International Journal of Ecotoxicology and Ecobiology
2016; 1(2): 60-66
http://www.sciencepublishinggroup.com/j/ijee
doi: 10.11648/j.ijee.20160102.15

Analysis of Population Genetics of the Endangered Nile Pufferfish *Tetraodon lineatus* (Linnaeus, 1758) in the Upper Egyptian River Nile

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To cite this article:

Khaled Mohammed-Geba, Aldoushy A. Mahdy, Ahmed S. A. Eissa, Alaa G. M. Osman. Analysis of Population Genetics of the Endangered Nile Pufferfish *Tetraodon lineatus* (Linnaeus, 1758) in the Upper Egyptian River Nile. *International Journal of Ecotoxicology and Ecobiology*. Vol. 1, No. 2, 2016, pp. 60-66. doi: 10.11648/j.ijee.20160102.15

Received: May 24, 2016; **Accepted**: July 12, 2016; **Published**: August 26, 2016

Abstract: Genetic population analyses for Nile fishes are very scarce. Nile pufferfish *Tetraodon lineatus* (Linnaeus, 1758), is a widely-distributed freshwater fish, with no known major widespread threats. It has been classed as 'Endangered'. Little is known concerning its biology and genetics in Egypt. Hence, this work was designed to study the genetic diversity and conservation status of *T. lineatus* for the first time in Egypt and Africa. DNA barcoding was carried out through PCR-amplification and sequencing of the cytochrome c oxidase gene barcode 5′ region of forty-five samples obtained from three different localities in Upper Egypt. Only three haplotypes could be characterized in all samples. The other population analyses showed clear population loss of extension and potential bottleneck, what may explain the severe drop of species records in the Northern areas of the Nile. Phylogenetic analysis exhibited the monophyletic origin *T. lineatus* and other African freshwater pufferfishes, more probably as descendants from an Indo-West Pacific ancestor. We highly recommend the fulfilling of more studies concerning the biology, ecology, and genetics of the species as major steps towards its proper conservation and understanding of its adaptation to different natural and man-made constraints in the River Nile system.

Keywords: Africa, COI, PCR, Population Genetics, Pufferfish, *Tetraodon lineatus*

1. Introduction

Tetraodontidae is a very diverse family of fishes that dwell many tropical and subtropical areas of the world. They are typically found in large rivers, open waters, weed beds and vegetated fringes in water with temperatures ranging from 24°C - 26°C [1]. Representatives of this family thrive in a wide range of habitats, being pelagic or benthic, and freshwater, brackish, or marine waters. The oldest fossils found for the family Tetrodontidae specifically were from the Oligocene in the Caucasus, Russia, 35 millions of years ago (MyA), sharing most of the typical features of the current modern tetraodontids (as 11 caudal fin rays, 18 vertebrae, and some broadened haemal and neural spines, and absence of ribs, see Reference 2). Fossil remains for other tetraodontiform families were even found in Egypt from the Middle Eocene, about 35 MyA [3].

Currently, few tetraodontifom species are known in Egypt. These include seven marine species *Lagocephalus sceleratus*, *L. suezensis*, *L. guentheri*, *L. lagocephalus*, *T. flavimaculosus*, *S. pachygaster* and *Arothron diadematus* [4, 5]. A single freshwater species, *Tetraodon lineatus*, is known from the Nile. Like all puffersfishes, the Nile pufferfish has the ability to inflate when threatened, and it carries the very famous alkaloid toxin, the Tetrodotoxin (TTX). TTX gives *T. lineatus* further importance as a neglected source of bioactive ingredients with potential therapeutic applications. In the written history, the ancient pharonic Egyptians identified the pufferfish of the Nile and draw it on their tombs (for example, see Reference 6). They could also identify the TTX [7, 8]. TTX is usually concentrated in the liver, gonads, and

skin, but other parts of the viscera may also be toxic. Some debate is still ongoing regarding its presence in the musculature, but other issues are less debatable, especially TTX thermostability, water-solubility, and severe human neuro- and respirotoxicity [9, 10, 11, 12]. Furthermore, increasing reports are being introduced about TTX potentials to enhance arterial blood pressure [13]; relief of acute, inflammatory, and neuropathic pains [14], and even to combat tumors [4].

TTX presence, however, rendered the fishing of all tetraodontids and their human consumption completely forbidden by the Egyptian authorities. TTX high toxicity presents decimating threat to several Egyptian consumers, even to the extent of intoxication of whole families (for example, see Reference 9). Panning of pufferfishes fishing and trade as food limited the works concerning the biology and genetics of these species, mainly the Nile pufferfish *T. lineatus*. This scarcity of studies led to possible gap in the knowledge about the species conservation status, what may threat its existence in the Nile and its role in the natural food webs. The species was classed early in 1997 as endangered [15], and the limited interest, information and number of studies available about it rendered its IUCN Red List category to be ¨Least Concern¨ [1].

For all the above reasons, we designed this work to study the genetic diversity and the conservation status of *T. lineatus* in Egypt, as a major tool for this natural resource conservation and authentication of possible future therapeutic derivatives from that fish. This study can be considered the first in Egypt and Africa in general to deal with the population genetics of this species.

2. Materials and Methods

2.1. Sampling and Samples Preservation

Forty five *T. lineatus* samples (Fig. 1) were obtained from the areas of Lake Nasser, Aswan, and Edfu in Upper Egypt during the period between August to November 2015.

Fig. 1. *Nile pufferfish Tetraodon lineatus from the river Nile (~23 cm). Photo courtesy Mr. Amr Abd ElHady (Environmental Affairs Agency, Egypt).*

Fig. 2 shows sampling areas. Fishing this species is prohibited in Egypt due to its high toxicity as previously mentioned, therefore necessary permissions were obtained from the local authorities for obtaining this species. The fishes were immediately dissected and 100 mg samples of liver tissue were preserved separately in 1.5 mL eppendorf tubes completely filled with absolute ethanol. For molecular analyses, the samples were transferred to the laboratories of Genetic Engineering and Molecular Biology Division, Faculty of Science, Menoufia University for further genetic analyses.

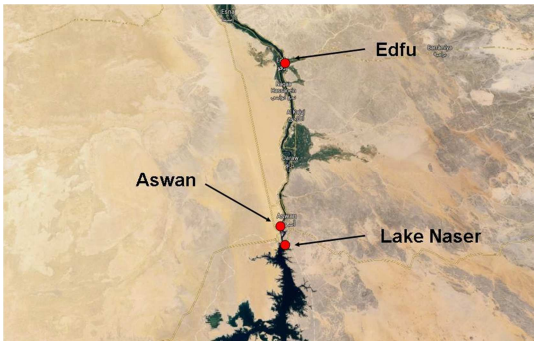

Fig. 2. *Map showing the sampling sites of T. lineatus in Upper Egypt.*

2.2. DNA Extraction

From each preserved liver sample, a 50 mg tissue sample was excised and placed in a 1.5 mL eppendorf tube containing 5% Chelex® 100 sodium form resin (Sigma-Aldrich, Madrid, Spain) in TE buffer (pH 8) according to the protocol described before [Wolff 2008]. 2.4 U of Proteinase K (ThermoFisher) were added to each tube. Samples were incubated at 55°C with shaking at 30 min intervals for 5 hours. Samples were then boiled in a 100°C water bath for 20 minutes and then finally stored at 4°C until DNA amplification.

2.3. PCR Amplification for COI Gene

The target region of the COI gene was amplified by PCR using the cycling conditions and universal primers pairs proposed as universal for fishes [16]. The amplification reactions were performed in a total volume of 25 µL, in which 3 µL of total genomic DNA, 0.4 µM of each primer, and 200 ng μL^{-1} of bovine serum albumin (BSA) were used. The PCR was performed MyTaq red master mix (Bioline) according to the manufacturer´s instructions. 3 µL of each PCR product were electrophoresed in a 2% agarose gel, containing 0.5 µg mL^{-1} of Ethidium bromide. The PCR samples with adequate size bands (~650 base pairs) and intensity were sent to MACROGEN Inc. (Seoul, South Korea) for standard Sanger sequencing.

2.4. Sequences Analyses

COI sequences chromatograms, received as .ab1 files, were reviewed and manually corrected and trimmed using the software Chromas lite version 2.5.1 (Technelysium Pty Ltd) whenever necessary. The quality of the sequences were checked for Phred quality scores, using region of interest PHRED quality scores calculation tool available in http://www.insilicase.co.uk/Desktop/ROI.aspx. Only sequences with quality scores above 30 were accepted for further analysis, as this stands for an erroneous 1 base call within each 1000 bases [17]). Edited sequences were compared to archived reference sequences in GenBank using

BLAST algorithm. The sequences were aligned using CLUSTALW integrated with the program Mega 7.0.14 software [18]. Nucleotide and amino acids composition were calculated for the Egyptian *T. lineatus* samples. The alignment created for *T. lineatus* COI sequences using Mega 7.0.14 software, which was 547 base-long for each of the used sequences after trimming of the non-informative nucleotide areas, was uploaded to DNAsp 5.0 Software [19] in Fasta format to determine different haplotypes. Also, DNAsp 5.0 was applied to identify private and shared haplotypes, haplotypes diversity index (Hd) and nucleotide diversity index (π).

Using the software ARLEQUIN 3.5.1.1 [20], several genetic population parameters were calculated. Neutrality analysis were performed applying the Fs statistic of Fu [21], with the negative values usually arising due to the excess of low-frequency haplotypes that result from selection or rapid population growth [21, 22]. The index of raggedness, r [23] as a measure for recent population expansion was also calculated, all of which are integrated in the software ARLEQUIN 3.5.1.1.

Finally, and in order to infer the genealogy among different freshwater pufferfishes, 49 African, Asian and South American freshwater pufferfishes COI sequences were retrieved from GenBank database, appended to the alignment of Egyptian *T. lineatus* COI sequences, and aligned all together using ClustalW algorithm. The alignment was saved as Fasta file and exported to JModelTest software V. 2.1.10 for detection of best nucleotide substitution model in order to construct a maximum likelihood phylogenetic tree between different species of freshwater tetraodontids. Then, the Fasta file was uploaded to Beauti software V. 1.8.3, with the substitution model determined using JModelTest, and 10,000,000 Markov chains were selected. The program was run once with this number of Markov chains, then two other runs were carried out, one with 50,000,000 chains and another with 100,000,000 chains. The resulting .xml files were opened using BEAST software V. 1.8.3 for estimating tree topologies. The resulting .log files from the three trials were uploaded to the program Tracer v 1.6 to assess the quality of the results, accepting only theses with Effective Sample Size (ESS) above 200. The resulting trees were combined using LogCombiner software V. 1.8.3, then uploaded to TreeAnnotator software V. 1.8.3 for summarizing the information retrieved from tree samples produced through BEAST. The resulting consensus tree was then finally obtained using FigTree software V. 1.4.2.

3. Results

Sequencing of the barcoding area of the COI gene in *T. lineatus* samples resulted in sequences of 547 bp (after sequences trimming). PHRED quality scores for all sequences were 46.5±2.5. 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. Amino acids composition was: Alanine (10.44%), Aspartic acid (2.75%), Glutamic acid (0.55%), Phenylalanine (6.59%), Glycine (8.79%), Histidine (2.20%), Isoleucine (8.24%), Lysine (0.55%), Leucine (14.84%), Methionine (5.50%), Asparagine (4.95%), Proline (7.69%), Glutamine (1.65%), Arginine (1.10%), Serine (5.50%), Threonine (6.58%), Valine (7.69%), Tryptophan (2.20%), and Tyrosine (2.20%). G+C content was 48.90%.

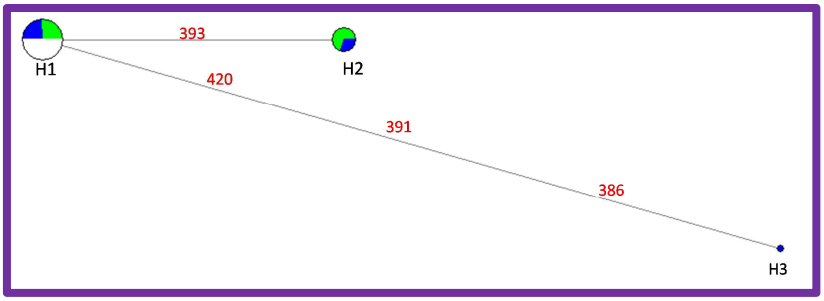

Fig. 3. *Reduced Median Network showing the interrelationships among the three haplotypes found. Green colour: Aswan, Blue color: Lake Nasser, White colour: Edfu. Red numbers above branches refer to mutated position in relation to the most abundant haplotypes. Letter H is designated for "haplotype".*

A total of three haplotypes could be found, all of which were present in Lake Nasser. Table 1 shows their major diversity criteria. Reduced Median Network showed the interrelationships among the three haplotypes found (Fig. 3). (Green: Aswan, Blue: Lake, White: Edfu). Analyzing the degree of independence (fixation) among the studied population, we found that the highest population Fst values were found between Edfu and Aswan populations, followed by Edfu and Lake Nasser populations ($p<0.05$) (Table 2). Moreover, No negative Fs values could be found at all for the whole three populations (Table 3). Non-significant raggedness values were found in all cases (Table 3).

Table 1. *Number of samples per site (N), haplotypes (Nh), private haplotypes (Ph), haplotypes diversity index (Hd) and nucleotide-diversity index (π).*

	N	Nh	Ph	Hd	π
Lake Nasser	12	3	1	0.53	0.00
Aswan	15	2	---	0.53	0.00
Edfu	14	1	---	0	0

Table 2. *Fst values and their corresponding p values. Below the diagnonal: Fst, above the diagonal: p-values of Fst.*

	Aswan	Edfu	Lake Nasser
Aswan		0.00+-0.00	0.26+-0.03
Edfu	0.41		0.018+-0.01
Lake Nasser	0.02	0.11	

Table 3. *Neutrality and mismatch analyses for T. lineatus samples.*

	Aswan	Edfu	Lake Nasser
Fu Fs	1.32	0	0.49
Fs p-value	0.68	0	0.55
Raggedness index	0.29	0	0.17
Raggedness p-value	0.11	0	0.57

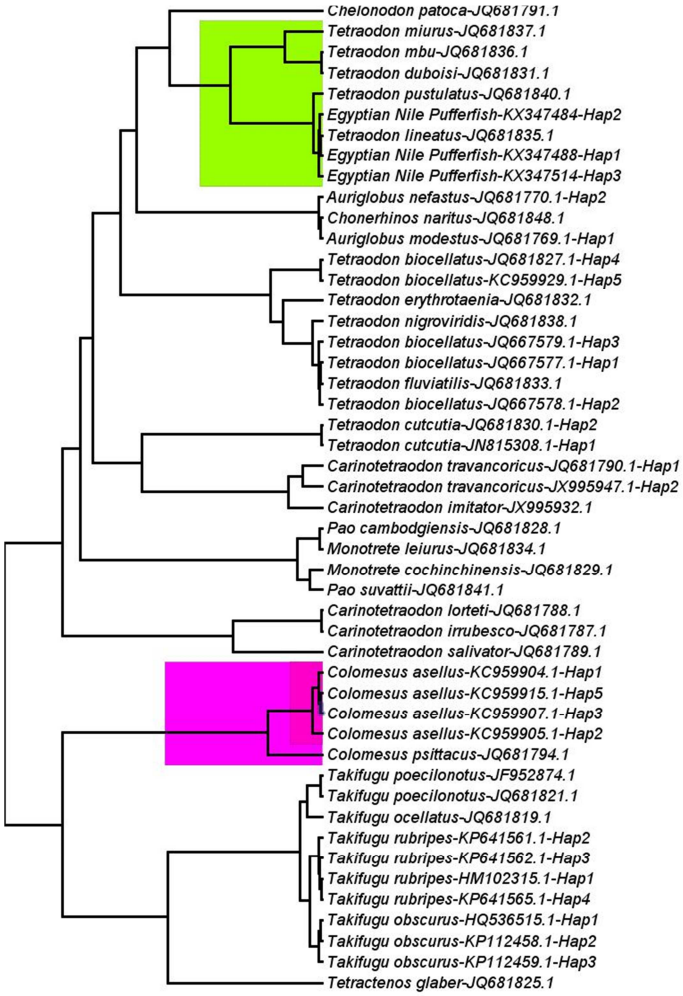

Fig. 4. *Consensus, un-rooted phylogenetic tree showing the relation between the Nile Pufferfish T. lineatus and other freshwater African (green highlight), Asian, and South American (purple highlight) pufferfishes. The abbreviation ¨hap¨ found in some species refers to ¨haplotype¨.*

Finally, the phylogenetic tree constructed for the African tetraodontids (Fig. 4) showed the close proximity of the Nile pufferfish investigated during this study and the other COI gene sequence of *T. lineatus* found in the GenBank database (accession number JQ 681835. 1).

Our tree also showed the clustering of *T. lineatus*, *T. pustulatus, T. miurus, T. mbu,* and *T. duboisi* together in a single clade, with the first two ones belonging to North Africa in a single subclade and the next three ones of Central and South Africa in the other. Interestingly, the same cluster for African freshwater pufferfishes contained also the West Indo-Pacific Milkspotted pufferfish *Chelonodon patoca*, that is one of the few euryhaline pufferfishes, found in both freshwaters and seawaters in the range from South West Africa to Australia and China. Finally, all African pufferfishes assessed were found in a single clade, different from that of the South American clade of pufferfishes. Asian freshwater pufferfishes, however, seemed to be more polyphyletic.

4. Discussion

In the current study, the Nile pufferfish *T. lineatus* was genetically analyzed for the diversity of its populations in the Egyptian Upper Nile part, and the results we found indicated severe loss of such diversity. In general, studies about population genetics of freshwater tetraodontids are very few. These fishes are labile to physico-chemical (e.g. turbidity, sweeping of natural chemicals from nearby vegetation, etc), and environmental modifiers of gene flow, as many other freshwater fishes upon comparison to marine ones [24, 25]. The role of changing water chemistry on species population

structuring is not studied for *T. lineatus*. However, other freshwater tetraodontid, the Amazonian pufferfish *Colomesus asellus*, showed consistent structuring with changing water colour and chemistry for example [25]. In the modern ages, construction of reservoirs and dams on the Nile main stem in Upper Egypt massively reduced the diversity and the abundance of different fish species. The decrease was more northwards. For example, fish species decreased from 47 to 25 in Assiut and 14 in Cairo, and from 11 to 3 in Mansoura governorate in the North [26, 27]. Our results showed clear population loss of extension and potential bottleneck for *T. lineatus* in the Nile, which coincided with the almost complete disappearance of the species from the Northern Nile areas.

Despite most pufferfishes of the world are found in marine waters, more than 30 genera belonging to three different tetraodontid lineages could adapt and inhabit freshwaters of Africa, South America, and South Asia [28, 29]. Freshwater tetraodontids do not to belong to a monophyletic group, rather than to independent invasions to freshwaters in each content by marine ancestors, with completely distant timings of such invasions or incursions (0–10 MyA in South America, 17–38 MyA in Central Africa, and 48–78 MyA in Southeast Asia) [28]. In Africa, marine tetraodontids invasions were attributed to the seawater incursions that peaked in the early and middle Eocene (37-54.8 MyA) [28, 30]. These incursions occurred much earlier in South America (in the Miocene, 11-12 MyA). Marine-to-Freshwater species transition due to marine incursions could be found for several finfish and shell fish species other than the tetraodontids [31-34].

Our phylogenetic results for *T. lineatus*, together with other African tetraodontids, coincided with the monophyletic origin of African freshwater pufferfishes as identified before [28, 35]. More important, the phylogenetic pattern of the African pufferfishes corresponded well with the geographical patterns of distribution of these species, with the Subtropical, West-Central African pufferfishes (*T. miurus*, *T. mbu*; and *T. duboisi*) are belonging to a single clade, and the North Western and Eastern representative (*T. lineatus* and *T. pustulatus*) are belonging to another one. These findings were congruent with those found before [35, 36] despite being analyzed using the mitochondrial 16 S rRNA gene sequences and full mitochondrial genomes. This further confirms the possibility of single origin of *T. lineatus* and *T. postulatus* from a common marine ancestor, but after isolation in Cross River the species *T. postulatus* seemed to develop separately [36].

The phylogenetic position of *C. patoca*, as the only euryhaline pufferfish species known in Africa and dominating the Indo-West Pacific area, attracted a strong attention. We, intentionally, added this species to our phylogenetic analysis in order to test the hypothesis of marine origin of the African freshwater pufferfishes, not pure freshwater origin such like other groups of fishes that thrive in the African continental waters like Siluriformes whose origin was in South America but they diverged due to the separation between South America and Africa [37]. Igarashi et al. (Ref. 36) accepted the theory of a Northern marine origin of *T. lineatus* and *T. postulatus*, but an Atlantic one for Congo River pufferfishes. Presence of *C. patoca* in a single monophyletic group with all these freshwater pufferfishes suggest a common marine ancestor for all African continent freshwater pufferfishes, possibly diverged from *Chelonodon* species, as we found in our phylogenetic analysis for COI gene and also suggested before [28] using full mitochondrial genomes analysis.

The assumption of common origin of the African tetraodontids based on an Indo-West Pacific ancestor may be acceptable. Early in the geological history, the first fossil record for *Tetraodon* sp. was obtained from Lake Turkana and belonged to the Mio-Pliocene period [38]. An ancient fluvial corridor from Lake Turkana to the Indian Ocean existed since the Plio-Pleistocene period in Turkana basin, and it was confirmed to be the route of introduction of some marine fish species to the African continental waters, such like the marine ancestors of the fossil Africa freshwater stingray *Dasyatis africana* that flourished in Lake Turkana for about half a million of years [39]. The lake suffered some changes in its outlet, shifting it from an eastern marine one to a fluvial Northern outlet to the Nile system in the Middle Holocene, what can be indicated by the excessive spread of the fluvial Nile oyster *Etheria elliptica* to the South of the lake´s basin [40].

Connection between eastern and western rivers of Africa is not known in the modern age. However, African Mega-palaeolakes may have provided such early connection, such like the mega-lakes that used to exist in the African Sahara, namely Chad and Congo lakes [41]. Another, more probable, possibility for the appearance of Congo River tetraodontids may come from a Nilotic migration pathway from Lake Tanganyika when an indirect link was established between Nile and Congo systems through the formation of Lukuga river during the Pleistocene, the system that possibly aided the interchange of Nilotic fishes, like *Lates* and *Polypteru* and cichlids, between Nile and Congo river systems [42, 43]. The general high African humidity in the Holocene period could provide means for connection and spread of species in the eastern and western Africa [38]. Their subsequent independent evolution should ensue upon disappearance of these lakes and closure of inter-connections bewtween African rivers.

In conclusions, and for the first time in Egypt and in the world in general, we used COI gene hypervariable 5´region sequencing to analyze the Nile pufferfish *T. lineatus* population genetics, and we could detect poor genetic diversity of its populations in Egypt after a long evolutionary history there and in the River Nile in general. We highly recommend carrying out of proper consetvation programs of this species in the Nile in Egypt. Also, it will be of great importance to extend the studies concerning the biology and ecology of the species as major steps towards its proper conservation, together with continuous genetic analysis for the state of its expansion in its different populations in

Africa. This recommendation is highly based on the importance of the fish in the environment as well as its importance as a natural source of bioactive therapeutic agents.

Acknowledgment

The authors would like to appreciate the roles of Genetic Engineering and Molecular Biology Division, Department of Zoology, Faculty of Science, Menoufia University (Shebin El-Kom, Menoufia Governorate), and Department of Zoology, Faculty of Science, Al-Azhar University, Assiut branch (Assiut Governorate), Egypt, for providing necessary lab facilities, materials and equipment's for carrying out this work.

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