# Genetic Relatedness and Phylogeny among Egyptian Cichlidae Species Based on mitochondrial D-Loop Region

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**Abstract:** Several species of fish, determined in the aquatic and saltwater habitats, shape the Cichlidae tilapia group. The common genera names for tilapia is Oreochromis, Sarotherodon, and Tilapia. Tilapias (family Cichlidae) are sizable in aquaculture and fisheries. The aim of the study is to estimate the genetic diversity, disclose polymorphism, and constructing the phylogenetic relationships between three tilapia species, *Tilapia zillii*, *Sarotherodon galilaeus*, and *Oreochromis niloticus*. Therefore, a total number of six fish specimens were sampled from different seven locations of Egyptian aquatic habitats and subsequently were assembled, aligned, and compared to GenBank databases based on the mtDNA D-loop region sequencing. In total, reported a length of 286 bp, 58 (20.2%) polymorphic sites, of them 51sites (17.8% two variants) and seven sites (2.4% three variants) and six haplotypes, 58 mutations were detected, the haplotype (Hd) and nucleotide variation ($\pi$) was calculated as 0.81and 0.10. The higher values of haplotype and nucleotide variation were observed in O. *niloticus* species. Based on the neighbor-joining (NJ) were assessed distances and genetic relationships. Among the three species, genetic distance representing different genera estimated, between *O. niloticus* and *S. galilaeus* was 0.97, *S. galilaeus* and *T. zillii* was 0.99, while between *O. niloticus* and *T. zillii* was 0.98, indicating that *S. galilaeus* and *O. niloticus* are closely related and the *T. zillii* is far more distant to *O. niloticus* than the *S. galilaeus*. Based on the data obtained from using the mtDNA D-loop region as candidate DNA markers, was confirmed accurate genetic diversity and phylogenetic relationships between three tilapiine species.

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## 1. Introduction

Tilapias are a group of aquaculture organisms of enormous potential and transplanted from their natural range in Africa to one form or another in most of the world. Although the typical environmental tolerances of various tilapia species, the species have minimal morphological variation, and the classification of species and its phylogenetic connections have been uncertain (McAndrew & Majumdar, 1983). In Egyptian waters, numerous tilapiine cichlid species were registered (*Oreochromis niloticus*, *O. aureus*, *Sarotherodon galilaeus*, and *Tilapia zillii*; Da Silva *et al.,* 1994). The tilapiine species are constituted an essential and economical fish in Egypt, especially *O. niloticus* (El-Sayed, 2006).

Statistics on molecular variation has shown to be very helpful in recent years in the solving of systematic problems, based on phylogenetic trees drawn from molecular studies (Mickevich & Johnson, 1976). Several researchers used tilapiine molecular data to support their classification (e.g., Saad *et al.* 2012; Wu & Yang 2012).

Variation and relationships between groups analyzed by developmental and genetic studies, form an integral part of farm programs, to use for reproductive stock choice and species identification for future conservation (De Silva, 2015). Tilapia are highly migratory and introgressive ratio fish that can change their genetic originality. Therefore, it becomes necessary to determine the genetic strategy and partnerships for the management and development of this essential organisms (Ekerette *et al.* 2017).

The D-loop sequences are non-coding regions of mtDNA, with no recombination and a high rate of evolution that became one of the most ordinarily used mtDNA sequences for processing the evolutionary relationship of closely related and/or subspecies (Romana *et al.,* 2004). Genetic studies of aquaculture organisms, originally concerned with genetic differences (Yokoyama & Goto 2002) and species validity (Genner *et al.,* 2007), phylogeny and molecular differentiation (Ozoje *et al.* 2018), were at this period extensively used in genetic analysis.

In the current study, the phylogenetic relationship was evaluated among three Egyptian tilapiine using D-loop mtDNA sequencing.

## 2. Material and Methods

### 2.1 Material sampling

Tilapias were sampled at 7 locations along the stream of the Nile, namely Suez Canal (SC), Ismailia aquafarms (IS), Nasser Lake (NL), El-Qanater (QN), Broulus Lake (BL), Qaroun Lake (QL) and Al-Sirw Canal (SS), species individuals were collected based on their morphological characterization (Trewavas, 1983) and bulked into four morpho-homogenate samples.

### 2.2 DNA extraction and PCR amplification

Genomic DNA was isolated from the fin tissue of two samples from each one tilapiine species studied (*O. niloticus, T. zillii,* and *S. galilaeus*). DNA extraction and purification were performed, according to (Al-Zafiri *et al*. 2018). The mtDNA region D-loop was amplified using 2x RedMix PCR master-mix (Bioline, UK) by applying the primer pair ormt-F (5'-CTA ACT CCC AAA GCT AGG AAT TCT-3') and ormt-R (5'-CTT ATG CAA GCG TCG ATG AAA-3'), the PCR conditions were set as standard with annealing Tm of 55 ◦C. Successful PCR products were cleaned and concentrated using ISOLATE II PCR and Gel kit (cat. no. BIO-52059, Bioline, UK); cleaned fragments sequenced by private service (Macrogene, Netherlands).

### 2.3 Data analysis

The chromatographs were assembled and collapsed into haplotypes using Bioedit Hall, 1998. Sequence ID was issued according to BLAST tool (NCBI). Mega X Tamura and Kumar, (2007) was used to perform pairwise alignment and construct the phylogenetic tree. DNASP6 Librado & Rozas, (2009) was used to estimate the genetic diversity within and between the Egyptian tilapiine.

## 3. Results

### 3.1 Identification by BLAST analysis

The sequence analysis of all the sequences sent to GenBank contributed to a clear correspondence of three studied organisms showing significant species-specific commonalities centered on GenBank databases. These databases reported conclusive identification matches for mtDNA D-loop consensus sequences in the Genebank with a distance of 96 –100 %. Accession numbers MG728029, MG728033, MG728035, and MG728040 for *O. niloticus*; KY940665, KY940662, KY940661, and KY940660 for *S. galilaeus*; EU163710, EU163714, and AF328853 for *T. zillii* (Table 1).

After alignment, mtDNA D-loop region for the 5 samples from the current study and additional 15 accessions retrieved from GenBank reported a length of 286 bp, in which 209 (73.0%) monomorphic sites, 58 (20.2%) polymorphic sites, 51 of them were parsimony informative sites, 17.8% were two-variants sites, and 7 were three-variant sites (Site position 18, 89, 92, 93, 225, 280, 285; Fig. 2).

Table 1. BLAST results for the D-loop region for the studied tilapiine species Including species, % pairwise, %, GC, accessions number.

| Species | Pairwise% | D-loop accessions | Sample code |
|---|---|---|---|
| *O. niloticus* | 99.8% | Sample 1 | *Oni* |
| | 99.5% | MG728029 | *Oni* |
| | 99.5% | MG728033 | *Oni* |
| | 99.5% | MG728035 | *Oni* |
| | 99.5% | MG728040 | *Oni* |
| *S. galilaeus* | 96.1% | Sample 2 | *Sga* |
| | 96.1% | KY940665 | *Sga* |
| | 96.1% | KY940662 | *Sga* |
| | 96.1% | KY940661 | *Sga* |
| | 96.1% | KY940660 | *Sga* |
| *T. zillii* | 100% | Sample 3 | *Tzi* |
| | 100% | Sample 4 | *Tzi* |
| | 100% | AF328853 | *Tzi* |
| | 100% | EU163710 | *Tzi* |
| | 100% | EU163714 | *Tzi* |

Based on D-loop sequences, the overall haplotype diversity and nucleotide diversity were 0.81, 0.10, respectively. Haplotypes number was 1-3 per species. For each species, the *O. niloticus* species, three segregating sites were detected, three haplotypes, and showed the highest level of haplotype diversity (0.7). The *S. galilaeus* species, one segregating site was detected, and one haplotype was

found, haplotype and nucleotide diversity were 0.4, 0.00, respectively. The *T. zillii* species had no segregating sites and recorded a single haplotype, and the haplotype and nucleotide diversity were 0.00 for both (Table 2).

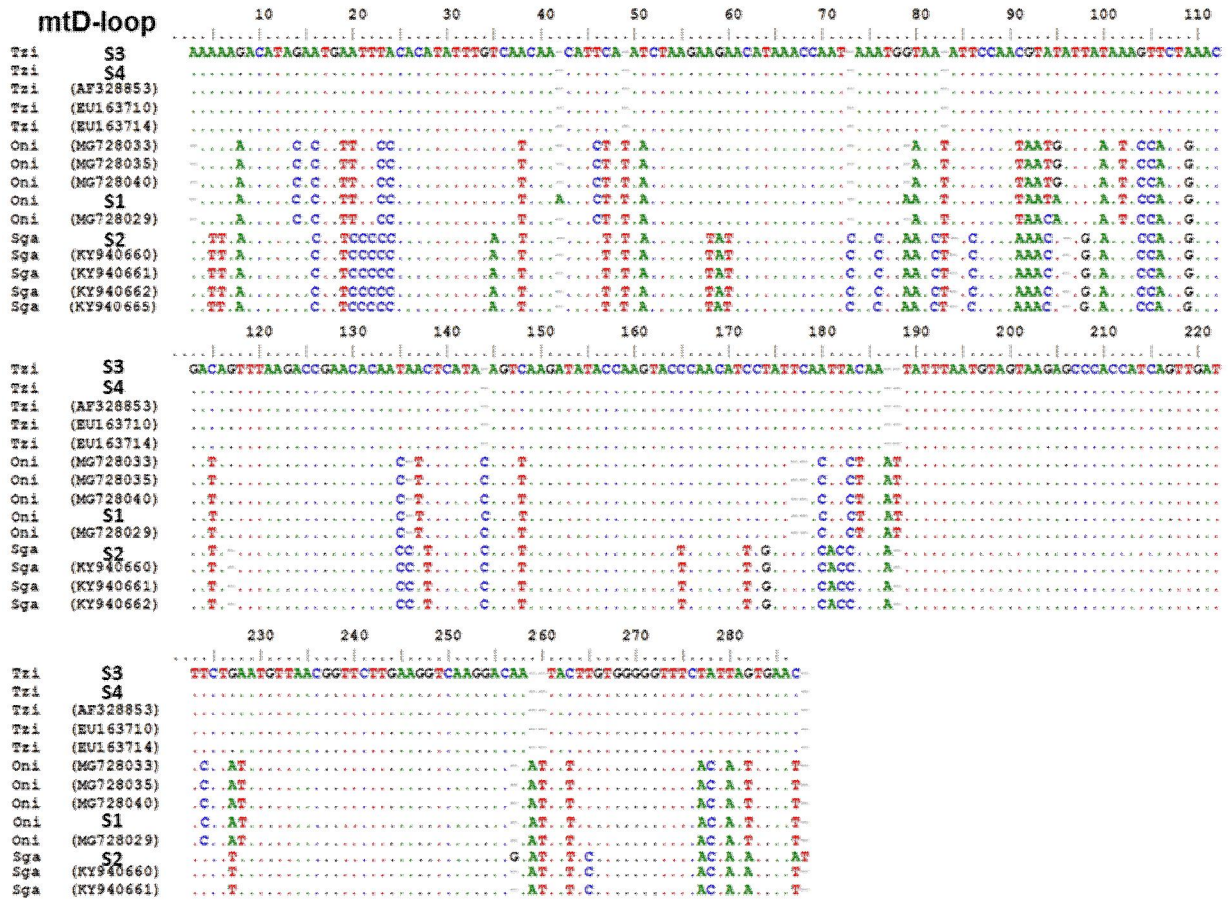


Figure 1. Comparative nucleotide alignment of the mtDNA D-loop region between tilapiine species, alignment length of 286bp shown. Abbreviations: *O. niloticus* (Oni), *S. galilaeus* (Sga), *T. zillii* (Tzi).

Table 2. DNA polymorphism indices between the studied tilapia species based on the mtDNA D-loop region. Number of sequence (N), haplotype diversity (Hd), number of haplotypes (H), number of segregation sites (S), nucleotide diversity (π).

| **Species** | **N** | **S** | **H** | **Hd** | **Pi** |
|---|---|---|---|---|---|
| *O. niloticus* | 5 | 2 | 3 | 0.70 | 0.00 |
| *S. galilaeus* | 5 | 1 | 2 | 0.40 | 0.00 |
| *T. zillii* | 5 | 0 | 1 | 0.00 | 0.00 |
| Total | 15 | 58 | 6 | 0.81 | 0.10 |

According to the mtDNA D-loop sequences, the genetic distances of the tilapiine species studied determined between *O. niloticus* and *T. zillii* were 0.98, *O. niloticus*, and *S. galilaeus* was 0.97, while between *S. galilaeus* and *T. zillii* was 0.99 (Table 3).

Table 3. The genetic distance between the studied tilapia species based on the mtDNA D-loop region.

| **Species** | *O. niloticus* | *S. galilaeus* | *T. zillii* |
|---|---|---|---|
| *O. niloticus* | - | - | - |
| *S. galilaeus* | 0.97 | - | - |
| *T. zillii* | 0.98 | 0.99 | - |

The phylogenetic tree was conducted in MEGA X and was generated using the Neighbor-Joining method, and the distances were computed using the Tamura 3-parameter method.

The tree divided into one main cluster rooted by an outgroup (flathead gray mullet fish, *Mugil cephalus*). Two main clusters were found, one cluster included at one branch, the *O. niloticus* of the current study (sample no. 1) grouped with the *O. niloticus* retrieved from GenBank (accession number: MG728029, MG728033, MG728035, and MG728040), and recorded a bootstrap value of 99. And another branch where the *S. galilaeus* of the current study (sample no. 2) and *S. galilaeus* retrieved from GenBank (Accession number: KY940665, KY940662, KY940661, and KY940660) were grouped together with bootstrap support of 100.

The second cluster included the *T. zillii* samples of the current study (samples no., 3 and 4) and *T. zillii* retrieved from the GenBank (accession number: EU163710, EU163714, and AF328853) together, with a bootstrap value of 100 (Fig. 3).

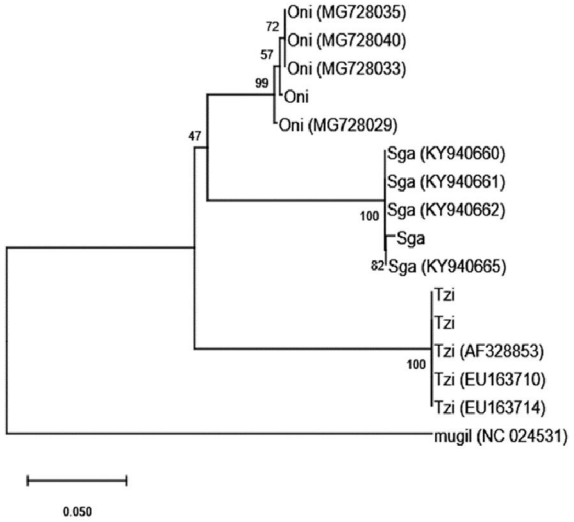


Figure 2. The phylogenetic tree showing the relationship between tilapiine fish according to mtDNA D-loop region *O. niloticus* (Oni), *S. galilaeus* (Sga), *T.zillii* (Tzi) the tree is rooted by the flathead grey mullet *Mugil cephalus* (mugil; accession number NC024531) from the family Mugilidae.

## 4. Discussion

Several molecular methods were used to identify tilapia species Liu & Cordes, (2004) e.g. Microsatellite. The mitochondrial DNA is known as a more accurate and functional molecular approach to species classification (Al-Zafiri *et al.,* 2018), because they characterized by high rate of mutations, and therefore used as a tool to build phylogenetic tree and identify species Cheng & Stoneking, (1994). The D-loop region is considered to be the most variable site in mtDNA and therefore represents the maximum level of contrast (Cheng *et al.,* 2015). Accordingly, haplotype analysis of the D-loop region is commonly used as a useful tool for classifying the genetic diversity of fish species (He *et al.*, 2011). The results we have achieved indicated that, the largest number of haplotypes and haplotype diversity was found among *O. niloticus* species, followed by *S. galilaeus* species. A previous study had indicated that the high levels of genetic diversity may ensure strong adaptability and survival skills of species (e.g. *Gasterosteus aculeatus*; Barrett & Schluter, 2008).

Tilapia fish are highly migratory species with a high ratio of introgression, which may affect their genetic originality, widely used to research aquaculture, phylogeny and molecular differentiation. Therefore, it is necessary to analyze their genetic characteristics and their relationship with other species to fully understand these genetic species (Ekerette *et al.,* 2017).

The results showed that there is a relationship between the two different genera of tilapia fish (*O. niloticus and S. galilaeus*) and are close the each other than *T. zillii* genus, in accordance with previous studies (Pouyaud & Agnese, 1995; Schwarzer *et al.,* 2009), the conclusion of the same results was confirmed using other markers such as SNP markers (Shirak *et al.,* 2009; Syaifudin *et al.,* 2019). This relationship can be explained by two Hypotheses: hypothesis one, the development of the mtDNA D-loop region is not environmentally affected, but it is possible to trace variations with other factors influences the genome (Ekerette *et al.,* 2017). Hypothesis two, the ancestors that lived before the extinction of both groups may be one of these species Duvernell & Aspinwall, (1995).

In conclusion, based on the data obtained from the mtDNA D-loop region results, an accurate genetic diversity and phylogenetic relationships between three tilapiine species were reached, reflecting the usefulness and importance in D-loop region, not only for population analysis, but further for species-based identification.

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