Recent developments have resulted in a linkage map and genetic markers that can be used to analyze the sex determination system. For genetic analysis of different genotypes of fish, microsatellite DNA marker ARO120 and ARO121 were used for studying the inheritance pattern for possible sex linkage using Polyacrylamide gel electrophoresis. In case of ARO120, it was observed that the Dam XX was heterozygous; 11 out of 22 female progeny and 10 out of 22 male progeny were found to be heterozygous. In case of ARO121, it was observed that the Dam XX was heterozygous; 16 out of 22 female progeny and 20 out of 22 male progeny were found to be heterozygous. Though the marker polymorphisms were observed in this study, these were excluded from the sex-linkage study due to limited extent of information as sex-linked markers in Nile tilapia BFRI strain. This study provides a baseline for further research using other suitable polymorphic markers for assisting marker-assisted selection.

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INTRODUCTION

The Nile tilapia (*O. niloticus*) is the most extensively cultured species among all tilapia (FAO, 2013). However due to the short life cycle and prolific breeding that result in uneven sized overcrowded population, most hatchery operators and farmers intend to culture monosex (male) Nile tilapia in commercial aquaculture practice because this system benefit the stakeholders to control unwanted reproduction by females of Nile tilapia, to obtain larger and even sized males and to gain profit. All male population of Nile tilapia can be produced by several ways. Today the most practiced method is hormone feeding in diets. Administrating androgen 17-α-methyltestosterone (MT) is considered to be the most effective method (Guerrero and Guerrero, 1988). Use of hormones in direct food chain is said to have negative impacts on public health and environment. In many countries, hormonal sex reversal is therefore unacceptable for this reason. Direct observation of external morphology (particularly male and female genital papillae) is cumbersome and time consuming. An alternative way could be production of supermales (YY males) in indirect method and rear them up to the sexual maturity to cross with the normal females (XX) so that the offspring is normal males (XY). This system is also known as the production of genetically male tilapia (GMT). A variety of evidence suggests that sex determination is principally monofactorial in tilapias (Wohlfarth and Wedekind 1991) but other autosomal influences (Hussain et al., 1998; Shirak et al., 2006; Cnanni et al., 2008), environmental effects particularly temperature (Baroiller et al., 2009) and polyfactorial mechanism including multiple allelism (Penman and Piferrer, 2008, Khan, 2011; Palaiokostas et al., 2013) and also override the simple chromosomal system to establish the sex.

Sex-linked markers could play potential role to locate the QTL for sex as the genetic map of this species is available (Lee et al., 2005). Microsatellites, also known as Simple Sequence Repeats (SSPs) or Short Tandem Repeats (STRs), are repeating sequences of 2-6 base pair of DNA. Microsatellites are typically co-dominant. They are used as molecular marker in genetics, for kinship, population and other studies. They can also be used to study gene depletion or deletion. Microsatellites are also known to be causative agents in human disease, especially neurodegenerative disorders and cancer. The repeat units are generally di-tri-tetra or pentanucleotides. They tend to occur in non-coding regions of DNA although a few human genetic disorders are caused by (trinucleotide) microsatellite regions in coding regions. On each side of the repeat unit are flanking regions that consist of “unordered” DNA. The flanking regions are critical because they allow us to develop locus-specific primers to amplify the microsatellites with PCR (Polymerase Chain Reaction). That is, given a stretch of unordered DNA 30-50 base pairs (bp) long, the probability of finding that particular stretch more than once in the genome becomes vanishingly small. In contrast, a given repeat unit (say AC19) may occur in thousands of places in the genome. The present study was performed to identify any sex linkage in the Nile tilapia by using DNA microsatellite ARO120 and ARO121. The goal of this experiment was to establish the suitability of these markers as sex linked polymorphic or monomorphic marker and identify the inheritance pattern of alleles in both sex of Nile tilapia.

MATERIALS AND METHODS

Study period and site

The experimental sites were Field Laboratory Complex, Bangladesh Agricultural University (BAU), Aquarium Facilities of Faculty of Fisheries, (BAU) and Fish Genetics and Biotechnology Laboratory of Department of Fisheries Biology and Genetics, (BAU), Mymensingh. The study was conducted for one year.

Sample collection

One day old spawns of first feeding stage of tilapia were previously collected from Agro-3 fish farm and hatchery, situated at Boilor of Trisal in the district of Mymensingh and stocked in three different ponds of Fish Field Laboratory Complex, (BAU) having an area of three decimals each. Initially a mixture of fingerlings (N=200) of Nile tilapia *O. niloticus* containing normal males (XY) and normal females (XX) were previously collected.

Feeding and management

Ponds were previously prepared using 3kg calcium carbonate per decimal and rotenone. The fingerlings were fed crumble feed daily at the rate of 4% body weight four times in a day up to thirty days. For another ninety days they were reared up to sex determination stage using pelleted feed twice a day.

Breeding among the different genotypes of *O. niloticus*

The sex of experimental species was identified by visual observation of genital papilla. For the purpose of breeding 15 pairs of male and female were selected after six months and kept in individual hapas (with 1:1 sex ratio (Male: Female). Among them offspring from five pairs of parents were collected after accomplishment of breeding. Twelve glass aquaria (each with 25L water holding capacity) were set to keep the fish fry out of the cross between XY males and XX females and also YY supermales and XX females.

Stocking of fry of *O. niloticus*

Derived from the cross between XY males and XX females

After crossing five pairs of XY males and XX females, the hatchlings were brought and kept at the aquaria after eleven days of fertilization. The fry were then fed DES hormone with the feed with different doses viz. T1 (100mg DES hormone /kg feed), Treatment T2 (200mg DES hormone/kg feed), Treatment T3 (300mg DES hormone/kg feed) and T4 (without hormonal feed). There were three replications for each of these three treatments that contained 300 fry in each aquarium. The hormonal treatment was conducted for a period of one month. The fry were released in cisterns after the completion of the hormonal trial. Normal feed had been provided to grow them up to sexual maturity in pond.

Derived from the cross between YY supermales and XX females

Three putative YY supermales (originally brought from the owner of the Agro-3 private hatchery fish hatchery Boilor, Trisal, Mymensingh) were successfully crossed with XX females (with 1:1 ratio of Male: Females). After incubation and yolk sac absorption period, they were stocked in glass aquaria each (N=100) under four hormone treatments viz. T1 (200mg DES hormone/kg feed), T2 (300mg DES hormone/kg feed), T3 (400mg DES hormone/kg feed), T4 (500mg DES hormone/kg feed) and a control (without hormonal feed). There were two replications for each of these four treatments that contained 100 fry in each aquarium. Hormonal treatment was done up to one month and the released in five different cisterns for rearing using normal feed up to maturity.

Collection of DNA Sample

Fin clips were collected from Family HRT 25 and dam samples of different genotypes (XX, XY, “XY” and YY) and preserved in separate eppendorfs containing 95% ethanol and stored at -18^{0}C. Before taking each sample, all scientific procedures were rigorously maintained.

Extraction of genomic DNA

Genomic DNA was extracted from fin clip tissues according to the method described by Islam and Alam (2005). Phenol-chloroform protocol (Sambrook and David, 2001) was used for this extraction. All DNA samples were tested qualitatively (presence of RNA or degradation of DNA) and quantitatively on 1% agarose gel.

Amplification of microsatellite markers by PCR

Primer selection

To perform amplification of microsatellite marker, two oligonucleotide primers were mixed with genomic DNA in presence of a thermostable Taq DNA polymerase and a suitable buffer. Microsatellite ARO120 and ARO121, marker was selected for this study (Table 1). These were used to determine the population genetic variation among three stocks of *O. niloticous.* Primers were evaluated based on intensity of bands, consistency within individual, presence of smearing and potential for population discrimination.

Table 1. The sequence and size of the microsatellite marker used for the study

Name of the primers	Expected PCR Product size (bp)	Primer sequence		Annealing Temp.(ºC)	Presence of mono-(M) or polymorphism (P)
ARO 120	-	For.	AAGGGAAAGTGGCTCAGCTC	60	-
		Rev.	GTTGCTTCCCCACAGTTTCA		
ARO 121	-	For.	GGTGGGACTGTGGTGTATGG	60	-
		Rev.	GGTGGATTGCAAGCAACATT		

PCR amplification

PCR was performed in a 12µl reaction volume containing 50ng templates DNA, 2.0µM of each primer, 0.25mM each of the dNTPs, 1 unit of Taq DNA polymerase, 1.5mM MgCl2 and 1µl 10X reaction buffer. During this experiment, DNA, PCR buffer, dNTPs, and primers solutions were thawed from its frozen state and placed on ice. Primers were diluted 100 times to take minute amount of primer. DNA samples were first taken into 0.2ml PCR tubes. For each DNA sample being tested, a pre-mix was prepared in 1.5ml eppendorf tube including, in the following order, PCR buffer, dNTPs, Primer, and sterile deionized water. Pre-mix was well mixed and placed on ice. Taq DNA Polymerase (GENEI, India) was then added to the pre-mix. The pre-mix was mixed by finger tapping and centrifuged for ≈5 seconds and placed on ice. The pre-mix was aliquoted into the PCR tubes already containing template DNA sample (2µl). The PCR tubes were then sealed and marked with permanent marker pen and centrifuged for ≈5 seconds. The tubes were then placed in an oil-free thermocycler (Master Cycler Gradient, Eppendorf) and the cycling was started immediately. The temperature profile consisted of 3 min initial denaturation at 94ºC followed by 35 cycles of 30 sec at 94ºC (denaturation), 30 sec at the respective annealing temperature, 1 min at 72ºC (extension) and ending with 5 min at 72ºC (final extension). When the PCR was completed, the PCR products were kept in a freeze (4ºC) for electrophoresis.

Polyacrylamide gel electrophoresis for microsatellite marker analysis

Glass plate preparation

At first the glass plate was washed clearly with detergent. Then the plate was washed with methanol and wiped out with tissue paper and air-dried. The glass plate was smeared with a solution containing 950µl 95% ethanol, 50µl 0.5% acetic acid and 3µl silane (γ MethacryloxypropylTrimethoxySilane) and wiped out with tissue paper and kept in air. After 3 min., the glass plate was sprayed with 95% ethanol and wiped carefully with tissue paper to remove excess silane. The same procedure was repeated three times. At the same time the vertical gel apparatus was washed with deionized water and wiped out with tissue paper and air-dried. Then the vertical gel apparatus was smeared with water repellent (Clear View, USA) and wiped after 2 min.

Polyacrylamide (6%) gel preparation

For preparation of 6% polyacrylamide, 25.24g urea (5M), 9ml of 40% acrylamide: bis-acrylamide (19:1) and 12ml 5×TBE buffer were taken in 100ml beaker and deionized water was added to make the solution approximately 60ml. The solution was stirred for few minutes with magnetic stirrer until the urea was no longer visible. Finally, the total volume of the solution was made into 60ml by adding deionized water. The gel chamber (38×30cm Sequi-Gen GT sequencing gel electrophoresis system BIORAD) was set horizontally and leveled properly with a magnetic leveler. The acrylamide gel solution was taken in 60ml injection syringe just after addition of 420µl of 10% APS (Ammonium per sulphate) and 84µl of TEMED and poured into the gel chamber. A clear level of gel at the sample loading edge was maintained by placing the comb directing opposite to the teeth to the gel. A gel with uniform 0.4mm thickness was used for separation of single microsatellite loci. The gel was kept for 30 minute for solidification. Then the upper side of the glass was cleaned with scissors and deionized water to have a clear level for sample loading.

Electrophoresis of PCR products in polyacrylamide gel

The gel was pre-run for 30 min at 120W to raise the temperature up to 50^{0}C before sample loading. After preheating, the air bubbles were removed carefully with micropipette. Meanwhile, PCR products and 4µl 100bp DNA ladder were preheated at 95^{0}C for 5 min. The preheated PCR-products were immediately kept on ice and spin for few seconds in a micro-centrifuge machine. The preheated PCR-products were loaded immediately between the teeth of the comb and the gel was run with the power set at 60W and temperature set at 50^{0}C for required length of time (1h 20 min) 40 according to the size of the DNA fragment. After completion of electrophoresis, the gel was stained with silver nitrate following Promega silver staining protocols.

Scoring and statistical analysis of Microsatellite data

The software DNA FRAG version 3.03 was used to estimate marker length and allelic length to identify any variation between the male and female haplotypes with microsatellite ARO120 and ARO121.

RESULTS AND DISCUSSION

Sex reversal rates in progeny derived from cross between XY normal males and XX normal females

The sex reversal rates of the survived progeny were determined upon sexual maturity after 6 months (five months after the first sampling on survivability). Although for one month, the survival rate was determined for each of the replication group, the replicates were merged afterward for observation of sex reversal after becoming sexual maturity in 6 months. On the basis of the number of fish after 6 months the sex reversal rates were 95, 95 and 94%, respectively for T1, T2 and T3 (Figure 1).

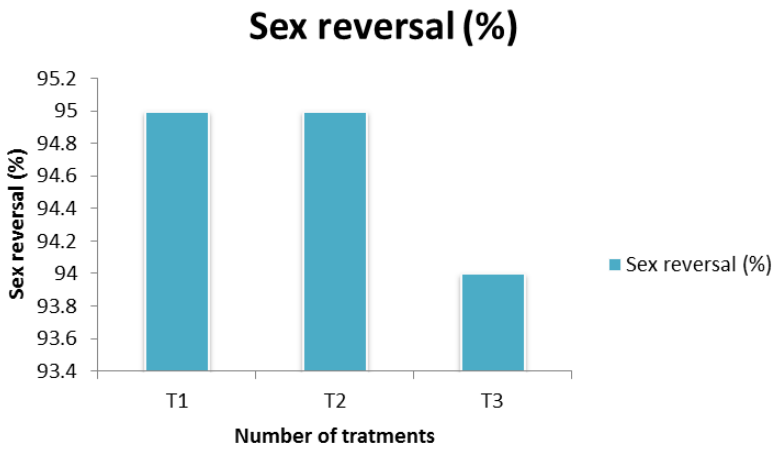

Figure 1. Sex reversal rates of Nile Tilapia (*Oreochromisniloticus*) from cross between normal males and normal females after 6 month on sexual maturity

Microsatellite analysis following polyacrylamide gel electrophoresis (PAGE) to identify polymorphism or monomorphism of markers

The PAGE deployed in the progeny (N=44) of family HRT 25 along with the Dam showed marker polymorphocity in both females and males with ARO120 and ARO121 (Figure 2 and Figure 3).

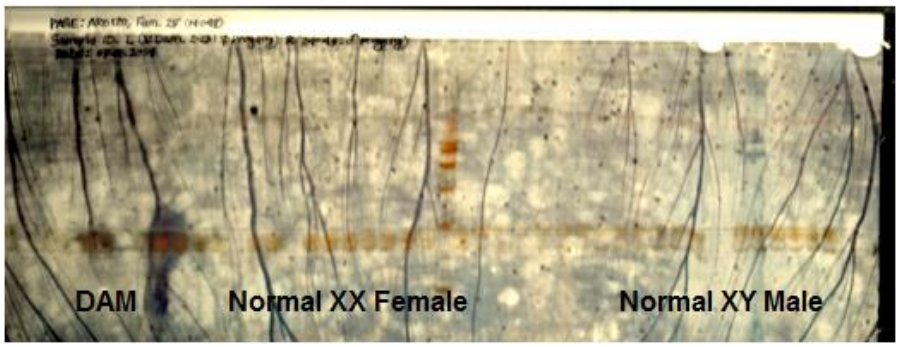

Figure 2. PAGE (Polyacrylamide gel electrophoresis) genotyping results with ARO120 using Dam XX (N=1) normal females XX (N=22), normal males XY (N=22)

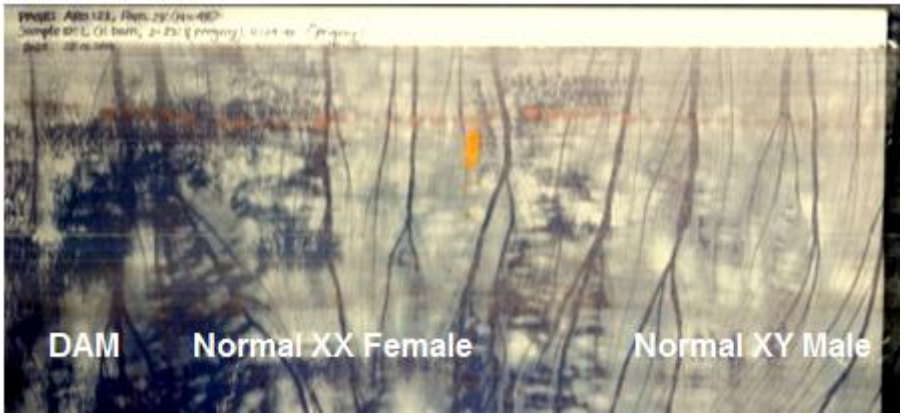

Figure 3. PAGE (Polyacrylamide gel electrophoresis) genotyping results with ARO121 using Dam XX (N=1) normal females XX (N=22), normal males XY (N=22)

Observation of allelic inheritance pattern in both sexes of Nile tilapia (*Oreochromisniloticus*), family HRT 25

The PAGE with ARO120 resulted in marker heterozygosity in the Dam of family HRT 25 in 11 out of 22 family progeny and 10 out of 22 family progeny. The rest were homozygous for one or the alternative allele (Figure 2). The genotyping with ARO121 resulted in marker heterozygosity in the Dam of family HRT 25 in 16 out of 22 family progeny and 20 out of 22 family progeny. The rest were homozygous for one or the alternative allele (Figure 3). The fragment size and marker monomorphocity/polymorphocity of ARO120 and ARO121 is presented in Table 2. Analysis of inheritance of allele in both sexes with two microsatellite markers is presented in Table 3 and Table 4.

Table 2. The fragment size and marker monomorphocity/polymorphocity of ARO120 and ARO121

Name of the primers	Expected PCR product size (bp)	Primer sequence		Annealing Temp (°C)	Presence of mono-(M) or polymorphism (P)
ARO120	200-300	For.	AAGGGAAAGTGGCTCAGCTC	60	P
		Rev.	GTTGCTTCCCCACAGTTTCA		
ARO121	200-300	For.	GGTGGGACTGTGGTGTATGG	60	P
		Rev.	GGTGGATTGCAAGCAACATT		

Table 3. Genotyping in PAGE of ARO120

Marker	Genotyped sample number					
ARO120	Dam	Sire	F_1 XX ♀	No.of samples	F_1 XY ♂	No.of samples
	175/163	185/163	175/163	11	185/163	10

Table 4. Genotyping in PAGE of ARO121

Marker	Genotyped sample number					
ARO121	Dam	Sire	F_1 XX ♀	No. of samples	F_1 XY ♂	No.of samples
	214/169	183/169	214/169	16	214/183	20
			183/169	3	Missing	1
			169/169	3	183/183	1

Marker assisted selection (MAS) can be applied in aquaculture species where sex control is important. One sex may grow faster than the other or has maturation features that are less desirable for production purposes. Microsatellite markers have great potential in terms of study of genetic variation within and among populations, germplasm classification, breeding and identification of genes that confer particular characteristics, selective breeding programs and gene mapping in fish species. The present study was conducted with a view to find out suitable markers whose inheritance pattern could play significant role to differentiate normal females and normal males in the first instance. Once the linkage pattern is known, the knowledge is supposed to be applied in differentiating normal and supermales. The quantitative Trait Loci (QTL) for SEX was looked for and the pattern of allelic inheritance in association with sex in progeny had been studied by a number of researchers. Inheritance of Linkage Group LG3 and LG23 marker alleles was studied on the basis of some research works, where for example, LG3 makers were useful in association studies (between sex and markers) in *O. aureus* and *O. karongae* (Lee *et al*. 2005). Besides, LG1 markers were found to suitable in sex linkage study on *O. niloticus.*

In the current study, two microsatellite markers were used separately to assess the genotype to discriminate the polymorphic allele and monomorphic allele. At first 22 normal female, 22 normal male and one parent (mother) were genotyped using ARO120, where 11 out of 22 female progeny and 10 out of 22 male progeny were found to be heterozygous or having polymorphic allele. It seemed the mother and father contains same allele at heterozygous state. While another genotype analysis using ARO121 shows mother in heterozygous, where 16 out of 22 female progeny and 20 out of 22 male progeny were found to be heterozygous or having polymorphic allele. Lee *et al.* (2004) found that sex determining locus lies within a few centimorgans of microsatellite markers GM354, UNH168, GM271 and UNH131 in Blue tilapia (*O. aureus*) which were all polymorphic. Although the markers used in the present study found to be polymorphic, but no specific sex-linked inheritance was observed. However, microsatellite marker ARO120 was interesting in respect of showing discrete bands in the female progeny in terms of allele 175 while in the male progeny 185 allele was distinct. In case of ARO121, the bands were not very clear though distinguishable. Many critical factors including buffer settings, PCR amplification with proper annealing temperature and pipetting could be the factors that might hamper the production of clear bands in case of ARO121.

Future work can be performed based on the observation on the allelic segregation with other primers, including primer ARO120. The allelic variation in the XX/XY model could demonstrate some alleles could be stronger in effect (producing close to all male) while some others are weaker giving intermediate sex ratios in the progeny. However this study excludes both the markers from being important sex-linked markers due to limited extent of genetic information.

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