ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Genetic Diversity in Egyptian Tilapia Species Using PCR-RFLP of D-loop Mitochondrial DNA Gene.

Zeinab G Abdel-Hamid, Heba A M Abd El-Kader, Dalia M Aboelhassan, and Karima F Mahrous*.

Cell Biology Department, National Research Center, Dokki, Giza, Egypt.

ABSTRACT

Genetic differentiation within and among three populations of Egyptian tilapia species (*Oreochromis niloticus*, *Oreochromis aureus* and *Tilapia zillii*) were examined using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of D-loop mitochondrial DNA gene. A total of 16 phenotypes corresponding to 11 different haplotypes were defined. Analysis of molecular variance (AMOVA) showed a significantly different genetic variation among tilapia species (ΦFst=0.2966, P=0.00). Gene flow (Nm) was high and ranged from 0.3046 to 8.5448. The value of net nucleotide divergence (dA) among species varied from 5.5000 to 8.8000%. A UPGMA tree based on the distance matrix of net interpopulation nucleotide divergence (dA) and haplotype of mtDNA D-loop divided tilapia species into two clades. *Oreochromis aureus* and *T. zillii* Qarun belonged to the first cluster whereas *O. niloticus* and *T. zillii* Manzalah aggregated to the second cluster. The neutrality and mismatch distribution tests explained that tilapia populations do not undergone population expansion. A high genetic diversity and significant genetic differentiation were observed between tilapia species.

Keywords: Genetic diversity, RFLP, mtDNA, tilapia species

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Tilapia is a cichlid fish which has become one of the most important species in aquaculture worldwide [1]. Nile tilapia production in world aquaculture reached two million tonnes in 2007 [2] covering about 83% of the total production of tilapia group [3]. Therefore, tilapia species is a great source of protein in human diets particularly in Egypt and its importance increases in aquaculture [4]. For more than 100 years, tilapia has received a wide attention from evolutionary biologists due to its extremely diverse morphology, behavior, ecology, easy breeding and high survival rates [4,5].

Several DNA marker systems have been reported for tilapia species identification, including the mitochondrial DNA (mtDNA) control region (D-loop) [5] the highly variable region in mammals and fish except salamon sp. [6]. Mitochondrial DNA control region is flanked by the tRNApro and tRNAphe mtDNA genes with length 927 and 932 bp in *Oreochromis. niloticus* and *Oreochromis. aureus*, respectively [7]. This region is sensitive for detecting genetic diversity and population genetic structure in various fish such as *Channa striatus* [8] and miiuy croaker [9]. Several molecular-based approaches for the identification of tilapia species were applied including Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) [10], Random Amplified Polymorphic DNA (RAPD)[11] and microsatellites [12].

Restriction fragment length polymorphism (RFLP) analysis of mtDNA has proved to be effective in distinguishing the population genetic structure and differentiation of many fish [8]. RFLP analysis in mtDNA studies has several advantages including quite high levels of detectable variation, evolving of a high mtDNA, high genetic drift, low gene flow, possibility of reconstructing the phylogenetic history of mtDNA and population analysis of very small tissues [13]. Patterns of genetic diversity among populations can provide clues to population's life history and degree of evolutionary isolation. Therefore, the current study used the mitochondrial genome of noncoding control region (D-loop) to examine the genetic diversity within and among three tilapia species *Oreochromis niloticus, Oreochromis aureus* and *Tilapia zillii* (Qarun & Manzalah) in Egypt using PCR-RFLP technique.

MATERIALS AND METHODS

Sampling and DNA extraction

For genetic variation investigation, 76 randomly chosen specimens of three tilapia species (*Oreochromis niloticus*, *Oreochromis aureus* and *Tilapia zillii*) were collected. *O. niloticus* and *O. aureus* samples were captured using gill nets from the Nile River in the region between Giza and Maser El –Kadima, while *T. zillii* samples were collected from Lake Qarun- El-Faiyum province and Manzalah. The live specimens were held in tanks filled with dechlorinated water, transported to the laboratory and carefully dissected. Genomic DNA was extracted using phenol/chloroform method according to John et al. [14].

PCR amplification of mtDNA control region

PCR amplification of 1 kb fragment in the control region of mtDNA was performed using primers HN20 (5'GTGTTATGCTTTAGTTAAGC 3) and LN20 (5'ACCACTAGCACCCAAA- GCTA 3) to amplify proline gene and phenylalanine tRNA genes, respectively [15]. A total volume reaction of 50 µl contained 5U/µl of DNA, 0.2 UM of each primer, 1x of Taq polymerase buffer, 2 units of Taq polymerase (Fermentas). PCR was performed under cycling conditions of 96°C for 4 min, followed by 35 cycles of 94°C for 30sec, 55°C for 1 min, 72°C for 1 min terminated with elongation at 72°C for 10 min. Length and purity of the PCR products were evaluated by agarose gel electrophoresis.

Digestion of the PCR products

Digestion of control region was carried out at 37ºC for *Hinf*I, *Ava*II, *Msp*I and *Rsa*I and 65Cº for *Taq*I restriction enzymes (fast restrictions, #ER0674, (5'...T↓C G A...3' 3'...A G C↑T...5'), Fermentas) for 6 min. For this reason a 12 µl mixture containing 8 µl PCR products, 2 µl of the restriction enzymes and 2 µl restriction buffers were used. The size of the resulting fragments of each restriction morphs was confirmed by 2% agarose

gel electrophoresis and estimated by comparing migration distances relative to a known standard 100bp DNA ladder Molecular Weight Marker VIII (Roche).

Statistical Analysis

Genetic variation

Haplotype frequencies under the infinite-allele model as predicted by Ewens [16] were performed. Sampling distribution, mtDNA haplotype frequencies, allele frequencies, average of nucleotide diversity (π) were computed from the PCR-RFLP genotype data using Arlequin [17]. To determine genetic variation within and among populations of tilapia sp., analysis of molecular variance (AMOVA) was performed to estimate the molecular variance at hierarchical levels as implemented in Arlequin with 10,100 permutations of mtDNA haplotype D-loop among populations for evaluation of Φ statistics. Two different structure analyses were considered, a cluster for three tilapia populations (*O. aureus*, *O. niloticus* and *T. zillii*) and a cluster for two groups of *T. zillii* species (Qarun & Manzalah). Genetic differentiation between population pairs was evaluated by pairwise fixation index (Fst) and the statistical significance of Fst was tested through 10,000 permutations for each pairwise comparison in Arlequin [18]. Degree of genetic isolation among populations was estimated by gene flow (Nm), which was calculated based on Wright's island model using Slatkin's linearized Fst values [19,20]. Genetic relationships in tilapia species (*O. niloticus*, *O. aureus* and *T. zillii* (Qarun & Manzalah) were diagrammatically presented in a UPGMA dendrogram based on pairwise nucleotide divergence (dA) [21]. The matrix of pairwise dA (net nucleotide diversity) values was used to construct a UPGMA dendrogram showing the relationship among populations using the Neighbor program as implemented in Phylip version 3.69 [22].

Neutrality and population expansion tests

Tajima's D [23,24], Fu's Fs [25] tests and Mismatch distributions [26] that conducted using Arlequin [18,27] were estimated by the bootstrap method using 10,000 permutations to determine the deviations from neutrality as would be expected under population expansion and discriminate whether a population had undergone sudden population expansion or remained stable over time [28]. Accordingly, the goodness-of-fit of the observed and expected mismatch distributions was tested by calculating the sum of squared deviation (SSD) for the estimated stepwise expansion models [29] as implemented in Arlequin and Harpending raggedness index (r) [27,30].

RESULTS AND DISCUSSION

Genetic variation

Genetic differences within and among three types of Egyptian tilapia species the Nile (*O. niloticus*), blue (*O. aureus*) and red belly tilapia (*T. zillii*) were demonstrated using PCR-RFLP of D-loop of mtDNA control region. Altogether, 5 restriction enzymes (*Hinf*I, *Rsa*I, *AvaII*, *Msp*I and *Taq*I) were cleaved the amplified mtDNA products (Fig. 1). A total of sixteen morphs were obtained. Eleven of them were detected by Agnese et al. [6] in addition to new five morphs, F* for *Hinf*I; C* and D* for *Rsa*I; C* for *Msp*I and C* for *Taq*I (table 1, 2). Two morphs D and E for *Hinf*I that reported by Romana-Eguia et al. [31] were absent in our analysis.

Haplotype analysis

Eleven haplotypes were resulted and distributed as five (h1, h4, h7, h8, and h6) in *O. niloticus*, five (h1, h2, h3, h4, and h5) in *O. aureus*, three (h3, h9, and h10) in *T. zillii* Manzalah and two (h5 and h11) in *T. zillii* Qarun (Table 3). *Oreochromis. niloticus* shared *O. aureus* in h1 and h4. *Oreochromis aureus* and *T. zillii* Manzalah exhibited the haplotype h3 whereas, h5 was found in both *O. aureus* and *T. zillii* Qarun. Both *T. zillii* populations from Qarun and Manzalah regions did not share any haplotype. The remaining haplotypes were monomorphic to their species. The haplotype diversity (hd) in each tilapia sp. ranged from 0.5714 to 0.8205. The highest hd (0.8205) was observed in *O. aureus*, followed by 0.800 in *O. niloticus*, in contrast to *T. zillii* Qarun, which showed the lowest hd (0.5714). The first three haplotypes h1, h2 and h3 were observed as mentioned by Agnese et al. [6] and Romana-Eguia et al. [31], who suggested the highly allele frequency haplotype h1 and the other two haplotypes h2 and h3 to be native haplotypes. Despite, the populations of *O.*

niloticus, *O. aureus* and *T. zillii* Manzalah indicated high variability, *T. zillii* Qarun detected the lowest variability. According to the study of Xiao et al. [32], a high hd suggests large, stable, and effective population sizes, while low diversity indicates the abundance of ancestral haplotype overlapping and fixation of high number of unique haplotypes for each population [33].

According to Grant and Bowen [34], analysis of mtDNA was classified into four demographic categories based on different combinations of small and large values of nucleotide (π) and haplotype diversity. The results hd and π in *O.aureus*, *O.niloticus*, *T.zillii* Manzalah and *T.zillii* Qarun (hd=0.8205,π= 0.276; hd=0.800, π=0.237; hd=0.7143 , π= 0.258 and hd=0.5714, π=0.054, respectively) were belonged to the second demographic category (hd＞0.5 and π＜0.5%). This pattern could be attributed to demographic expansion after a period of low effective population size [28], nearly as reported by Romana-Eguia et al. [31] (Nile tilapia, hd =0.344 - 0.805 and red tilapia, hd=0.183-0.499).

Tilapia zillii Qarun showed moderate hd and low π (hd=0.5714 & π=0.054), this strong geographic structure pattern in the distribution of mtDNA haplotypes and relatively low level of genetic diversity within Qarun region may indicate that *T. zillii* populations (Qarun) undergone a subsequent genetic drift during isolation time. Genetic drift usually reduces the total amount of genetic variation and this effect is comparably stronger for the lower levels of gene flow when spatial structure becomes more pronounced in agreement with the findings of Wu and Yang [5] and Ikedia and Taniguchi [35]. In contrast to the study of Rahim et al. [8], which revealed a high level pattern of hd (0.6789–0.8737) and from low to moderate level pattern of π (0.008–0.0181) in *Channa striatus* populations. The overall haplotype analysis showed that *T. zillii* Qarun is closer to *O. aureus*, which may reflect the common ancestral mtDNA haplotype [6]. Among tilapia populations, the highly level of nucleotide divergence ranged from 0.2968 to 5.7396 and the value of net nucleotide divergence ranged from 5.5000 to 8.8000% were exhibited in comparison to Romana-Eguia et al. [31], the mean nucleotide divergence value (dA) was 0.009 among the Nile tilapia stocks and 0.004 among the red tilapia.

Population genetic structure

Pairwise Fst statistics and gene flow (Nm) showed a significant genetic differentiation between *O. aureus* and *T. zillii* Manzalah (Fst, 0.6213), *O. niloticus* and *T. zillii* Qarun (Fst, 0.6213), O. aureus and *O. niloticus* (Fst, 0.5752) and *O. aureus* and *T. zillii* Qarun (Fst, 0.4930), unlike non significant genetic differentiation between *O. niloticus* and *T. zillii*, Manzalah (Fst, 0.0552). Gene flow (Nm) demonstrated intermediate level (0.3691) between and within T.zillii regions [36], which suggested a relatively genetic exchanges between populations according to the infinite Wright's island model.

Two clusters were reflected from AMOVA analysis; the first cluster covered the three species with 29.66 % genetic variation among the populations and 70.34% within each species. The significant value of fixation index (Φst) (0.2966) across the populations prevents the rejection of non hierarchical genetic structure, suggesting that these populations were consistent with very large genetic heterogeneity [8]. These findings revealed a lower genetic variation among populations (37.57%) rather than within populations (62.43%). The second cluster was specific for *T. zillii* Manzalah and Qarun, the genetic variation percentage between and within their individuals was 57.52% and 42.48%, respectively. A significant Φst value was 0.57325, which led to a very large genetic heterogeneity [37] and different genetic structure between two populations of *T. zillii*. A dendrogram for tilapia species was constructed using unweighted pair-group method (UPGMA) [38] producing two branches (branch I and II). Branch I clustered the populations of *O. aureus* and *T. zillii* Qarun, while the populations of *O. niloticus* and *T. zillii* Manzalah were belonged to branch II. This topology describes the genetic structure among three species of tilapia and between two groups of *T. zillii* conformity to the geographical location of the populations (Fig.2).

Significance of mismatch distribution parameters (SSD) and raggedness index values (r) and non significance of Tajima's D and Fu's Fs tests (p>0.05) indicated that the populations of tilapia species are not under the selection or expansion, at least in the control region of mitochondrial DNA except populations of *T. zillii* Qarun (SSD=0.220, P-value=0.16, r = 0.83673, P-value=0. 14) which may be under the expansion. Lake Qarun is a closed system and acts as indicators for the effect of various anthropogenic reservoirs for agricultural and sewage drainage water of pollutants on organisms and a reflection of the overall El-Faiyum province [39]. On the other side, the largest brackish coastal lakes fringing the Nile Delta (Manzalah) is opened

ISSN: 0975-8585

system and connects to the Mediterranean Sea via three outlets, which permit the exchange of water and biota between the lake and the sea [40]. A highly genetic diversity in Manzalah population indicates large, stable, and effective population sizes [32]. The data of the present work showed that tilapia is a species with high potential for dispersal, suggesting that these species might have population structuring over large geographic areas. The analysis of a larger length of the control region DNA fragment is a good marker, which help to understand the genetic differentiation and population genetic structure, in addition to raise the challenging issue of how the genetic data can help fisher managers to manage the genetic fish resources in the face of anthropogenic environmental changes such as habitat loss and overfishing.

Table 1: Five restriction enzymes (R.E.) were cleaved the amplified mtDNA control region of three tilapia species producing 16 enzyme morph fragments with their digested inserts (bp).

R.E.	*Hinf*I				*Rsa*I				*Ava*II		*Msp*I			*Taq*I		
Enzyme morphs	A	B	C	F*	A	B	C*	D*	A	B	A	B	C*	A	B	C*
Digested insert (bp)	660 320	400 290	440 320	660 220 220	710 290	710 260	650 290	400 290	1000	550 450	820 180	520 300 180	500 220 110	700	520 300	1000 480

*Indicates new restriction morphs.

Table 2: Polymorphic types of tilapia species, which resulted from restriction enzymes cleavage of mtDNA products.

Restriction enzymes	*Oreochromis niloticus*	*Oreochromis aureus*	*Tilapia zillii*	
			Manzalah	Qarun
HinfI	A,C	A,B,C,F	B	F
RsaI	A,D	A,C	A,B,C	C
AvaII	A,B	A,B	A,B	A,B
MspI	A,B	A,B,C	A,B	C
TaqI	A,B	A,B,C	A,C	C

Table 3: Haplotypic frequencies of mtDNA are represented in the following table for three species of tilapia.

Haplotypes		*O. niloticus*	*O. aureus*	*T. zillii*	
Haplotype code	Composite* haplotype			Manzala*h*	Qarun
h1	AABBB	6(0.300)	8(0.308)	0.000	0.000
h2	AAABB	0.000	2(0.077)	0.000	0.000
h3	BAAAA	0.000	8(0.308)	8(0.5)	0.000
h4	CAAAA	8(0.4000)	4(0.154)	0.000	0.000
h5	FCCBC	0.000	4(0.154)	0.000	8(.571)
h6	CAAAB	2(0.100)	0.000	0.000	0.000
h7	AABBA	2(0.100)	0.000	0.000	0.000
h8	CDAAA	2(0.100)	0.000	0.000	0.000
h9	ACABA	0.000	0.000	4(0.25)	0.000
h10	ABBBC	0.000	0.000	4(0.25)	0.000
h11	FCCAC	0.000	0.000	0.000	6(0.429)
Number of samples		20	26	16	14
Nucleotide diversity (π)or Average of gene diversity		0.237	0.276	0.258	0.054
Number of haplotypes		5	5	3	2
Haplotype or Gene diversity(hd)		0.800	0.8205	0.7143	0.5714
Number of polymorphic sites		11	14	11	2

* Composite genotypes are denoted by capital letters for restriction morphs in the following order: *Hinf*I, *Rsa*I, *Ava*II, *Msp*I *and Taq*I.

ISSN: 0975-8585

Figure 1: Digestion of amplified PCR products from different tilapia species (L: 1-5) by restriction enzymes *Hinf*I, *Ava*II, *Msp*I. L7, L14 100 bp ladder.

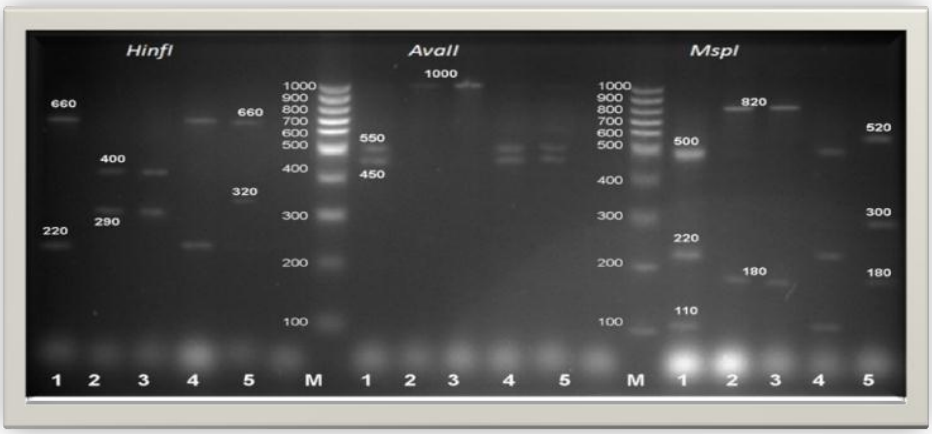
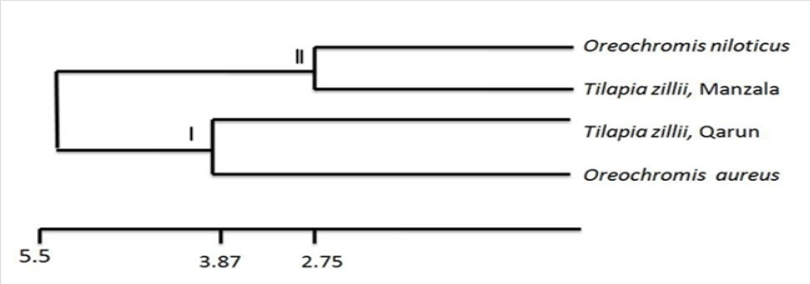


Figure 2: A dendrogram for tilapia species was constructed using UPGMA producing two branches. Branch I clustered the populations of *O. aureus* and *T. zillii* Qarun and branch II clustered *O. niloticus* and *T. zillii* Manzalah populations.



REFERENCES

[1] Amy, H R. Ph. D. Thesis, Institute of Aquaculture, University of Stirling, Scotland, and UK 2011.
[2] FAO. Fishery and Aquaculture Statistics. Rome, Italy 2007.
[3] FAO.World aquaculture production by species groups. Rome, Italy 2008.
[4] Awaad MH. The Egyptian J Hospital Med 2002; 6: 15 – 26.
[5] Wu L and Yang J. PLoS ONE 2012; 7(12): e51731.
[6] Agnese JF, Adepo-Gourene B, Abban EK and Fermon Y. Heredity 1997; 79: 88–96.
[7] He A, Luo Y, Yang H, Liu L, Li S and Wang C. Mol Biol Rep 2011; 38: 2015–2021.
[8] Rahim MHA, Ismail P, Alias RB, Muhammad N and Jais MA. Gene 2012; 494:1–10.
[9] Cheng Y, Jin X, Shi G, Wang R and Xu T. Biochem Systemat Ecol 2011; 39: 718–724.
[10] Toguyeni A, Thévenon AB, Soara SB, D'Cotta EC, Baroiller HCJF and Rogn X. Aquaculture 2007; 272S1, S238–S321.
[11] Heba AMA, Z G Abdel-Hamid and Karima FM. J App Biol Sci 2013; 7 (1): 01-04.
[12] Nyingi, D, Vos, LD, Aman, R and Agnèse, JF. Aquaculture 2009; 297:57– 63.
[13] Hansen MM, Ruzzante DE, Nielsen EE and Mensberg KLD. Mol Ecol 2000; 9: 583-94.
[14] John SWM, Weitzner G, Rozen R and Scriver CR. Nucleic Acid Res 1991; 19: 408-412.
[15] Bernatchez L and Danzmann RG. Mol Biol Evol 1993; 10: 1002–1014.
[16] Ewens W J. Theor Pop Biol 1972; 3: 87-112.
[17] Schneider, S, Roessli, D and Excoffier, L. Genetics and Biometry Lab, Dept. of Anthropology, University of Geneva 2000.
[18] Excoffier L and Lischer HEL. Linux and Windows Molecular Ecology Resources 2010; 10 (3): 564–567.
[19] Slatkin M. Genetics 1995; 139: 457-462.
[20] Slatkin M and Barton NH. Evolution 1989; 43: 1349–1368.

[21] Nei M. Molecular evolutionary genetics. Columbia University Press, New York 1987; p.512.
[22] Felsenstein J. Philosophical Transactions of the Royal Society of London, series B. 2005;360: 1427-1434.
[23] Tajima F. Genetics 1989; 123: 597–601.
[24] Tajima F. Genetics 1989; 123: 585–595.
[25] Fu YX. Genetics 1997; 147 (2): 915–925.
[26] Harpending HC, Sherry ST and Rogers AR et al. Curr Anthropol 1993; 34: 483-496.
[27] Excoffier L, Laval G and Schneider S. Evol Bioinform 2005; 1: 47–50.
[28] Rogers AR and Harpending H. Mol Biol Evol 1992; 9 (3): 552 -569.
[29] Schneider S and Excoffier L. Genetics 1999; 152 (3): 1079-1089.
[30] Harpending HC. Hum Biol 1994; 66 (4): 591-600.
[31] Romana-Eguia MRR, Ikeda M, Basiao ZU and Taniguchi N. Aquaculture 2004; 236: 131- 150.
[32] Xiao Y, Takahashi M, Yanagimoto T, Zhang Y, GAO T, Yabe M and Sakurai Y. Afr J Biotechnol 2008; 7(21): 3836–3844.
[33] Zhou G, Yung L, Li C, Xu W and Chen G. Biochem Syst Ecol 2010; 38(5): 911–916.
[34] Grant WAS and Bowen BW. J Hered 1998; 89 (5): 415–426.
[35] Ikeda M and Taniguchi N. Fisheries Science 2002; 68: 18–26.
[36] Fikiru E, Tesfaye K and Bekele E. Afr J Biotechnol 2007; 6 (12): 1460–1468.
[37] Wright S. Evolution and the genetics of population's variability within and among natural populations, vol. 4. University of Chicago Press, Chicago, 1978; p. 580.
[38] Sneath IHA and Sokal RR. Numerical taxonomy. W. H. Freeman and Company, San Francisco 1973.
[39] Fatma ASM. World Journal of Fish and Marine Sciences 2009;1(1): 29-39.
[40] Elewa AA, Saad EA, Shehata MB and Ghallab MH. J Aquat Biol Fish 2007; 11(2): 65 – 78.