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Cytochrome Oxidase I (COI) gene analysis of the Nile Puffer Fish (*Tetraodon lineatus*) from Lake Nasser, Aswan, Egypt.

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ABSTRACT

The study of the Nile Puffer Fish (*Tetraodon lineatus*) from Lake Nasser, Aswan, Egypt is still at a very early stage. Samples from two locations (Khor El Ramla and Tushka East) from Lake Nasser were studied. Partial sequences of the COI gene (barcode) for 20 Nile Puffer Fish from Lake Nasser were successfully amplified and analyzed. The average of nucleotides composition percent of both locations samples was 25.88, 31.54, 24.53, and 18.05 for T, C, A, and G respectively. The base composition analysis of both locations samples of the COI sequence revealed that AT content (50.41%) was higher than GC (49.59%). The current results of the phylogenetic analysis of Lake Nasser samples indicate that there are three groups of Nile Pufferfish in Lake Nasser. Two of them are presented in the south and the last one in the north and south of the Lake. Conversely, the GenBank and Lake Nasser samples indicate that there are two haplotypes from *Tetraodon lineatus* of the two locations from Lake Nasser based on the partial sequence of the COI gene. The first haplotype includes different samples from the two studied locations (North and south Lake) and the second includes five samples from the North Lake. Finally, the present phylogenetic results for Nile Pufferfish in Lake Nasser (*Tetraodon lineatus*), together with other African freshwater groups, matched with the monophyletic origin of African freshwater Puffer fishes. As a conclusion, overall data collected from this study will be fundamental for the Lake Nasser fishery and the handling of the problems caused by Nile pufferfish. Therefore, proper utilization of this species towards sustainable management, nutritional composition, and biosecurity issues will lead to achieving a sustainable blue economy.

INTRODUCTION

In Africa, Aswan Reservoir is the second greatest artificial freshwater lake. The lake is approximately 500 km long and almost 35 km wide. The lake is about 300 km long in Egypt (Lake Nasser). The identified Lake Nasser aquatic animals' community contains 52 species within 15 families of fishes including Tetraodontidae (*Tetraodon*

lineatus) (**Van Zwieten *et al.*, 2011**). In Egypt, Lake Nasser capture fisheries represented about 66% of the total inland lakes production (**GAFRD, 2016**).

Due to their strange morphological characters, tetraodontiforms fishes have attracted the attention of biotechnologists, ichthyologists and ecologists.

The Nile puffer fish (*Tetraodon lineatus*) is a tropical freshwater puffer fish found in Egypt (upper Nile) and several basins in Africa (**Akinyi *et al.*, 2010**; **Froese and Pauly, 2017**). It can grow up to 42 cm in length. Also, they could inflate during threatening and have the tetrodotoxin (TTX) mainly in internal organs (**Çaklı and Yılmaz, 2017**).

Barcoding system was lately confirmed in differentiated between species of aquatic animals (**Galal *et al.*, 2016**). The mitochondrial cytochrome oxidase subunit I gene (COI) is the standard barcode for puffer fish and fishery products. Mitochondrial DNA is quite heat-resistant, and it has a great level of recovery as the cell has higher quantities of mitochondrial DNA. The reference standard sequence library for seafood identification, GenBank, and FISH-BOL databases are used for aquatic animals' identification (**FAO, 2018**). Therefore, the studying of genetic diversity of *Tetraodon lineatus* by using Cytochrome Oxidase I (COI) gene is needed to change the negative impact of the puffer fish population on Lake Nasser's fisheries.

So, this study aims to study the genetic diversity of *Tetraodon lineatus* by using Cytochrome Oxidase I (COI) gene sequencing.

MATERIALS AND METHODS

Samples collection

A total of 20 Nile Puffer fish samples were collected using commercial nets from Lake Nasser, Aswan, Egypt (10 fish samples from Khor El Ramla, North Lake and 10 samples from Tushka East, South Lake) as shown in the **Figure 1**.

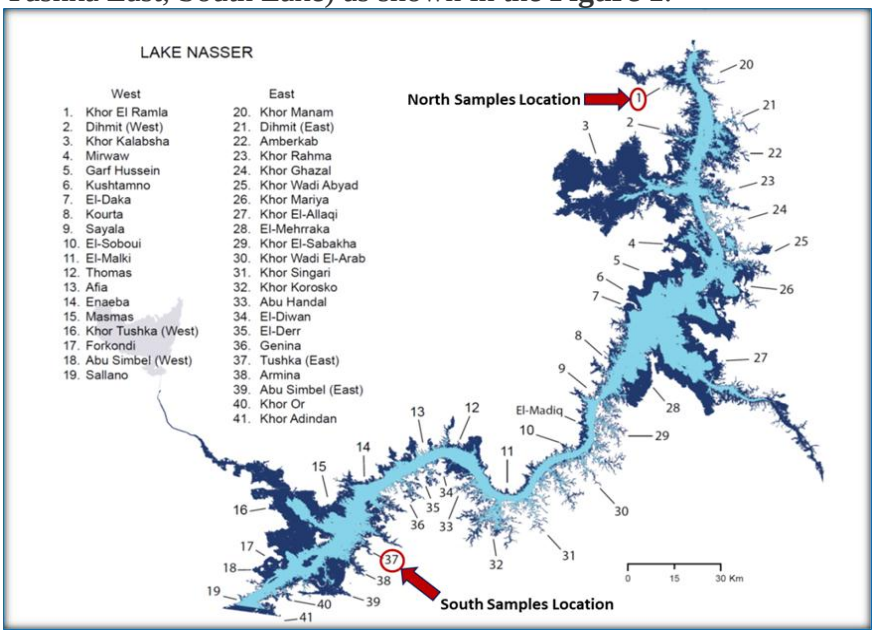

Figure 1: The two sampling locations from Lake Nasser, Aswan, Egypt. Location number 1 is Khor El Ramla and number 37 is Tushka (East). (**Van Zwieten *et al.*, 2011**).

The fishes were individually placed in plastic bags, labeled and placed inside coolers with ice pads for transport to the aquatic biotechnology laboratory where they were stored in the freezer at -20^{o}C for further analysis.

DNA extraction

DNA samples were extracted from the muscles tissues by using DNA extraction kit (iNtRON Biotechnology, Inc.; Korea) through following the manufacturer's instructions.

The extracted DNA quality was determined through 1% agarose gel electrophoresis To determine the extracted DNA concentration, 1µl of DNA was used in NanoDrop™ 2000 spectrophotometer, thermo scientific. The extracted DNA samples concentrations ranged from 50 to 80 ng/ µl.

COI specific primers

The COI specific primers were used as described by **Ward *et al.* (2005)**. The nucleotide sequences of the primers are shown in **Table 1**.

Table 1: Primers sequences that were used for CO1 gene amplificarion.

Primers	Sequence (5' 3')
FishF	5'-TCAACCAACCACAAAGACATTGGCAC-3'
FishR	5'-ACTTCAGGGTGACCGAAGAATCAGAA-3'

PCR amplification

All PCR reactions were carried out in a volume of 25 µL containing 100 ng of template DNA, 12.5 µL of PCR master mix (2X TOPsimpleTM PreMIX-nTaq, enzynomics, Korea) and 0.4 µM of each primer, then the volume completed to 25 µL by de-ionized water. The PCR amplification was achieved in an Applied Biosystems® ProFlex™ PCR System in the following conditions: pre-denaturation step at 94°C for 4 min., followed by 35 cycles of 60 s at 94°C, 55°C for 30 s and 60 s at 72°C and a final extension at 72 °C for 7 min.

The amplified PCR products were analyzed by 2% agarose gel electrophoresis. DNA ladder (50bp) was used to detect the COI fragment size. The electrophoresis run was done at 80 V in horizontal electrophoresis unit (Bio-Rad) for 90 mins. To visualize the obtained DNA fragments, UV-trans-illuminator (ELETTROFOR, Italy, EU) was used and the gel was photographed by digital camera with orang filter.

For elution, PCR product (40 µl) from each sample was injected in 0.8% agarose medium. DNA fragments of COI gene were eluted from the agarose gel according to the kit manufacturer's instructions of iNtRON Biotechnology, Inc. Korea.

COI gene sequencing and phylogenetic analyses

Partial sequencing of CO1 gene were performed through 373xl automated DNA sequencer (Applied Biosystems, Korea) using the COI gene forward primer.

For phylogenetic analyses, the related closed sequences of COI gene from GenBank, NCBI, USA (http://www.ncbi.nlm.nih.gov/Blast) were retrieved by using Blast program. The phylogenetic analyses were conducted using MEGA version 10.1.7 (**Kumar *et al.*, 2018**). Firstly, a Neighbor-Joining tree was constructed between the all samples of Lake Nasser (20 samples) and secondary between the Lake Nasser samples and that from GenBank. In MEGA program, Alignment is done by using the ClustalW. Pairwise deletion option was used for phylogenetic construction. The positions containing

alignment gaps and lost data were eliminated and 500 replicas bootstrapping (**Felsenstein, 1985**) in MEGA was used.

In addition to phylogenetic analyses and to detect the different haplotypes of fish from Lake Nasser, multiple sequence alignments version 5.4.1 with hierarchical clustering (http://multalin.toulouse.inra.fr/multalin/) was used. As well as, the multiple alignments were used for determination any differences in the nucleotide sequences between the all studied samples of the two locations of Lake Nasser.

RESULTS

1- COI gene amplification

For COI gene amplification, about 650 bp fragments were obtained for the all studied samples as observed from **Figure 2**.

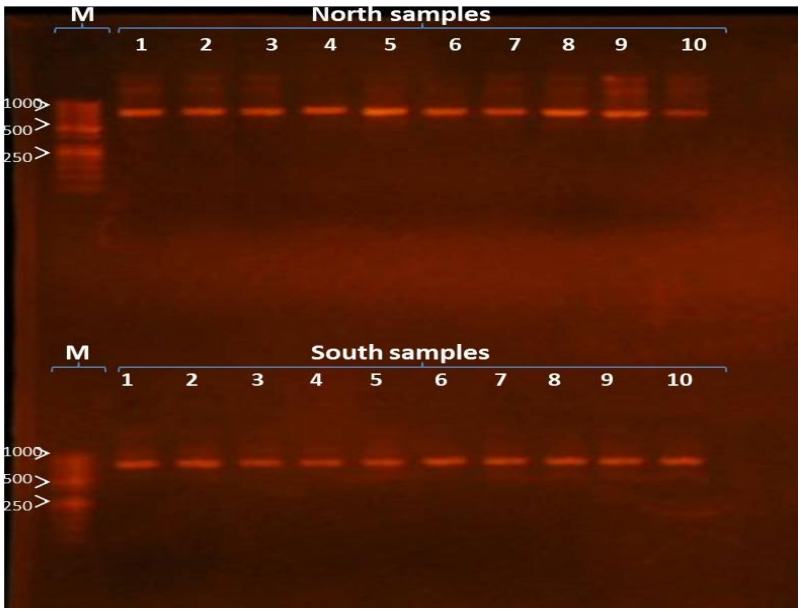

Figure 2: Agarose gel electrophoresis for amplified CO1 gene of *Tetraodon lineatus* from two locations of Lake Nasser (North and south of the lake), M: Molecular marker.

2- Multalin analyses

After sequences editing, 550 nucleotides of CO1 gene from PCR product of every sample were used in the diversity and sequences variations studying. Eight nucleotide variations were found between Lake Nasser samples at positions 23, 27, 31, 62, 91, 176, 507 and 531 **(Figure 3).** Mainly, the sequence variations were related to the south samples (4, 5, 6 and 8).

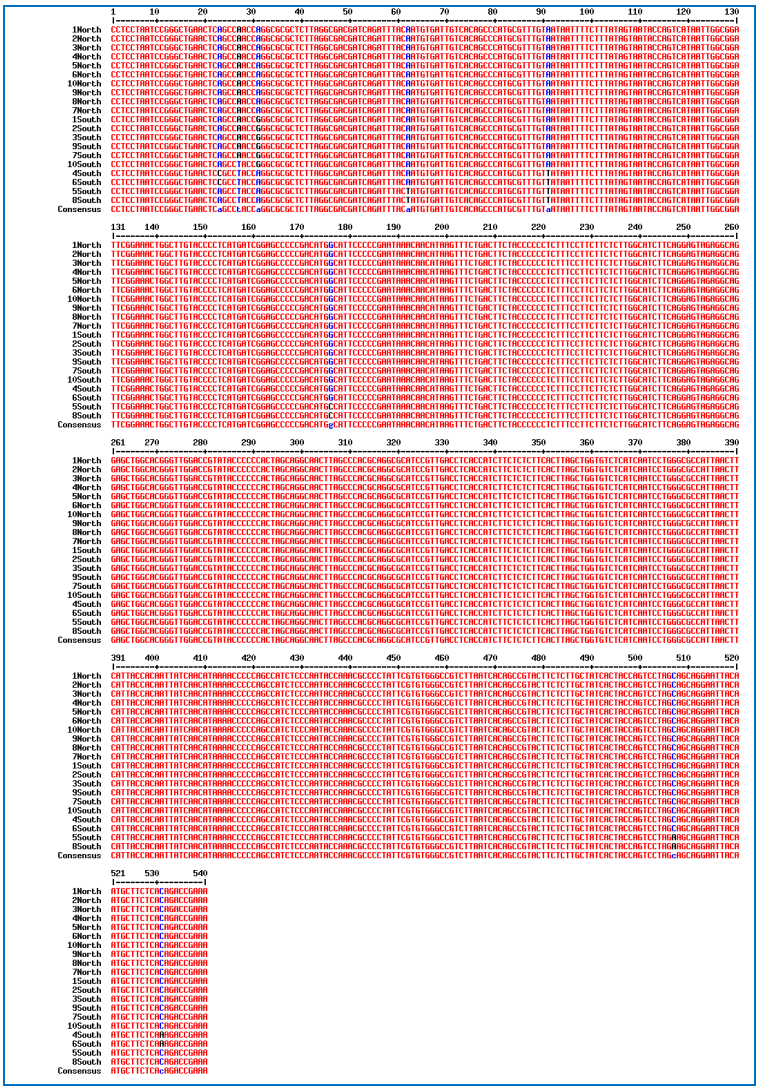

Figure 3: Nucleotide sequence comparison of Nile Puffer Fish (*Tetraodon lineatus*) COI gene partial sequence between Lake Nasser, Aswan, Egypt samples (10 fish samples from Khor El Ramla, North Lake and 10 samples from Tushka East, South Lake).

3- Phylogenetic analysis

3.1- Phylogenetic tree between the studied samples

Phylogenetic tree between the studied samples is presented in **Figure 5**. The tree branched into two main clusters (C1 and C2). The first cluster includes four samples from the south of lake (4S, 6S, 5S and 8S) whereas there are 16 samples in the second cluster that include samples from both locations (5N, 8N, 4N, 3N, 9N, 10S, 2N, 6N, 10N, 1N, 7N, 1S, 2S, 3S, 7Sand 9S). The second cluster includes two groups. The first contains 5 samples from the south while the second one has the rest samples from the two locations.

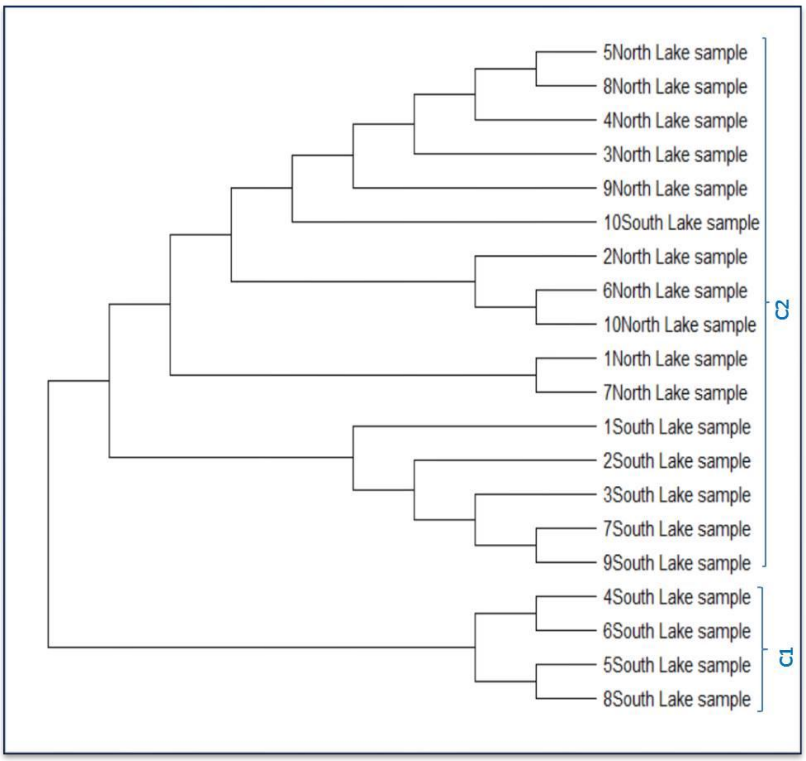

Figure 5: Neighbor-Joining tree of *Tetraodon lineatus* COI gene sequences from the north and south of Lake Nasser, Aswan, Egypt. The bootstrap test (500 replicates). Evolutionary analyses were conducted in MEGA.

The average of nucleotides composition percent of North Lake samples was 25.80%, 31.45%, 24.75% and 18.00% for T, C, A and G, respectively. While, the total average of nucleotides composition percent of South Lake samples was 25.96%, 31.62%, 24.31% and 18.11%. The average of nucleotides composition percent of both locations samples was 25.88, 31.54, 24.53 and 18.05 for T, C, A and G respectively.

Amino acids composition average was: Alanine (4.28%), Aspartic acid (1.70%), Glutamic acid (2.46%), Phenylalanine (3.81%), Glycine (3.17%), Histidine (5.57%), Isoleucine (2.52%), Lysine (1.85%), Leucine (9.85%), Methionine (0.76%), Asparagine (4.10%), Proline (12.54%), Glutamine (7.00%), Arginine (7.62%), Serine (14.92%), Threonine (8.29%), Valine (1.52%), Tryptophan (1.76%), Cysteine (2.46%) and Tyrosine (3.81%).

3.2- Phylogenetic tree between the GenBank and the studied samples

The all obtained sequences of *Tetraodon lineatus* from Lake Nasser and much closed 10 sequences from GenBank were used in Neighbor-Joining tree construction as observed in **Figure 6**.

Neighbor-Joining tree shows that different samples of each location from Lake Nasser distributed on different clusters. The tree mainly branched into 3 clusters.

Just, the first cluster (C1) includes samples from both locations of Lake Nasser and GenBank. Also, cluster (C1) shows that two samples from the north (5N and 3N) from current studied samples are closely to three *T. lineatus* samples from GenBank (LC487196.1, LC487195.1 and LC487194.1). These three samples were identified as

Egyptian freshwater fishes. At the same time, C1 presents that three north samples (4N, 8N and 9N) are nearly to *T. lineatus* sample (MG913990.1) from GenBank that was detected in Lake Turkana in East Africa. Moreover, cluster two (C2) contains samples from South Lake Nasser only.

On the other hand, five samples from North Lake were together in cluster three (C3). C3 indicates that (10N, 6N, 2N, 1N and 7N) North Lake samples are closely to MG824681.1 that was studied and identified from north-central Nigeria.

These results indicate that there are three haplotypes from *T. lineatus* of the two locations based on the partial sequence of COI gene. The first haplotype includes different samples from the two studied locations (North and south Lake) and the second includes four samples from the south. Whereas, the third one includes five samples from the south Lake.

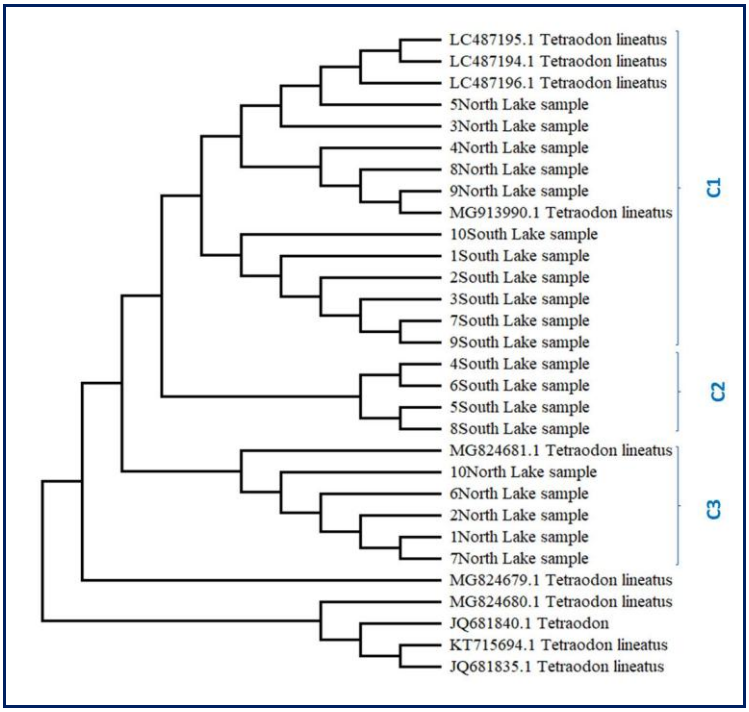

Figure 6: Neighbor-Joining tree of *Tetraodon lineatus* COI gene sequences from GenBank and Lake Nasser, Aswan, Egypt. The bootstrap test (500 replicates). Evolutionary analyses were conducted in MEGA.

DISCUSSION

DNA barcoding systems depend on a fragment of COI gene in the mitochondrial genome is commonly applied in species identification and biodiversity studies **(Bingpeng *et al.*, 2018)**.

The COI gene is widely utilized as a species barcoding, and it has been reported its high efficiency in species identification in Japanese oceanic fishes (**Zhang & Hanner, 2011**), Indian freshwater (**Chakraborty** & **Ghosh, 2014**), ray-finned fish in Taiwan (**Chang *et al.*, 2017**) and Mediterranean Sea fishes (**Karahan *et al.*, 2017**).

In the present study, COI sequences for 20 Nile Puffer Fish (*T. lineatus*) from Lake Nasser, Aswan, Egypt was successfully amplified. The results indicated to slight differences between the studied samples of aswan lake. The differences mainly releated

to the south samples. The results of **Mohammed-Geba *et al.* (2016)** showed a clear extension population loss and potential bottleneck of *T. lineatus* in the Nile River, which corresponded with the almost complete loss of this species from the Northern Nile areas.

The average of nucleotides composition percent of both studied locations samples was 25.88, 31.54, 24.53 and 18.05 for T, C, A and G respectively. The base composition analysis of the both locations samples COI sequence revealed that AT content (50.41%) was higher than GC content (49.59%). **Mohammed-Geba *et al.* (2016)** found that the percent of AT content was 51.1% and GC was 48.9% when they anlaysis a partial sequence of Co1 gene of *T. lineatus* samples from three areas of the Nile River including Lake Nasser. Moreover, **Gong *et al.* (2016)** determined the content of AT (52.57%) and GC (47.43%) of *T. lineatus* (freshwater fish in China), which was similar to *T. mbu* and *T. miurus* (**Yamanoue *et al.*, 2011**).

The current results of phylogenetic analysis of Lake Nasser samples indicate that there are three haplotypes of Nile Puffer fish in Lake Nasser. **Mohammed-Geba *et al.* (2016)** reported that there were only three haplotypes could be characterized in the all samples they studied from River Nile. Phylogenetic results for Nile Puffer fish in Lake Nasser, together with other African freshwater group (from East Africa and Nigeria), matched with the monophyletic origin of African freshwater Puffer fishes. This results is in agreement as identified before by **Yamanoue *et al.* (2011) and Igarashi *et al*. (2013).**

CONCLUSION

Finally, the present phylogenetic results for Nile Puffer fish in Lake Nasser (*T. lineatus*), together with other African freshwater group, matched with the monophyletic origin of African freshwater Puffer fishes. Overall data collected from this study will be fundamental for the Lake Nasser fishery and the handling of the problems caused by Nile Puffer fish. Therefore, proper utilization of this species towards sustainable management, nutritional composition and biosecurity issues will lead to achieve sustainable blue economy.

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