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CYTOCHROME OXIDASE SUBUNIT I GENE BASED IDENTIFICATION OF THE COMMON EGYPTIAN TILAPIINE SPECIES

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Cichlidae is one of the most prominent vertebrate families, new species are discovered annually, and many species stay undescribed (Froese and Pauly, 2006). Increasing the scientific interest about Cichlid due to the rapid adaptive radiation, which has led to a significant ecological diversity and their vast importance to tropical and subtropical aquaculture (Poletto *et. al*., 2010).

Tilapia is the common name currently applied to three genera and species of fish within the family Cichlidae: *Oreochromis*, *Sarotherodon*, and *Tilapia*. Native to Africa are distributed throughout the globe and became the second most significant consumable fishes within the world (Fitzsimmons, 2016). Not much is known about the genetic relatedness among the tilapiine species, even though they form an essential component of the African aquaculture (Nagl *et. al*., 2001).

In the last decade, mtDNA has been used as a genetic marker for genetic structures, species identification, molecular phylogenetics and population studies due to its patterns of maternal inheritance, absence of recombination, relatively rapid

evolutionary rate and a high degree of mutation rate (Boore, 1999; Meng *et. al*., 2008). Molecular diagnostic techniques are now commonly employed in species identification; it can provide a mean for correct identification when morphological identification is uncertain or impossible and also in case of possible hybrids among species (Meyer and Paulay, 2005; Sogbesan *et. al*., 2017). The proposition of a single gene sequence as the mitochondrial DNA *cytochrome oxidase subunit I* (*COI*) gene to be a global bio-identifiar marker for animals was proven to be efficient to differentiate all, or at least the vast majority of animal species (Hebert *et. al*., 2003). The *COI* gene was utilized as the determiner in several Cichlidae species characterization studies (e.g. Saad *et. al*., 2019; Sogbesan *et. al*., 2017). The objective of this study was to identify different species of Egyptian tilapiine (*Oreochromis niloticus, Tilapia zillii*, and *Sarotherodon galilaeus*) using *cytochrome oxidase subunit I* (*COI*) gene and to determine the genetic relatedness among those species.

MATERIALS AND METHODS

DNA extraction, PCR amplification and sequencing

All the specimens for the current study were collected from different locations in Egypt. The specific species of the study were identified using the tail morphological aspect (the fin tail spot arrangements). DNA from modern specimens was extracted from muscles of different Egyptian tilapiine species (*O. niloticus, T. zillii* and *S. galilaeus*) according to the protocol of Li *et. al*., (2015). The *CO1* gene which located in the mitochondrial genome was amplified using the pair of primers COI-FF2D-1 (5'-TTC TCC ACC AAC CAC AAR GAY ATY GG-3') and COI-FR1D-1 (5'-CAC CTC AGG GTG TCC GAA RAA YCA RAA-3'). PCR amplification was performed using EasyTaq® DNA polymerase (Trans, China); all reactions were performed in a total volume of 25 µl, containing 2.5 µl of EasyTaq® 10X buffer, 2 µl of dNTPs (10mM), 0.5 µl of each primer (Forward and Reverse, each of 10mM), 0.2 µl of EasyTaq® DNA polymerase and 1 µl of extracted DNA (~100 ng/µl). The thermal program consisted of initial step of denaturation at 95°C for 5 min followed by 30 cycles of 95°C/ 30s, annealing at 55°C/ 30s, extension at 72°C/ 30s, and a final extension segment at 72°C/ 10min. PCR products were separated by electrophoresis in a 1.5% agarose gel and visualized under UV transilluminator. The resulted *COI* gene fragments were purified using EasyPure PCR Purification Kit (Trans, China). Finally, *COI* fragments were sent for sequencing (Macrogen Inc., South Korea).

Data analysis

Sequences were evaluated, assembled, and aligned using Geneious V10.2.5 software *(*Kearse *et. al*., 2012). Based on the *COI* genesequences, the matches from the GenBank (NCBI) database were retrieved using BLAST search tool (Table 1). The molecular diversity such as polymorphic sites, the average number of nu-

cleotide differences, haplotype diversity (h), Number of segregating sites (S), and nucleotide diversity (л) were estimated using DnaSP v. 6.12.01 (Rozas *et. al*., 2017). The analysis of molecular variance (AMOVA; Excoffier *et. al.*, 1992) was calculated to estimate the levels of genetic divergence among the common Egyptian tilapiine species. The phylogenetic tree was performed by MEGA X software (Tamura *et al*., 2018) using the Neighbor-joining method (Saitou and Nei, 1987) with 500 bootstrap replicates.

RESULTS AND DISCUSSION

Polymorphism and sequence divergence

Multiple sequences of examined samples were characterized, isolated, purified, sequenced and analyzed; similarities between sequences were calculated based on the sequence alignments. GenBank databases were searched for matches in the range of 93% - 99.9% similarity and above 90% query coverage. The amplified sequences of the *COI* gene from the Egyptian tilapiine species were aligned, forming 556 bp alignment (Figs. 1-3). A total of 462 sites were monomorphic, and 94 sites were polymorphic; 5 sites of 94 were singleton variable included two variants, and 89 sites were parsimony informative; 84 sites included two variants, and five sites included three variants (28, 58, 86, 179, 392).

Intraspecific sequence variation

Intrapopulation diversity indices in Egyptian tilapiine species were detected in the examined range based on the *COI* gene. Seven samples; three *O. niloticus*, two *T. zillii*, and two *S. galilaeus*; which were compared with a similar sequence obtained from GenBank (NCBI) database (Table 1). The number of haplotypes (Table 2) (h) was 4, 2, 1 for *O. niloticus, T. zillii*, and *S. galilaeus,* respectively. The haplotype diversity (Hd) was 0.21, 0.17, and 0.00 for *O. niloticus, T. zillii*, and *S. galilaeus*, respectively. While the nucleotide diversity (л) was 0.0012, 0.0007, and 0.00 for *O. niloticus*, *T. zillii,* and *S. galilaeus,* respectively. The AMOVA analysis denoted a strong level of genetic structure between the Egyptian tilapiine species. The highest percentage of genetic variation was detected between populations of *T. zillii* and *S. galilaeus* where the F-statistics (Fst) was equal to 0.996; the variation between *O. niloticus* and *T. zillii* comes after, with Fst equal to 0.992, and finally the variation between populations of *O. niloticus* and *S. galilaeus* Fst was equal to 0.991 (Table 3). Several studies used the region of *COI* gene to indicate intraspecific variation; for example, Mohammed-Geba *et al*., (2017) identified the Nile tilapia (*Oreochromis niloticus*) from different water systems in Egypt. Additionally, sometimes the *COI* proved to be more useful when combined with other mitochondrial markers, Wu and Yang (2012) compared intraspecies variation between the mitochondrial DNA control region and *COI* gene for captive

and wild tilapia populations in Oahu and Hawaii successfully.

Phylogenetic relationship

A total of 43 *COI* sequences were aligned and utilized to construct a Neighbor-Joining tree at 500 bootstrap replicates under Kimura 2-Parameter (K2) substitution model (Kimura, 1980). The 43 *COI* alignment formed 556 base pairs in length. All studied species displayed clades of conspecific sequences and showed a match between the present study and the GenBank (NCBI) database. The tree displayed significant separation between the three species of Egyptian tilapiine (*O. niloticus*, *T. zillii*, and *S. galilaeus*); each species clustered into a unique branch. The *COI* gene tree separated all the three species of Egyptian tilapiine into three clusters group based on their genetic distance (Fig. 4). The tree showed *T. zillii* as the most distant; while *O. niloticus* was closer to *S. galilaeus* (Fig. 4). All groups clustered with 100% bootstrap value and showed next to each branch. The overall distance value within the evaluated Egyptian tilapiine sample was 0.07. In this study, based on the basis of the mitochondrial lineage, the divergence between the three species of Egyptian tilapiine clusters was sufficient to distinguish each group as a separate species, which was in concordance with the work of Sogbesan *et. al*., (2017) who identified four species that belong to Cichlidae family (*Oreochromis niloticus*, *Sarotherodon galilaeus*, *Sarotherodon galilaeus boulengeri* and *Coptodon zillii*) from Upper Benue River and Lake Geriyo. Another exmple, Maranan *et. al*., (2016) applied the *COI* gene to delimit the species within the genus *Oreochromis* (i.e, *O. niloticus*, *O. mossambicus* and *O. aureus*).

In Conclusion, the present study highlighted to reinforce the usefulness of the mitochondrial *COI* gene for fish species identification and to estimate genetic relationships, especially when the morphological characteristics are unreliability or inaccurate.

SUMMARY

DNA barcoding has become a massively applied tool for accurate and rapid identification of various taxa using *COI* gene. The current study aimed to identify different species of Egyptian tilapiine using *cytochrome oxidase subunit I* (*COI*) gene. Total seven specimens were sampled, representing three species *Oreochromis niloticus*, *Tilapia zillii* and *Sarotherodon galilaeus*. DNA was extracted, PCR was performed, a conventional assay using gel electrophoresis, purified the amplicons, sequenced and analyzed. This study has validated the efficacy of *COI* gene for identifying fish species. The Egyptian tilapiine identity was confirmed and stated that this marker is suitable for its molecular identification. Additionally, it was successful to identify closely related species and determine the genetic relationship among them.

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Table (1): Blast results for the *COI* gene; for the seven samples of the common Egyptian tilapiine species. Including species name, percentage of pairwise, GC content, the accession number, and sample code.

Species name	Pairwise%	GC%	*COI* accessions	Sample code*
O. niloticus	98.7%	46.4%	MF280061	Oni 1
	98.7%	46.4%	MF509597	Oni 2
	96.5%	46.4%	MH515205	Oni 3
	96.5%	46.4%	MH515206	Oni 4
	96.5%	46.4%	MH515207	Oni 5
	96.5%	46.4%	MH515211	Oni 6
	96.5%	46.4%	MH515213	Oni 7
	96.5%	46.4%	MH515214	Oni 8
	96.5%	46.4%	MH515215	Oni 9
	96.5%	46.4%	MH515217	Oni 10
	96.5%	46.4%	MH515219	Oni 11
	96.5%	46.4%	MH515225	Oni 12
	96.5%	46.4%	MH515234	Oni 13
	96.5%	46.4%	MH515235	Oni 14
	96.5%	46.4%	MH515237	Oni 15
	96.5%	46.4%	MH515238	Oni 16
	93.7%	46.4%	MH515239	Oni 17
	96.5%	46.4%	MH515261	Oni 18
	96.5%	46.4%	MH515271	Oni 19
	96.5%	46.4%	MH515278	Oni 20
	96.5%	46.4%	MH515289	Oni 21
	96.5%	46.4%	MH515294	Oni 22
	93.7%	46.4%	MH721191	Oni 23
	93.7%	46.4%	MK049493	Oni 24
	93.7%	46.4%	MK448187	Oni 25
	92.9%	46.6%	current study	Oni 26
	87.5%	46.0%	current study	Oni 27
	97.4%	46.6%	current study	Oni 28

Table(1):Cont'

T. zillii	97.5%	50.2%	FJ348132	Tzi 1
	97.6%	50.2%	MG438464	Tzi 2
	97.5%	50.2%	HM882904	Tzi 3
	97.5%	50.2%	KJ552862	Tzi 4
	99.9%	50.2%	KJ938166	Tzi 5
	99.9%	50.2%	KJ938169	Tzi 6
	97.6%	50.2%	KJ938219	Tzi 7
	97.6%	50.2%	KJ938220	Tzi 8
	97.5%	50.2%	KY465478	Tzi 9
	99.8%	50.2%	current study	Tzi 11
	94.6%	50.2%	current study	Tzi 12
S. galilaeus	99.5%	47.3%	KM438544	Sga 1
	99.5%	47.3%	KM438546	Sga 2
	99.8%	47.8%	current study	Sga 3
	99.8%	47.8%	current study	Sga 4

**O. niloticus* (Oni), *T. zillii* (Tzi), *S. galilaeus* (Sga)

Table (2): Basic parameters of the *COI* gene of mitochondrial DNA in *O. niloticus, T. zillii,* and *S. galilaeus.*

	Species	NS	S	h	Hd	л
	O. niloticus	28	9	4	0.21	0.0012
	T. zillii	11	2	2	0.17	0.0007
	S. galilaeus	4	0	1	0.00	0.000

Number of sequence (NS); Number of segregating sites (S), Number of haplotypes, haplotype diversity (Hd), Nucleotide diversity (л).

Table (3): The genetic differentiation estimation among the common Egyptian tilapiine species based on *COI* gene sequence.

F_{ST}	*T. zillii*	*O. niloticus*
T. zillii	0	
O. niloticus	0.992	0
S. galilaeus	0.996	0.991

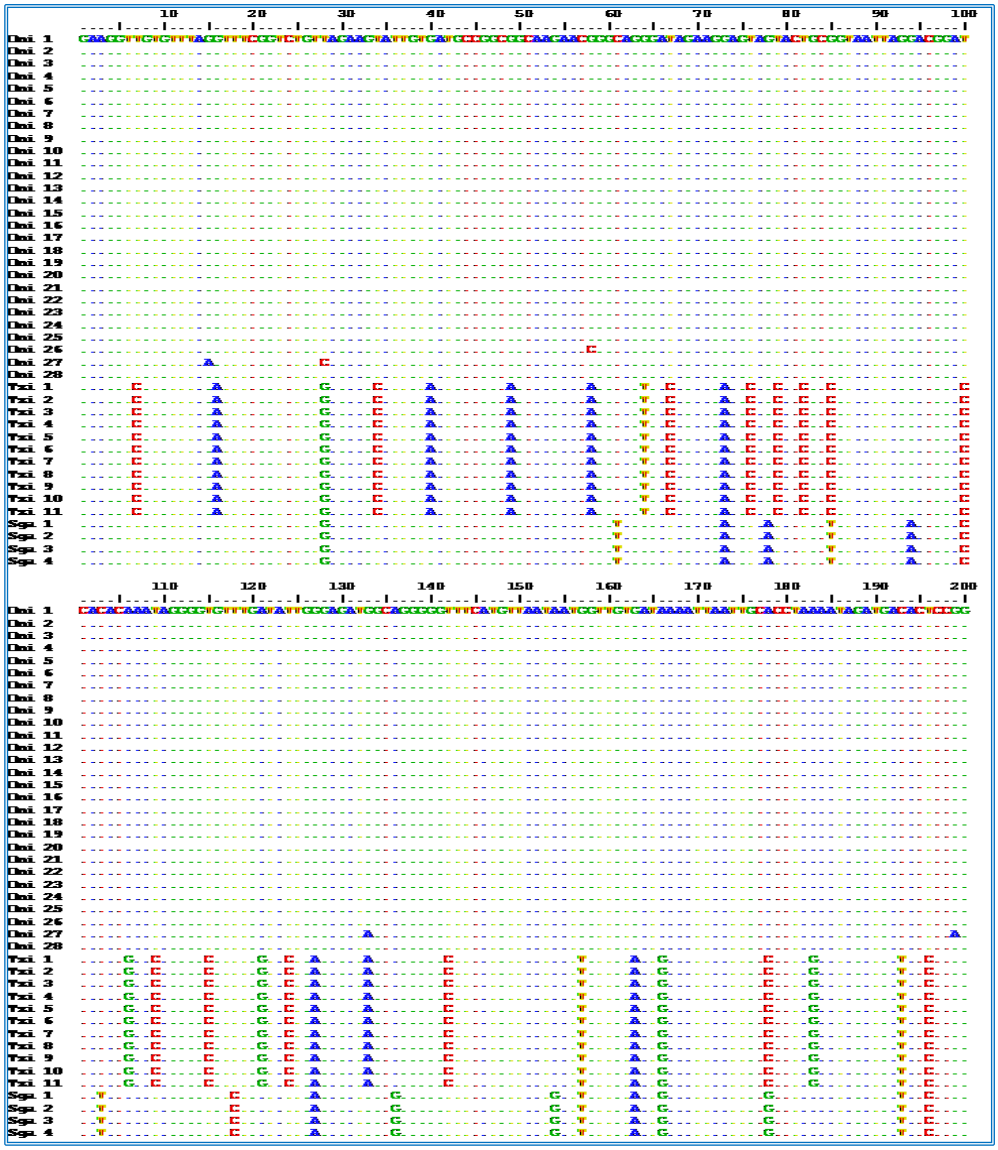

Fig. (1): Comparative alignment of *COI* gene sequence (part 1: from 1-200 bp); between different species of the common Egyptian tilapiine; *O. niloticus*, *T. zillii*, and *S. galilaeus* and matched accession from Genbank. Dotes (.) represents identical nucleotide. Numbers from 1 to 200 represents the ruler for bp length.

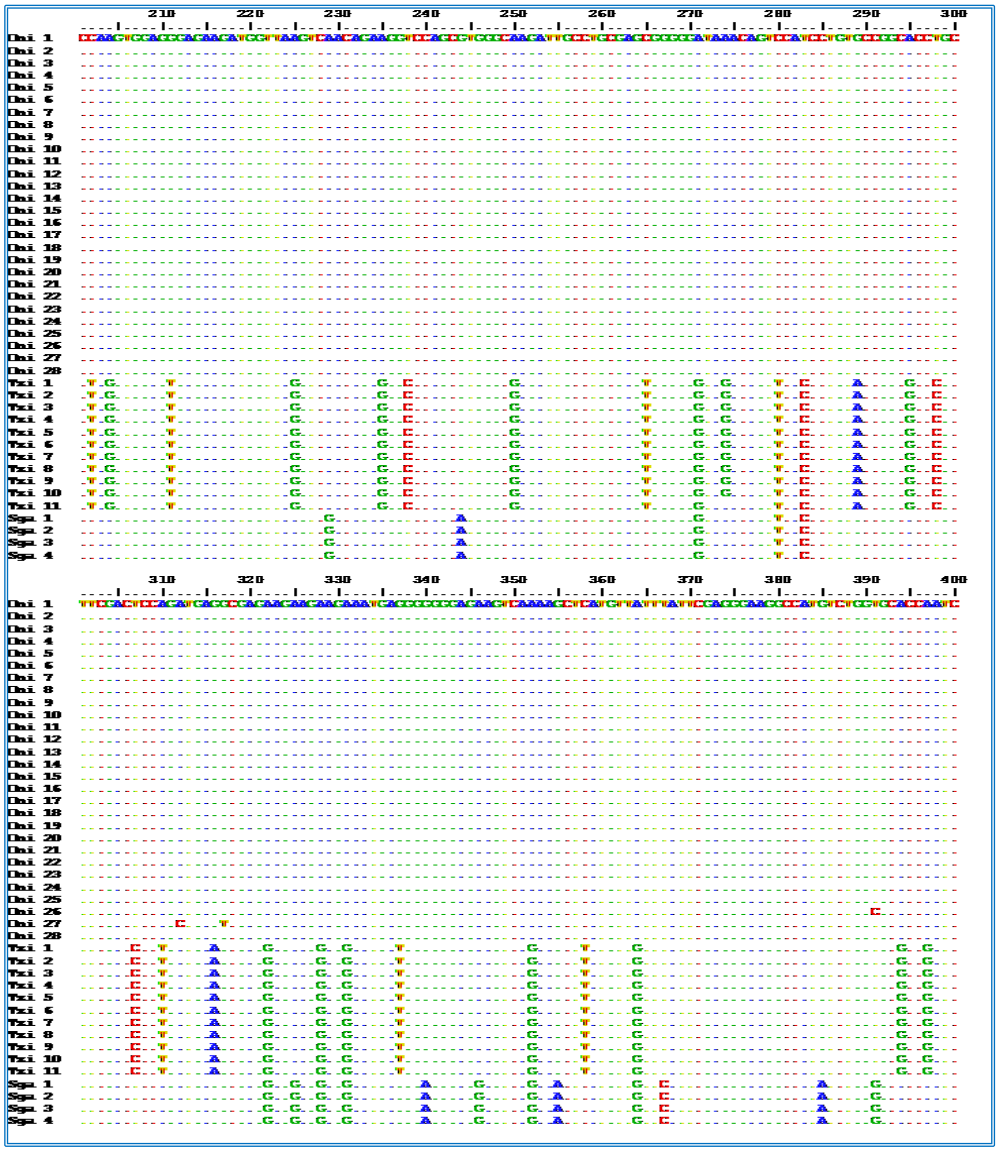

Fig. (2): Comparative alignment of *COI* gene sequence (part 2: from 201-400 bp); between different species of the common Egyptian tilapiine; *O. niloticus*, *T. zillii*, and *S. galilaeus* and the matched accession from Genbank. Dotes (.) represents identical nucleotide. Numbers from 201 to 400 represents the ruler for bp length.

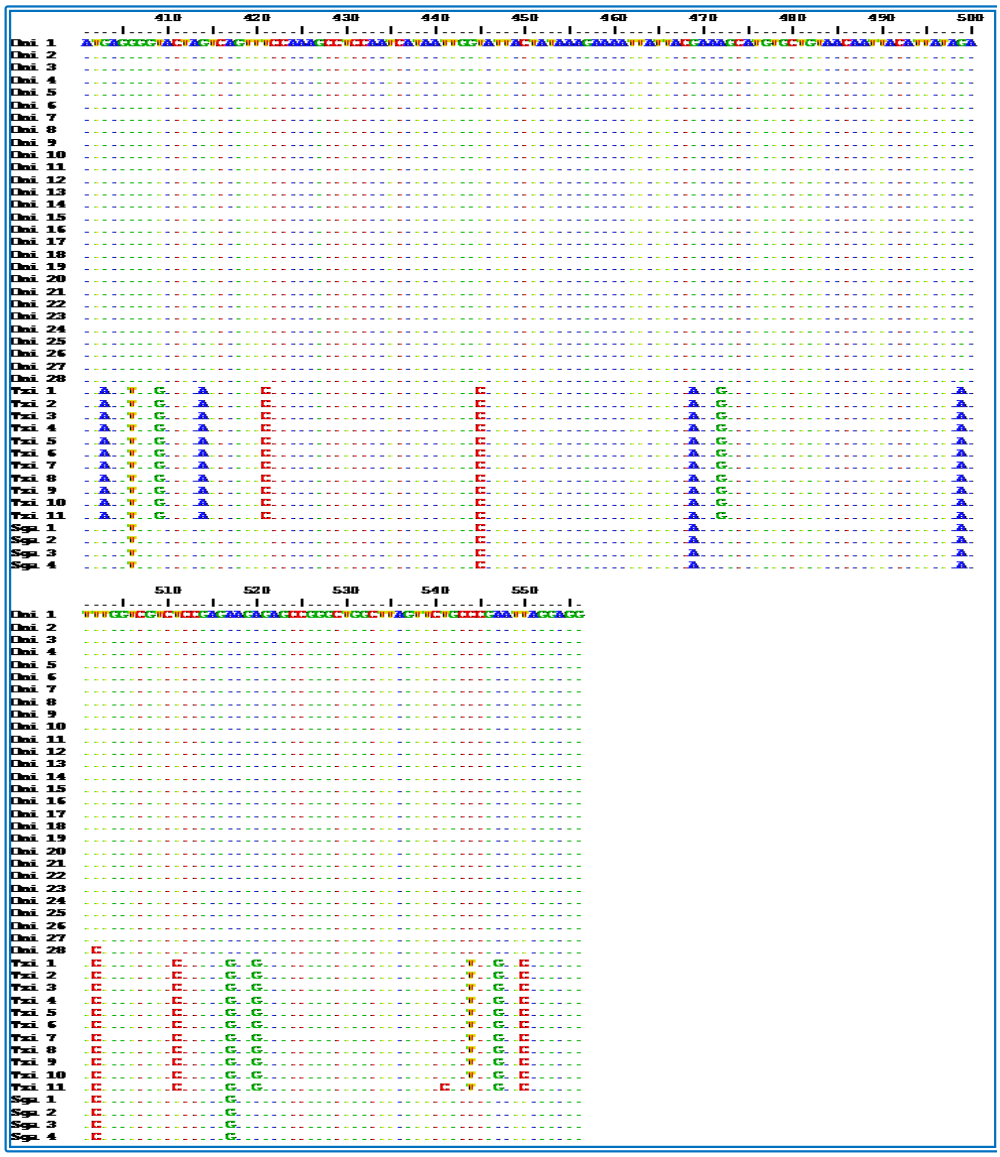

Fig. (3): Comparative alignment of *COI* gene sequence (part 3: from 401-556 bp); between different species of the common Egyptian tilapiine *O. niloticus*, *T. zillii,* and *S. galilaeus*, and the matched accession from Genbank. Dotes (.) represents identical nucleotide. Numbers from 401-556 represents the ruler for bp length.

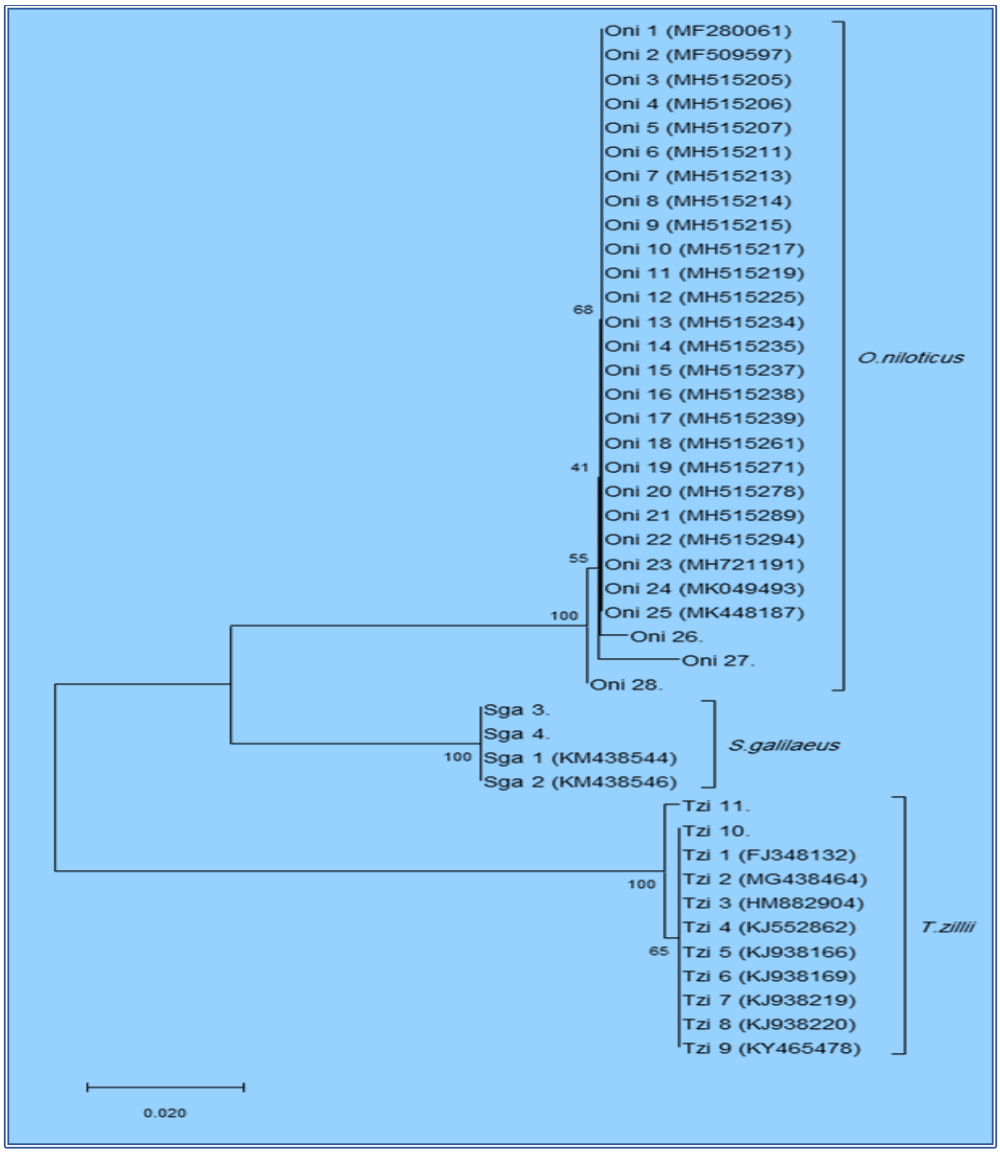

Fig. (4): The phylogenetic tree between different species of Egyptian tilapiine *O. niloticus, T. zillii,* and *S. galilaeus*, showing a match between the present study and sequence in the GenBank database, using the Neighbor-Joining method (Kimura 2-parameter substitution model) based on the mitochondrial *COI* gene. Bootstrap support percentages based on 500 replicates are shown.