Genetic Diversity among Some Tilapia species Based on ISSR Markers

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Abstract: Inter-simple sequence repeat (ISSR) analysis was used to develop fish species-specific molecular markers for four Tilapia species (*Oreochromis niloticus, Oreochromis aureus, Tilapia zillii and Sarotherodon galilaeus*). 12 ISSR primers were tested to assess the effectiveness of ISSR analysis in discriminating among the four applied fish species. Some ISSR markers were detected as species-specific for *O. niloticus*, *O. aureus*, *S. galilaeus* and *T. zillii* species. The Phylogenetic relationships among applied fish species were reconstructed using deferent methods (Sokal & Sneath, Dice and Simple match coefficients). The percentages of polymorphism were ranged from 0% to 67% within *S. galilaeus*, while polymorphism values were ranged from 0% to 100% in the other studied fish species. The highest genetic dissimilarity value was observed between *O. aureus* and *T.zillii*. In contrary, the lowest dissimilarity value was observed between *O.niloticus* and *O. aureus*. ISSR analysis was an attractive tool for species identification. These markers were recommending when coupled with appropriate statistical analyses in fish species identification and classification.

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1. Introduction

Genetic markers are fundamental tools for monitoring fish populations (Rashed *et al.,* 2008) and fish species genetic variability (Saad *et al*., 2009). Genetic markers should be conducted to provide the information needed for a sound management of farming fish (such as Tilapia fish) and wild fish stocks (Saad *et al*., 2009). This way will be useful, especially in fish breeding programs which use genetic markers as marker-assisted selection to improve the fish performance and/or economic traits (Rashed *et al*., 2009).

Tilapias have become the most important fish species for freshwater aquaculture in tropics and subtropics areas because of the relative ease of culture in variety of aquaculture systems and their favorable attributes as fish food (EL-Tawil, 1984; Ladewig and Shwantes, 1984; Bezrukov, 1987 and Rashed *et al*., 1998).

Inter-simple sequence repeat (ISSR) as a molecular technique does not require the knowledge of flanking sequences (Zietkiewicz *et al*. 1994). This technique uses a single primer containing the repetitive sequence of a microsatellite. The amplified DNA segments include the nucleotide sequence situated between two microsatellites blocks, yielding a multilocus marker system useful for genetic diversity analysis (Maltagliati *et al.,* 2006). The variable lengths of these amplified DNA sequences allow for the identification of differences between closely related species, although they reveal little information about the genetic variability within a single species (Reddy *et al.,* 2002).

ISSR analysis has been successfully applied in fish gene tagging (Ammiraju *et al.,* 2001), variety fingerprinting or genetic diversity analysis (Archak *et al*., 2003). This analysis is widely used for studying the genetic background of some plants (Blair *et al*., 1999), some animal such (Kol and Lazebny, 2006) and fish such as *Paralichthys* sp. (liu *et al.,* 2006).

The present study aims to assess the molecular genetic variability among four Tilapia species (*O. niloticus, O. aureus, T. zillii* and *S. galilaeus*) using ISSR analysis. In addition this technique was used to detect some Tilapia species-specific DNA markers. ISSR markers will be useful, especially in Tilapia breeding programs which use genetic markers as marker-assisted selection to improve the Tilapia performance and/or economic traits.

2. Material and Methods

Tilapia zillii (Z), *Sarotherodon galilaeus* (G), *Oreochromis niloticus* (N) and *Oreochromis aureus* (A) species individuals were collected based on their morphological characterization (Trewavas, 1983) from an Egyptian fish farm (National Institute of Oceanography and fisheries, EL-Qanatair EL-Khairia station, Egypt). Ten fish individuals were sampled from each collected tilapia species.

From each specimen, approximately 1 x 1 cm of caudal fin tissue was excised, placed in a 70 %

isopropanol and held at 4°C for subsequent DNA extraction. DNA extraction and purification were performed according to (Hills *et al*., 1996).

Twelve ISSR primers (Table 1) were originally selected (Biotechnology Laboratory, University of British Columbia, primer set 9) to measure the genetic variability among applied Tilapia species.

PCR reaction was prepared in a 10 µl contained a 50 ng of DNA, a 0.3 µM of primer, a 0.2 mM of dNTPs, a 25 mM of $MgCl_2$, a 0.5 unit of Taq polymerase and a 1 X buffer. PCR program was consisted of one cycle for 2 min. at 94°C, 30 cycles for (30 sec. at 94°C, 45 sec. at 44°C & 1.5 min. at 72°C) and one cycle for 10 min. at 72°C.

Gel images were analyzed using *GelAnalyzer3* software to determine molecular sizes, presence (1) or absence (0) in addition, frequencies and polymorphism type of the amplified fragments, the mean of band frequency and the polymorphism percentage for each primer were calculated.

Data analysis:

Data were analyzed as reported by Rashed et al., (2011). *NTSYSpc2.01b* and *SPSS* (10, and 15) software were used to estimate the similarity percentages between the four Tilapia species and reconstructing the phylogenetic relationships using Sokal & Sneath, Dice and Simple match coefficients (Dice, 1945, Nei and Li 1979 and Rohlf 1997 .

3.Results

ISSR analysis was successfully used to examine the genetic polymorphism, to detect species-specific ISSR markers and to determine the variability among the four applied Tilapia species.

The Molecular sizes (bp) of the specific ISSR markers for the four applied Tilapia species were presented in Table (2). *O. niloticus* species had eight specific ISSR markers at molecular size (MS) ranged from 1560 bp (814A primer) to 240 bp (HB10 primer). *O. aureus* species had eight specific ISSR markers at MS ranged from 1500 bp (814A primer) to 390 bp (HB13 primer). In addition, *S. galilaeus* species had ten specific ISSR markers at MS ranged from 1450 bp (814A primer) to 420 bp (HB11 primer). Finally, *T. zillii* had five specific ISSR markers at MS ranged from 1350 bp (814A primer) to 340 bp (HB15 primer).

A total of 2426 detected bands were produced (628, 603, 638 and 557 bands were produced in the species of *O. niloticus*, *O. aureus*, *S. galilaeus* and *T. zillii*, respectively). The number of bands with different molecular sizes which amplified by the 12 tested ISSR primers were ranged from 10 (HB12 in *T. zillii*) to three (HB13 in *O. niloticus* and *O. aureus* and HB15 in *S. galilaeus*, *O. niloticus* and *O. aureus*) with the mean of six bands per primer. The numbers of detected ISSR bands were varied among the four studied tilapia species. Bands were ranged from eight to three bands for *O. niloticus*, *O. aureus* and *S. galilaeus* species and from ten to four bands for *T. zillii* species (Table 3).

Table (1): ISSR primer names and sequences

Code	Sequence	Code	Sequence
814A	5' $[CT]_8$TG 3'	17899A	5' $[CA]_6$AG 3'
844A	5' $[CT]_8$AC 3'	HB10	5' $[GA]_6$CC 3'
844B	5' $[CT]_8$GC 3'	HB11	5' $[GT]_6$CC 3'
17898A	5' $[CA]_6$AC 3'	HB12	5' $[CAC]_3$GC 3'
17898B	5' $[CA]_6$GT 3'	HB13	5' $[GAG]_3$GC 3'
HB14	5' $[CTC]_3$GC 3'	HB15	5' $[GTG]_3$GC 3'

Table 2. The Molecular sizes (bp) of the specific ISSR markers for the four studied tilapia species.

O. niloticus		*O. aureus*		*S. galilaeus*		*T. zillii*	
Primer	bp	Primer	bp	Primer	bp	Primer	bp
814A	1560	814A	1500	814A	1450	814A	1350
844A	590	844A	770	814A	730	814A	690
844A	460	844A	550	844A	710	844A	1530
17898A	450	844A	410	844B	870	HB11	940
17898A	330	844B	540	844B	500	HB15	340
HB10	240	17898A	410	17898A	940		
HB11	1090	HB10	500	HB10	1010		
HB13	340	HB13	390	HB11	420		
				HB14	770		
				HB14	470		

* N, A, G & Z refer to *O. niloticus*, *O. aureus*, *S. galilaeus* and *T. zillii*, respectively.

The highest polymorphism percentages were detected in *T. zillii*. Within this species, five ISSR primers (844B, 17898A, 17898B, 17899A and HB14) generated a 100% of polymorphism (Table 3).

Table (3): Average of band frequencies (A.B.F), polymorphism percentages (P%) and number of detected ISSR bands (n) within each studied tilapia species.

Primer code	*O. niloticus*			*O. aureus*			*S. galilaeus*			*T. zillii*		
	A.B.F.	P%	n	A.B.F.	P%	n	A.B.F.	P%	n	A.B.F.	P%	n
814A	0.86	20	5	0.84	20	5	1.00	0	5	0.92	20	5
844A	0.96	20	5	0.80	40	4	0.83	33	6	1.00	0	5
844B	0.90	40	5	0.84	60	5	0.98	17	6	0.65	100	4
17898A	1.00	0	4	1.00	0	4	0.88	20	5	0.80	100	4
17898B	1.00	0	5	1.00	0	4	0.98	20	5	0.68	100	6
17899A	0.95	25	8	0.95	13	8	1.00	0	4	0.80	100	5
HB10	0.95	13	8	0.82	44	8	0.96	33	8	0.82	56	9
HB11	0.97	17	6	0.98	17	6	0.97	29	7	0.90	20	5
HB12	0.90	100	7	0.87	100	7	0.96	13	8	0.74	80	10
HB13	1.00	100	3	1.00	100	3	0.74	60	5	0.83	50	4
HB14	0.99	14	7	0.99	14	7	0.93	29	7	0.86	100	8
HB15	1.00	0	3	1.00	0	3	0.73	67	3	0.68	75	4
Mean ± Se	0.96 ± 0.01	29 ± 10	6 ± 0.5	0.92 ± 0.02	34 ± 10	5 ± 0.5	0.91 ± 0.03	27 ± 5.9	6 ± 0.45	0.81 ± 0.03	67 ± 11	6 ± 0.6

The Similarity mean and standard error (Se) values were calculated within the studied Tilapia species based on the 12 ISSR markers via the three similarity coefficients Table (4). Relatively, the lowest values were detected within *T. zillii.*

Table (4) Similarity mean and standard error (Se) values within the studied tilapia species based on the 12 ISSR markers via the three similarity coefficients.

Tilapia species	Dice (Nei and Li)		Simple matching		Sokal and Sneath I	
	Mean	Se	Mean	Se	Mean	Se
O. niloticus	0.961	0.004	0.966	0.003	0.982	0.002
O. aureus	0.984	0.004	0.955	0.003	0.977	0.002
S. galilaeus	0.951	0.004	0.955	0.003	0.977	0.002
T. zillii	0.830	0.019	0.875	0.012	0.931	0.007

The highest genetic dissimilarity value was detected between *O. aureus* and *T.zillii* species. In contrary, the lowest dissimilarity value was determined between *O.niloticus* and *O. aureus* species (Table 5). The dendrogram (Figure1) showed the genetic relationships among the four applied Tilapia species based on ISSR analysis. However, the lowest genetic distance was noticed between *O. niloticus* and *O. aureus* species.

Table (5): Similarity values among the studied tilapia species based on the 12 ISSR markers via the three similarity coefficients.

	Dice (Nei and Li)	Simple matching	Sokal and Sneath I
O. niloticus & *O. aureus*	0.666	0.704	0.827
O. niloticus & *S. galilaeus*	0.496	0.541	0.702
O. niloticus & *T. zillii*	0.354	0.450	0.620
O. aureus & *S. galilaeus*	0.471	0.527	0.691
O. aureus & *T. zillii*	0.324	0.436	0.607
S. galilaeus & *T. zillii*	0.435	0.514	0.679

Comparing the results of SPSS10 and NTSYSpc2.01b, we found that the relationships between *O. niloticus* and *O. aureus* are slightly differed among the three used similarity coefficient and this appears in the NTSYSpc2.01b dendrograms but not appeared in the SPSS10 dendrograms where the same distance between *O. niloticus* and *O. aureus* although the different similarity measures. Also, the

distance between the combined cluster of *O. niloticus*, *O. aureus* and *S. galilaeus* and *T. zillii* reflects the similarity between the four species in the case of NTSYSpc2.01b dendrogram but in the SPSS10 the dendrogram showed *T. zillii* distantly related from other applied fish species (Fig. 1). The same results were found using SPSS10, SPSS13 and SPSS15 versions. We suggest the use of NTSYSpc2.01b to deduce the phylogenetic relationships.

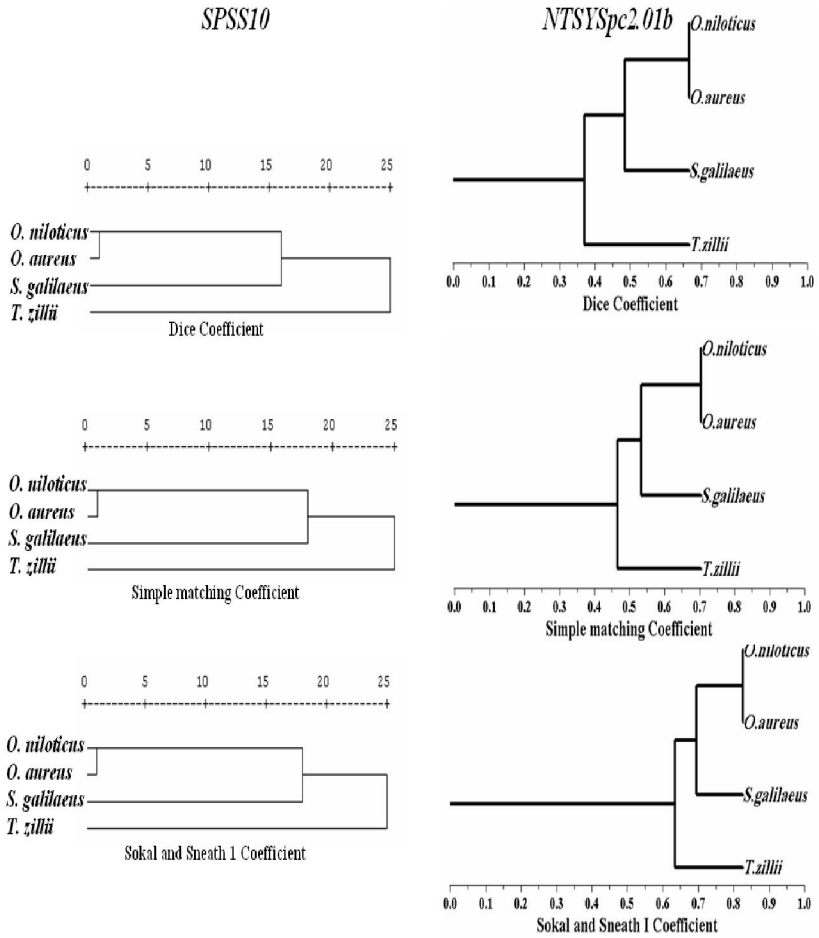

Figure 1. Dendrograms represent the comparison of *SPSS10* and *NTSYSpc2.01b* via three similarity coefficients in assessing the phylogenetic relationships using ISSR markers

4.Discussion

ISSR technique was used because it is simple and reliable tool for assessing the molecular genetic variability within and among many living organisms with highly reproducible results and abundant polymorphism (Kol and Lazebny, 2006 and Lalhruaitluanga and Prasad, 2009). Moreover, the potential applications of ISSR analysis for diverse aims is depend on the variety and frequencies of microsatellites within the specific genomes. (Chunjiang *et al*., 2005). In addition, variable ISSR patterns have potentials as dominant markers for studying genetic diversity of many fishes (Tong *et al*., 2005).

In the present study, ISSR analysis was offered some species-specific markers. The numbers of these molecular markers were varied from species to species. These DNA markers will be useful value, especially in fish breeding programs, which use genetic markers as marker-assisted selection to improve the fish performance (Rashed *et al.,* 2009).

The same idea was tested by Rashed *et al*., (2011). They used RAPD marker to detect the genetic variations among some tilapia species. They found that, the values of similarity among the four studied Tilapia species were high in Sokal and Sneath I, moderately in Simple matching and the low in Dice due to the use of shared present and absent fragments between each two estimated Tilapia species.

The molecular genetic markers are widely used to identify lines or strains, define stock diversity, monitor inbreeding, diagnose simply inherited traits

and even to improve stocks (Rashed *et al*., 2008 and 2009). The application of DNA-based genetic analysis as marker-assisted selection in fish research (such as tilapia) and stock development and management is still not fully maximized (Kocher *et al*., 1998 and Rashed *et al*., 2009).

ISSR analysis of the genetic diversity among the four applied Tilapia species showed that, the lowest genetic distance was noticed between *O. niloticus* and *O. aureus*. This conclusion was previously confirmed using another analysis such as RAPD. However, Saad *et al*. (2002) used bulked segregate analysis to reconstruct the phylogenetic relationships among three tilapia species (*O. niloticus, O. aureus* and *T. zillii*). They found that *T. zillii* species was distantly related from both *O.aureus* and *O.niloticus* species.

Liu *et al*., (2006) studied the genetic diversity in three *Paralichthys olivaceus* populations using ISSR analysis, which was confirmed to be a reproducible and sensitive tool for the study of population genetics of these fish. The genetic variability of domestic hatchery populations has implications to the conservation of natural *Paralichthys olivaceus* resources (Yun-Guo *et al.,* 2006).

The use of ISSR primers consisting of degenerate anchors or degenerate motifs increased the number of amplified markers. Since ISSR analysis is an easy to perform, high flow-through technique may represent it an alternative for the RAPD, better reproducibility was characterized due to the elevated annealing temperatures.

An especially attractive feature of ISSR analysis is its flexibility in terms of experimental design, where the number of generated amplicons may be optimized by changing the number of the core repeat units and anchoring bases (Liu and Wendel, 2001).

We suggest that ISSR analysis should be a standby choice for genome mapping or gene tagging and marker-assisted selection. For its high simplicity, ISSR analysis should be the first choice for genome mapping or gene tagging for organisms (which genomic knowledge is limited).

The above-mentioned exploitation and further studies would be significant for the basic and applied research on fisheries & aquaculture genetics and extend the knowledge of microsatellite conservation and evolution in Tilapia fish.

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