Investigating Nuclear DNA Microsatellites in the Nile Tilapia (*Oreochromis niloticus*): Insights into Association Genetics

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ABSTRACT

One of the key challenges in aquaculture is the selection of individuals with superior traits, including rapid growth rate, high flesh quality, and disease resistance. Marker-assisted selection (MAS) using molecular markers (e.g., simple sequence repeats; SSR) is known to be more effective in identifying individuals with specific traits based on their genetic makeup. The current study aimed to examine SSR markers across different linkage groups in their efficiency in characterizing the Nile tilapia fish with superior growth performance traits for usage as effective tools for MAS. A total of 152 Nile tilapia samples with identical ages but contrasting growth performances were collected from a fish farm in Kafr El-Sheik Governorate, Egypt. The collected genotypes were evaluated for growth performance metrics such as weight and length, and their microsatellite allelic patterns were also analyzed. A total of 13 microsatellite markers were assessed in the two sampled Nile tilapia categories. The *t*-test of growth performance traits between the two fish categories revealed highly significant differences in body weight and length. The average number of alleles per locus in the large and small populations was 2.6 and 2.2, respectively. The analyzed populations showed a significant deviation from Hardy-Weinberg equilibrium.Only seven markers showed private alleles unique to either small or large populations, indicating the suitability of these markers for association genetic studies. The studied markers showed low to moderate gene diversity (*H*), which ranged between 0.25 and 0.48, with an average of 0.41. The discrimination power of the studied loci was relatively high (*D* = 0.888). The discrepancies in growth parameters between the investigated populations were aligned with the disparities in allele frequency, indicating a possible correlation between certain allele(s) and growth performance characteristics. The present study highlighted the effectiveness of specific SSRs in addressing growth parameters during the planning for the Nile tilapia selection in breeding programs.

INTRODUCTION

Aquaculture has become an important sector in the global food production; hence, it fills the gap between the growing demand for seafood, caused by the continuous population surge, and the steady decline in wild captures due to overfishing and habitat destruction. Therefore, enhanced aquaculture techniques are indispensable (**Aquaculture, 2020**).

One of the main targets in aquaculture is the discovery and selection of individuals with superior phenotypes, for instance, an accelerated growth rate, elevated immunity, and improved

carcass quality **(Gjedrem & Robinson, 2014; Song *et al*., 2023)**. Traditionally, selective breeding approaches involved selecting broodstock based on their phenotypes, such as body measurements and shape **(Gjedrem, 2010)**. But this approach was time-consuming and non-cost effective, as it required several succeeding generations to decide whether the trait of interest was successfully inherited or not **(Chavanne *et al*., 2016; Gjedrem, 2016)**. In addition, traditional breeding was ineffective in polygenic trait selection, where the trait of interest is influenced by multiple genes and the surrounding environmental conditions (pleiotropic effects) **(Ozaki *et al*., 2012; Zenger, 2019)**.

In contrast, MAS has made it possible to accurately identify individuals with hidden codes of superiority based on their DNA sequence **(Gjedrem, 2010)**. PCR based techniques such as SSRs, and single nucleotide polymorphisms, SNPs, can precisely identify individuals with unique phenotypes based on their genetic content, while saving time and lowering the input cost of the breeding cycle **(Baranski, 2014; Haldar, 2018)**.

Simple sequence repeats, SSRs, are non-coding short-tandem repeats of DNA; they represent the most prevalent DNA marker in aquaculture since these repetitions are evenly distributed throughout the genome, hypervariable, reproducible, affordable, and of a co-dominant inheritance **(Mojekwu & Anumudu, 2013; Haldar, 2018)**.

Investigating the genetic makeup and diversity between and within fish populations is essential for a successful aquaculture **(Cheng *et al*., 2016)**. Therefore, SSR markers have been adopted in several improvement programs in aquaculture, including population genetics, pure line establishment, and genetic improvement programs **(Mojekwu & Anumudu, 2013)**. Furthermore, SSR markers have been used for the establishment of accurate pedigree records, parental identification, and innovative pure line creation for several economically cultivated species, especially the Nile tilapia, *Lates calcarifer*, the Asian seabass, and the mandarin fish, *Siniperca chuatsi* **(Gjedrem, 2005; Abdul-Muneer, 2014; Yang *et al*., 2014; Zhang *et al*., 2016; Thomas *et al*., 2021)**. Moreover, SSR markers have been used in projects of genetic resource conservation by identifying hybrid individuals in mixed populations, which is essential for assessing the genetic structure and developing effective management strategies **(Gjerde & Villanueva, 2003; Guan *et al*., 2020; Regan *et al*., 2021)**.

Several successful genetic improvement programs were previously accomplished in economically cultivated species based on SSR markers; for example, the MAS programs of crustaceans including the red swamp crayfish, *Procambarus clarkii*, and the blue crab, *Portunus trituberculatus*, and the bivalve Mollusca such as the west African mangrove oyster, *Crassostrea tulipa* **(Ali *et al*., 2017; Guo *et al*., 2022; Duan *et al*., 2023; Tine *et al*., 2023; Ukenye & Megbowon, 2023)**. Additionally, SSR markers associated to growth and immunity have been identified in several commercially important fish species, including *Oreochromis niloticus*, *Oncorhynchus mykiss, Salmo salar*, *Salvelinus alpinus*, and *Psetta maxima* **(Das & Sahoo, 2014; Kessuwan*et al.,* 2016; Ali *et al*., 2017; Zafrin & Alam, 2020; Chen *et al*., 2022)**. Furthermore, sex-related SSR markers have been identified in countless cultivated species, *viz. Clarias gariepinus*, *Scleropages formosus*, *Megalobrama amblycephala*,*Cyprinus carpi*, *Oncorhynchus mykiss*, *Pylodictis olivaris*, and *Oreochromas niloticus* **(Sun *et al*., 2014; Xu *et al*., 2015; Zheng *et al.,* 2020)**. These sex related SSR markers are fundamental in aquaculture because they make it possible to perform a reliable and quick sex determination at an early stage of development. This is vital in aquaculture owing to the presence of sexual dimorphism during growth performance, age of sexual maturation, flesh quality, and immunity, which render one sex more valuable than another **(Chen *et al*., 2018)**.

Finally, SSR markers of tolerance to suboptimum conditions for instance, high stocking density, low rate of water exchange, and suboptimum temperatures have been discovered and applied in aquaculture **(Gjerde &Villanueva, 2003; Zhu *et al*., 2015; Regan *et al*., 2021)**.

Several SSR markers are mapped to the same linkage group, thus showing different extents of linkage. To prevent such linkages among markers in any association study, it is essential that the chosen SSRs are mapped to distinct linkage groups. The idea behind the selection of different linkage groups is to confirm that the markers are not linked; hence, the significant marker–trait associations are not false positives due to linkage. Second, the markers used were previously reported to be associated with different economically important traits in other tilapia populations (*Oreochromis mossambicus* x *O. aureus*). Third, the selected markers showed a linkage with QTLs affecting body weight, length, and thickness in addition to cold tolerance. The selected markers were formerly reported as associated with QTLs underlying genes with pleiotropic effects **(Lin *et al*., 2016)**.

In this context, the primary objectives of the present study were first to utilize SSRs distributed across various linkage groups to characterize the Nile tilapia fish with superior traits, particularly growth performance, and second, to explore their potential as efficient tools for MAS.

For these purposes, two categories of the Nile tilapia of the same age, but with contrasting growth performances, were collected to assess the differences in fish growth (length and weight) relative to their microsatellite allelic pattern. This represents a primary step for determining the SSR markers associated with growth performance.

MATERIALS AND METHODS

Sample collection and phenotypic measurements

A total of 152 Nile tilapia of identical age were collected from a fish farm in Kafr El-Sheik Governorate, Egypt. The growth traits (weight and length) of the collected individuals were recorded.

DNA extraction and SSR marker genotyping

The caudal fins ($0.5cm^2$) of the collected samples were dissected and preserved in 100% ethanol at -20°C for further molecular analysis. DNA was isolated from the preserved tissues using the phenol-chloroform method according to **Asahida *et al*. (1996)**, with some modifications, as described in **Ali *et al*. (2019)**.

A total of 13 SSRs were used for genotyping **(Ali *et al*., 2017, Tibihika *et al*., 2019)**. The selected markers were chosen according to many criteria. First, the markers used were spread across different linkage groups (i.e., LG 2, LG 8, LG 18, and LG 23) **(Cnaani *et al.*, 2004)**. The selection of markers from different linkage groups serves several purposes. First, it ensures that the markers are not genetically linked, thereby minimizing the risk of false positive associations between markers and traits due to linkage. Second, the chosen markers have been previously linked to various economically significant traits in other tilapia populations (*Oreochromis mossambicus* x *O. aureus*). Third, the selected markers demonstrated linkage with quantitative trait loci (QTLs) affecting body weight, length, thickness, and cold tolerance. Moreover, these markers associated with QTLs govern genes with pleiotropic effects **(Lin *et al*., 2016)**. PCRs were performed following the method of **Tibihika *et al*. (2019)**. A list of the 13 SSR markers used for genotyping of the Nile tilapia (*O. niloticus*) are presented in Table (1).

Statistical analysis of body characteristics

The collected weight and length data were statistically analyzed using GraphPad Prism software version 9.5.1 (GraphPad Software Inc., San Diego, CA), and the values were expressed as the means $\pm$ SE; the unpaired Student's *t*-test was used for comparisons, and $P<0.05$ was considered to indicate statistical significance.

Genetic diversity analysis

Diversity estimates including the number of alleles per locus, allelic richness remedied for the unequal sample size through the rarefaction approach **(El Mousadik & Petit, 1996)**, as fulfilled in MSA 4.05 **(Dieringer & Schlötterer, 2003)**. In addition, observed heterozygosity (H_o) and Nei's unbiased estimate of expected heterozygosity (H_e)**(Nei, 1987)** were also calculated through MSA 4.05. Departure from Hardy–Weinberg equilibrium was calculated within each fish category based on precise tests as fulfilled in GENEPOP 4.0 **(Raymond & Rousset, 1995)**. Finally, the fixation indices estimates (F_{IS}) **(Wright, 1965)** were obtained using MSA 4.05.

Since the ability of each marker to discriminate among genotypes and populations varies, different differentiation potential estimates were determined for each microsatellite marker. The gene diversity (H) **(Nei, 1973)**, polymorphism information content (*PIC*) **(Botstein *et al.*, 1980)**, and discriminating power (*D*) **(Tessier *et al.*, 1999)** were calculated using the iMEC program **(Amiryousefi *et al.*, 2018)**.

When choosing markers for genetic research, the *PIC*, which measures a marker's capacity to identify polymorphisms, becomes extremely significant **(Serrote *et al.*, 2020)**. On the other side, *D* stands for the likelihood that two randomly selected people will have distinct banding patterns and can be distinguished from one another.

To quantify the extent of population structure, the analyses of molecular variance (AMOVA) were accomplished using GenAlex 6 **(Peakall & Smouse, 2012)**, and by applying permuting the data 999 times, the significance of the obtained results was tested.

Table 1. List of 13 SSR markers used in the tilapia genotyping and their sequence information

Marker	Linkage Group	Forward sequence (5´-3´)	Reverse sequence (3´-5´)	Product length (bp)	Tm °C
OMO160	2	AGGATTTCCTGAAAGTGTTTTT	ACTCTACGTGACCTCTGACAATAG	210	55
OMO177	2	AGTGATGACCGGCCAGAAAGAGA	CAGGGATGGATAAACGTGACAATG	345	55
OMO238	8	ATATTACGTCCAAACATCCAGAGC	GAGCCAAAGGCAGAAGTAAACAGT	208	55
OMO312	8	ATAGTTTGGCAGGTCATTTTCAGA	GGGGTAGTTTTGTTGTGCTTTTT	345	55
GM104	8	ACTAACCCCTGCTCTGTGCTT	GAACCCAGCGATGTCCC	273	55
OMO341	18	TGGAGCTCTACTTTGCCCCTACTA	ACGCTATAGATGGACCCTGGATTT	369	60
OMO426	18	ATGCGTGGTTATTAGGTGTGGTAT	TAATAGGATCGGTGACTTCAAACA	143	55
UNH130	23	AGGAAGAATAGCATGTAGCAAGTA	GTGTGATAAATAAAGAGGCAGAAA	191	58
UNH879	23	GCATAAGGTGACTGGCTGGT	ACAAAGGGGTCCTGCAATTT	202-224	56
UNH848	23	TCCCCCGTAATAAATTAAACCA	GCCTGTGAATAACAATGTATTTCCT	199	54
UNH898	23	GATGTCCCCACAAGGTATGAA	TAATCCACTCACCCCGTTTC	237	56
UNH907	23	CAGGACCGACTCTGCAAGAT	GAGCTCTTTTGTTGTTCAAAATC	124	55
UNH183	Not mapped	CTTTCAGGCTGTGTGTT	CCTCACTTGGCGTTTAC	196	50

RESULTS AND DISCUSSION

1. Statistical analysis of SSRs
1.1. Genetic diversity assessment

Thirteen microsatellite markers were assessed in the two sampled Nile tilapia categories. Two of the investigated markers showed monomorphic patterns, GM104 and OMO341, and hence were removed from further analyses. The studied markers showed similar degrees of polymorphism within the two fish categories. The number of alleles per locus was between two and three in the large fish category, while the same estimate ranged between one and five in the small fish category. The microsatellite diversity estimates for the 11 SSR markers in the large and small Nile tilapia categories are presented in Table (2).

Table 2. Microsatellite diversity estimates for 11 SSR markers in two categories of the Nile tilapia

Category	Locus	N	N_a	N_e	I	H_o	H_e	F_{IS}
	OMO160	28	2	1.96	0.68	0.00	0.49	1.00
	OMO177	30	2	1.99	0.69	0.00	0.50	1.00
	OMO238	36	2	1.12	0.21	0.11	0.10	-0.06
	OMO312	32	2	1.82	0.64	0.19	0.45	0.58
	OMO426	36	3	2.22	0.87	0.00	0.55	1.00
Large	UNH130	34	3	2.32	0.96	0.47	0.57	0.17
	UNH879	28	3	1.84	0.74	0.29	0.46	0.37
	UNH848	30	2	1.80	0.64	0.00	0.44	1.00
	UNH898	36	2	1.12	0.21	0.11	0.10	-0.06
	UNH907	36	2	1.53	0.53	0.00	0.35	1.00
	UNH183	32	2	1.28	0.38	0.00	0.22	1.00
Average		32.545	2.273	1.727	0.595	0.106	0.385	
	OMO160	26	2	1.17	0.27	0.00	0.14	1.00
	OMO177	32	3	2.17	0.86	0.00	0.54	1.00
	OMO238	30	3	1.74	0.76	0.00	0.43	1.00
	OMO312	28	2	1.15	0.26	0.00	0.13	1.00
	OMO426	32	5	2.98	1.30	0.00	0.66	1.00
Small	UNH130	28	3	2.18	0.88	0.00	0.54	1.00
	UNH879	24	4	1.56	0.73	0.08	0.36	0.77
	UNH848	32	1	1.00	0.00	0.00	0.00	nd[1]
	UNH898	34	2	1.84	0.65	0.24	0.46	0.48
	UNH907	24	2	1.60	0.56	0.00	0.38	1.00
	UNH183	30	2	1.64	0.58	0.00	0.39	1.00
Average		29.091	2.636	1.730	0.623	0.029	0.366	

N: number of genotypes, N_a: number of alleles, N_e: effective number of alleles, I: Shannon's information index, H_o: observed heterozygosity, H_e: unbiased estimate of expected heterozygosity (**Nei, 1987**), F_{IS}: fixation index measuring the correlation of alleles within individuals relative to that within categories, [1]: not defined.

The average number of alleles per locus in the large and small populations was 2.2 and 2.6, respectively. Among the studied markers, OMO426 had the greatest number of alleles per locus (i.e., 5) in the small fish category, while UNH848 had only one allele in the same fish category. In the large fish category, the studied markers could be classified into two categories. The first category included markers with two alleles per locus (i.e., OMO160, OMO177, OMO238, OMO312, UNH848, UNH898, UNH907, and UNH183), while the second category included markers with three alleles per locus (OMO426, UNH130, and UNH879). In general, the Shannon information indices (I) of the studied fish groups for the studied markers were relatively low to moderate, except for that of UNH130, which was 0.96 and 1.30 for large and small fish categories, respectively. A similar trend of low I was also observed in twelve *Tilapia guineensis* populations using eight SSRs **(Ukenye *et al*., 2016)** and in a study of the wild and farmed tilapia using eight SSRs **(Ukenye *et al*., 2023)**. Across all markers, a highly substantial heterozygote deficiency pattern was consistently present. The studied markers showed a relatively low observed heterozygosity (H_o) in the large versus small fish category (Table 3).

Table 3. Average microsatellite polymorphisms in two categories of the Nile tilapia. All standard errors are displayed in parenthesis unless otherwise specified

Data set	N[a]	A[b]	$r(10)$[c]	H_o[d]	H_e[e]	F_{IS}[g]
All categories	74	2.45	2.23(0.05)	0.057(0.03)	0.43(0.06)	0.815*
Large	36	2.27	1.86(0.03)	0.090(0.04)	0.33(0.06)	0.732*
Small	38	2.63	2.00(0.06)	0.025(0.02)	0.32(0.06)	0.923*

[a]N: number of genotypes, [b]A: average number of alleles per locus detected in each group, [c]$r(10)$: allelic reaches, defined as the number of alleles that would have been detected if 10 alleles (i.e., 5 fishes: rarefaction cutoff) had been sampled in each population, [d] H_o: observed heterozygosity, [e]H_e: unbiased estimate of expected heterozygosity **(Nei, 1987)**, [f] I: Shannon's information index, [g] F_{IS}: fixation index measuring the correlation of alleles within individuals relative to that within populations, [*]Not in Hardy–Weinberg equilibrium at $P<0.001$.

The observed heterozygosity (H_o) in the large group ranged between 0 and 0.47, with an average of 0.10, while the H_o in the small group ranged between 0 and 0.24, with an average of 0.026. Significant departure from the Hardy-Weinberg equilibrium was observed in the markers under study ($P<0.001$). The average polymorphism estimates showed a significant heterozygote deficiency, $F_{IS} = 0.732$ and 0.923 in the large and small groups, respectively. In general, the small group showed greater inbreeding than did the large group. The observed pattern of low heterozygosity (H_o) compared to the expected heterozygosity (H_e) reflects a significant heterozygote deficiency, a phenomenon common in the Nile tilapia farms since only a few genotypes with favorable growth traits are kept as parents for subsequent generations.

1.2. Private alleles frequency

Among the studied markers, only seven showed private allele(s) relative to small and/or large fish categories (Fig.1).
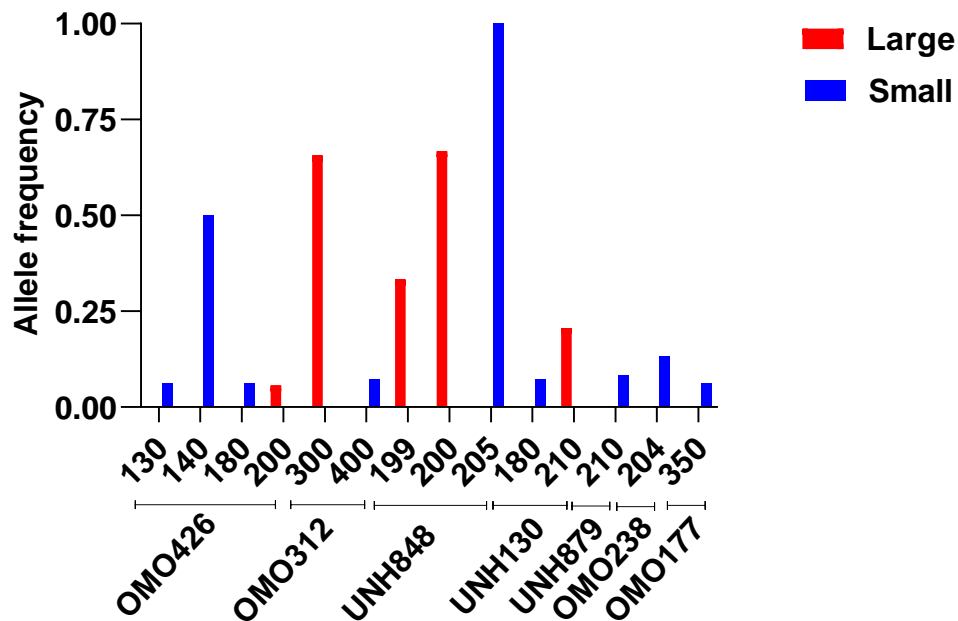
Fig.1. Private alleles unique to the large and small Nile tilapia categories and its associated frequencies at seven SSR loci

In the large population, OMO426 had one private of 200bp, while it had three alleles specific to the small population (130, 140, and 180bp). Additionally, OMO312 had one allele (300bp) specific to a large population and one allele (400bp) specific to a small population. UNH848 had two alleles specific to large populations (199 and 200bp), while only one allele (205bp) was specific to a small population. UNH130 had one allele specific to large population (210bp) and one allele specific to small population (180bp). The three markers UNH879, OMO238, and OMO177 showed private alleles present only in small populations with molecular weights of 210, 204, and 350bp, respectively. The presence of specific alleles could serve as specific markers related to desired genotypes. These markers allow early-stage screening, consequently eliminating undesirable genotypes as early as possible.

1.3. Differential capability of SSRs

Differential potential is an important aspect when selecting DNA markers for diversity assessment. Different estimates were used to address the ability of the SSR markers to differentiate among genotypes. The studied markers showed low to moderate gene diversity (H), which ranged between 0.25 and 0.48, with an average of 41 (Table 4).

Table 4. Differentiation potential of 11 microsatellites in the two Nile tilapia categories

Marker	H	PIC	D
OMO160	0.463	0.302	0.868
OMO177	0.403	0.328	0.923
OMO238	0.432	0.316	0.902
OMO312	0.418	0.322	0.913
OMO426	0.259	0.376	0.977
UNH130	0.378	0.338	0.936
UNH879	0.331	0.354	0.957
UNH848	0.403	0.328	0.923
UNH898	0.496	0.286	0.702
UNH907	0.482	0.293	0.837
UNH183	0.487	0.291	0.826
Average	0.414	0.321	0.888

H: gene diversity, *PIC*: polymorphism information content,
D: discriminating power.

Eight markers, OMO177, UNH848, OMO312, OMO238, OMO160, UNH907, UNH183, and UNH898, exhibited $H \geq 0.40$, while three markers, OMO426, UNH879, and UNH130, exhibited $H \leq 0.40$. The polymorphic information content (*PIC*) ranged between 0.286 for UNH898 and 0.376 for OMO426, with an average of 0.321. Additionally, the discriminating power(*D*) ranged between 0.702 for UNH898 and 0.977 for OMO426, with an average of 0.888.

The differentiation potential of the studied loci was moderate with respect to gene diversity (*H*) and polymorphic information content ($0.25 \geq PIC \leq 0.50$)(**Botstein *et al*., 1980**). The observed pattern of moderately informative SSR loci was also reported in the wild and farmed *tilapia* (average *PIC* = 0.390)(**Ukenye*et al*., 2023**). Additionally, comparable results were reported in similar Cichlidae species, *Geophagus brasiliensis* (average *PIC* = 0.502) (**Ferreira *et al*., 2013**), and in the crayfish *Procambarus clarkia* (average *PIC* = 0.390) (**Sun *et al*., 2023**). On the other hand, the *PIC* values observed were lower than those obtained for 40 SSRs in the Nile tilapia populations in Eastern Africa (average *PIC* = 0.668) (**Tibihika *et al*., 2019**). The observed pattern of moderate *PIC* might be associated with the low genetic diversity in the two populations. The *PIC* evaluates the discriminatory ability of a genetic locus by considering both the number of alleles and their respective frequencies (**Serrote *et al*., 2020**).

On the other hand, the discrimination power of the studied loci was relatively high (*D* = 0.888) compared to that of SSRs in other fish species (*Poecilia vivipara*, *D* = 0.202 - 0.872) (**Tonhatti *et al*., 2014**). The use of different estimates to assess the differential potential of any genetic marker is crucial and provides a comprehensive understanding of the ability of a marker to determine the genetic diversity of a population. The studied markers showed a low gene diversity and moderate polymorphic information content; moreover, they showed an increased discriminating power. Although the genetic polymorphisms were relatively low and most likely associated with the low genetic diversity of the studied populations, the high discriminating power of the studied loci

could be utilized in marker comparisons and marker efficiency prediction when used in combination **(Tessier *et al.*, 1999)**.

1.4 Analysis of molecular variance (AMOVA)

AMOVA is considered a significant partitioning approach for genetic variation among and within populations. AMOVA revealed a greater level of genetic variation within rather than among populations (Table 5), which is in accordance with the results of the inbreeding coefficient (F_{IS}).

Table 5. Analysis of molecular variance (AMOVA) of the Nile tilapia categories using SSR

Source	df	Sum of squares	Mean squares	Estimated variance	Percent of variation	Fixation index	Value
Among categories	1	202.53	202.53	5.27	40		
Within categories	72	560.16	7.780	7.78	60	F_{ST}	0.404[*]
Total	73	762.70	210.31	13.04			

df; degrees of freedom.[*]P<0.05.

The presence of such differences is expected, as factors such as genetic drift, mutation, gene flow, and local adaptation within populations can contribute to the observed genetic diversity within populations. A similar trend of greater genetic variance within than among populations was also observed in the Nile tilapia populations in Brazil when 11 SSR markers were applied **(Da Silva *et al.*, 2020)**.

CONCLUSION

The current research emphasizes the significance of assessing SSRs in the Nile tilapia populations exhibiting varying growth performances. Variances in body length and weight between the two Nile tilapia populations corresponded with differences in allele frequency, suggesting a potential link between specific allele(s) and growth performance traits. Confirming such associations through an association genetics approach could streamline the early identification of desirable tilapia genotypes for aquaculture, facilitating the implementation of marker-assisted selection (MAS) programs.

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