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Genetic Diversity among Three Species of Tilapia in Egypt Detected by Random Amplified Polymorphic DNA Marker

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Abstract
Genetic diversity of the different tilapia species collected from Cairo, Giza and Fayuom in Egypt was examined by the analysis of randomly amplified polymorphic DNA (RAPD).The polymorphisms within and between three Tilapia species were assayed using 15 random primers, and 215 loci were amplified ranging from 118 to 2556bp. The results indicated that the percentage of polymorphic loci recorded 51.8%, 58.6% and 60.3% for *Oreochromis niloticus*, *Oreochromis aureus* and *Tilapia zilli* species, respectively. Total genetic diversity (Ht) was (0.304) and the average coefficient of genetic differentiation (Gst) was (0.583). The genetic diversity within population was the highest for *T.zilli* (0.249) compared to *O.niloticus* and *O. aureus* 0.219, 0.238, respectively and the gene flow between three species was 0.357. Among the three populations, *Tilapia zilli* appeared to be an outgroup while *Oreochromis niloticus* and *Oreochromis aureus* were genetically closer as revealed by Nei's biased measure of genetic identity and genetic distance values. This study concluded a relatively high level of polymorphism and genetic diversity within and between the three tested *Tilapia* species which may be required for the adaptation of the populations to the environmental changes.
Keywords: Polymorphism, RAPD, PCR, Tilapia

INTRODUCTION

Tilapias were an important component of subsistence fisheries for thousands of years but have gained prominence in recent years in areas where they are not endemic(Shair et al. [1]).The family Cichlidae includes the tilapias, a group of 40–50 nominal species that are important food fishes in their native habitats in Africa and the Middle East. Several species of tilapia have been introduced to tropical and subtropical countries around the world as a means of increasing supplies of animal protein (Bo-Young et al. [2]). The tilapiines have been divided into three major genera primarily on the basis of breeding habits: *Oreochromis*, the maternal mouth brooders (31 species); *Sarotherodon*, the biparental and paternal *mouthbrooders* (9 species); and Tilapia, the substrate *spawners* (Trewavas [3]) .Tilapias have become one of the most important groups of fish, with worldwide production of _ 2 million metrictons in 2002 (FAO [4]). About 75% of this production is from aquaculture. The most important species is the Nile tilapia, *Oreochromis niloticus* (Bo-Young et al. [2]), because they grow fast, are easy to feed, are resistant to poor water quality and disease and are easily reproduced (Argue and Phelps [5]).

DNA-level investigations were thus developed for fish genetic studies. Such approach provides direct investigations of the genetic make-up of several fish species, thus eliminating the effects of extraneous factors. Furthermore, polymorphisms (variant forms) in the DNA are highly numerous as compared to that of isozymes. This required DNA-level analysis with a lot of genetic markers (Omitogun [6]).Various methods of genetic markers for stock identification and assessment in Nile tilapia *O.niloticus* have historically been used, including: allozymes (Macaranas et al. [7]; Pouyaud and Agnese[8]; Rognon et al.[9]), mitochondrial DNA restriction fragment length polymorphisms (mtDNA-RFLPs)(Agnese et al.[10]; Rognon and Guyomard [11]) and random amplified polymorphic DNA (RAPD) (Naish et al. [12]; Dinesh et al.[13]; Hassanien et al. [14]).

Random amplification of polymorphic DNA (RAPD) markers are polymorphic DNA separated by gel electrophoresis after PCR using short random oligonucleotide primers (Williams et al. [15]) and without any previous genome information on the target species (Rashed et al. [16]).The primer randomly anneals to an unknown segment on one of the DNA strands. When two species, strains, or individuals are compared, polymorphism between them will be revealed on agarose electrophoresis gels by presence or absence of an amplification product, this method has been applied to the discovery of genetic markers for mapping studies (Postlethwait et al. [17]) and to elucidate the phylogenetic relationships between species (Baradakci and Skibinski [18]) and for determining genetic diversity and similarity in various organisms (Salem et al. [19]).

The RAPD technique has further advantages over other systems of genetic documentation because it has a universal set of primers, no preliminary work such as probe isolation, filter preparation, or nucleotide sequencing is necessary (Williams et al. [15]). This technique can be used to differentiate individuals or breeding stocks with a given species but also to differentiate among different species. The conditions of the analysis can be optimized to reveal only species specific differences, since all individuals belonging to a given species have more of their genetic material in common than with individuals from other species (Elvevoll et al. [20]).

This study attempts to identify and estimate polymorphisms and genetic diversity between and within three Tilapia species; *Oreochromis niloticus, Oreochromis aureus* and *Tilapia Zilli* using RAPD assay.

MATERIALS AND METHODS

The present study was carried out at Cell Biology Department, National Research Centre, Egypt.

Fish origin

Forty five individuals representing three species of Tilapia were used in the present investigation. Tilapia fishes (*O.niloticus* &*O.aureus*) was collected with nets from the Nile in the region in Helwan (Cairo) and Monibe (Giza) while *T.zilli* was collected from Qarun Lake in Fayuom, Egypt. The live specimens were held in tanks filled with dechlorinated water, transported to the laboratory and carefully dissected.

DNA Extraction

Genomic DNA was isolated from forty five blood sample of three Tilapia fish (fifteen for each species) by phenol/chloroform method described by (John et al. [21]). DNA presence was verified by agarose gel electrophoresis and its concentration and purity were determined with a spectrophotometer set at 260 and 280 nm absorbance's.

RAPD-PCR

Fifteen commercially available decamer random primers, designed and chosen arbitrarily for these experiments, were obtained from Operon Technologies (Operon, Almeda, CA, USA) and used to initiate PCR amplifications. Primers were randomly selected on the basis of GC content (60-70%), primer codes and sequences are presented in Table 1.

PCR amplification and agarose gel electrophoresis

The PCR protocol for RAPD analysis was followed as described by (Williams et al. [15]; Plotsky et al. [22]). Briefly, the amplification reactions were performed in volume of (15µl) consisted of 1.5 µl (50ng genomic DNA), 1.5 µl of 10X PCR reaction buffer, 1.5 µl DNTPs (200µM), 1.5 µl primer (1pmol) (operon, CA, USA), 1U Taq DNA polymerase .The final reaction mixture was placed in a DNA thermal cycler (ependorff). The PCR programme included an initial denaturation step at 94°C for 4 mins followed by 45 cycles with 94°C for 1 min for DNA denaturation,1 min at 36°C, at 72°C for 2min and final extension at 72°C for 5 min were carried out. Approximately 10 µl of RAPD-PCR amplified product plus 3 µl of 1X loading dye were loaded and then subjected to electrophoresis in 1X TBE buffer (0.5M Tris-HCL, 0.5M Boric acid and 0.5M EDTA, pH 8.0) on 2.0% agarose gel stained with ethidium bromide (0.5µg/ml).The amplified pattern was visualized on an UV transilluminator and photographed by Gel Documentation system, Gel-Pro analyzer (Media Cybernetics), version 3.1 for windows3. DNA fragments sizes were estimated by their comparison with standard molecular size marker (ô x 174 DNA Hae III digest).

Scoring and analysis of RAPDs

The DNA profiles generated for all samples were compared within and between the three Tilapia species. Comparisons were carried out between samples amplified by the same primer and the amplified fragments were scored as binary data, i.e. presence as 1 and absence as 0, for homologous bands. All calculations were carried out using the population genetic analysis software, POPGENE 1.31(Nei [23]). The UPGMA dendrogram and genetic distances of population was constructed based on Nei's genetic identity among the three species. Overall observed number of alleles and effective number of alleles was calculated according to (Kimura and Crow [24]). Genetic differentiation (Gst) was calculated by using formula: Genetic dif (Gst) = 1- Hs/Ht, Where, (Hs) is sample gene diversity and (Ht) is total gene diversity (Nei [25]). Gene flow was indirectly estimated among the populations by using the formula:Nm =0.5(1 - Gst)/ Gst. (McDermott and McDonald [26]). Shannon's diversity index (I) was calculated to provide a relative estimate of the degree of genetic variation within each population (Lewontin [27]).

RESULTS

RAPD Polymorphisms

The banding patterns generated through RAPD assay in the present study were used to differentiate between and within three Tilapia species *O.niloticus*, *O.aureus* and *T.zilli* and to deduce genetic diversity among them. The number of amplified bands detected varied, depending on the primers, species and individuals. Fifteen different decamer primers possessing (60-70% G+C content) were screened on a group of fifteen individuals for each of the three Tilapia species. Totally, 345 bands were scored within the three populations. The numbers of detected bands were 108, 121 and 116 in *O.niloticus*, *O.aureus* and *T.zilli*, respectively. The percentages of polymorphic RAPD bands were ranged from 0% to 100% in all studied Tilapia species; the percentages of polymorphism between the individuals within population were (51.8%, 58.6% 60.3%) in *O.niloticus*, *O.aureus* and *T. zilli*, respectively (Table 2).

As presented in Table 3, the fifteen primers generated total of 215 scorable loci among the three Tilapia species, of these 201 bands were polymorphic (93.94%). Number of total bands generated per primer varied from 5 to 30 with a

Table 1. The sequence of fifteen primers used RAPD analysis.

Primer	Sequence	GC%	Primer	Sequence	GC%
OPA01	5'-CAGGCCCTTC-3'	70%	OPB09	5'-TGGGGGACTC-3'	70%
OPA05	5'-AGGGGTCTTG-3'	60%	OPB15	5'-GGAGGGTGTT-3'	60%
OPA06	5'GGTCCCTGAC-3'	70%	OPB18	5'-CCACAGCAGT-3'	60%
OPA07	5'-GAAACGGGTG-3'	60%	OPB20	5'-GGACCCTTAC-3'	60%
OPA08	5'-GTGACGTAGG-3'	60%	OPC07	5'- GTCCCGACGA-3'	70%
OPA09	5'-GGGTAACGCC-3'	70%	OPC08	5'-TGGACCGGTG-3'	70%
OPA12	5'-TCGGCGATAG-3'	60%	OPC09	5'-CTCACCGTCC-3'	70%
OPA13	5'-CAGCACCCAC-3'	70%			

Table 2. Total no. of bands, no. of polymorphic bands and %percentage of polymorphic loci within three Tilapia species.

Primer	Total no.of bands			No. of polymorphic bands			% of polymorphic loci		
	O.niloticus	*O.aureus*	*T.zilli*	*O.niloticus*	*O.aureus*	*T.zilli*	*O.niloticus*	*O.aureus*	*T.zilli*
OPA01	18	17	18	13	15	12	72.2	88.2	66.6
OPA05	10	15	11	5	8	8	50	53.3	72.7
OPA06	5	9	8	3	6	7	60	66.6	87.5
OPA07	5	7	4	1	6	4	20	85.7	100*
OPA08	8	9	9	2	3	0	25	33.3	0
OPA09	5	4	6	3	4	5	60	100*	83.3
OPA12	6	7	8	0	1	4	0	14.2	50
OPA13	5	10	6	2	9	0	40	90	0
OPB09	5	2	2	4	1	1	80	50	50
OPB15	6	9	8	2	6	6	33.3	66.6	75
OPB18	4	9	7	1	8	5	25	88.8	71.4
OPB20	8	6	5	5	0	1	62.5	0	20
OPC07	3	4	8	2	0	6	66.6	0	75
OPC08	6	7	5	1	4	4	16.6	57.1	80
OPC09	14	6	11	12	0	7	85.7	0	63.6
Total	108	121	116	56	71	70	51.8	58.6	60.3

size range (118-2556pb), the results showed highly genetic polymorphic percentage range per primer (70 to 100%). Among the primers, primer OPA01 & OPA02 gave DNA profiles with more bands than the other primers among the three Tilapia species. The ranges of RAPD markers were (2556-134pb), (2037-118pb), (20197-118pb) in *O. niloticus*, *O.aureus* and *T.zilli*, respectively.

RAPD fragments were examined for the presence of species specific band, all tested RAPD primers generated 201 different loci, some of them 35 were considered as species specific band which present in one species and absent in the other Tilapia species. These species specific band were analyzed: (11) sp.specific band detected in *O.niloticus*, 10 sp.specific band in *O.aureus* and 14 sp.specific band in *T.zilli* (Table 4).

The observed number of alleles (Na) and the effective number of alleles (Ne) were used as a measure of genetic polymorphism and it was varied among the present studied three Tilapia populations, however they were higher in *T.zilli* population (1.603 ± 0.491, 1.437 ± 0.381, respectively) than that in *O.niloticus* (1.518±0.502, 1.389±0.394, respectively) and *O.aureus* (1.587 ± 0.495, 1.418±0.389, respectively) populations. The total average number of observed alleles and effective number of alleles among three Tilapia species was (1.935 ±0.247 & 1.506 ±0.321, respectively) (Table5).

Genetic variability parameters

Genetic variation holds the key to the ability of populations and species to persist over evolutionary time through changing environments. Therefore, it is necessary to assess the amount of genetic diversity among populations and the distribution of diversity within populations.

Table 3. Percentageof polymorphic loci and size range generated by 15 random primers

Primer	Total no. of bands	Number of polymorphic loci	% of polymorphic loci	Size range Max.	Min.
OPA01	30	27	90	2197	344
OPA05	29	29	100	2556	156
OPA06	13	13	100	1015	231
OPA07	10	10	100	872	118
OPA08	14	10	71.4	1254	162
OPA09	10	10	100	990	241
OPA12	10	7	70	584	146
OPA13	14	13	92.8	1180	294
OPB09	5	4	80	674	232
OPB15	13	13	100	846	164
OPB18	13	13	100	789	206
OPB20	11	10	90	843	215
OPC07	8	7	87.5	840	220
OPC08	14	14	100	919	266
OPC09	21	21	100	1764	262
Total	215	201	93.49	2556	118

Table 4. Range of detected molecular weight (MWR) generated by 15 RAPD primers and Tilapia species specific band (SP.S.B).

RAPD primers	*O.niloticus*		*O.aureus*		*T.zilli*	
	MWR	**SP.S.B**	**MWR**	**SP.S.B**	**MWR**	**SP.S.B**
OPA01	2099-294	0	2037-344	0	20197-294	694,1647
OPA05	2556-483	1724,1333	1917-246	1217,872,676,619 ,440	1598-156	522,352
OPA06	1015-372	0	1015-226	0	1015-231	0
OPA07	872-134	0	872-118	0	477-118	0
OPA08	872-162	548	1254-162	0	1254-162	820,725
OPA09	752-241	626	725-241	0	990-263	0
OPA12	584-146	181	584-146	264	584-146	0
OPA13	888-445	580	1180-404	0	888-294	413,350,294
OPB09	674-219	0	232-219	0	232-219	0
OPB15	771-262	603,380	846-164	369	846-262	358
OPB18	531-206	0	789-206	0	667-206	0
OPB20	843-224	0	763-224	0	690-215	341,215
OPC07	699-285	0	699-285	0	840-220	0
OPC08	919-266	919,641	888-266	0	936-555	936
OPC09	1764-262	445	1698-310	1163,540,398	1698-262	903

Table 5. Estimates of genetic variation: overall observed number of alleles (Na), Effective number of alleles (Ne), Nei's (1972) gene diversity (H) and Shannon's Information index (I).

Sample	Na	Ne	H	I
O.niloticus	1.518±0.502	1.389±0.394	0.219±0.216	0.318±0.311
O.aureus	1.587±0.495	1.418±0.389	0.238±0.210	0.348±0.302
T.zilli	1.603±0.491	1.437±0.381	0.249±0.209	0.363±0.301
overall	1.935±0.247	1.506±0.321	0.304±0.152	0.464±0.195

Table 6. Genetic identity (above diagonal) and genetic distance (below diagonal) between three Tilapia Populations (Nei's 1972).

Tilapia Populations	***O.niloticus***	***O.aureus***	***T.zilli***
O.niloticus	****	0.736	0.667
O.aureus	0.306	****	0.686
T.zilli	0.405	0.377	****

The data obtained demonstrated that the highest value of Nei's gene diversity (H=0.249±0.209) and Shannon's information index (I=0.363±0.301) were observed in *T.zilli* population while the lowest values were found in *O. niloticus* population (H=0.219±0.216 and I=0.318±0.311) (Table5).The genetic diversity was calculated according to heterozygosity (Ht) for the total populations, sample heterozygosity (Hs), the genetic differentiation (Gst), gene flow (Nm), genetic distance (D) and genetic identity. The overall estimated total diversity (Ht) was (0.304±0.023); sample diversity (Hs) was (0.127±0.011). Genetic differentiation (Gst) between all loci in three Tilapia species was (0.583) and the gene flow value was (0.357).

The genetic identity and distance between three Tilapia species are presented in (Table 6).*O.niloticus* was closest to *O.aureus* with a genetic identity of (0.736), whereas *T.zilli* was farthest to *O.aureus & O.niloticus* with a genetic distance of (0.377, 0.405, respectively). The UPGMA-tree dendogram (Fig. 1) indicates the relationship among the three Tilapia species which are of family Cichlidae, taking into consideration the close relationship between *O.niloticus* and *O.aureus.*

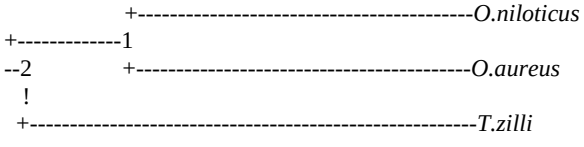

Fig1. Dendrogram Based Nei's (1972) Genetic distance: Method = UPGMA Tree (1000 replications) generated from RAPD data of the three Tilapia species.

DISCUSSION

Several authors (Baradakci and Skibinski [18]; Naish et al.[12]) have demonstrated that the RAPD PCR method is a powerful tool for the assessment of genetic markers that are capable of discriminating between species or subspecies in a wide range of organisms, including fishes. This capacity was confirmed by the results of the present study because, for all screened primers, different RAPD banding patterns were observed within and between the three Tilapia species**.**

In the present study fifteen primers generate a total number of 215 fragments, with the approximate size ranging from 118 to 2556 bp. This wide range of band sizes is comparable to the results of studies carried out by other authors. For example, Chandra et al. [28], observed 45 fragments with size range (250-2000bp), also in Das et al. [29], investigation, the 15 decamer primers generated 449 bands, ranging in size between 400 and 3000 bp**.** However, Liu et al. [30], observed 462 amplified fragments (200-1500bp) by using 75 primers in Catfish. In Native American maize accessions, amplified fragments obtained by RAPD ranged in size from 220 to 3,000 bp for all primers (Moeller and Schaal [31]). The presence of more numbers of fragments might be due to the presence of more priming site at the template DNA with the particular series of operon primers employed in this study.

In the present study, the RAPD profile shows (93.49%) polymorphic loci among the three Tilapia species. These results in agreement with previous studies reported by Soufy et al. [32], where high polymorphism was detected between three Nile Tilapia species (*O.niloticus*, *Sarotherodon galilaeus* and *T.zilli*). Nebauer et al. [33], observed high polymorphic percentage (98.9%) among the species *Digitalis.* However, Tamanna et al. [34], calculated very high (100%) polymorphic loci between three wild populations of the striped dwarf catfish; *Mystus vittatus (Bloch*).On the other hand, a very low level of polymorphism (18.75%) was obtained in two Indian populations of *H. fossilis* in RAPD loci (Garg et al. [35]). Generally high levels of polymorphism were found, because they offer random polymorphic regions in the genome.

In the present study the percentage of polymorphic bands within population appears to be more in *Tilapia zilli* (60.3%) than *O.aureus* (58.6%) and *O.niloticus* (51.8%), This result also coincides with previous results of Mwanja et al. [36], where the percentage of polymorphism in *T.zilli* was 65% followed by *O. niloticus* (59.0%), also Shifat et al. [37], observed high degree of polymorphism within and between two populations of *Hilsa shad*. Meanwhile, lower polymorphic loci (42.6%, 31.7%, 30%, 19.2%, 16.8% and 14.3%) were observed within different *carp species* (Das et al.[29]). Generally, polymorphic bands generated by RAPD-PCR using random primers had good merits for

detecting DNA identity and diversity between life organisms.

In our results, fifteen primers generated (201) polymorphic fragments among three Tilapia species which is higher than results indicated by Das et al. [29] , where 15 primers generated 114 polymorphic loci among six *Labeo species* , Shifat et al. [37], where fifteen primers yielded 98 polymorphic bands in two populations of *Hilsa shad* and Upadhya et al. [38],where 20 primers produced 121 polymorphic bands in yellow grouper (*Epinephelus awoara*) and lower than that recorded in study of Garg et al. [39], by using five primers, 513 polymorphic bands were generated in two populations of catfish *Aorichthys seenghala (skyes*).The results in our study indicate a large number of polymorphic fragments detected per primer and suggest high genetic variation among three Tilapia species.

All tested primers in our work generated 35 species specific band analyzed as follow (11sp.s.b in *O. niloticus*, 10 sp.s.b in *O.aureus* &14 sp.s.b in *T.zilli*) which is higher than that recorded by Rashed et al. [16], 26 loci were detected in four Tilapia species analyzed 6 sp.s.b in *O.aureus* and *T.zilli*, 4&10 sp.s.b in *O. niloticus* and *S.galilaeus*, respectively and that stated by Yoon & Kim [40], the total number of specific fragments in *catfish* and *bullhead* population were 76 and 64, respectively. Also, Parveen et al. [41] demonstrated that the total scorable bands in *Damselfishes* were 164, under which the total number of species-specific bands were 99**.**

While considering the Nei's gene diversity (H), higher diversity was found in *T.zilli* (0.249) than in *O.niloticus* and *O.aureus* populations (0.219, 0.238, respectively), this means that *T.zilli* population has a higher proportion of heterozygous genotypes than *O.niloticus* and *O.aureus* populations, which is in accordance with the results of Shannon's Information index (0.363) for *T.zilli* and that (0.318, 0.347) for *O. niloticus* and *O.aureus* respectively. These results are similar to the findings of Chandra et al. [28],where genetic diversity within *Madhepura* populations (0.203) was higher than the *Patna* populations (0.189) and the Shannon index ranged from 0.280 (*Patna)* to 0.300 *(Madhepura)*, also Semra [42], observed genetic variations of *guppy* interspecies ranged from 0.074 to 0.320. Upadhya et al. [37], recorded high level of genetic diversity between two populations of *Xiamen and Guangdong* of *Epinephelus awoara*. Higher gene diversity and Shannon's Information Index values are also an indicative of relatively higher level of genetic variation exists in the populations, which is highly important for the adaptation to changing environments and, as a consequence, for long-term survival of a species.

In the present study the total genetic diversity (Ht) and sample diversity (Hs) among the three Tilapia species is (0.304,0.127, respectively).Genetic differentiation (Gst) is (0.583) and the gene flow value is (0.357),which is consistent with the results obtained by Upadhya et al. [37], among two populations of *Epinephelus awoara* in China, total heterozygosity (H_T) values ranged from (0.219 to 0.462), sample heterozygosity (H_S) from (0.123 to 0.316), an estimate of gene flow (N_m) from (0.371 to 4.172) and the proportion of total genetic differentiation (G_{ST}) ranged from (0.107 to 0.534). Carvalho et al. [43], observed a very high differentiation (G_{ST} =0.648) in the guppy (*Poecilia reticulata*) in northern Trinidad. In our study, the differences between gene diversity (Hs) and total gene diversity (Ht) indicate high genetic differentiation among the populations and are explained by the low rate of gene flow.

Thorpe [44], determined Nei's genetic identity indices and found standard genetic diversity values for co-specific populations (0.95 to 1.0), for species of the same genus (0.35 to 0.85) and for genera of the same family (0.0 a 0.60). In our study the genetic identity between *O. niloticus* and *O.aureus* (0.736) is higher compared with that between *O. niloticus*, *O.aureus* and *T. zilli* (0.667, 0.686, respectively) and *O. niloticus* is closest to *O.aureus* with a genetic distance of (0.377). These results are similar to the findings of Rashed et al. [16], who observed that *T.zilli* was 100% distantly from *O. niloticus, O.aureus* and *S. galilaeus* Also, Soufy et al. [32], stated that the genetic similarity between *O. niloticus* and *S.galilaeus* was higher compared with that between *O. niloticus*, *S. galilaeus* and *T. zilli*, respectively. Degani et al. [45], showed that the genetic distance was highest between *T. zilli* and *O.mossambicus*, followed by *O. aureus*, *O.niloticus* and *O.aureus x O.niloticus*, respectively. These findings are an indication of the distinct character of *T.zilli* territoriality, in contrast to the other Tilapia species.

In conclusion, this study curried to investigate the genetic variation in three Tilapia species by using different random primers from different operon series in RAPD analysis. This study revealed a relatively high level of polymorphism and genetic diversity within and between three Tilapia species *(O.niloticus, O.aureus and T.zilli*) which is required for populations to be more adaptive with the environmental changes.

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