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Optimization of detection of sexual genotype through microsatellites in populations of Nile Tilapia (*Oreochromis niloticus*) from the breeding pools

Gustavo Naranjo[1]*, Darwin Rueda Ortiz[1*], María Augusta Chávez[1], Bangeppagari Manjunatha[1], Juan Ortiz Tirado[1], Daysi Muñoz[1], Rajesh R. Kundapur[2] and Muchakayala Ravi[3]

[1]Department of Life Sciences, Universidad de las Fuerzas Armadas-ESPE, Sangolqui, Quito, Ecuador, South America
[2]Department of Zoology, University of Pune, Pune, India
[3]Shenzhen Key Laboratory of Advanced Materials, Department of Materials Science and Engineering, Shenzhen Graduate School, Harbin Institute of Technology, Shenzhen, China

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ABSTRACT

The culture of Nile tilapia (Oreochromis niloticus) is one of the most important aquaculture activities in Ecuador in specific and the world in general, where the cultivation of males is transcendental. The use of breeding with a sexual genotype YY guarantee the viability of male offspring in captivity, avoiding the use of invasive technologies for the environment, and possible interaction with human health. However the absence of rapid detection methods limits the application of this technology. Therefore, the present study was sought optimizing a method for detecting sexual genotypes using microsatellite markers. Genotyping was performed with UNH104, UNH898 and UNH995 microsatellites; of individuals with a known and sexual confirmed genotype, and their relationship was assessed by a test of independence chi-square, and finally the reliability as a diagnostic test was analyzed. Being the UNH898 marker which gave the highest dependency ($p = 2.92 \times 10^{-28}$); showing two alleles (with a size of 264 and 296 bp) present mainly in males, forming a genotype to individuals YY (264/296) and XY (264/296 / X allele). To differentiate males from females this marker showed sensitivity, specificity, PPV and NPV of 96.97%, 82.35%, 91.43% and 93.33% respectively, while differentiating YY from XY individuals; it was about 100.00%, 85.29%, 85.71% and 100.00% respectively.

Keywords: *Oreochromis niloticus*, Microsatellites, Sexual genotype, Male YYA*.

INTRODUCTION

Tilapia farming is one of the most important aquaculture activities around the world. It is the second most produced fish species after carp, in 2012 the world production was 4.5 million tons, of which 3.8 million tons (~75%) are *Oreochromis niloticus*, commonly known as Nile tilapia [1]. Its success lies in its ease of cultivation and its good organoleptic properties, which have been widely accepted in local and international markets [2].

This native of rivers and lakes, especially from warm northeast African species [3], is very attractive for cultivation. It can adapt to different environmental conditions, systems and culture densities [4]. It can easily live in both freshwater and brackish water, and may even become adapted to seawater. No need for high quality water to survive and can also adapt to low oxygen concentrations. Being basically omnivores, they can be fed almost any product, and may even feed on zooplankton in the water [5]. All these advantages have led to a great expansion of cultivation of this species in over 135 countries around the world and especially in developing countries, where it has been an alternative food source in order to improve the income and quality of nutrition in rural areas of high poverty [4].

However, the culture presents a big problem, especially, an early reproduction and the difference in the size of the female, which causes uncontrolled reproduction in individuals who have not yet reached the commercial size, causing uneven and much slower growth because of competition for food. Concomitantly, decreases the profitability of the crop [6].

That is why unisex growing groups of males, is the best way to maintain a healthy and growing which is profitable. To achieve this goal people have developed several technologies around them, which includes manual sexing, inter specific hybridization, transgenesis, androgenesis, YY male technology and use of sexual hormones [7, 2].

According Beardmore et al. [7], hormonal sex reversal is the most effective and easiest way to obtain only males with a higher success rate of 98%. However, the application of environmentally friendly technology is one of the important goals in production systems and trying to avoid contamination of aquatic systems. Therefore, the development and improvement of technology for producing genetically male tilapia using YY males is paramount [8, 2].

The YY male technology has advantages over other methods, such as it is not being polluted or dangerous and successful in obtaining true males with better growth, better feed conversion, improved quality of steak, etc. However, this technology depends on obtaining these players, which is very complicated and expensive and because of lack of testing for sexual genotype were only progeny testing is used [7, 9].

Due to the absence of sexual dimorphism and ignorance of chromosomal regulatory genes, other screening methods such as karyotyping or sexual genotypic identification of regulatory genes could not be applicable in this species [10]. To address this issue molecular markers can be used. Fortunately, different microsatellite markers have been identified and connection between the predictions of sex with certain markers proved to be about 95 % effective. So, it is the most viable option to identify the sexual genotype of an individual [11, 12, 13].

The objective of this study was to optimize the detection of sexual genotype by different microsatellites in populations of the species *O. niloticus*, present in breeding pools of the Aquatilgen Company CIA. Ltd. Simultaneously, all the methodology applied in the present study was evaluated.

MATERIALS AND METHODS

This study was conducted in cooperation between Aquatilgen cia. Ltd. and Bioaquatic Resources Laboratory at the University of the Armed Forces.

Specimens of Study

The Nile Tilapia (*Oreochromis niloticus*) which were donated by the company Aquatilgen Cia. Ltd. 100 individuals with a known sexual genotype were selected and previously verified by progeny testing, within which 30 XY males, 30 females and 40 males YY were used. Each individual was identified by a metal ring and a sample of about 1 cm long pelvic fin was collected from individual fish and stored at- 20°C.

DNA extraction

DNA was extracted from fins using Pure Link Genomic DNA™ Kit (Invitrogen, USA kit, Cat. No. K1820-02); followed according to the manufacturer's instructions with necessary modifications. A sample of about 25 mg of finely cut and dried fin tissue digested about 4 hrs and the same was eluted in 150 uL of elution buffer (1X TE). Subsequently, thus obtained DNA was quantified using dsDNA kit Qubit® BR (Invitrogen, USA, Cat. No. Q32850) and the DNA quality was evaluated by horizontal electrophoresis in 0.8% agarose containing gel and an analysis of the relationship between the absorbance at 260 and 280 nm, in µQuant™ spectrometer was performed.

Genotyping

Based on previous studies three markers were selected for microsatellite genotyping [12,11]. They are: UNH104 (F: GCAGCACAACCACAGTGCTA; R: CCAGCCCTCTGCATAAAGAC); UNH898 (F:GGTATATGTCTAACTGAAATCC; R:GCAGTTATTTGTGGTCACTA) and UNH995 (F: TAATCCACTCACCCCGTTTC; R: GATGTCCCCACAAGGTATGAA). The procedure for amplification of the markers was followed as described by Khan [2]. The 20 µl reaction mix contained 0.05 U/ ml of Platinum® Taq Polymerase (Invitrogen, USA), PCR Buffer (1x) without $MgCl_2$, 1.5 mM magnesium chloride ($MgCl_2$). 1.5 mM dNTP mix (Invitrogen, USA) 0.3 µM of forward primer and reverse primer mix, approximately 50 ng of genomic DNA and finally the volume were adjusted with ultrapure water (Invitrogen, USA). The conditions for amplification were as follows: Initial denaturation at 94°C for 10 min, 35 cycles of amplification (denaturation at 94°C for 10 sec;

annealing for 30 sec at 47°C for *UNH104*; 59°C for *UNH898* and *UNH995* and extension at 72° C for 1 min) and final extension at 72°C for 7 min.

The PCR product was validated by horizontal electrophoresis in 1.5 % agarose gel containing 0.05 µg / ml ethidium bromide [14]. Subsequently, marker genotyping was performed using 6% native polyacrylamide [6% of Acrylamide (29): Bisacrylamide (1) mixture in 1X TBE] gel (CBS Scientifics, USA), DNA was visualized by immersing the gel in distilled water containing 0.5 µg / ml of ethidium bromide [15] and the same was photographed and documented. The gel photographs were modified by Bioinformatics software ImageJ 1.4 R (National Institute of Health, USA) and analyzed using Bioinformatics software Quantity One™ (BioRad, USA), where each marker genotypes and allele size for each sample was identified.

Statistical Analysis

Allele and genotype obtained derivative thereof, were analyzed by frequency tables and graphs of frequency according to its presence in the different genotypes in order to find alleles associated chromosomes. Subsequently the degree of relationship between genotype obtained by molecular markers and the sexual genotype was measured using a chi-square test of independence between variables with categorical data, so one can select the bookmark which is statistically dependent. The same was evaluated for sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) to differentiate between males and females showing the reliability of the marker for sexual genotype.

RESULTS AND DISCUSSION

DNA Extraction and Evaluation

The DNA obtained using DNA extraction kit (PureLink™ Genomic DNA) is sufficient for genotyping. The genomic DNA was found to be intact evident by gel electrophoresis, which is similar to that of the silica affinity kit [16], slight contaminations (possibly RNA) which does not significantly interfere in the development of the PCR [17], was also observed. The intensity of the bands was not fully uniform, it is due to the possible variation of the amount of sample used. Furthermore, elution buffer without sample as a negative control excluded the possibility of external contamination (Figure 1).

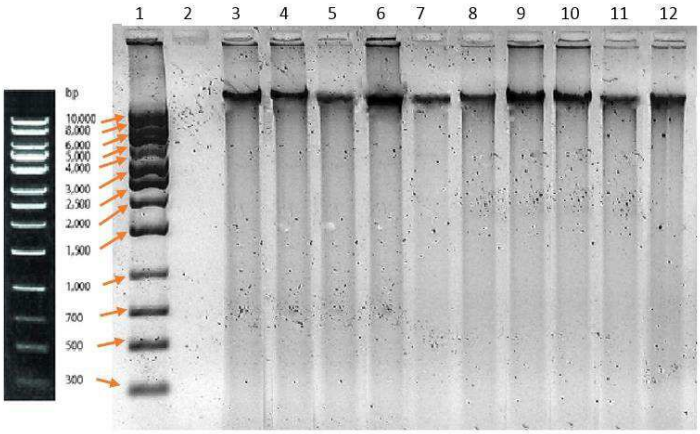

Figure 1: Electropherogram showing the intact genomic DNA isolated from the fin of *O. niloticus* using Genomic PureLink ™ kit. 1: 10Kb Ladder, 2: 1X TE, 3-12: DNA samples

An average concentration of 47.54 ng / µl of DNA were obtained from fin samples with a standard deviation of 13.23 ng / µl, which contrasts with the expected concentration in the kit instructions using 150 µl of elution buffer, about 20 ng / µl. However, the concentration mentioned in the kit is based on human blood, where there is less amount of nucleated cells, unlike animal tissues the larger amounts of DNA obtained in this case is due to the use of animal tissues [18].

The recovery rate of DNA is a more reliable on extraction, from animal tissues, it is expected to obtain a recovery rate of 1-4 µg DNA / mg sample [16], but we obtained only 284.38 ng of DNA / mg sample. This low amount of DNA recovered due to the method employed separation of DNA, by the use of silica columns [19]. The 260/280 nm ratio was measured to access the purity of the genomic DNA, ideally DNA considered pure when the ration shows the value between 1.80 to 2.00 [14]. But in our study, we obtained an average ratio of 1.63 this is due to the protein contamination. However, this low purity is not a major issue in conventional PCR [16].

Validation of microsatellite

The methodology for the present study showed a good amplification, nonspecific amplification of products was not formed because of proper annealing of primers at selected temperatures [20], and the absence of contamination in reagent controls (Figure 2).

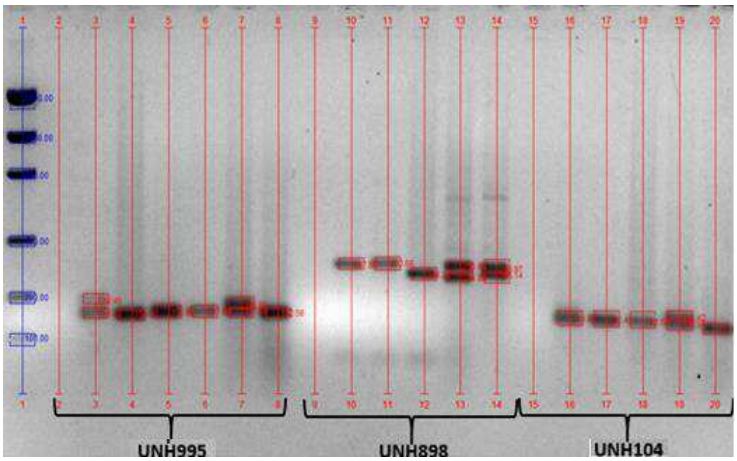

Figure 2: Electropherogram of PCR products for each marker. 1: Invitrogen Low Mass DNA Ladder (100bp, 200bp, 400bp, 800bp, 1000bp, 2000bp); 2, 9, 15: reagent control; 3-8: UNH995 amplifications; 10-14: UNH898 amplifications; 16-20: UNH104 amplifications

Similarly, the range in allele band sizes was found to be very much similar to that of the work carried out by Lee et al. [11] Khan [2] and Eshel et al. [12,13]. For the microsatellite *UNH104* ranging from 120 to 170 bp, whereas *UNH995* in the range of 180 to 250 bp, which is coinciding with the results of Lee et al. [11] and Khan [2] i.e. 130-189 bp for *UNH104* and 160 to 240 bp for *UNH995*. While for the microsatellite *UNH898* it was in the range of 220-300 bp which coincides with the range (250 to 300 bp) mentioned by Eshel et al. [12] and Khan [2].

Genotyping and dependence to sexual genotype

It was Lee et al. [11], Khan [2] and Eshel et al. [12, 13], used *UNH104*, *UNH898* and *UNH995* microsatellites and showed their nature of high sex dependency. The results obtained in the present study substantiate the same. The three markers were sexual genotype dependent at different level, the marker *UNH898* is the most dependent and selected as an indicator of sexual genotype (Table 1).

Table 1: Analysis of dependence between each marker and sexual genotype in the study population

Marker	Genotype	Genotype Sexual			X2	g.l	p-value
		XX	XY	YY			
UNH104*	A	22	17	6	46.29	4	2.14×10^{-09}
	AB	8	20	6			
	B	0	3	18			
UMH898**	A	28	6	0	135.26	4	2.92×10^{-28}
	AB	2	29	0			
	B	0	5	30			
UNH995	200/215	3	0	0	42.76	6	1.30×10^{-07}
	182/200	1	20	17			
	182/215	19	16	1			
	182/200/252	7	4	12			

**A: genotipes involving alleles 131,140,154 and170bp; genotypes involving alleles 123and 127bp and AB: Genotypes combined.*
**A: genotypes involving alleles 224, 227, 236, 247, and 274bp; genotypes involving alleles 246, 264 and 296bp; and AB: genotypes combined.*

According to Eshel et al. [12], allele 276 is mostly present in males and is related to chromosome Y. In the present study the allele 264 is identical to that of allele 276 is because the size difference between the two alleles is merely of about 10 base pairs and it resolved as single band in vertical gel electrophoresis (Wang et al. [21]. Alleles found from a particular genotype combination for each chromosome, being present exclusively 296/264 in YY individuals, while genotype 296/264/x allele is mostly in XY individuals, and other alleles are dual combinations for XX individuals (Figure 3).

The high dependence of this marker with sexual genotype may be because it is closely linked to *amh* gene [12]. Similarly, Shirak et al. [22] found that this gene is located 1 cm away from the linkage group 23 (LG 23). The *amh*

encodes an anti - mulleriana hormone, which inhibits the development of the Mullerian ducts that later forms the uterus and Fallopian tubes [23]. Additionally, Poonlaphdecha et al. [24] demonstrated that *amh* is expressed predominantly in males of *O. niloticus* at 14 days post-fertilization (dpf), just before the start of the sexual differentiation.

Reliability Assessment of microsatellite UNH898 to detect sexual genotype

The microsatellite *UNH898* was selected to serve as a marker that can detect the sexual genotype due to the high dependency ($p = 2.91 \times 10^{-28}$). In addition to the analysis of allele frequencies showed highly associated with each chromosome allele, especially the Y chromosome, which is of primary interest. Alleles 296 and 264 were considered as chromosome markers, since having a high relationship therewith. While the other alleles were regarded as X chromosome markers, thus the combination of alleles indicates the genotype of the individual sex. The sexual genotype obtained by allelic study was contrasted with the sexual genotype previously identified by progeny testing to generate a comparison chart and get the sensitivity, specificity, positive predictive value and negative predictive value. This analysis was first performed to differentiate between the genotypes of male (XY / YY) and female and secondly to differentiate between super males (YY) and that of heterogametic males (XY). Its importance for selection in a population both males in the first case and super males in the second case are considered.

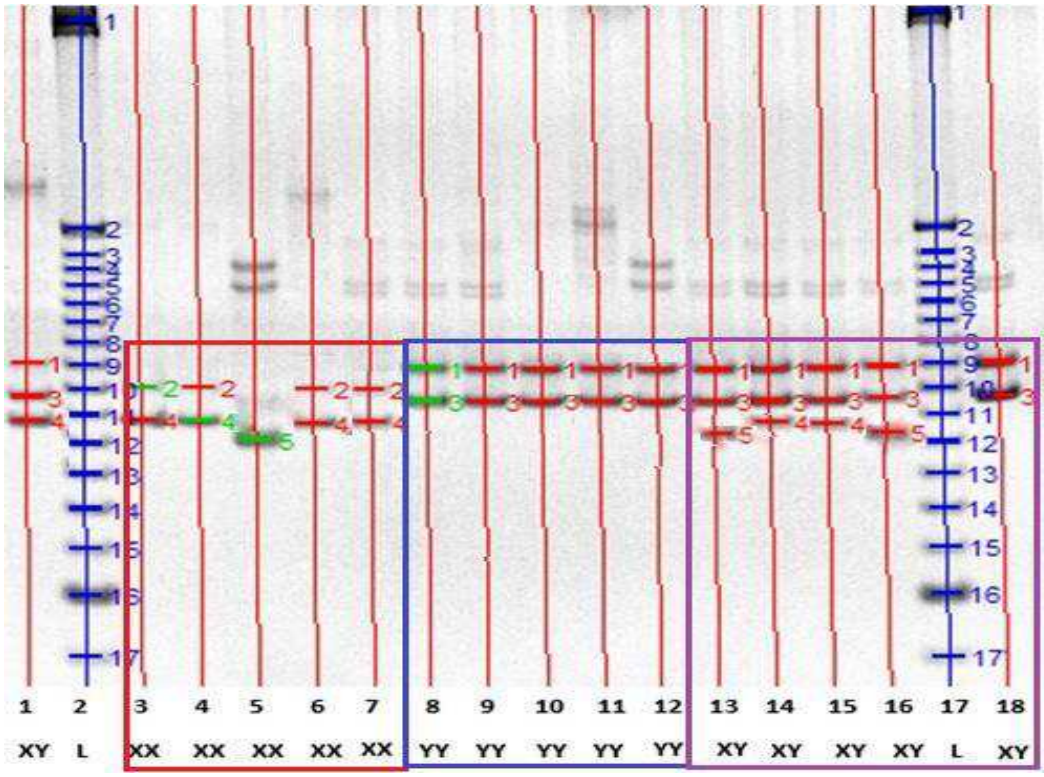

Figure 3: The alleles and genotypes found for *UNH898* marker where the alleles 1 and 3 (296 and 264 bp respectively) and exclusively found in males

Table 2: A) Sexual genotype differentiation between males (XY/YY) and females (XX). B) Sexual genotype differentiation between YY and XY individuals

A)

Indicator	Probability
Sensitivity	96.97 %
Specificity	82.35 %
VPP	91.43 %
VPN	93.33 %

B)

Indicator	Probability
Sensitivity	100.00 %
Specificity	85.29 %
VPP	85.71 %
VPN	100.00 %

These results show that *UNH898* is a highly reliable sexual marker for the identification of genotypes in tilapia.

Research on the reproduction of tilapia, whose priority obtains a unisexual growing more efficient and nowadays people seek novel methodologies that generate the least environmental impact [25]. The development of technology to use YY males was based on this principle, but its application has been diminished by the absence of diagnostic tests to identify quickly and good reliability of sexual genotype during the production of these players [2]. In this regard, our study would be a great help.

The progeny test can predict sexual chromosome load with high confidence. In this test the ratio between males and females from a specimen of interest (sexual genotype is already known) is measured and compared with an expected ratio. For example, if an individual is a female reversed (XY), to cross it with normal male (XY), the resulting progeny should be composed of 25% females and 75% males [7]. People sought other ways too, to identify sexual genotype in tilapia sp. Like Crosetti et al. [10] performed cytogenetic studies in some species of the genus *Oreochromis* including *O. niloticus*, to identify possible candidates as sex chromosomes. Their study revealed that this species has 44 chromosomes (2n) and no significant differences in any pair met. So the cytogenetic study is not successful in recognizing the sex chromosomes in tilapia.

Subsequently, Foresti et al. [26] and Carrasco et al. [27] found differences in the mating of a pair of chromosomes through synaptonemal complex analysis by electron microscopy. Their studies revealed that in XY individuals a section of longer chromosome was absence during mating, while in XX and YY individuals there was a normal mating. It is presumed that in this chromosomal section there is an accumulation of heterochromatin, which inhibits mating, and which acts as a sex regulators.

The development of genetic maps in tilapia, prompted the search for potential markers related to sex, which could serve for marker-assisted selection for rapid identification of sexual genotype. Studies by Lee et al. [11] revealed markers for the first time that are highly linked to gender in the linkage group 8 (LG8) and subsequently, Khan [2], used to identify YY males for breeding, although the alleles showed much disparity and could not be identified with great fidelity in such individuals.

Concomitantly, Eshel et al. [12], found a strong connection with microsatellite sexual genotype can predict with high reliability, which later used to validate YY individuals in subsequent research [13]. Our results further confirm that UNH898 a microsatellite marker can be used as a tool to distinguish between male and female individuals, and subsequently identify YY individuals with high level of confidence.

CONCLUSION

Sexual identification using microsatellite genotyping was shown to be highly sensitive in the population studied. Where the microsatellite *UNH898* was selected as the perfect candidate for this purpose because it showed to be highly related to the sex-determining genes in the species (p = 2.91 x 10-28), presenting the alleles 296 and 264 which are closely linked to the Y chromosome, forming the 264/296 genotype in individuals and genotype YY 264/296 / x in XY individuals. The marker proved to be highly sensitive in identifying the sexual genotype, allowing to differentiate males from females (sensitivity = 96.97% and VPP = 91.43%), and also differentiate YY males from XY males (sensitivity = 100% and PPV = 85.71%) with high reliability. Therefore, its application in production level is highly recommended and thus shortens production time of YY males. We conclude that the various optimized methodologies in the recent study produced good results, so they can also be applied on a larger scale. Based on the above results in future there is a scope to develop highly sensitive Loop Mediated Isothermal Amplification assay (LAMP-assay) to sexually differentiate tilapia.

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