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Determination of Sex-Linked by Fluorescent Amplified Fragment Length Polymorphism Molecular Marker in *Oreochromis niloticus* L. Fish

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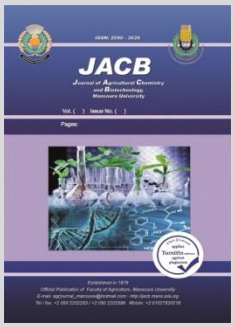

ABSTRACT

Nile tilapia (*Oreochromis niloticus* L.) is a worldwide significant aquaculture species rapidly gaining rank as a farmed commodity. Six fish specimens (three males and three females) of Nile tilapia were collected from Kafr El-Sheikh Egypt (31.3ºN and 30.93ºE). Fluorescent Amplified Fragment Length Polymorphism (F-AFLP) analysis was applied to qualitatively discriminate sex-dependent genomes, was successfully able to determine sex-linked marker. Principal Coordinate Analysis (PCA) identified sex-linked marker easily. PCR amplification was obtained using three pairs of primers. Total number of scored bands detected the specimens was 1243 as 129, 154, 291, 148, 165 and 356 bands for the three primer pairs, respectively. All bands ranged between 50 to 542 bp including 919 polymorphic. Percentage of polymorphic loci was 73.93%. The PCA test revealed different distribution of male and female samples according to the degree of similarity. The difference in PCA revealed genetic variation among the studied samples. Such observations reflect the success of the F-AFLP markers. A PCA variable showed clustering in four quadrants variables for female and male samples in different quadrants.

Keywords: Epigenetics, Fluorescent, AFLP, Nile tilapia, *Oreochromis niloticus*

INTRODUCTION

Reproduction of Nile tilapia (*Oreochromis niloticus*) is usually controlled by culturing males of same phenotype through hormonal sex reverse, converting females, physiologically to males (Citations) or produced by YY broodstock. However, some factors may affect this (Bezault *et al*., 2007). The tilapia genetic sex may be XX/ XY male heterogametic affected by a major gene (Campos-Ramos *et al*., 2001); with no big variation among sex chromosomes (Majumdar and McAndrew 1986). Sex chromosomes showed synaptonemal complex in the meiotic chromosomes (Harvey, 2002 and Ocalewicz *et al*., 2009).

Epigenetics refers to a modification of phenotypic characters, with no change in the inherited DNA sequence. The DNA methylation as well as the non-coding RNAs (ncRNAs) and histone modifications are primary epigenetic mechanisms connected with gene expression maintenance (Gunes *et al*., 2016). Regulation of gene expression differentiates between cells during development and is essential in determination of sex (Gunes *et al*., 2016 and Ortega-Recalde *et al*., 2020). Genetic as well as epigenetic change in the phenotype of a generation is essential for aquaculture management (Konstantinidis *et al*., 2020).

Fingerprinting through AFLP (Amplified Fragment Length Polymorphism) is used for genetic variability and genomic comparison in fish, *e.g. Liza ramada*, *Oreochromis niloticus* and *Oreochromis aureus*, (Bagley *et al*., 2001; Barreto and McCartney 2008). The technique is a sensitive DNA fingerprinting (Vos *et al*., 1995) which needs no data on the required genome It is used in evaluating genetic diversity (Maldini *et al*.,2006) and phylogenies (Bagley *et al*., 2001 and Wang *et al*., 2009).

In the current study, AFLP was used to extend information about sex-related genome markers in Nile tilapia through determination of sex-linkage in *Oreochromis niloticus* L. fish by Fluorescent Amplified Fragment Length Polymorphism (AFLP) molecular marker.

MATERIALS AND METHODS

1. Sampling

Three adult males and three females *Oreochromis niloticus* L. were used (collected from Kafr El-Sheikh Governorate, Egypt; 31.3ºN and 30.93ºE).

2. Molecular analyses

DNA extraction and purification

Isolation of DNA was done on caudal fin tissue (Bardakci and Skibinski ,1994).

AFLP fluorescence technique

The AFLP technique involves one preparatory step in addition to three major steps according to Vos *et al*., (1995). Preparations were for adaptors and primers, Restriction "R" and ligation "L" of adaptors, Pre-selective PCR and Selective PCR. Then the product was visualized using the gene scan method. The AFLP technique was carried out fluorescently (Table 1).

Table 1. The 5´- 3´ sequence of primers and adaptors to establish the F- AFLP technique.

Primer/Adaptor	5´ - sequence - 3´
EcoRI - A1	CTCGTAGACTGCGTACC
EcoRI - A2	AATTGGTACGCAGTC
Mse I - A1	GACGATGAGTCCTGAG
Mse I - A2	TACTCAGGACTCAT
Eco + A	GACTGCGTACCAATTCA
Mse + C	GATGAGTCCTGAGTAAC
Eco + ACA	*FAM*-GACTGCGTACCAATTCACA
Eco + AGG	*HEX*-GACTGCGTACCAATTCAGG
Eco + ATA	*CY3*-GACTGCGTACCAATTCATA
Mse + CAA	GATGAGTCCTGAGTAACAA
Mse + CTC	GATGAGTCCTGAGTAACTC

Adaptors and primers preparation.

For preparation of adaptors, the EcoR1 and Mse1 were re-suspended to 100µM with distilled water followed by mixing and 5-min heating at 95°C to denature, then left to

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cool completely for 1 h. Preparation of primers was through re-suspension to 100 **µ**M with water.

Adaptors restriction and ligation (RL).

RL preparation: 10 µL DNA added to a mix of Msel (Biolabs, R0525S), EcoR1 (Fermentas, ER0271), both Msel and EcoR1 adaptors, ATP (Fermentas, R0441), T4 ligase (Fermentas, EL0015) and Tango buffer (Fermentas, BY5) to allow enzymes activity, then adding distilled to 20 µL. RL reactions were incubated at 37°C 4 hours then placed on ice. Testing the reaction of RL was by 1% agarose gel electrophoresis with visualization by with 1X RedSafe®. Loading 5 µL RL containing the 5x Green GoTaq®Flexi on the gel was done. Also 1 lane was loaded with 2.5 µL GeneRuler™ 100bp DNA ladder plus (Fermentas, SM1153).

PCR preselection:

Reactions of PCR reactions were through the use of adaptor specific primer having 1 base extension. Preparation of a master mix was done and distributed. Application of Green GoTaq®Flexi DNA polymerase (Promega, M8295) was performed in 25µL. The followings were added on iced sterile, nuclease–free micro-centrifuge tubes:

Component	Stock concentration	Reaction concentration
GoTaq® Flexi buffer	5X	1X
$MgCl_2$	25 mM	4 mM
DNTPs	10Mm	0.2mM
Eco + A primer	10µM	1µM
Mse + C primer	10µM	1µM
GoTaq™	5 U/ µl	1.25 U
RL mix	0	2.0 µL
Distilled water	0	added up to 25 µL

An aliquot of 0.2 µL RL mix was added for every 25µL pre–selective PCR reaction in centrifuge tubes in a Flex Cycler (Analytic Jena) 96 Thermo-cycler. Control treatments (negative and positive) were conducted and reactions were tested by the agarose gel and visualized with 1X Red Safe® (5µLper 100 mL). An amount of 5 µL PCR containing the 5 x Green GoTaq® Flexi buffer was loaded on the gel after amplification, along with one lane with 2.5 µL GeneRuler™ 100bp DNA plus; Fermentas, SM1153.

Three different labeled PCR products of the same sample were mixed together (triplex analysis) as follow:

Selective PCR product	To be added
FAM labeled	2 µl
HEX labeled	2 µl
CY3 labeled	2 µl
Dist. Water	4 µl
Total	10 µl

Analysis of fluorescent - AFLP data.

Analysis of fragments

Since AFLP data are sensitive to bands, stutter bands, dimers, noisy background and other factors, automated scoring was adopted using PEAK SCANNER and RAWGENO V2 band technique (Arrigo *et al*., 2009).

Data analysis

Data analysis was by band-binary and allele frequency (Bonin *et al*., (2007).

Genetic polymorphism

Genetic variability was shown by indices measured adopting FAMD (Fingerprint Analysis with Missing Data) Software (Schlüter and Harris 2006).

RESULTS AND DISCUSSION

Fluorescent Amplified Fragment Length Polymorphism (F- AFLP) fingerprint

Amplification, scoring, and readability

The epigenetic changes epigenetic modulation associated with growth is sex-specific were assessed in order to avoid imprecision (Podgorniak *et al*., 2019 and Ortega-Recalde *et al*., 2020). PCR amplification was obtained using three pairs of primers. The total number of scored bands was 1243 as 129, 154, 291, 148, 165, and 356 bands for the three primer pairs, respectively. All bands ranged between 50 – 542 bp.

DNA polymorphism

Genetic diversity and polymorphism

A total of 1243 bands were detected from all six specimens; the number of polymorphic bands was 919 and the percentage of polymorphic loci 73.93% are shown in Table 2.

Such results resemble those obtained by Maldini *et al*., (2006) who measured the percentage of polymorphic loci among 20 species and ranged between 2.75 - 37.27% and the highest percentage was between two Liza species (31.19 - 37.27%). Wang *et al*., (2000) obtained 299 selectively amplified DNA fragments of which 66.2% were polymorphic using three primer set combinations in Common carp (*Cyprinus carpio* L.).

Table 2. Number of polymorphic and monomorphic loci for three pairs of primers from six samples (three males and three females) representing *Oreochromis niloticus* L.

Primer/Adaptor combinations	Polymorphic loci	Monomorphic loci	Total	% of polymorphic
Eco + ATA / Mse + CAA	67	62	129	51.94
Eco +AGG / Mse + CAA	110	44	154	71.43
Eco + ACA / Mse + CAA	232	59	291	79.73
Eco + ATA / Mse + CTC	107	41	148	72.30
Eco + AGG / Mse +CTC	142	23	165	86.06
Eco + ACA / Mse + CTC	261	95	356	73.31
Total	919	324	1243	73.93

Comparative analysis of F- AFLP product

Sex Linked Fluorescent AFLP molecular marker in *Oreochromis niloticus* L. fish

Data in Table 3 indicate that the first combination of sex-linked marker was at 70.86 Mw for males. The second combination showed sex-linked marker at 100.13, 123.35, 128.44 and 168.03 Mw for males, while 115.77 Mw for females. The third combination detected sex-linked marker at 100.11, 112.92, 167.99, 193.47, 263.56, 339.92, 507.27 Mw for males, and 262.16 Mw for females. The fourth combination showed sex-linked marker at 56.46, 94.66 and 101097 Mw for females. The fifth combination confirmed sex-linked marker at 106.94 and 142.09 Mw for females. Finally, the sixth combination obtained sex-linked marker at 127.5 Mw for males, and 56.46, 57.39, 58.41, 94.71, 140.29, 144.42, 265.04 Mw for females.

According to work done by Ezaz *et al.,* (2004) XX and YY were identified in Nile tilapia through genotypical methods with screening of diploid checks; also screening and segregant analysis indicated identification of X-linked and Y-linked markers.

Lee *et al*., (2011) developed new AFLP markers of the tilapia by analysis of the mapping families. In the current study three AFLP markers were identified implying sex segregation patterns. Sun, *et al*., (2014) used AFLP fingerprinting in Nile tilapia fish for sex-linked markers.

Similarity and dissimilarity

Data in Table 4 show that calculated Jaccard similarity coefficients (based on AFLP data) ranged from

0.57 between male 2 and female 1 which display low genetic similarity, to 0.85 between male 1 and female 3 which display a higher genetic similarity. Below diagonal pairwise Fst values were according to standard Jaccard distance transformation (1-similarity).

Table 3. Primer / Adaptor combinations and size of F-AFLP marker for three pairs of primers from six samples (Three males and three females) which represented *Oreochromis niloticus* L. as '1' for presence and '0' for absence of bands.

Primer / Adaptor combinations	Size of F-AFLP Marker	Male1	Male2	Male3	Female1	Female2	Female3
Eco + ATA / Mse + CAA	70.86	1	1	1	0	0	0
Eco +AGG / Mse + CAA	168.03	1	1	1	0	0	0
	128.44	1	1	1	0	0	0
	123.35	1	1	1	0	0	0
	115.77	0	0	0	1	1	1
	100.13	1	1	1	0	0	0
Eco + ACA / Mse + CAA	507.27	1	1	1	0	0	0
	339.92	1	1	1	0	0	0
	263.56	1	1	1	0	0	0
	262.16	0	0	0	1	1	1
	193.47	1	1	1	0	0	0
	167.99	1	1	1	0	0	0
	112.92	1	1	1	0	0	0
	100.11	1	1	1	0	0	0
Eco + ATA / Mse + CTC	101.97	0	0	0	1	1	1
	94.66	0	0	0	1	1	1
	56.46	0	0	0	1	1	1
Eco +AGG / Mse + CTC	142.09	0	0	0	1	1	1
	106.94	0	0	0	1	1	1
Eco + ACA / Mse + CTC	265.04	0	0	0	1	1	1
	144.42	0	0	0	1	1	1
	140.29	0	0	0	1	1	1
	127.5	1	1	1	0	0	0
	94.71	0	0	0	1	1	1
	58.41	0	0	0	1	1	1
	57.39	0	0	0	1	1	1
	56.46	0	0	0	1	1	1

Table 4. Genetic similarity matrix among six samples (Three males and three females) which represented the *Oreochromis niloticus* L. under study, as computed according to Jaccard & Bayesian similarity coefficient from F- AFLP generated data.

	Male1	Male2	Male3	Female1	Female2	Female3
Male1	0					
Male2	0.76	0				
Male3	0.58	0.72	0			
Female1	0.75	0.57	0.76	0		
Female2	0.80	0.63	0.82	0.58	0	
Female3	0.85	0.63	0.84	0.63	0.62	0

Inter species analysis

Principal Coordinate Analysis (PCA)

The PCA analysis depends mainly on computing the principal components only (Abdi and Williams 2010). In the current study the analysis showed clustering in four quadrant variables relating the male 2, female 1 and female 2 set in quadrant I. Male 1 and male 3 set in quadrant II displayed a distance from the other studied samples, while Female 3 set in quadrant IV indicates that the 6 fish specimens are apart from each other (Figure 1).

Applying the same method revealed the ability of PCA to reveal the genetic variation among the studied fish specimens. Such observation indicates a success of the F-AFLP markers.

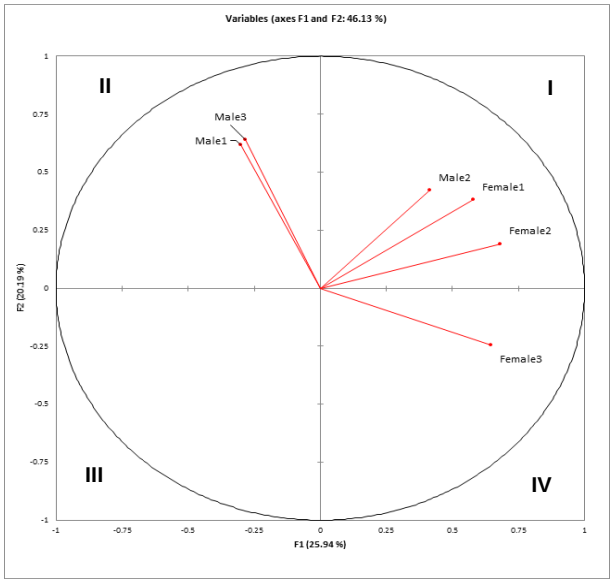

Figure 1. PCA plot explained by 2 axes. Axis 1 = 50.76 % and axis 2 = 33.63%. 4 quadrants were defined.

The hypothesis of Artemov *et al*., 2017 and Ortega-Recalde *et al*., 2020 supposes that a challenge a minimum of genetic diversity would make the population respond by adaptation and adopt a level of phenotypic variation high level of variability. Sønstebø *et al*., (2007) mentioned that PCA in brown trout (*Salmo trutta* L.) described 50% of the variation. Lucek *et al*., (2010) mentioned PCA variations on the scale between two axes, the first (17.4%) and the second axis (10.8%) in stickleback freshwater fish. Kajungiro, *et al*., (2019) used PCA to visualize relationships in Nile tilapia. The first and second principal components accounted for 62 and 14% of total variations respectively. The current study show assessed the effect of epigenetic processes on determining the breeding values and accuracy of genomic selection for genetic improvement of fish.

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تقدير الإرتباط الجنسى فى أسماك البلطى النيلى باستخدام AFLP الفلوروسنتى

احمد محمد سراج الدين*

قسم الوراثة والهندسة الوراثية ـ كلية الزراعة ـ جامعة بنها ـ مصر

يعتبر سمك البلطي النيلي (*Oreochromis niloticus* L.) أحد أنواع تربية الأحياء المائية ذات الأهمية العالمية التي تكتسب مكانة سريعة كسلعة مستزرعة. في هذه الدراسة تم استخدام ست عينات من الأسماك (ثلاثة ذكور وثلاث إناث) والتي تمثل أنواع البلطي النيلي ذكورا واناثا تم جمعها من محافظة كفر الشيخ ، مصر (31.3 درجة شمالاً - 30.93 درجة شرقاً). وتمت دراسة التباين الوراثى بإستخدام تقنية البصمة الوراثية Fluorescence AFLP والتي نجحت في تحديد المعلمات المرتبطة بالجنس في البلطي النيلى (XX,XY) *Oreochromis niloticus* L.) قيد الدراسة. حيث كان العدد الإجمالي للحزم المتحصل عليها من العينات الست التي تمثل *Oreochromis niloticus* L. 1243 حزمة موزعة كالآتى 129 ، 154 ، 291 ، 148 ، 165 و 356 حزمة باستخدام ثلاثة أزواج من البادئات ، على التوالي. تراوحت جميع الحزم بين 50 - 542 bp. بينما بلغ عدد الحزم متعددة الأشكال 919، النسبة المئوية للمواقع متعددة الأشكال 73.93٪. علاوة على ذلك فإن تحليل مكونات التباين الأساسية على مستوى الأنواع (PCA) أظهر تحديد العلامة المرتبطة بالجنس بسهولة مما يسهل عملية التمييز الجنسى. ويكشف PCA عن التباين الجيني بين العينات المدروسة، وهذا يؤكد نجاح معلمات F-AFLP. وقد أظهر اختبار PCA توزيعا مختلفا للعينات الذكرية والأنثوية للأسماك حسب درجة التشابه والإختلاف وهذا يحدد درجة القرابة بين أسماك البلطى النيلى .