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Reconstruction of Phylogenetic Relations Among Four Tilapia Species

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Abstract: The objective of this study is to select the suitable method for reconstruction of phylogenetic relationships among four Tilapia species (*Oreochromis niloticus, Oreochromis aureus, Sarotherodon galilaeus and Tilapia zillii*). Data revealed from RAPD-PCR were analyzed to select the suitable method(s) for calculating the similarities and inferring the dissimilarities among applied Tilapia species. Similarity degrees within all studied Tilapia species were slightly different among the three the estimated equations (Dice, Sokal and Sneath and simple match coefficients). RAPD was an appropriate technique to differentiate among the four applied Tilapia species. The similarity values among the four studied Tilapia species were high in Sokal and Sneath I, moderately in Simple matching and the low in Dice coefficient.

Key words: Tilapia · Genetic diversity · RAPD-Markers · Phylogeny

INTRODUCTION

Determining true genetic dissimilarity between individuals is a decisive point for clustering and analyzing diversity within and among fish species, because different dissimilarity indices may yield conflicting outcomes. Generally, there is no rigorous well-founded solution in the case of dominant markers [1] such as RAPD markers.

Different measures were relevant to genetic markers [2] depending on the ploidy of organisms [1]. The Dice and simple match coefficients are commonly employed in the analyses of similarity for individuals in the absence of knowledge of ancestry of all individuals of fish species and sub species such as in Tilapia species.

Groups of research were carried out separately and independently to provide information regarding the discreteness of Tilapia fish stocks, establish genetic variation or relatedness of different Tilapia stocks [3-6] and elucidate evolutionary trends within the Tilapia genera [7, 8].

The application of DNA-based genetic analysis in Tilapia research, stock development and management is still not fully maximized. Such information is indeed valuable to the overall scientific study of Tilapia and to the management programs [9, 10] for its genetic resources necessary for farming, breeding and development of superior strains and breeds through marker-assisted selection [2].

The advantage of RAPD to generate fish molecular characterization is the production of molecular markers without any previous genome information on the target species. RAPD markers have been extremely useful in studies on population structuring for *O.niloticus* [2] and *Brycon hilarii* [11].

The main objective of this study is to select the suitable method for reconstruction of phylogenetic relationships among four Egyptian Tilapia species (*O. niloticus, O. aureus, S. galilaeus and T. zillii*).

MATERIALS AND METHODS

Forty fish samples belong to four Tilapia species (*O. niloticus, O. aureus, S. galilaeus and T. zillii*) 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 [12]. Eleven RAPD primers were used to study the genetic diversity within and among the four applied Tilapia species.

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Table 1: RAPD Primers used in the study and their sequences

code	sequence	code	sequence
OPA1	(5'CAGGCCCTTC3'),	OPA06	(5'GGTCCCTGAC3')
OPA14	(5'TCTGTGCTGG3')	OPA16	(5'AGCCAGCGAA3')
OPA17	(5'GACCGCTTGT3'),	OPA20	(5'GTTGCGATCC3')
OPB03	(5'CATCCCCCTG3')	OPB12	(5'CCTTGACGCA3')
OPC09	(5'CTCACCGTCC3')	OPC11	(5'AAAGCTGCGG 3')

The RAPD primer codes and sequences are presented in Table (1).

RAPD-PCR Reaction Mixture Was Carried out as the Following:

100 ng DNA, 0.4 μM Primer, 0.25 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 unit Taq polymerase and H2O (up to 10 μl).

The reaction conditions involved initial denaturation of DNA for 4 minutes at 94 °C, 30 cycles of 45 sec (denaturation) at 94° C, 45 sec (annealing) at 37° C, 45 sec (extension) at 72° C and one 10 min cycle at 72° C for final extension.

The amplification products were separated on 1.5 % agarose gels according to Rashed *et al.* [6] with some modifications. DNA Ladder (1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp) was used to estimate the molecular sizes of each generated RAPD bands.

Data Analysis: *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 and Sneath, Dice and Simple match coefficients.

RESULTS

Genetic Polymorphism Generated by RAPD Markers: A total of 2589 detected bands were generated by the 11 RAPD primers in all studied Tilapia species. The numbers of detected bands were 631, 643, 690 and 625 in *O. niloticus*, *O. aureus*, *S. galilaeus* and *T. zillii* respectively. The percentages of polymorphic RAPD bands were ranged from 0% to 100% in all studied Tilapia species (Table 2).

Table 2: Total number of bands, % of polymorphic bands and average of band frequencies generated by 11 RAPD primers in the four studied Tilapia species

Fish primer		*O. niloticus*	*O. aureus*	*S. galilaeus*	*T.zillii*
	TNB	60	60	46	62
Ao1	%PB	0%	0%	17%	33%
	ABF	1.00	1.00	0.66	0.78
	TNB	50	50	65	52
A06	%PB	0%	100%	33%	100%
	ABF	1.00	0.71	0.81	0.58
	TNB	65	56	60	56
A14	%PB	50%	50%	33%	78%
	ABF	0.93	0.80	0.86	0.80
	TNB	52	47	83	58
A16	%PB	86%	67%	67%	92%
	ABF	0.74	0.78	0.75	0.64
	TNB	74	60	56	40
A17	%PB	56%	40%	100%	75%
	ABF	0.93	0.67	0.80	0.67
	TNB	53	80	76	59
A20	%PB	88%	0%	20%	20%
	ABF	0.88	1.00	0.95	0.74
	TNB	60	89	80	69
Bo3	%PB	0%	20%	0%	90%
	ABF	1.00	0.99	1.00	0.77
	TNB	33	39	42	73
B12	%PB	100%	38%	22%	22%
	ABF	0.83	0.78	0.84	0.81
	TNB	59	42	54	47
C09	%PB	100%	50%	57%	50%
	ABF	0.98	0.84	0.77	0.78
	TNB	67	66	55	60
C11	%PB	14%	60%	50%	0%
	ABF	0.84	0.94	0.69	1.00
	TNB	58	54	73	49
C13	%PB	63%	88%	33%	60%
	ABF	0.64	0.77	0.91	0.61

TNB=Total number of detected bands, PB= polymorphic bands, ABF= average of band frequencies

Table 3: Range of detected molecular weight (RMW) generated by the RAPD primers and Tilapia species specific RAPD bands (sp. S.B)

sp. primer		*O. niloticus*	*O. aureus*	*S. galilæus*	*T.zilli*
Ao1	RMW (bp)	640-190	640-190	930-270	690-190
	sp. S. B	0	0	0	0
A06	RMW (bp)	910-210	1070-210	1380-250	2430-210
	sp. S. B	910 and 470	0	0	0
A14	RMW (bp)	670-200	1450-200	950-280	2130-280
	sp. S. B	0	0	0	0
A16	RMW (bp)	1780-260	1350-210	2570-260	1610-260
	sp. S. B	0	210	1060	0
A17	RMW (bp)	1190-260	1480-260	1190-310	1770-400
	sp. S. B	950	0	0	400
A20	RMW (bp)	1140-150	1070-150	1220-210	1960-210
	Sp. S. B	0	660	1220,1020 and 710	600
Bo3	RMW (bp)	1190-220	1120-220	1190-220	1340-290
	sp. S. B	0	870	320	0
B12	RMW (bp)	640-190	640-190	930-270	690-190
	sp. S. B	0	700, 390 and 150	1480, 1030 and 670	950 and 770
C09	RMW (bp)	1930-320	2110-320	1880-320	1680-320
	Sp. S. B	0	0	0	0
C11	RMW (bp)	860-140	950-220	1490-180	1060-220
	Sp. S. B	270	0	0	1060 and 670
C13	RMW (bp)	1250-150	1180-150	2080-150	2680-480
	sp. S. B	0	0	1330 and 600	0

RMW=Range of detected molecular weight and sp. S.B= species specific RAPD bands

Table 4: Average of similarity values and standard error (SE) within the studied Tilapia species based on the 11 RAPD markers via the three similarity coefficients

	Dice (Nei and Li)		Simple matching		Sokal and Sneath I	
Tilapia species	Mean	SE	Mean	SE	Mean	SE
O. niloticus	0.913	0.008	0.941	0.005	0.969	0.003
O. aureus	0.912	0.005	0.938	0.003	0.968	0.002
S. galilaeus	0.906	0.004	0.928	0.004	0.962	0.002
T. zillii	0.797	0.016	0.866	0.009	0.927	0.005

The averages of band frequencies were calculated for each tested RAPD primer in all studied fish species. The ranges of these values were (0.64 to 1), (0.67 to 1), (o.66 to 1) and (0.58 to 1) in *O. niloticus*, *O. aureus*, *S. galilaeus* and *T. zillii* respectively (Table 2).

Analysis of RAPD Markers for the Applied Tilapia Species: The ranges of RAPD markers were (140-1930bp), (150-2110bp), (150-2570bp) and (190-2680bp) in *O. niloticus*, *O. aureus*, *S. galilaeus* and *T. zillii* respectively. All tested RAPD primers generated 180 different loci. Some of them (26 loci) were detected in one species (Band frequency =1) and absent in the other studied Tilapia species (Band frequency =0). These loci are considered as species specific DNA markers. The 26 Tilapia species specific RAPD markers were analyzed. Six species specific bands were detected in both *O. aureus* and *T. zillii*. Four and ten species specific bands were detected in *O. niloticus* and *S. galilaeus* respectively. The molecular weights of these bands were presented in (Table 3).

Similarity Values Within Applied Tilapia Species: Similarity degrees within all studied Tilapia species were slightly different among the three estimated similarity equations as shown in Table (4). Similarity degrees within *O. niloticus* were almost the same of those of *O. aureus*. The highest similarity value within Tilapia species was for *O. niloticus* (0.913±0.008, 0.941±0.005 and 0.969±0.003 for Dice, Simple matching and Sokal and Sneath I, respectively) and *O. aureus* (0.912±0.005, 0.938±0.003 and 0.968±0.002 for Dice, Simple matching and Sokal and Sneath I, respectively.

Table 5: Similarity values among the studied Tilapia species based on RAPD markers via three similarity coefficients

	Dice (Nei and Li)	Simple matching	Sokal and Sneath I
O. niloticus and *O. aureus*	0.560	0.691	0.817
O. niloticus and *S. galilaeus*	0.395	0.559	0.717
O. niloticus and *T. zillii*	0.303	0.517	0.682
O. aureus and *S. galilaeus*	0.425	0.574	0.729
O. aureus and *T. zillii*	0.331	0.529	0.692
S. galilaeus and *T. zillii*	0.321	0.504	0.670

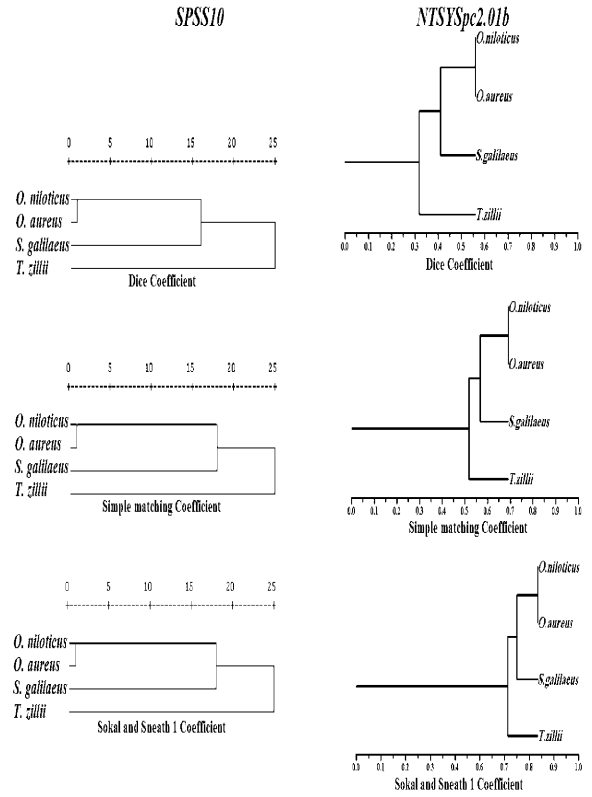

Fig. 1: Reconstruction of Phylogenetic relationships generated by the 11 RAPD markers using three similarity coefficients (Dice, Simple matching and Sokal and Sneath.

Reconstruction of Phylogenetic Relationships among Applied Tilapia Species: To assess the genetic distances among the four studied Tilapia species, three most frequently similarity equations were used: Dice, simple matching and Sokal and Sneath I. As presented in Table (5) and Figure (1).

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. On the other hand, in case of Simple matching and Sokal and Sneath I equations the calculated similarity values are increased (between each two estimated Tilapia species). In addition, Sokal and Sneath I equation gives double weight to shared present and absent fragments, therefore its similarity values were higher than those of Simple matching (Figure1).

In comparison between *SPSS10* and *NTSYSpc2.01b*, outputs to deduce the phylogenetic relationships among the studied Tilapia species, (based on molecular data): we found that there are no differences between the similarity values revealed from the two software, but in the plotted dendrogram there is a problem in the *SPSS10* program.

The *SPSS10* program draw the phylogenetic tree on the whole scale of the program whatever the relationships between the individuals covering the scale or not. In other words, the relationships between *O. niloticus* and *O. aureus* are slightly differed among the three used 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.*

The distance between the cluster of *O. niloticus* and *O. aureus* and *S. galilaeus* is smaller in the case of *NTSYSpc2.01b* dendrogram than those of *SPSS10*. Finally, 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* dendrograms but in the case of *SPSS10* the dendrograms show *T. zillii* as 100% distantly from the other three species. In this study we focused on the version ten of the software *SPSS*, but actually we tested three versions of this program: *SPSS10*, *SPSS13* and *SPSS15* and we found the same results. We suggest unusing *SPSS* to deduce the phylogenetic relationships because of their incorrect results.

DISCUSSION

In the present study, four Tilapia species (belong to three genera) were studied as models to select the suitable method for calculating similarity values within and among fish genomes.

Similarity degrees within *O. niloticus* were almost the same of those of *O. aureus*. The highest similarity value within Tilapia species was for *O. niloticus* (0.913±0.008, 0.941±0.005 and 0.969±0.003 for Dice, Simple matching and Sokal and Sneath I, respectively) and *O. aureus* (0.912±0.005, 0.938±0.003 and 0.968±0.002 for Dice, Simple matching and Sokal and Sneath I, respectively.

The analyses of genetic dissimilarity and/or similarity between diploid organisms with dominant markers should be viewed with caution unless the organism is inbred and therefore relatively homozygous.

The problem with dominant markers for diploids is that, without genetic data from segregation patterns after selfing, it would be impossible to distinguish bands that represent two alleles at a homozygous locus from bands that represent only one allele at a heterozygous locus [1]. So, the DNA polymorphism detected by RAPD can be seen from two viewpoints. First, the presence (or absence) of one or more RAPD fragment which possess particular size from the RAPD patterns. Second, changes in the intensity of fragments having the same size. So, as RAPD enables arbitrary amplification of genomic sites, it can generate unlimited number of markers which are inherited mainly as dominant markers [6, 8, 13].

Dice equation uses only the shared present fragments so its equation estimates the similarity based on the observed fragments between two Tilapia species and ignores any other fragments in all studied species. Dice ignores the shared absent fragments and gives double weight for the shared-present matched fragment between any two estimated individuals. Simple matching includes both shared present and absent fragments and gives equal weight for shared and un-shared fragments. Sokal and Sneath I include both shared present and absent fragments and gives double weight to shared fragments.

Kosman and Leonard [1] did not show any acceptable universal approaches to assessing the dissimilarity between individuals with molecular markers. On the other hand, our results suggested using Dice equation in this way. Kosman and Leonard [1] concluded that, Dice coefficient is the suitable measure for haploids with co-dominant markers and it can be applied directly to (0, 1) data representing banding profiles of individuals. The data of the present study showed that Dice coefficient is suitable measure for diploids with RAPD (as a dominant) markers.

In the present study, Dice coefficient is appropriate for diploids with (RAPD) dominant markers.On the other hand, Kosman and Leonard [1] found that, none of the common measures, Dice and simple mismatch coefficient is appropriate for diploids with co-dominant markers.

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 Tilapia species in case of Simple matching and Sokal and Sneath I equations which increase the estimated similarity between every two species.

The values of Sokal and Sneath dissimilarity are always differ from those of the Dice dissimilarity and the simple mismatch coefficient. On the other hand, values of the Dice dissimilarity may be greater or smaller than the corresponding values of the simple mismatch coefficient depending on whether the number of positions with shared bands a is less or greater than the number of positions with shared absence of bands.

No differences were detected between the similarity values that calculated using *SPSS10* and *NTSYSpc2.01b*. On the other hand, in the plotted dendrogram there is a problem in the *SPSS10* program. *SPSS10* program draw the phylogenetic tree on the whole scale of the program whatever the relationships between the individuals covering the scale or not. In other words, the relationships between *O. niloticus* and *O. aureus* are slightly differed among the three used coefficient and this appears in the *NTSYSpc2.01b* dendrogram but not appeared in the *SPSS10* dendrogram.

The distance between the cluster of *O. niloticus*, *O. aureus* and *S. galilaeus* in the case of *NTSYSpc2.01b* dendrogram is smaller than those revealed from *SPSS10*. Finally, 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 case of *SPSS10* the dendrogram show *T. zillii* as 100% distantly from the other three species.

In this study we focused on the version ten of the software *SPSS*, but actually we tested three versions of this program: *SPSS10*, *SPSS13* and *SPSS15* and we found the same results. Finally, *SPSS* is not recommended to deduce the phylogenetic relationships between species because of their incorrect results.

These data suggested that RAPD analysis was an appropriate analysis to differentiate between the four studied Tilapia species and other fish species or sub

species as reported by Saad [8] to measure the polymorphism within and among them. This point is in agreement with Bardakci and Skibinski [14] who used RAPD analysis to identify three species of the genus *Oreochromis* and four subspecies of the Nile tilapia (*O. niloticus*). Naish *et al.* [15] used RAPD markers to evaluate the genetic diversity between six aquacultural strains of *O. niloticus* from the Philippines. Müller *et al.* [16] proved that RAPD analysis can be used to detect the hybrids between two pikeperch and Volga perch. Rastogi *et al.* [17] reported that RAPD was proved to be a discriminatory, accurate and efficient method to identify the vertebrate animal tissues like buffalo, cow, pig, goat, chicken, frogs, fishes and snakes etc. Rashed *et al.* [6] estimated the gene flow, detect the genetic diversity and construct phylogenetic relationships of three populations of Egyptian Nile tilapia *O. niloticus* which collected from Aswan-Nasser lake, Giza and Qanater using RAPD markers.

Khalil *et al.* [18] differentiated between samples of *Tilapia zillii* which treated with and without Metronidazole the mutagen and carcinogen of the rodent using RAPD analysis. Si-Fa *et al.* [19] identified two strain-specific RAPD markers to the NEW GIFT Nile tilapia strain (*Oreochromis niloticus niloticus* L.).

Hassanien *et al.* [4] differentiated between some Egyptian *O. niloticus* populations (Cairo, Assuit and Qena) and two Delta lakes (Burullus and Manzalla) using RAPD analysis. In the present study, the RAPD analysis was an appropriate analysis to measure the homogeneity values within the applied Tilapia species. RAPD is an efficient tool in allowing multiple loci to be analyzed for each individual in a single gel run.

CONCLUSION

Dice (Nei and Li) coefficient is suitable measure of similarity and/or dissimilarity values from fish species fingerprint profiles.

RAPD is a suitable tool for fish species characterization. In this technique (as dominant markers), there is a direct identity assumed between the number of unique bands observed and the number of identifiable loci for the sample of individuals. The interpretation of shared absences of specific bands by two individuals depends on the degree of genetic similarity among individuals within the sample. The interpretation will be different when the individuals are drawn from different taxa in a phylogenetic tree.

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