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Genetic Diversity Assessment and Association Analysis of Body Characters of the Nile tilapia (*Oreochromis niloticus*) using AFLP

Fawzia S. Ali[1], Ashraf M. A.S. Goda[1], Mohamed Ismail[2*], Mohamed A. Rashed[3], Ahmed Mamoon[4], Khaled Mohammed-Geba[5]

[1]Aquaculture Division, National Institute of Oceanography and Fisheries, NIOF, Egypt, 13621
[2]Genetics Department, Faculty of Agriculture, Menoufia University, Shibin El-Kom, Egypt
[3]Genetics Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt
[4]Fish Production Department, Faculty of Agriculture-Al-Azhar University, Nasr City, Cairo, Egypt
[5]Department of Zoology, Faculty of Sciences, Menoufia University
[*]**Corresponding Author: mohamed.ismail@agr.menofia.edu.eg**

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ABSTRACT

The Nile tilapia, *Oreochromis nilotiucs*, is a species of prime importance in local and international aquaculture. It has been receiving an increasing attention in genetic markers development for improving aquaculture. The current study assayed the genetic diversity and population structure of the Nile tilapia in three populations in Egypt (Kafr El-Sheikh, El-Fayoum, and El-Dakahlia). Amplified fragment length polymorphism (AFLP) markers were developed, applying two restriction enzymes (MseI and EcoRI) in three primer combinations (MM1, MM2, MM3). Samples from El-Fayoum exhibited higher genetic richness than El- Dakahlia and Kafr El-Sheikh. Among the studied markers, MM2 showed a higher allele frequency in males compared to females of all locations combined. Furthermore, the analysis of molecular variance (AMOVA) indicated higher genetic variance within locations, as well as within males, and females, rather than among them. Using association genetics between AFLP makers and body weight traits, 22 significant associations were detected after false discovery rate correction (FDR) at $P< 0.05$. Phenotypic variance (R^2) explained by each marker was moderate and ranged between 13.2% for the MM1 (M126, associated with body length) and 30.7% for MM3 (M459, associated with body weight). The current study showed that El-Fayoum population was the most diverse compared to Kafr El-Sheikh and El-Dakahlia populations, with lesser aquaculture activity, calling for efforts to diversify Kafr El-Sheikh and El-Dakahlia populations. The significant marker-trait associations identified in the current study would contribute to the acceleration of tilapia breeding programs.

INTRODUCTION

Aquaculture is the fastest-growing food production sector worldwide, providing nearly 50% of the global fish supply. By 2030, it is estimated that aquaculture production would increase by 40% to satisfy global fish demand. Tilapia fish, the second most farmed fish in the world, have played an essential role in the growth of aquaculture **(FAO, 2018; GAFRD, 2018)**. Asian countries (e.g., China) ranked first in tilapia production and exportation of tilapia products; they produce one-half of the global supply **(Zhao *et al*., 2011; Matlala *et al*., 2013; FAO, 2018)**. Egypt is Africa's

leading producer of tilapia **(GAFRD, 2018)**. However, the contribution of natural resources (River Nile and freshwater lakes) in the tilapia production is significantly low compared to fish farms **(GAFRD, 2018)**. The tilapia fish produced by aquaculture are approximately seven-fold higher than those from natural sources **(GAFRD, 2018)**. The farmed tilapia fish represent 68.6% of the total Egyptian aquaculture, 90% of the Nile tilapia, *Oreochromis niloticus*, is produced from fish farming and only 10% from natural resources. Among the 70 known species of tilapias, the Nile tilapia, *Oreochromis niloticus* is the primary cultured species responsible for the global expansion of tilapia aquaculture production **(Gupta & Acosta, 2004)**. However, poor productivity in tilapia farms due to inadequate seed supply and the deteriorating performance of fish in many aquaculture systems are considered significant obstacles that can hinder tilapia growth and expansion **(GAFRD, 2018)**. Genetic variability provides a key element in fundamental criteria in the life of fish, especially when combined with different environmental factors. Genetic variations in disease resistance and tolerance can control the survival of a fish species **(Debes *et al.*, 2017)**. The broodstock' high genetic diversity and inbreeding reduction are recommended for economic aquaculture species. Additionally, hybrid process methodologies are crucial for crossbreeding production to ensure the continuity of genetic variability and inbreeding reduction **(Wang *et al.*, 2013; Astorga, 2014; Hillen *et al.*, 2017)**. Several genetic improvement programs concerning *O. niloticus* that favored traits enhancement, mainly growth and body weight, led to varying degrees of genetic gain per generation, as reviewed by **Gjedrem *et al.* (2018)**. A high level of genetic gain per generation of *O. niloticus* was previously reported in the studies of **Bentsen *et al.* (2002)**, **Charo-Karisa *et al.* (2006)**, **Khaw *et al.* (2012)** and **Ali *et al.* (2017)**. However, a clear alert was created against the increase of inbreeding (diversity loss) of favored growth owing to the continuous crossing within the same selected strains for the enhanced growth that is practically attained or predicted. Such reduced diversity is common in numerous species of prime importance for aquaculture, including, for example, the Pacific white shrimp, *Litopenaeus vannamei* **(De Donato *et al.*, 2005)**, the rainbow trout, *Oncorhynchus mykiss* **(Kause *et al.*, 2005)**, and the silver-lipped pearl oysters, *Pinctada maxima* **(Lind *et al.*, 2009)**. Hence, it is necessary to use genetically diverse sources of tilapia breeders for selection improvement, a process that is achievable through marker assisted selection (MAS).

Marker-assisted selection (MAS) is a powerful tool in fish breeding **(Chen *et al.*, 2018)**. It represents an indirect selection operation that is based on a molecular marker associated with the desired trait **(Rothschild *et al.*, 2007)**. Employing DNA markers to define a specific genotype and predict an animal's behavior is crucial in general animal breeding **(Beuzen *et al.*, 2000)** and in the genetic improvement of desirable traits in aquaculture fishes, in particular **(Chen *et al.*, 2018)**. A versatile milieu of molecular markers has been established and extensively applied for the enhancement of aquaculture production through the selection of favorable traits in fish that are mainly resistant to diseases **(Liu *et al.*, 2005)**, growth and sex control, and induction of gynogenesis **(Ma *et al.*, 2018)**. These markers include AFLP, ESTs, microarrays, microsatellites, RAPD, RFLP, RNA sequencing and SNPs **(Dong *et al.*, 1999; Liu &**

Cordes, 2004; Maqsood *et al.*, 2017), all of which target either the nuclear or mitochondrial genomes of the fish, with the final objective of identifying the genes or genomic loci responsible for quantitative traits (QTLs). Among many DNA markers, AFLP was used on a large scale in different fish species for breeding and diversity assessment purposes **(Khatei *et al.*, 2021)**. The Mendelian inheritance pattern of AFLP made it applicable for gene mapping and marker-assisted selection **(Agresti *et al.*, 2000; Sun *et al.*, 2014)**.

This technique could characterize a reduction of genetic variability in a hatchery-produced disease-resistant population of the olive flounder *Paralichthys olivaceus* compared to disease-resistant and common populations **(Liu *et al.*, 2005)**. Also, AFLP efficiently revealed high within- and among-population genetic variability in the endangered aquatic lycophyte, *Isoëtes yunguiensis*, in China **(Dong, 2018)**. AFLP was also used to assess the population structure of several fishes, including *Oreochromis* **(Firmat *et al.*, 2013)** and *Mugil cephalus* **(Magdy *et al.*, 2016)**. Moreover, AFLP is highly reproducible and provides remarkable genome coverage **(Vos *et al.*, 1995)**.

The main objectives of the current study were to evaluate the genetic structure of three populations of the Nile tilapia in Egypt (Kafr El-Sheikh, El-Fayoum, and El-Dakahlia), and search for potential AFLP markers associated with body traits using association genetics approach.

MATERIALS AND METHODS

1. Sampling

Oreochromis niloticus samples were collected from three different governorates, Kafr El-Sheikh, El-Fayoum, and El- Dakahlia (Fig. 1). After unifying the age **(Penttila & Dery, 1988)**, 91 samples of 2-year-old fish were retained to ensure that the recorded differences in fish sizes are not age-dependent. These samples were sorted according to their sexes, weights and lengths into 26 large females (average; 271g, 260cm), 26 small females (average; 184gm, 23cm) in addition to 21 large males (average; 402g, 270cm) and 18 small males (average 265g, 240cm). Male samples weighing more than 270gm were considered large males, and female samples weighing more than 200gm were regarded as large females. The condition factor (CF) was calculated by using the formula CF= W*100/ L of **Tesch (1971) and Weatherley (1972)**, where W is the fish weight (gram), and L is the fish total length (cm).

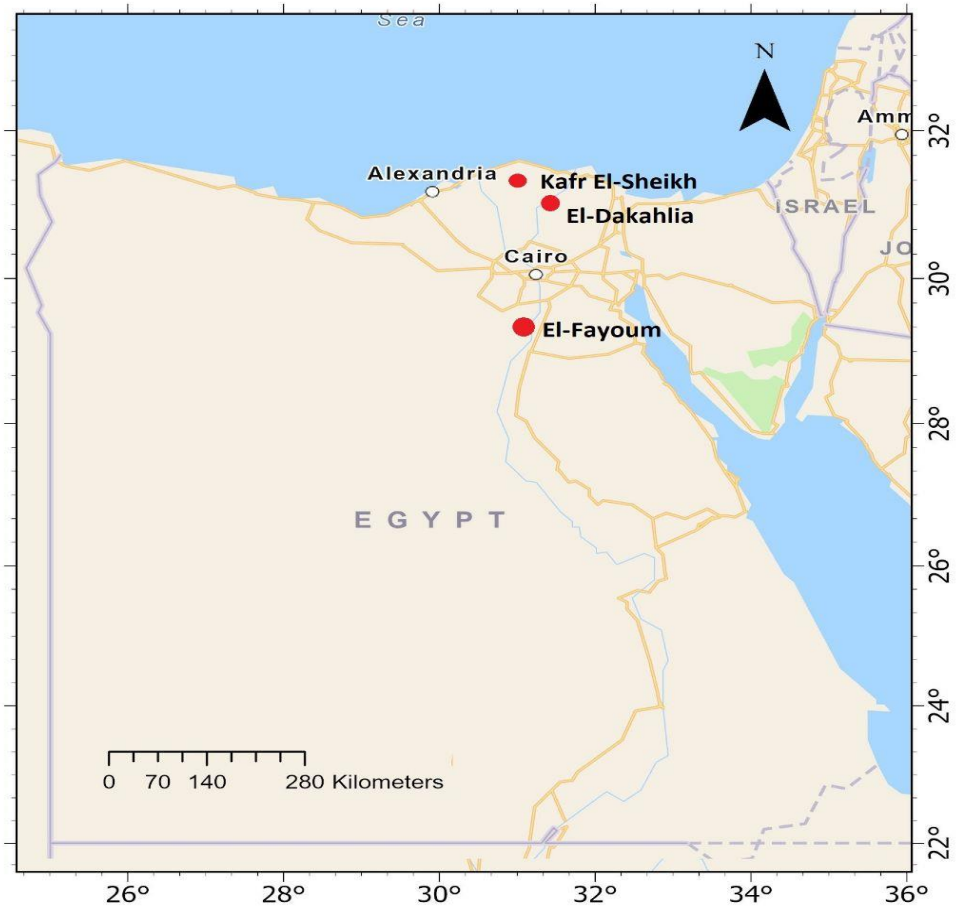

Fig. 1. Sampling sites of *O. niloticus* populations throughout Egypt

2. DNA extraction and AFLP protocol

The Nile tilapia, *O. niloticus* caudal fin tissues were used for DNA extraction as described by **Ali *et al.* (2021)**. The DNA quantity and quality were checked using a nano drop spectrophotometer (Implen, Nanophotometer, NP80 touch, Germany).

AFLP procedure was conducted using the original protocol of **Vos *et al.* (1995)**. The DNA was cut using two restriction enzymes; EcoRI and MseI, with the recognition motifs GAATTC and TTAA. The enzyme T4 DNA Ligase was applied to ligate EcoRI and MseI adapters to the restricted fragments. A pre-selective PCR was then performed using Eco+A and Mse+C primers as a preparatory step for the final selective PCR. Finally, the selective PCR was performed using three combinations of fluorchromes' labelled primers (MM1, MM2, and MM3) instead of radioactive agents, as described by **Magdy *et al.* (2016)**. The applied primers combinations are presented in Table (1). All primers were manufactured using a nucleotide synthesizer and purified through HPLC (Life Technology, England). The amplified products were visualized using the ABI3730 DNA analyzer (Applied Biosystems, USA), with a size standard GS500-LIZ (Macrogen Genescan Service, Korea).

Table 1. Primer sequences and adaptors used to amplify EcoRI and MseI-restriction fragments

	Adaptor/Primer	5'- 3'	Adaptor/Primer	5'- 3'
Adaptor	EcoRI-Adap1	CTCGTAGACTGCGTACC	MseI-Adap1	GACGATGAGTCCTGAG
	EcoRI-Adap2	AATTGGTACGCAGTC	MseI-Adap2	TACTCAGGACTCAT
Fragments for pre-selective PCR	Eco-A	GACTGCGTACCAATTCA	Mse + C	GATGAGTCCTGAGTAA**C**
Primer combination (Selective PCR) (Mse1+CAG)	Eco-AGG	**HEX**-GACTGCGTACCAATTC**AGG**	Mse1-CAG (MM1*)	GATGAGTCCTGAGTAA**CAG**
	Eco-ATA	**CY3**-GACTGCGTACCAATTC**ATA**		
	Eco-ACA	**FAM**-GACTGCGTACCAATTC**ACA**		
	Eco-AGG	**HEX**-GACTGCGTACCAATTC**AGG**	Mse1-CGT (MM2)	GATGAGTCCTGAGTAA**CGT**
	Eco-ATA	**CY3**-GACTGCGTACCAATTC**ATA**		
	Eco-ACA	**FAM**-GACTGCGTACCAATTC**ACA**		
	Eco-AGG	**HEX**-GACTGCGTACCAATTC**AGG**	Mse1-CTC (MM3)	GATGAGTCCTGAGTAA**CTC**
	Eco-ATA	**CY3**-GACTGCGTACCAATTC**ATA**		
	Eco-ACA	**FAM**-GACTGCGTACCAATTC**ACA**		

*(MM1, MM2, and MM3) refer to different primer combinations applied for restriction fragments amplification. Selective nucleotide, fluorescent dyes are in bold.

3. Genetic diversity analyses and marker discrimination power

Prior to genetic diversity analysis, all loci were subjected to filtering procedures that removed loci with 100% presence across all samples (0 or 1), using redundant loci filtering criteria implemented in AFLPOP 1.1 **(Duchesne *et al.*, 2002)**. Subsequently, the filtered results were used to estimate genetic diversity parameters using POPGEN 1.32 **(Yeh, 1997)**. The genetic diversity parameters determined for three populations included Shannon's information index (I) **(Lewontin, 1972)**, the percentage of polymorphic loci, the observed number of alleles (Na), the effective number of alleles (Ne) **(Kimura & Crow, 1964)**, and Nei gene diversity **(Nei, 1987)**. The estimated genetic diversity parameters Na, Ne, H, and I for each population were further analyzed using one-way analysis of variance (ANOVA) using Statgraphics Centurion XVI. To test which primer combination (s) has discrimination ability between males and females, ANOVA was performed on allele frequency data of MM1, MM2, and MM3 for all males and females using Statgraphics Centurion XVI.

4. Analysis of molecular variance (AMOVA)

Hierarchical analysis of molecular variance (AMOVA) using different groups was used to detect genetic variance breakdown across the three populations using Arlequin 3.5 **(Excoffier & Lischer, 2010)**. AMOVA was used to partition the total genetic variance into components explaining divergence among populations (regardless of sex or size), among males (small versus large), and females (small versus large) across the sampling locations.

5. Genetic association between AFLPs and fish body characters

The association genetic analysis was performed to determine any putative association between AFLP markers and fish weight, length and ratio. First, genotypic data from AFLP markers were used to determine population structure (Q matrix) using the model-based Bayesian clustering algorithm (STRUCTURE) **(Pritchard *et al.*, 2000)**. Second, marker-trait associations were determined using a general linear model (GLM with Q-matrix as correction for population structure), as implemented in TASSEL version 5.0 (released October 2018, http://www.maize genetics.net). Including population structure in the assertion model would eliminate false positives due to the population-based relationship among genotypes **(Cappa *et al.*, 2011)**. In addition, following association analysis, false discovery rate (FDR) approach was used to eliminate the false positive associations **(Benjamini & Hochberg, 1995)**. Moreover, using the FDR approach is essential if many markers (i.e., AFLPs) are in strong LD, which might result in a loss of statistical power and generate a false negative. Positive associations were determined at the $P < 0.05$ level.

RESULTS

1. Genetic diversity indices and marker discrimination power

The three primers combinations, MM1, MM2, and MM3 showed a high level of polymorphism (Table 2). The highest was attributed to MM3 with 100% average polymorphism, followed by MM1, then MM2 with 95.24 and 94.34%, respectively. Among the studied populations, El-Fayoum showed the highest average polymorphisms of 98.97%, followed by El-Dakahlia and Kafr El-Sheikh with 97.73 and 92.98%, respectively.

Table 2. AFLP primers polymorphism across three *O. niloticus* populations

Primer combination		MM1	MM2	MM3	Average
Total number of loci		161	218	57	
Percentage of polymorphic loci	Kafr El-Sheikh	93.17	85.78	100.00	92.98
	El-Fayoum	97.52	99.08	100.00	98.87
	El-Dakahlia	95.03	98.17	100.00	97.73
Average		95.24	94.34	100.00	

The effective number alleles (*Ne*) was higher in El-Fayoum than El-Dakahlia and Kafr El-Sheikh (Fig. 2). Similarly, Shannon information index (I) was higher for El-Fayoum as compared to El-Dakahlia and Kafr El-Sheikh. Overall gene diversity (*H*) was low across all three populations (≈ 0.4) with El-Fayoum having slightly higher *H*, as compared to El- Dakahlia and Kafr El-Sheikh.

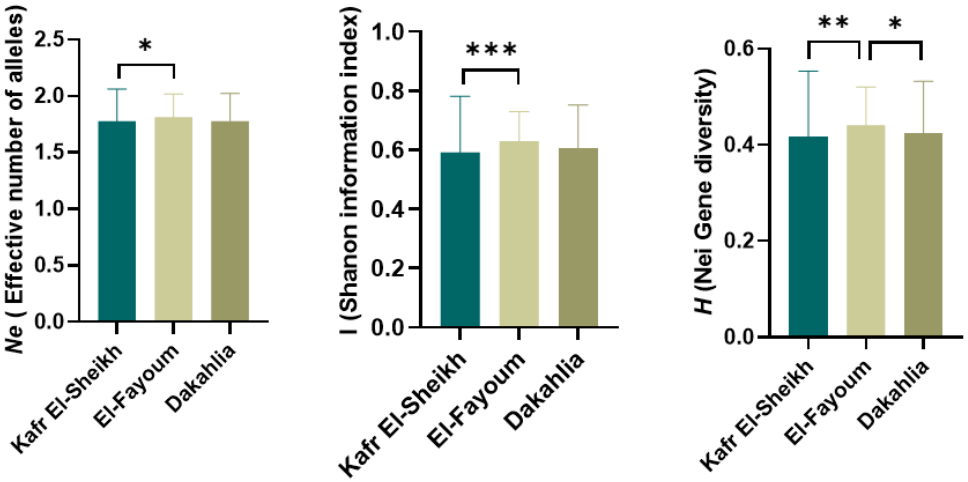

Fig. 2. Diversity estimates across three sampling locations. Columns with asterisk are significantly different

Across all locations, males showed a higher significant allele frequency than females (Fig. 3a). Among the studied markers, MM2 showed higher discrimination power as indicated by highly significant allele frequency differences between males and females ($P< 0.001$) (Fig. 3b).

(**a**) (**b**)

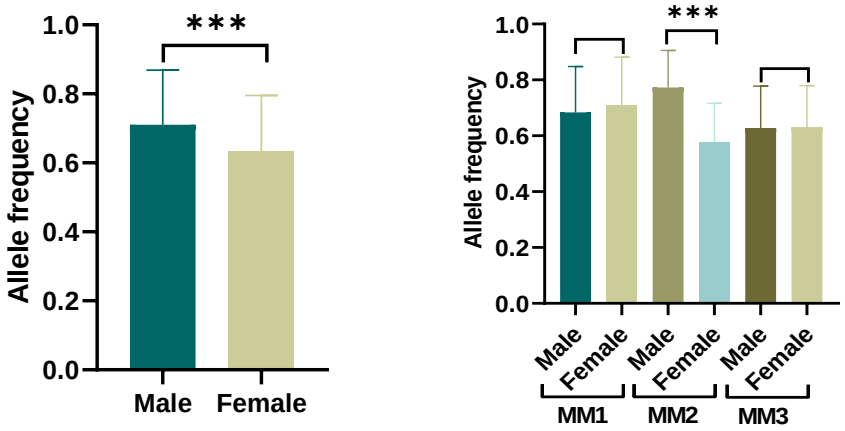

Fig. 3. Allele frequency differences among males and females across three locations using all primer combinations (**a**) combined and (**b**)separately. Columns with an asterisk are significantly different

2. Analysis of molecular variance (AMOVA)

Analysis of molecular variance indicated no significant difference among locations, males, and females; most of the genetic variance was attributed to within rather than among locations, males, and females (Table 3). Most genetic differences are attributed to the differences among males and females within locations rather than among locations.

Table 3. Analysis of molecular variance (AMOVA) of *O. niloticus* populations using AFLP

	Source of variation	df	SS	Est. Var.	%	P
Location	Among	2	197.84	-0.38	-0.37	0.48
	Within	29	2986.25	102.97	100.37	
	Total	31	3184.09	102.59	100.00	
Males	Among	1	100.79	-1.32	-1.22	0.53
	Within	12	1320.57	110.05	101.22	
	Total	13	1421.36	108.72	100.00	
Females	Among	1	96.11	0.22	0.23	0.42
	Within	16	1506.44	94.15	99.77	
	Total	17	1602.56	94.37	100.00	

df; Degree of freedom, *SS*; Sum of Squares, *MS*; Mean squares; %; Percentage of variance, *P*; Significant level.

3. Association genetics analysis

The current study determined significant associations between AFLP markers and body weight traits. The three body weight traits were significantly associated with MM1, MM2, and MM3; however, only body length/body weight ratio was significantly associated with MM2. A total of 22 significant marker-trait associations ($P< 0.05$) for three body characters were discovered after FDR correction (Table 4). With eight significant associations, body length (cm) was significantly associated with MM1 and MM2. With seven significant associations, body weight (gm) was significantly associated with MM2 and MM3. Body length/ body weight ratio was only associated with MM2, with seven associations. The phenotypic variance (R^2) explained by each marker was moderate and ranged between 13.2% for MM1 (M126, associated with body length) and 30.7% for MM3 (M459, associated with body weight).

Table 4. Marker-trait associations of body characters identified using AFLP primer combinations and its phenotypic variance (R^2) and significant level (P) for over-all means of three traits

Trait	Primer combination	Locus	R^2	P
Body length (cm)	MM1	M1	0.143	0.033
		M72	0.127	0.045^{ns}
		M78	0.125	0.047^{ns}
		M126	0.132	0.037
	MM2	M223	0.145	0.032
		M225	0.145	0.032
		M228	0.145	0.032
		M242	0.147	0.030
		M332	0.145	0.032
		M345	0.138	0.037
Body weight (gm)	MM2	M188	0.136	0.038
		M238	0.133	0.040^{ns}
		M255	0.136	0.038
		M271	0.131	0.042^{ns}
		M322	0.137	0.037
		M345	0.139	0.035
		M373	0.137	0.037
	MM3	M416	0.128	0.045^{ns}
		M464	0.127	0.045^{ns}
		M456	0.128	0.044^{ns}
		M459	0.307	0.001
		M478	0.142	0.033

		M489	0.125	0.047^{ns}
Body length/body weight ratio	MM2	M173	0.147	0.030
		M219	0.126	0.046^{ns}
		M223	0.132	0.041^{ns}
		M225	0.132	0.041^{ns}
		M228	0.148	0.030
		M229	0.146	0.031
		M239	0.160	0.023
		M271	0.131	0.042^{ns}
		M280	0.142	0.033
		M313	0.300	0.001
		M322	0.128	0.044^{ns}
		M358	0.142	0.034
		M373	0.129	0.043^{ns}

^{ns} = Rejected significant associations by FDR test.

DISCUSSION

It is crucial to assess the level of genetic diversity of wild parental populations of the Nile tilapia in Egypt since it contributes significantly to fish production. The Nile tilapia fish were previously studied in Africa using different marker systems **(Lind *et al.*, 2019; Tibihika *et al.*, 2020)**.

In this study, AFLP markers was developed to assess the genetic richness of wild tilapia populations in Egypt, which will be potential candidates for genetic association analysis of body characters. These populations represented a sample from the key northern Egyptian tilapia-producing governorates, namely Kafr El-Sheikh, El-Fayoum, and Dakahlia. Three primer combinations, MM1, MM2 and MM3, were developed and used in genetic diversity evaluation in tilapia populations. Different patterns of genetic diversities were shown in the studied localities. Remarkably, the same population, El-Fayoum, showed a striking difference in all diversity estimates compared to Kafr El-Sheikh and El-Dakahlia. The presence of such higher polymorphism in El-Fayoum population would make it an ideal source for diverse genotypes in the Nile tilapia improvement programs.

In this sense, males exhibited a higher allelic frequency at all sizes compared to females, reflecting higher genetic diversity of males versus females. This tendency of high male genetic diversity appeared upon using all combined primers. The results of AMOVA indicated an absence of population structure between locations, males and females and genetic variance mainly attributed to within each category. Our results indicate that more genetic variance exists between two randomly selected individuals from the same group than between two individuals from a different group. The current findings contrast with the previous study by **Nayfa *et al.* (2020)**, who reported clear population differentiation among the wild and domesticated Nile tilapia populations. The

discrepancies between the two studies are probably associated with the widespread sampling area **(Nayfa *et al.* 2020)** and the inclusion of the domesticated Nile tilapia in their study.

Additionally, the differences in molecular markers used are most likely contributed to the disparities in population structure inferences. Most importantly, the Nile tilapia farming industry has increased rapidly in Egypt, particularly around the Nile Delta region, in the past three decades **(Soliman & Yacout, 2016)**. As such, increased fish movement among hatcheries and farms has occurred significantly **(Nayfa *et al.*, 2020)**, probably resulting in a higher rate of gene flow among regions that led to low population differentiation.

An exciting aspect found in the current study is the reduced genetic diversity of Kafr El-Sheikh and El- Dakahlia *O. niloticus* populations compared to Fayoum populations. A possible explanation for such low genetic diversity is mainly associated with the role of Kafr El-Sheikh Governorate as the first Egyptian tilapia-producing location in Egypt, followed by Behera, Fayoum and Dakahlia **(Macfadyen *et al.*, 2012)**. The absence of clear bases for selecting spawners and escapes to the main Nile stem is common in Kafr El-Sheikh and El- Dakahlia. This may result in continuously introducing genetically exogenous populations to the wild, a common cause that might lead directly to the reduction of genetic diversity in wild populations **(Liu *et al.*, 2005)**. The source of the peculiarity of the farmed fish's genetic material compared to the wild ones is that the former group is subjected to frequent inbreeding, selective breeding and domestication **(Hindar *et al.*, 1991)**. The introgression between wild and farmed populations, as a result of escape or intentional release for purposes of stocking or sports, can induce the reduction of wild genetic diversity, evolutionary potentials, tolerance to environmental changes, and eventually, the increase of extinction risk **(Gozlan *et al.*, 2010; Kelly *et al.*, 2010; Létourneau *et al.*, 2018)**. However, there is an evidence now that ceasing stocking, as one of the inducers of introgressive breeding, can assist the recovery of natural genetic diversity. Such recovery is expected to improve with time, providing that no further stocking happens, and the wild populations are resilient enough to alleviate such genetic contamination **(Perrier *et al.*, 2013; Valiquette *et al.*, 2014; Létourneau *et al.*, 2018)**.

Association genetics aim to determine significant associations between genetic markers and a trait of interest. One of the major detrimental factors of any marker-trait association study is the abundance of genetic markers/ alleles, a criterion of AFLP. AFLP can also be genotyped arbitrarily from the genome, without prior knowledge of the structure and sequence of the genome **(Vos *et al.*, 1995)**.

The current study identified significant associations between MM1, MM2, and MM3 and body traits. Association genetic analysis in the Nile tilapia became of interest to many researchers recently applying genome-wide association studies (GWAS) **(Yoshida *et al.*, 2019)** or individual marker-based genetic association **(Chu *et al.*, 2021)**. Identifying marker-trait associations in the current study would provide guidelines for selecting the tilapia parental population in any breeding program.

CONCLUSION

In conclusion, we successfully provided a molecular tool that evaluated the genetic diversity of the three major Nile tilapia populations. AFLP markers used in the current study showed that the El-Fayoum population was the most diverse compared to Kafr El-Sheikh and El-Dakahlia, making it a suitable candidate for any prospective breeding tilapia program. The decreased genetic diversity in Kafr El-Sheikh and Dakahlia calls for an urgent need to diversify the genetic resources of Egyptian *O. niloticus* aquaculture in Kafr El-Sheikh and Dakahlia populations. Furthermore, the current study identified significant marker-trait associations between AFLP markers and body traits. The identified molecular makers will contribute to accelerating breeding tilapia programs, and mining for such markers in parental populations will significantly reduce tilapia aquaculture efforts.

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