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Comparison between different populations of Nile Pufferfish (*Tetraodon lineatus*) in Lake Nasser, Aswan, Egypt using Mitochondrial CO1 gene

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Abstract

The study about Nile Pufferfish (Tetraodon lineatus) and its growth conditions in Lake Nasser, Aswan, Egypt which is still at a very early stage. Recently, Pufferfish might be considered nutritious food due to its content of good amount of oil, protein, minerals. A total of 30 Nile Pufferfish samples were collected using commercial nets from Lake Nasser (15 fish samples from Khor El Ramla as a North Lake and 15 samples from Tushka East as a South Lake). The total lengths of North and south Lake samples ranged between 16 – 39 and 16 - 28cm with a mean of 27.1 and 21.3 cm, respectively. While, the total body weight of North and south Lake samples ranged between 136 – 1636 and 129-608 g with a mean of 680.8 and 265.7 g, respectively. In addition, the results indicate that there are different groups from T. lineatus of the two locations based on the partial sequence of CO1 gene. The nucleotide frequencies of CO1 gene of North Lake samples were 25.80%, 31.45%, 24.75% and 18.00% for T, C, A and G, respectively. While, the Nucleotide frequencies of South Lake samples were 25.96%, 31.62%, 24.31% and 18.11% for T, C, A and G, respectively. The habitat and location of fish has significant effects on the weight and length of Pufferfish but the origin has the same father. Segregation was happened in the deep water which leads to more haplotypes in the same area. Therefore, proper utilization of this species towards sustainable management, nutritional composition, and biosecurity issues will lead to achieving a sustainable blue economy.

Keywords: Nile Pufferfish; nucleotides composition; CO1 gene; location.

1. Introduction

Fish and fishery production is an important Seafood source. It having high protein, long chained unsaturated fatty acids (mono and poly); besides, poor in saturated fatty acids and cholesterol. 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 Nasser is about 300 km long in Egypt (Hashem et al., 2020). The identified Lake Nasser aquatic animals'' community contains 52 species within 15 families of fishes including Nile Pufferfish (*Tetraodon lineatus*) (Van Zwieten et al., 2011). The Nile Pufferfish belonging to the genus (Tetraodon) one of the greatest groups in the family Tetraodontidae (CAO et al., 2019).

Selected species of Pufferfishes (Order: Tetraodontiformes) of Puducherry coastal waters along south-east coast of India and other places of the world were identified by mitochondrial CO1 gene sequencing (Kaleshkumar *et al*., 2015). Mitochondrial genes encoding 16S rRNA and cytochrome b for 17 Tetraodon species collected from Africa and Southeast Asia were sequenced. Supermatrix analysis based on the complete sequences of the two genes from Tetraodon species was performed using a tree with 50 tetraodontid species (including 7 Tetraodon species) as a backbone. The obtained phylogenetic tree classified Tetraodon species into three groups, correlating well with their habitats, such as Asian freshwater, Asian brackish water, and African freshwater. They also showed that In the African freshwater group, there are a clear segregation of *T. lineatus* and *T. pustulatus* from other African freshwater Tetraodon species, which was supported by the highest bootstrap probability (100 %), and then T. mbu diverged from the common ancestor (Igarashi *et al*., 2013; Kaleshkumar *et al*., 2017).

The cytochrome c oxidase I (CO1) gene was used as the target gene for identification of the Pufferfish species in the samples. Based on the previous results, it suggest that the DNA barcoding as taxonomy and conservation tools of fish fillet should be strengthened and the processing procedures should be standardized to recognize poisoning caused by Pufferfish (Karahan *et al*., 2017).

The effect of changes in an environmental factor on the physiology of an organism is known, it will be difficult to evaluate the outcome of this organism-level physiological response at the population or ecosystem level (MacKenzie and Koster, 2004). Statistical analysis of available time-series revealed changes in

distribution and abundance of fish species that correlate with environmental variables (Perry *et al.*, 2005; Weijerman *et al*., 2005; Heath, 2007). However, statistical correlations do not necessarily indicate underlying processes (Koster *et al*., 2005).

The Nile Pufferfish was considered a significant issue in Lake Nasser as they have flourished to the detriment of more valuable species, they destroy fishing nets, and they also attack the economic species (e.g. Nile Tilapia) that are caught by fishing nets which severely affect their value (Mohammed-Geba *et al*., 2016).

Currently, in Egypt, few species of tetraodontifom are known. These involve seven marine species *L. suezensis, L. guentheri, Lagocephalus sceleratus, L. lagocephalus, T. flavimaculosus, Arothron diadematus and S. pachygaster*. *Tetraodon lineatus* is a single freshwater species that is known from the Nile. When threatened, the Nile pufferfish has the capability to inflate. Also, it carries the Tetrodotoxin (TTX), the well-known alkaloid toxin. TTX gives T. lineatus an additional importance in the therapeutic applications (Khaled *et al*., 2016 and Hashem *et al*., 2020).

Therefore, the studying of genetic diversity of the Pufferfish species by using an innovative approach is needed to change the negative impact of the Pufferfish population on Lake Nasser's fisheries and the validation of the future therapeutic application of TTX derived from this fish. There are a rear studies in Egypt and Africa in the field of the genetic diversity of *Tetraodon lineatus*. So, this work aimed to study the genetic diversity of the Nile Pufferfish (*Tetraodon lineatus)* individuals by using Cytochrome Oxidase 1 (CO1) gene sequencing. As well as, the sample locations effect on the size as length and weight of the fish were investigated.

2. Materials and methods

The present study was carried out in Aquatic Biotechnology Laboratory, Animal Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Sadat City, Minoufiya, Egypt; during the period of 2019 to 2020. A total of 30 Nile Pufferfish samples were collected using commercial nets from Lake Nasser, Aswan, Egypt (15 fish samples from Khor El Ramla as North Lake and 15 samples from Tushka East as South Lake) as show in the Figure 1. The first group includes eight samples from the South Lake. Whereas there are 22 samples in the second one that include the fifteen north samples in addition to seven south samples

Mitochondrial cytochrome Oxidase 1 (CO1) gene

PCR method was performed for the amplification of partial sequence of mitochondrial CO1 gene of T. lineatus by using specific primers. Twenty samples of fish (15 samples from each location) were used. All samples were obtained and immediately placed on ice, transported to the laboratory and kept at −20°C until DNA extraction (Hashem et al., 2020).

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 in TBE buffer, pH 6.8. For DNA fragments staining, Ethidium bromide was added to the gel before pouring in the electrophoresis unit, to a final concentration of 0.5 µg/ml. The obtained DNA fragments were visualized by using UV-trans-illuminator (ELETTROFOR, Italy, EU). To determine the extracted DNA concentration, 1 µl of DNA was used in NanoDrop™ 2000 spectrophotometer (thermo scientific, USA). The extracted DNA samples concentrations ranged from 50 to 80 ng/µl.

CO1 specific amplification

The CO1 specific primers were used as described by Ward *et al*. (2005). The nucleotide sequences of the primers are shown in Table 1.

Table 1: Primer sequences that were used in this study for amplifying and sequencing the CO1 gene of the Nile Pufferfish Tetraodon lineatus from Lake Nasser, Egypt.

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

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 TOP simple TM 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 electrophoresis with 2% agarose gel. DNA ladder (50bp) was used to detect the CO1 fragment size. The electrophoresis run was done at 80 V in horizontal electrophoresis unit (Bio-Rad) for 90 min (Hashem *et al*., 2020).

CO1 gene sequencing and phylogenetic analyses

Partial sequencing of CO1 gene were performed through 373xl automated DNA sequencer (Applied Biosystems, Korea) using the CO1 gene forward primer. For elution, PCR product (40 µl) from each sample was injected in 0.8% agarose medium. DNA fragments of CO1 gene were eluted from the agarose gel according to the kit manufacturer"s instructions of iNtRON Biotechnology, Inc. Korea. For phylogenetic analyses, the related closed sequences of CO1 gene from GenBank, NCBI, USA (http://www.ncbi.nlm.nih.gov/Blast) were retrieved by using Blast program. The phylogenetic analyses were conducted using MEGAX (Kumar *et al*., 2018). Firstly, a Neighbor-Joining tree was constructed between the all samples of Lake Nasser (30 samples) and secondary between the Lake Nasser samples and that from GenBank.

Data processing

In MEGAX program, Alignment is done by using the Clustal W. Pairwise deletion option was used for phylogenetic construction. The positions containing alignment gaps and lost data were eliminated and 500 replicas boots trapping (Felsenstein, 1985) in MEGAX 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.

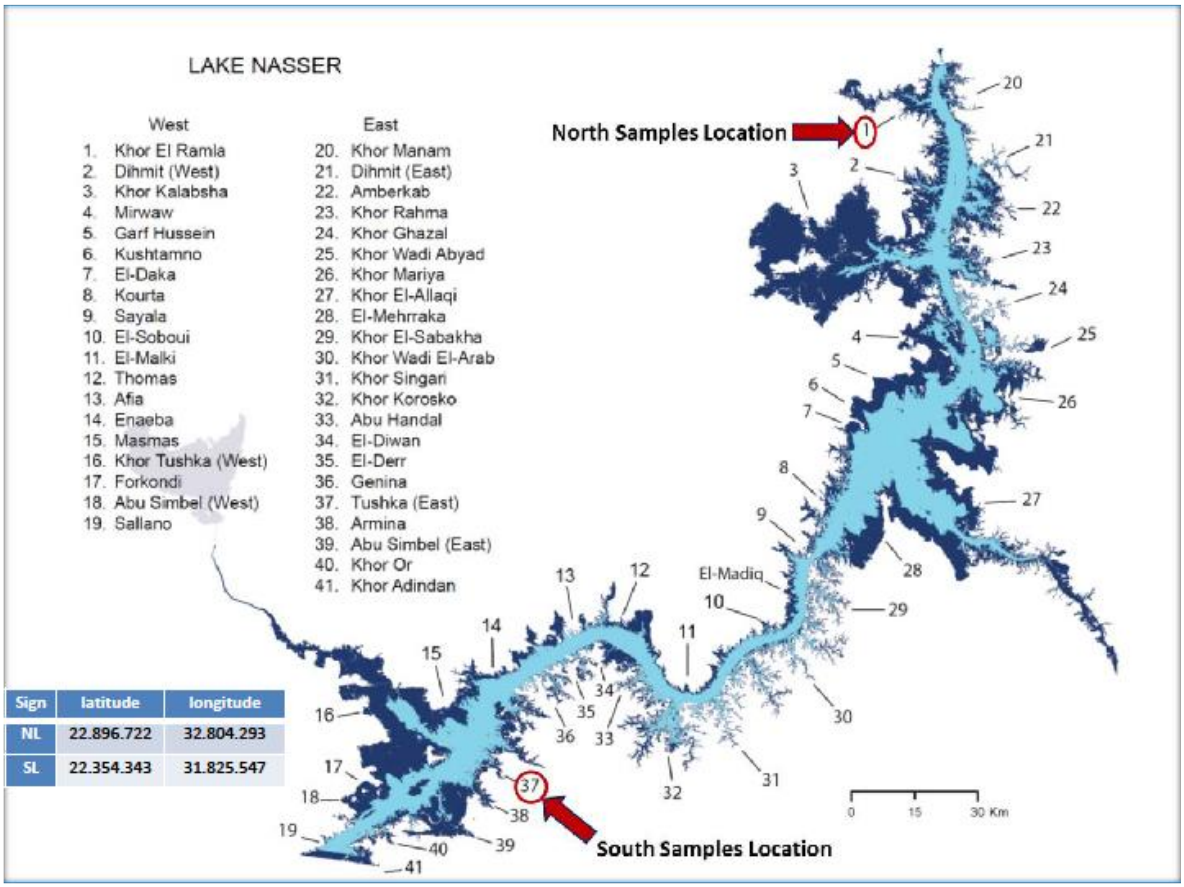

Figure 1: The two sampling locations from Lake Nasser, Aswan, Egypt and the Location geographic dimensions of Khor El Ramla (North) and Tushka East (South).

RESULTS AND DISCUSSION

Length-Weight relationship was calculated for a total of 30 Nile Pufferfish (15 samples from each location) as present in Table 2 and the distribution pattern was figure out in the Figure 2. The total lengths of North Lake samples ranged between 16 and 39 cm with a mean of 27.1 cm and the body weights ranged from 136 g to 1636 g with a mean 680.8 g. While, the total lengths of South Lake samples ranged from 16 to 28 cm with a mean of 21.3 cm and the body weights ranged from 129 g to 608 g with a mean of 265.7 g.

Table 2: The total length (cm) and body weight (gm) of the Nile Pufferfish samples caught from two locations of Lake Nasser, Aswan, Egypt.

	Body weight (g)		Total length (cm)	
	NL	SL	NL	SL
Min	136.0	129.0	16.0	16.0
Max	1636.0	608.0	39.0	28.0
Average	680.8	265.7	27.1	21.3

The relationships between the body weight (g) and the total length (cm) were calculated by the exponential curve fitted using Microsoft® Excel® 2010 (14.0.4734.1000), Microsoft Windows 10 as described as below as show in Figure 2.

The obtained constant q in the relationship was identical for both samples.

North Lake samples: Weight = 2.97(Total length) $^{0.35}$

South Lake samples: Weight = 2.97(Total length) $^{0.36}$

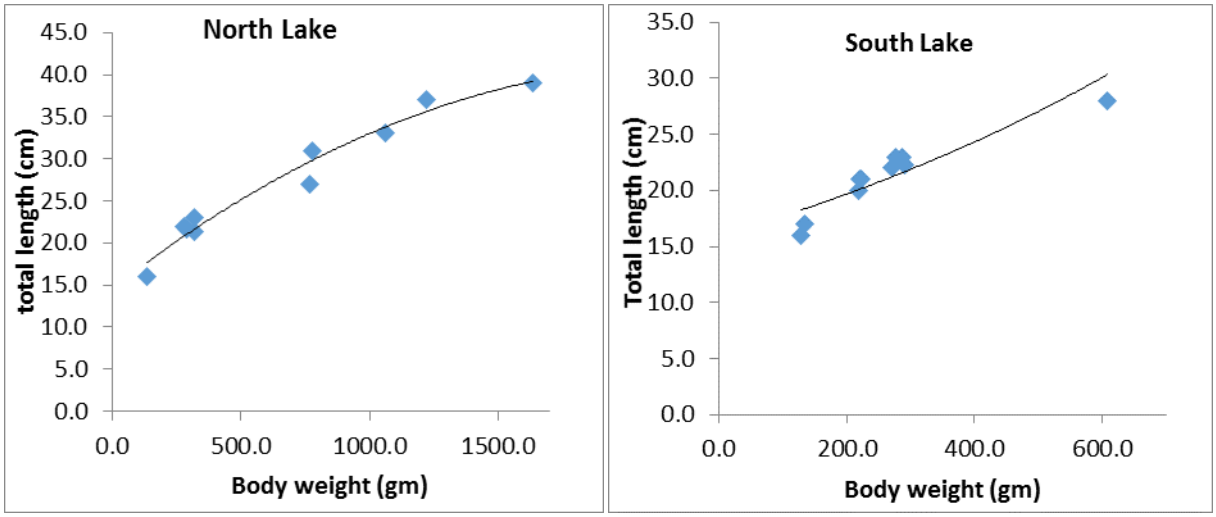

Figure (2) Distribution and Length-Weight relationship of the Nile Pufferfish samples caught from two points of Lake Nasser, Aswan, Egypt.

Therefore, the present study provides reference point information on length-weight relationships for the Nile Pufferfish (*Tetraodon lineatus*) collected from Lake Nasser, Aswan, Egypt. These results will be useful for fisheries biology of Lake Nasser and aquatic biotechnologists in future. Likewise, Minimum, maximum as well as mean total lengths and weights were shown by Bilge *et al.* (2017) as (16.7, 63.8, 29.34) cm, (64.36, 2968.42, 342.39) g from Muğla coasts for *L. sceleratus*, respectively. In addition, Mohamed-Geba *et al.* (2016) discuss the clear loss of extension of *T. lineatus* population in the Nile that the almost complete disappearance of the species from the Northern Nile areas.

Fishes play a pivotal role as functional and structural mediators in aquatic ecosystems. These lower order consumers couple the planktonic and benthic pathways and thus constitute a crucial trophic node for energy transfer to higher order consumers (Pikitch et al., 2012). Outside of the littoral zone, deep waters as north Lake Nasser are characterized by spatial distribution of nutrients and planktons (Mustama et al., 2016).

CO1 gene sequencing analysis

DNA barcoding systems depend on a CO1 gene fragment in the mitochondrial genome, is commonly applied in species identification and biodiversity studies (Bingpeng *et al.*, 2018). For CO1 gene amplification and sequencing (barcoding), about 650 bp fragments were obtained for the all studied samples as observed from Figure 3.

The thirty tested samples were studied for the diversity and sequence variations. After sequence editing, sequence of 541 nucleotides of CO1 gene from PCR product was used in the analysis. Seven nucleotide variations were found between the studied samples sequences at positions 25, 29, 33, 64, 93, 178, and 533 (Figure, 4). The sequences variations distributed between north and south samples. This result is agreement with Hashem *et al.* (2020), who found eight nucleotide variations between twenty studied samples from Lake Nasser.

In MEGAX, pairwise deletion option was used in the comparison between the studied sample sequences. A total of 541 positions in the final dataset were found. The total nucleotides composition percent of both locations samples is illustrated in Table 4 and figure 5.

There were slightly differences in the nucleotides composition between the studied locations samples. The total average of nucleotides composition percent was 25.80, 24.75, 31.45 and 18.00 for T, A, C and G for North Lake samples respectively. While it was 25.96, 24.31, 31.62, and 18.11 for South Lake samples. Khaled *et al.* (2016) reported that the average for nucleotide composition for *T. lineatus* populations was 26.5% for T, 31.6% for C, 24.6% for A, 17.2% for G for 45 samples from Lake Nasser, Aswan, and Edfu in Upper Egypt. The content of AT (52.57%) and GC (47.43%) of *T. lineatus* the freshwater fish in China was reported by Gong *et al.* (2016) determined. Whereas Hashem *et al.* (2020) obtained the same nucleotides composition percent of the present study.

In the following study, phylogenetic tree between the studied samples showed that the samples are distributed on two main clusters (Figure 6). The first cluster includes eight samples from the South Lake. Whereas there are 22 samples in the second cluster that include the fifteen north samples in addition to seven south samples. The south lake samples are spread on the two different clustered comparisons with the north samples. This result suggests that the *T. lineatus* in the south lake is more divergence than that of the north. This may be related to the water flow and the migration of the fish from south to the north especially with the long distance of Lake Nasser. As well as, in the south, the new migrations of Pufferfish from Nile Basin countries will contribute a separated population. The results of Khaled *et al.* (2016) indicated to severe loss of Pufferfish diversity among three locations along the Nile. In general, studies about the population genetics of freshwater tetraodontids includes are very few (Cooke *et al.*, 2012 and Khaled *et al.*, 2016).

On the other hand, the phylogenetic tree between the closed related sequence of GenBank and the consensus sequence of CO1 gene partial sequence of north and south Lake samples is presented in Figure 7.

The phylogenetic tree shows that the CO1 gene sequence of Nile pufferfish samples from the Lake Nasser are the close proximity and the other CO1 gene sequence of *T. lineatus* from Africa and was found in the GenBank database (accession number MG913990.1). This indicates that Nile Pufferfish together with African freshwater fish matched with the monophyletic origin of African freshwater Pufferfishes. This obtained result is in agreement with Yamanoue *et al*. (2011), Igarashi *et al*. (2013) and Khaled *et al*. (2016). In addition, the obtained phylogenetic tree by Igarashi *et al*. (2013), classified Tetraodon species based on mitochondrial DNA analysis into three groups that relating well to their habitats, such as African freshwater, Asian brackish water and Asian freshwater. Rijnsdorp *et al*. (2009) indicated that understanding the complex effects of different locations on a Pufferfish will require an integrated life-cycle approach that examines the importance of different mechanisms acting on all life stages and identifies those developmental stages and mechanisms most critical for life-cycle closure and recruitment.

As a conclusion of the present study, the data collected from this study will be fundamental for the Lake Nasser fishery and the handling of the problems caused by Nile Pufferfish collected from Lake Nasser, Aswan, Egypt. The habitat and location of fish has significant effects on the weight and length of Pufferfish but the origin has the same father. Segregation was happened in the deep water which leads to more haplotypes in the same area. Therefore, proper utilization of this species towards sustainable management, nutritional composition, and biosecurity issues will lead to achieving a sustainable blue economy.

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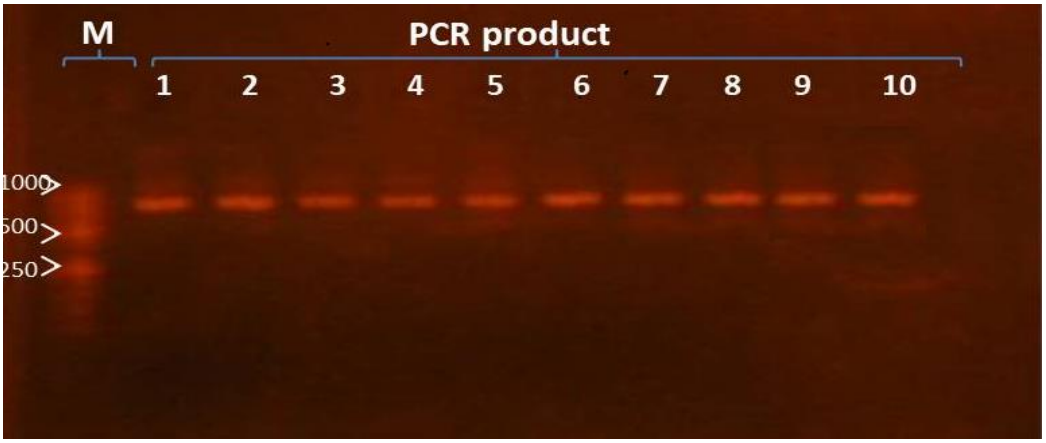

Figure 3: Example of agarose gel electrophoresis for *Tetraodon lineatus* CO1 gene amplificatin from Lake Nasser, M: Molecular marker.

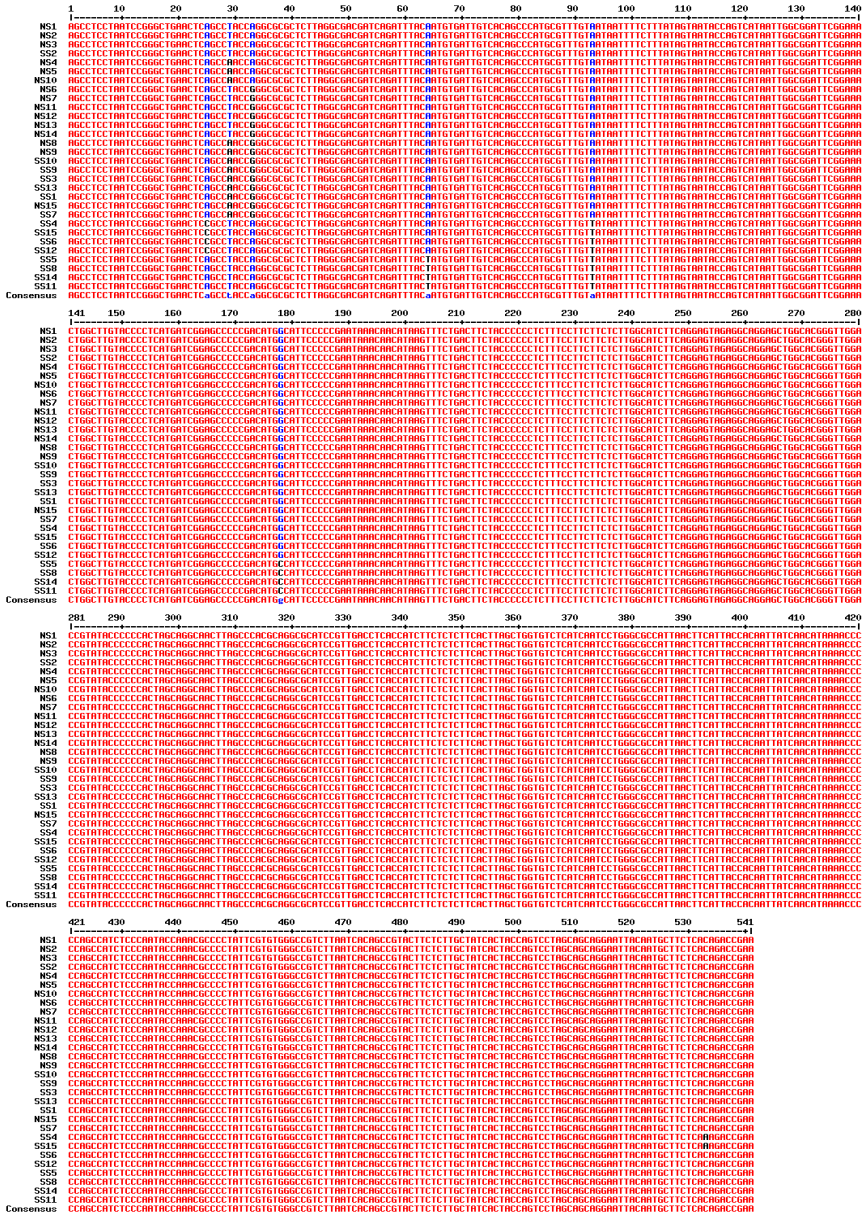

Figure 4: Nucleotide sequence comparison of the Nile Puffer Fish CO1 gene partial sequence between the studied samples. NS: North Samples and SS: South samples

Table 4: Percent of nucleotides composition of CO1 sequences of Lake Nasser samples.

	North Lake samples				South Lake samples			
	T(U)	**A**	**C**	**G**	**T(U)**	**A**	**C**	**G**
Minimum	25.64	24.55	31.45	18.00	25.82	24.18	31.27	17.82
Maximum	26.00	24.91	31.45	18.00	26.18	24.55	31.82	18.18
Average	25.80	24.75	31.45	18.00	25.96	24.31	31.62	18.11

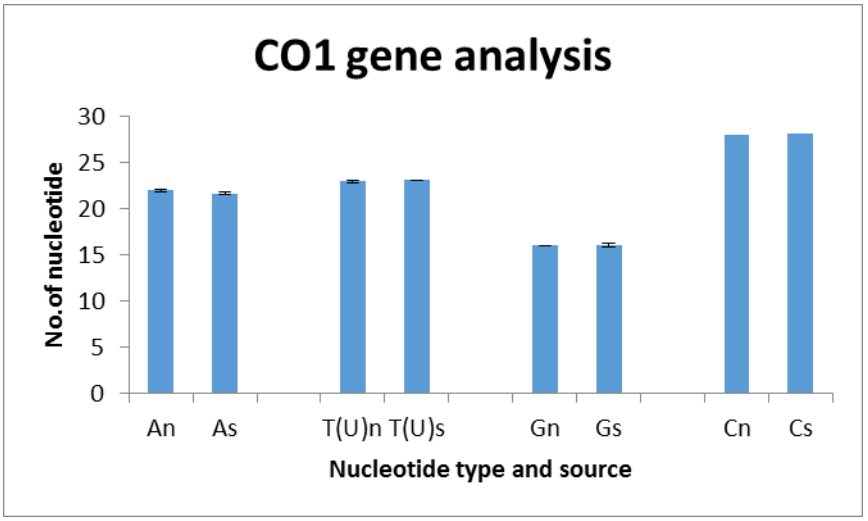

Figure (5) Distribution of nucleotides of CO1 gene of Pufferfishes from North (n) and south (s) regions of Lake Nasser.

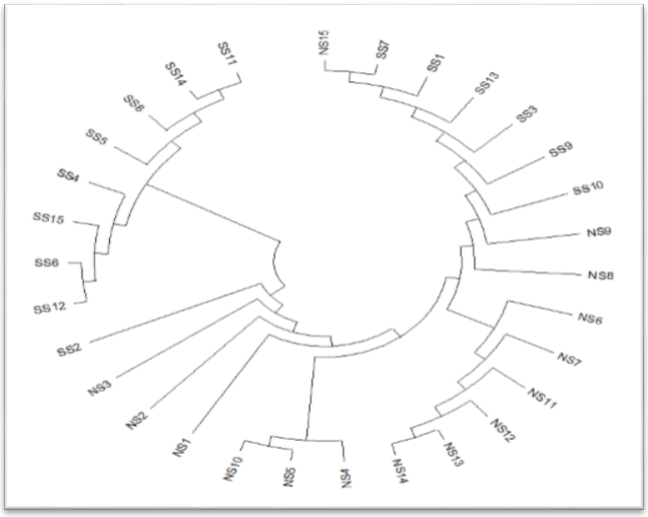

Figure 6: Neighbor-Joining tree of *Tetraodon lineatus* studied samples from the Lake Nasser based on CO1 gene partial sequence. NS: North Samples and SS: South samples.

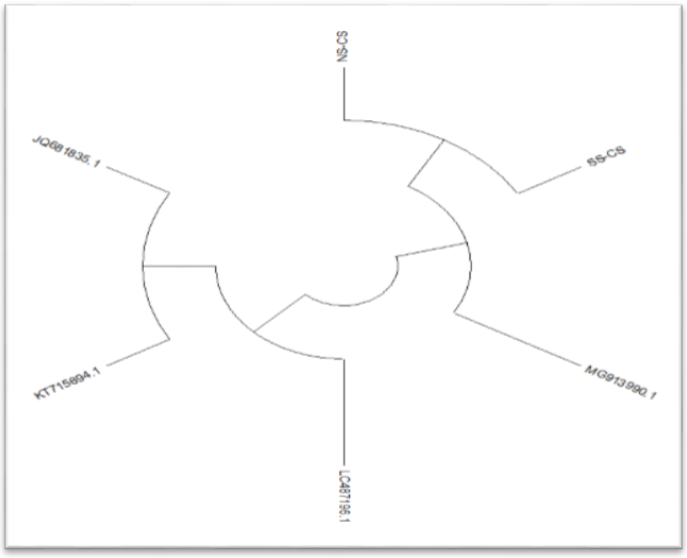

Figure 7: Neighbor-Joining tree between related sequences of GenBank and the consensus sequence of CO1 gene partial sequence of *Tetraodon lineatus* from north and south Lake. NSCS and SSCS: North and south samples consensus sequence.

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