The efficiency of microsatellite DNA markers for estimating genetic polymorphism in some Tilapia species

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Abstract: Microsatellite DNA markers were used to study the genetic diversity and characterizing some Tilapia fish species (*Oreochromis niloticus*, *O.aureus*, *Sarotherodon galilaeus* and *Tilapia zillii*). In addition, this study was designed to test the efficiency of such markers for estimating genetic diversity and reconstructing the phylogenetic relations among the applied Tilapia species. The allele numbers were ranged from seven (for GM538 locus in *O. niloticus* and *O. aureus*, UNH106 locus in *O.niloticus*, UNH123 locus in *O.aureus* and UNH995 locus in *O. aureus*) to zero (for UNH104, UNH185 and UNH995 loci in *T. zillii*). Most studied microsatellite loci were polymorphic. The highest similarity value was calculated between *O. niloticus* and *O. aureus*. The lowest similarity value was calculated between *O. niloticus* and *T. zillii* (0.131). The developed microsatellite markers were powerful tools to estimate the genetic diversity and detecting genetic polymorphism in the applied Tilapia fish species**.** The significance of these data is that they reflect and lead to new inferences regarding the culture methods used in managing these fish species in farms and nature.
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1. Introduction

Up to date, fish species identification fall into some categories such as separation of problematical species and accurate identification of early live stages. Generally, the genetic markers should be conducted to provide the information needed for a sound management of popular fish (such as Tilapia) farming and wild fish stocks (Saad *et al.,* 2009 and Saad *et al*., 2012).

Tilapia is the common name for three fish genera: *Oreochromis*, *Sarotherodon* and *Tilapia* (Trewaves, 1983).

Different molecular tagging methods (Rashed *et al.,* 2011 and Saad *et al.,* 2012) have been designed to study fish species characterization and population structure. Among these tagging methods, microsatellite or simple sequence repeats (SSR) markers are become accurate and convenient tools in studying genetic structure and phylogenetic relationships among fish species and sub species (Rashed *et al.,* 2009).

The use of molecular genetic techniques such as SSR in fisheries research was increased due to the increasing availability of such techniques in research labs. The objectives of this study are maximization the efficiency of microsatellite DNA markers for estimating genetic diversity, detecting polymorphism and reconstructing phylogenetic relationships among some Tilapia species (*O. niloticus, O. aureus, S. galilaeus and T. zillii*).

2. Material and Methods

Forty fish samples belong to four Tilapia species (*Oreochromis niloticus*, *O.aureus*, *Sarotherodon galilaeus* and *Tilapia zillii*). Samples were collected from NIOF (National Institute of Oceanography and Fisheries, Egypt) for DNA extraction, purification and molecular analysis. Ten fish samples were applied from each collected Tilapia species. DNA extraction and purification were carried out according to **Hillis (1996).**

The microsatellite loci were selected and originally developed by **Lee *et al*.**, (**2005**) except the UNH 207. The UNH 207 was selected from **Shirak *et al*.,** (2006). The primer codes, sequences and annealing temperature were presented in Table (1).

Each 10 L PCR reaction contained 100ng of template DNA, 0.3 L forward primer, 0.3 L reverse primer, 0.25mM dNTPs, 1.5mM MgCl2 1X Buffer L 0.5 U Taq polymerase. PCR program was designed as the following: 2 min initial 96^{o}C denaturation, 30 cycles of 94^{o}C for 30sec, 30sec at the appropriate annealing temperature (Table 1), and 30sec at 72^{o}C, followed by a 6 min extension at 72^{o}C.

Gel images which revealed via SSRs were analyzed using free software (*GelAnalyzer3*) to determine molecular sizes of the amplified fragments, their frequencies through samples, their polymorphism type either monomorphic or polymorphic, the mean of band frequency and the

polymorphism percentage for each SSR primer pairs.

Population genetic analysis:

For population genetic analysis, the *PopGene1.32* software was used to estimates genotypic and allele frequencies, polymorphism percentage, observed and expected heterozygosity.

Table (1): SSR primer code, sequences and annealing temperature.

Primer code	Sequence	Annealing temperature
GM211	Forward 5' GCAAGTTGAGAGGCTACTGT 3'	**65°C**
	Reverse 5' AAACAACCCACAACCTTAGTT 3'	
GM531	Forward 5' AAAGCCAACGGTCTGAATTG 3'	**65°C**
	Reverse 5' AGCAGAGGACACCCCTCAT 3'	
GM538	Forward 5' CAGCATGTTGTCTGGATCTTG 3'	**65°C**
	Reverse 5' TTTGTTGCTGTGGTCTGTTCTT 3'	
UNH104	Forward 5' GCAGTTATTTGTGGTCACTA 3'	**65°C**
	Reverse 5' GGTATATGTCTAACTGAAATCC 3'	
UNH106	Forward 5' CCTTCAGCATCCGTATAT 3'	**65°C**
	Reverse 5' GTCTCTTTCTCTCTGTCACAAG 3'	
UNH123	Forward 5' CATCATCACAGACAGATTAGA 3'	**65°C**
	Reverse 5' GATTGAGATTTCATTCAAG 3'	
UNH146	Forward 5' CCACTCTGCCTGCCCTCTAT 3'	**65°C**
	Reverse 5' AGCTGCGTCAAACTCTCAAAAG 3'	
UNH185	Forward 5' CAGACACACTAGACACATTCTA 3'	**55°C**
	Reverse 5' GTGTTTCCATGTGTCTGTAC 3'	
UNH207	Forward 5' ACACAACAAGCAGATGGAGAC 3'	**55°C**
	Reverse 5' CAGGTGTGCAAGCAGAAGC 3'	
UNH995	Forward 5' CCAGCCCTCTGCATAAAGAC 3'	**65°C**
	Reverse 5' GCAGCACAACCACAGTGCTA 3'	

3. Results

Ten SSR primer pairs (Table 1) were used to characterize the four applied Tilapia species (*O. niloticus*, *O. aureus*, *S. galilaeus* and *T. zillii*).

The number of alleles generated by the SSR primers:

The number of alleles through the ten SSR loci ranged from seven (for GM538 locus in *O. niloticus* and *O. aureus*, UNH106 locus in *O. niloticus*, UNH123 locus in *O. aureus* and UNH995 locus in *O. aureus*) to zero alleles (for UNH104, UNH185 and UNH995 loci in *T. zillii*). The highest number of alleles across the four applied Tilapia species was detected in the locus GM538 while the lowest number was detected in UNH185 locus (Fig.1).

Estimating allele frequencies generated by each SSR primer:

The averages of allele frequencies values were calculated and presented in Table (2). These values were ranged from 0.143 to 0.333 in both *O. niloticus* and *O. aureus*. On the other hand, averages of allele frequencies were ranged from 0.2 to 0.5 and from 0.148 to 0.333 in *S. galilaeus* and in *T. zillii* respectively.

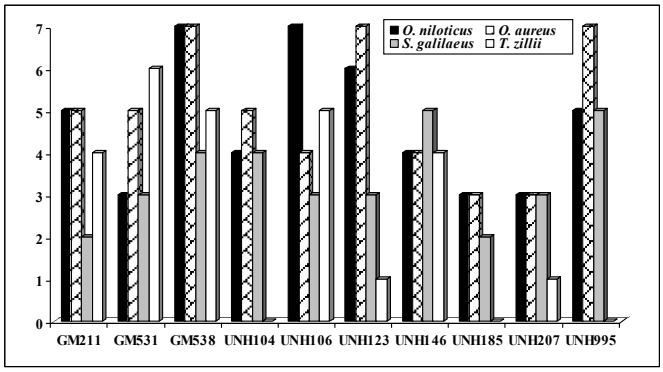

Fig. (1): Number of alleles which generated by SSR primer pairs.

Table (2): Average of allele frequencies ± standard error generated by each SSR primer pairs for each studied Tilapia species.

Primer code	Tilapia species			
	O. niloticus	***O. aureus***	***S. galilaeus***	***T. zillii***
GM211	0.200±0.045	0.200±0.045	0.50±0.000	0.250±0.036
GM531	0.315±0.098	0.200±0.069	0.333±0.140	0.148±0.031
GM538	0.143±0.029	0.143±0.038	0.250±0.080	0.200±0.045
UNH104	0.250±0.048	0.200±0.065	0.250±0.066	0.250±0.048
UNH106	0.143±0.047	0.250±0.123	0.333±0.111	0.200±0.054
UNH123	0.167± 0.045	0.143±0.038	0.333±0.195	0.167±0.045
UNH146	0.250±0.073	0.250±0.048	0.200±0.045	0.250±0.036
UNH185	0.333±0.064	0.333±0.0147	0.50±0.313	0.333±0.064
UNH207	0.333±0.163	0.333±0.278	0.333±0.150	0.333±0.163
UNH995	0.200±0.038	0.143±0.038	0.20±0.052	0.200±0.038

Microsatellite polymorphism:

All the ten microsatellite loci were polymorphic in the four applied Tilapia species except the locus GM211 (in *S. galilaeus*) which showed only two alleles in all *S. galilaeus* samples.

Three microsatellite loci (UNH104, UNH185 and UNH995) were totally absent in the *T. zillii* samples.

Microsatellite heterozygosity:

The observed heterozygosity values were higher than expected values in most estimated microsatellite loci (Table3). The observed heterozygosity values were higher than the expected values in all *O. niloticus* microsatellite loci except three of them (GM211, GM531 and UNH207).

Half of the tested SSR loci in *O. aureus* (GM211, GM531, GM538, UNH123 and UNH146) showed observed heterozygosity equal 1.

The observed heterozygosity values were higher than those of expected heterozygosity values in all estimated SSR loci in *S. galilaeus*. Both the observed and expected heterozygosity values for five SSR loci (UNH104, UNH123, UNH185, UNH207 and UNH995) were equal (0) in *T. zillii*.

Similarity values within each studied Tilapia species:

The similarity value within each applied Tilapia species was estimated. The averages of these values were calculated. The lowest average of similarity values (0.405±0.045) was detected within *T. zillii* samples. The highest average similarity values was calculated within *S. galilaeus* samples (0.632±0.022). The average of similarity values within *O.niloticus* was 0.440±0.026 whereas average similarity in *O.aureus* was 0.496±0.028.

Phylogenetic relationships among the applied Tilapia species:

The similarity values (Table 4) and phylogenetic relations (Fig.2) among the four studied Tilapia species were estimated. The highest similarity value was observed between *O. niloticus* and *O. aureus* (0.344). The lowest similarity value was observed between *O. niloticus* and *T. zillii* (0.131).

Table (3): Observed (H_o) and expected (H_e) heterozygosity for the four Tilapia species across the ten estimated microsatellite loci.

Locus	***O. niloticus***		***O. aureus***		***S. galilaeus***		***T. zillii***	
	H_o	**H_e**	**H_o**	**H_e**	**H_o**	**H_e**	**H_o**	**H_e**
GM211	0.667	0.804	1.000	0.804	1.000	0.529	0.889	0.778
GM531	0.556	0.712	1.000	0.745	0.778	0.582	1.000	0.876
GM538	1.000	0.869	1.000	0.843	0.889	0.712	1.000	0.804
UNH104	0.889	0.765	0.889	0.758	0.889	0.739	0.000	0.000
UNH106	1.000	0.811	0.778	0.601	0.889	0.628	0.889	0.784
UNH123	1.000	0.825	1.000	0.843	0.556	0.464	0.000	0.000
UNH146	0.889	0.726	1.000	0.765	1.000	0.804	1.000	0.778
UNH185	0.889	0.680	0.778	0.569	0.375	0.325	0.000	0.000
UNH207	0.500	0.542	0.222	0.216	0.750	0.567	0.000	0.000
UNH995	1.000	0.817	0.556	0.843	0.889	0.791	0.000	0.000

Table (4): Similarity values within each studied Tilapia sp. (diagonal) and among the studied Tilapia species (under diagonal) based on the DNA microsatellite polymorphism.

	O. niloticus	***O. aureus***	***S. galilaeus***	***T. zillii***
O. niloticus	**0.440**			
O. aureus	0.344	**0.496**		
S. galilaeus	0.206	0.303	**0.63**	
T. zillii	0.131	0.245	0.133	**0.40**

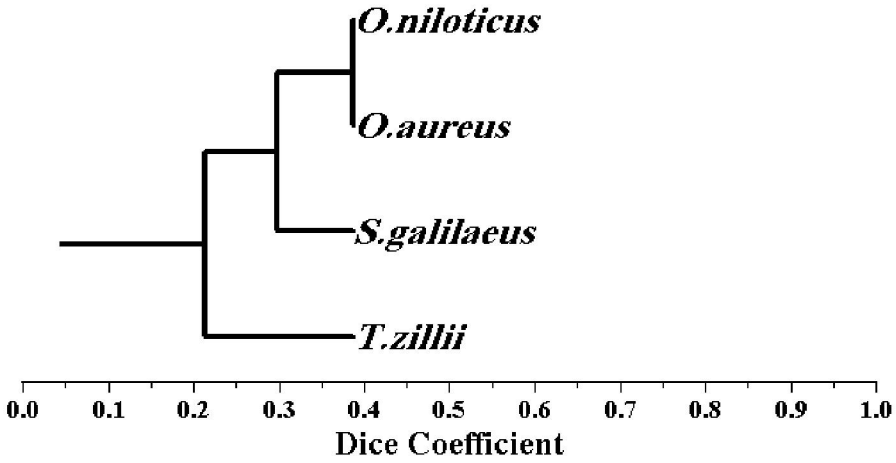

Fig. (2)*: NTSYSpc2.01b* dendrogram represents the phylogenetic relationships among the four Tilapia species based on microsatellite polymorphism.

4. Discussion

The future of Tilapia stock management (documentation, characterization, evaluation and utilization of Tilapia genetic resources) is depend on appropriate stock choice, development of sound management techniques and selective breeding (**Appleyard** and **Mather, 2000 and Rashed *et al.*, 2009**).

In the present study, ten SSR primer pairs were used to characterize four applied Tilapia species (*O. niloticus*, *O. aureus*, *S. galilaeus* and *T. zillii*).

The application of DNA markers has allowed rapid progress in aquaculture investigations of genetic variations and inbreeding, parentage assignments (**Rashed *et al.*, 2009**), species (**Saad *et al.*, 2012**) and strain identification (**Liu and Cordes, 2004** and **Saad *et al.*, 2011**).

In the present study, the number of alleles through estimated SSR loci ranged from seven (for GM538 locus in *O. niloticus* and *O. aureus*, UNH106 locus in *O. niloticus*, UNH123 locus in *O. aureus* and UNH995 locus in *O. aureus*) to zero alleles (for UNH104, UNH185 and UNH995 loci in *T. zillii*). All the ten microsatellite loci were polymorphic in the four Tilapia species except the locus GM211 in *S. galilaeus*. Also, *T. zillii* samples were totally absent in three microsatellite loci (UNH104, UNH185 and UNH995). DNA microsatellites are valuable markers for fish stock discrimination (**Rashed *et al.*, 2009**) due to the high polymorphism levels compared with other genetic markers such as RAPD (**Rashed *et al.*, 2008 and Saad *et al.*, 2011**) and ISSR (**Saad *et al.*, 2012a and Saad *et al.*, 2012b**).

In the present study, the observed heterozygosity was higher than the expected one in all estimated microsatellite loci for *O. niloticus* except three of them (GM211, GM531 and UNH207).The majority of molecular variants that become polymorphic are affected primarily by the interplay of **mutation** (generate new variation) and **genetic drift** leading to the eventual fixation of these variants" **(Hedrick 2000).**

Saad *et al.* (2012b) used Inter-simple sequence repeat (ISSR) analysis to develop fish species-specific molecular markers for the same estimated Tilapia species (*O.niloticus*, *O.aureus*, *Tilapia zillii* and *S.galilaeus*). The ISSR primers were tested to assess the effectiveness of ISSR analysis in discriminating among the four applied fish species. The Phylogenetic relationships among these fish species were reconstructed using deferent methods (Sokal & Sneath, Dice and Simple match coefficients). The highest genetic distance value was observed (by **Saad *et al.*, 2012b**) between *O. aureus* and *T.zillii*. In contrary, the lowest genetic distance value was observed between *O.niloticus* and

O.aureus. The same conclusion was revealed from SSR data analysis in the present study. We found that, the highest similarity value was observed between *O. niloticus* and *O.aureus*. The lowest similarity value was observed between *O. niloticus* and *T. zillii*. Generally, the similarity values were small due to the high polymorphism nature of the microsatellite loci.

In the present study nine microsatellite primer pairs were selected and originally developed by **Lee *et al.*, (2005**). They developed the second-generation genetic linkage map of Tilapia (*Oreochromis niloticus* and *Oreochromis aureus*). These primers were tested for matching with another Tilapia species (*T.zillii* and *S. galilaeus*). The tenth primer pair was chosen from **Shirak *et al.*, (2006).** They tested this this primer pair with *O.aureus* genome.

Microsatellite variation at most estimated loci (In the present study) was more informative in characterizing Tilapia species (*O.niloticus, O.aureus and S. galilaeus*) differences than the ISSR markers (estimated by **Saad *et al.*, 2012b**). On the other hand, ISSR markers (Dominant markers) were an attractive tool for general characterization of all studied Tilapia species.

SSR analysis was an efficient tool for Tilapia species identification and characterization because SSR markers are considered co dominant markers, species and sub species reporter. Microsatellite DNA markers coupled with appropriate statistical analyses are recommended in fish species identification and classification. The significance of these data is that they reflect and lead to new inferences regarding the characterization methods used in managing these fish species in farms and nature.

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