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Molecular Phylogenetic Linkage for Nile and Marine Puffer Fishes Using Mitochondrial DNA sequences of Cytochrome b and 16S rRNA

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ABSTRACT
Based on two mitochondrial DNA sequences; cytochrome b and 16S rRNA with using a total DNA template from muscle tissues of two puffer fishes species *Arothron hispidus* and *Tetraodon lineatus*, our study was performed to reveal a general view about the phylogenetic linkages among River Nile *Tetraodon lineatus* and Red Sea *Arothron hispidus* to other marine puffer fishes. The nucleated sequence lengths based on cytochrome b gene in *Arothron hispidus* and *Tetraodon lineatus* were (337 and 367 bp. respectively), while using the 16S gene in *Arothron hispidus* and *Tetraodon lineatus* revealed nucleated sequence of length (186 and 237 bp. respectively). The sequenced regions of cytochrome b in *Arothron hispidus* and *Tetraodon lineatus* were submitted in the GenBank/NCBI under accession numbers MN186251 and MN186252 respectively, also the sequenced regions of 16S in *Arothron hispidus* and *Tetraodon lineatus* were submitted in the GenBank/NCBI under accession numbers MN186287 and MN175976 respectively. The phylogenetic linkages were designed using three phylogenetic methods; Maximum Likelihood (ML), Neighbour Joining (NJ) and Minimum Evolution (ME). The Pairwise genetic distances of both (cyt-b) and (16S) illustrated that, the Nile puffer fish *Tetraodon lineatus* was closely related to marine puffer fishes of genera *Arothron* and *Canthigaster* more than other marine puffer fish genera. Our results showed high efficiency of cyt-b and 16S rRNA in the phylogenetic analysis, so we strongly support the usefulness of them for taxonomy studies of puffer fishes.

INTRODUCTION

Puffer fishes or blow fishes, are named due to their capability to inflate their bodies due to pull the water into the abdomen, or air when they found out of the water, tough scale less skin often with small spindles, jaws with break-like dental plates having a medium structure, a small slit-like gill opening (Randall, 1982). Puffer fishes are among the most poisonous vertebrates in the world, many parts of the body contain toxin tetrodotoxin and one highly toxic to most animals when eaten (McClane, 1977). Sabrah, *et al*. (2006) reported that, the puffer fishes are widespread in the Red Sea and about 120 species of them are mostly distributed in tropical seas. They also called blowfish and globefish, due to their ability of expanding their bodies with water or air when intimidate, and become difficult to swallow. Brenner *et al*. (1993) said that the genome of puffer fishes seem to be uniquely compact, because it includes unusually small introns and lack extensive repetitive sequences and pseudogenes. However, the puffer fish genome preserves the structural complexity reflected in the intron and exon arrangements observed in homologous genes of higher vertebrates (Elgar *et al*., 1996 and Koop and Nadeau, 1996) and has become

a common model for sequencing and mapping the genomes of higher-taxa vertebrates (Song *et al*., 2001).

Tetraodontiform lineages posses' extremely compact genome among the smallest of all vertebrates (Hinegardner, 1968 and Brainerd *et al*., 2001), puffer fishes became a major model organism in vertebrate genomics, where *Takifugu rubripes* and *Tetradon nigroviridis* became the first non-human vertebrates to have their complete genomes sequenced (Brenner *et al*., 1993 and Jaillon *et al*., 2004). Comparative study for the gene sequences of puffer fish and other related species can furnish the help to knowledge the answer for genome structure and evolution (Kai *et al*., 2003). Pepe *et al*. (2005) reported that, the improvement of molecular biological techniques may solve problems about fish identification through enabling forthright analysis for the nucleotide sequences of either nuclear or mitochondrial DNA.

In fishes, Karaiskou *et al*. (2003) estimate the phylogenetic linkages among some species of genus *Trachurus* using partial sequences of cyt-b and the 16S rDNA gene. The mitochondrial cytochrome b (cyt-b) and 16S rRNA (16S) DNA sequences are among the very widely used genetic markers in fish species identification and have been employed for population genetic studies (Peng *et al*., 2004; Kartavtsev *et al*., 2007; Kochzius, 2009 and Teletchea, 2009).

Within many mtDNA genes, cytochrome b has been widely used to estimate the genetic variation and inferring phylogenetic relationships (Irwin *et al*., 1991; McVeigh and Davidson, 1991; Johns and Avise, 1998; Callejas and Ochando, 2000 and Bajpai and Tewari, 2010).

The 16S ribosomal RNA, a component of the 30 S small subunit of the mitochondrial ribosome in vertebrate, has been reported 1640 bp. long in fish (Naock *et al*., 1996), which is highly conserved. The mitochondrial (16S) gene is very commonly used to examine the phylogenetic linkages of fishes at different taxonomic levels (Faddagh, *et al*., 2012). Segments of sequence of this region are efficiently used for constructing phylogenies (Singh *et al*., 2015).

The primary target of this work was to reveal the phylogenetic relationships of Nile puffer fish *Tetraodon lineatus* and Red Sea *Arothron hispidus* to other marine puffer fishes, using Mitochondrial DNA sequences of Cytochrome b and 16S rRNA. Additionally, we used three phylogenetic methods; Maximum likelihood (ML), Neighbour Joining (NJ) and Minimum Evolution (ME) to compare results with more widely used methods.

MATERIALS AND METHODS

Collection of the samples:

The puffer fishes of two genera; *Arothron* and *Tetraodon* belong to Family Tetraodontidae, Order Tetraodontiformes were collected from Red Sea and River Nile respectively, in Egypt, then brought to the laboratory for the morphologically identification according to (Randall, 1982 and Bishai and Khalil, 1997). The muscles tissues were individually isolated and preserved in -80ºC until genomic DNA extraction.

DNA Extraction:

The genomic DNA from each puffer fish was extracted from the muscles tissues using the DNA extraction method of QIAamp DNA Mini kit (Qiagen, Hidden, Germany) depending on the manufacturer's instructions.

PCR Conditions:

Polymerase chain reaction (PCR) amplification of genomic DNA was performed using forward and reverse primers of cyt-b (cytb F: 5'-CCCTACTCGGACTTTGCCTG-3') and (cytb R: 5'-AATCGGGTGAGAGTTGCGTT-3'), also using forward and reverse primers of 16S rRNA (16S F: 5'-GCCTGCCCTGTGACTATACG-3') and (16S R: 5'-TCGCCCCAACCAAAGACATT-3'). All primers were designed based on the (cyt-b and 16S rRNA) genes sequences described for the under study puffer fish *Arothron hispidus* using the National Center for Biotechnology Information (NCBI) database.

The PCR reactions were carried out with 10 pmoles of each primer, ~100 ng of genomic DNA and 12.5 µL PCR master mix in a final reaction volume of 25 µL. PCR reaction was carried out with, an initial denaturation for 2 minutes at 95 ºC, followed by 34 cycles for 30s at 95 ºC, annealing: for 30s at 55ºC and an extension at 72 °C for 10 min. The PCR products were run on 1.3% agarose gel stained with ethidium bromide.

The Sequencing of PCR Products:

The PCR amplification and agarose gel electrophoresis resulted in a single band with each species; *Arothron hispidus* and *Tetraodon lineatus* of (337 and 367 bp., respectively) in cytochrome b and of (186 and 237 bp., respectively) in 16S rRNA.

The sequenced regions of cytochrome b in *Arothron hispidus* and *Tetraodon lineatus* were submitted in the National Center for Biotechnology Information (GenBank/NCBI) under accession numbers (MN186251 and MN186252, respectively). Also the sequenced regions of 16S rRNA in *Arothron hispidus* and *Tetraodon lineatus* were submitted in the (GenBank/NCBI) under accession numbers (MN186287 and MN175976, respectively). Sequences of (cyt-b) were subjected to BLAST/N at the National Center for Biotechnology Information (NCBI) and revealed 13 related marine puffer fishes species in additional to two species as outgroup. Sequences of (16S rRNA) after BLAST/N at (NCBI) revealed 9 related marine puffer fishes and four related marine Boxfish species, Order Tetraodontiformes, in addition to three outgroup species (Table 1). The marine ecology of the selected fishes were detected according to (Randall, 1982) and the FishBase website.

Table1: The understudying puffer fishes and its related marine puffer fishes with outgroups from the GenBank/ NCBI based on (cyt-b) and (16S) genes sequences.

	(cyt b gene		(16S) genes	
No.	Species	Accession umber	Species	Accession umber
1	*Tetraodon lineatus*	MN186252	*Tetraodon lineatus*	MN175976.1
2	*Arothron hispidus*	MN186251	*Arothron hispidus*	MN186287.1
3	*Arothron meleagris*	JQ681861.1	*Arothron hispidus*	AB742006.1
4	*Arothron nigropunctatus*	JQ681862.1	*Arothron reticularis*	AB742012.1
5	*Arothron diadematus*	JQ681856.1	*Arothron diadematus*	AB742004.1
6	*Arothron firmamentum*	GU057265.1	*Arothron firmamentum*	AB742005.1
7	*Arothron stellatus*	JQ681863.1	*Arothron stellatus*	AB742013.1
8	*Arothron mappa*	JQ681860.1	*Arothron mappa*	AB742009.1
9	*Arothron immaculatus*	JQ681858.1	*Lagocephalus gloveri*	KT718806.1
10	*Arothron manilensis*	JQ681859.1	*Lagocephalus lunaris*	KT718807.1
11	*Arothron hispidus*	FJ434546.1	*Canthigaster valentini*	AY679666.1
12	*Canthigaster valentini*	JQ681881.1	*Ostracion cubicus*	DQ532926.1
13	*Sphoeroides spengleri*	JQ681909.1	*Lactophrys bicaudalis*	AY679656.1
14	*Sphoeroides testudineus*	JQ681910.1	*Acanthostracion polygonius*	KT600989.1
15	*Lagocephalus lunaris*	MG817076.1	*Acanthostracion quadricornis*	AY679659.1
Out group	*Lutjanus analis* isolate 1	HQ162430.1	*Cymolutes praetextatus*	AY279696.1
	Lutjanus analis isolate 2	HQ162431.1		
	Lutjanus analis isolate 3	HQ162432.1	*Cymolutes torquatus*	AY279697.1

Sequence Alignments:

Sequences of (cyt-b) and (16S rRNA) were aligned with homologous sequences of related species from the GenBank database. Phylogenetic analyses were performed with MEGA version 7.0 18 (Kumar *et al*., 2016), using Maximum likelihood (ML), Neighbour Joining (NJ) and Minimum Evolution (ME) methods of trees construction and using 1000 bootstrap iterations (Felsenstein, 1985). Sequence divergences were calculated using Kimura 2-parameter distances (Kimura, 1980) to provide a graphical representation of divergence between species.

RESULTS

We wanted to used the (mtDNA) sequences of cytochrome b and 16S rRNA genes, to detect the best phylogenetic relationship between the species under study and its related marine puffer fishes. Our analysis inclusive *Tetraodon lineatus* species of puffer fish, represented species of Nile puffer fish and *Arothron hispidus* species of marine puffer fish.

Sequence variation using cytochrome b:

Genetic distance:

The nucleated sequence lengths using the cytochrome b gene in marine puffer fish *Arothron hispidus* and Nile puffer fish *Tetraodon lineatus* were (337 and 367 bp. respectively). The average content of A+T contents and C+G contents were similar in the two species (Table 2).

Table 2: Nucleotide frequencies and its average of cyt-b sequence in River Nile *Tetraodon lineatus* and marine *Arothron hispidus*.

Species	Base pair length	Nucleotide Number %				A+T Content (%)	C+G Content (%)
		A%	T%	C %	G%		
Arothron hispidus	337	25.5	24.4	32	18.1	49.9	50.1
Tetraodon lineatus	367	25.1	24.8	32.7	17.4	49.9	50.1
Average *%*	-	25.3	24.6	32.3	17.8	49.9	50.1

Pairwise genetic distances among Nile puffer fish *Tetraodon lineatus*, marine puffer fishes and the out group, were estimated by MEGA version 7.0 18 (Kumar *et al*., 2016) using the K^2P method with gamma correction. The P-distances among all fish species ranged from 0.000 to 0.060 %. Overall the distance value among all fish species was 0.156%.

Among puffer fishes the highest P-distance (0.060) was found between *Arothron mappa* and *Canthigaster valentini*. While, the lowest value (0.000) was found between *Tetraodon lineatus* and understudied marine puffer fish *Arothron hispidus*, likewise between *Arothron meleagris* and *Arothron nigropunctatus*, also between *Arothron immaculatus* and *Arothron manilensis* (Table 3).

The low genetic distance values among Nile puffer fish *Tetraodon lineatus* and both of marine puffer fishes; the understudied *Arothron hispidus* (MN186251) and *Arothron hispidus* from the GenBank (FJ434546.1) (0.000 and 0.014, respectively), reflected the closely genetic linkage between Nile puffer fish *Tetraodon lineatus* and *Arothron hispidus*.

The results of (cyt-b) revealed that, the genetic distance value (0.035) between Nile puffer fish *Tetraodon lineatus* and marine *Canthigaster valentine* was lower than the value (0.038) between Nile puffer fish *Tetraodon lineatus* and marine *Arothron mappa*. This expressed closely genetic relation among *Canthigaster valentine* and both of marine species of genus *Arothron* and Nile puffer species *Tetraodon lineatus*.

Compared to all marine puffer fishes, *Lagocephalus lunaris* showed the greatest sequence divergence for Nile puffer fish *Tetraodon lineatus*.

Table 3: Pairwise distances based on (cyt-b) sequence using Kimura 2- parameter among River Nile *Tetraodon lineatus* and marine puffer fishes additional to the outgroup.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
MN186252 *Tetraodon lineatus*	1		0.000	0.014	0.028	0.028	0.029	0.029	0.029	0.030	0.030	0.038	0.035	0.056	0.040	0.040	0.043	0.045	0.045	1
MN186251 *Arothron hispidus*	2	0.000		0.014	0.028	0.028	0.029	0.029	0.029	0.030	0.030	0.038	0.035	0.056	0.040	0.040	0.043	0.045	0.045	2
FJ434546.1 *Arothron hispidus*	3	0.030	0.030		0.028	0.028	0.029	0.038	0.034	0.031	0.031	0.039	0.043	0.056	0.039	0.044	0.043	0.045	0.045	3
JQ681861.1 *Arothron meleagris*	4	0.087	0.087	0.095		0.000	0.010	0.024	0.022	0.027	0.027	0.028	0.042	0.049	0.050	0.042	0.045	0.047	0.047	4
JQ681862.1 *Arothron nigropunctatus*	5	0.087	0.087	0.095	0.000		0.010	0.024	0.022	0.027	0.027	0.028	0.042	0.049	0.050	0.042	0.045	0.047	0.047	5
JQ681856.1 *Arothron diadematus*	6	0.095	0.095	0.103	0.018	0.018		0.026	0.020	0.027	0.027	0.027	0.044	0.042	0.051	0.039	0.043	0.045	0.045	6
JQ681863.1 *Arothron stellatus*	7	0.095	0.095	0.138	0.071	0.071	0.079		0.029	0.030	0.030	0.034	0.043	0.047	0.046	0.048	0.044	0.046	0.046	7
GU057265.1 *Arothron firmamentum*	8	0.100	0.100	0.126	0.063	0.063	0.056	0.100		0.026	0.026	0.022	0.047	0.040	0.051	0.040	0.043	0.045	0.045	8
JQ681858.1 *Arothron immaculatus*	9	0.102	0.102	0.110	0.093	0.093	0.086	0.102	0.092		0.000	0.022	0.046	0.037	0.049	0.041	0.039	0.041	0.041	9
JQ681859.1 *Arothron manilensis*	10	0.102	0.102	0.110	0.093	0.093	0.086	0.102	0.092	0.000		0.022	0.046	0.037	0.049	0.041	0.039	0.041	0.041	10
JQ681860.1 *Arothron mappa*	11	0.138	0.138	0.147	0.095	0.095	0.087	0.120	0.070	0.063	0.063		0.060	0.050	0.047	0.046	0.044	0.046	0.046	11
JQ681881.1 *Canthigaster valentini*	12	0.144	0.144	0.190	0.180	0.180	0.190	0.180	0.207	0.192	0.192	0.256		0.058	0.050	0.044	0.048	0.050	0.050	12
MG817076.1 *Lagocephalus lunaris*	13	0.242	0.242	0.253	0.209	0.209	0.179	0.199	0.163	0.153	0.153	0.220	0.251		0.057	0.043	0.042	0.042	0.042	13
JQ681909.1 *Sphoeroides spengleri*	14	0.170	0.170	0.170	0.220	0.220	0.230	0.199	0.227	0.211	0.211	0.209	0.211	0.261		0.036	0.054	0.057	0.057	14
JQ681910.1 *Sphoeroides testudineus*	15	0.186	0.186	0.205	0.195	0.195	0.176	0.226	0.186	0.187	0.187	0.215	0.189	0.197	0.139		0.035	0.037	0.037	15
HQ162430.1 *Lutjanus analis* isolate_1	16	0.203	0.203	0.212	0.212	0.212	0.203	0.212	0.203	0.175	0.175	0.203	0.215	0.185	0.249	0.151		0.006	0.006	16
HQ162431.1 *Lutjanus analis* isolate_2	17	0.212	0.212	0.222	0.222	0.222	0.212	0.222	0.212	0.184	0.184	0.212	0.226	0.185	0.261	0.160	0.006		0.000	17
HQ162432.1 *Lutjanus analis* isolate_3	18	0.212	0.212	0.222	0.222	0.222	0.212	0.222	0.212	0.184	0.184	0.212	0.226	0.185	0.261	0.160	0.006	0.000		18
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	

The Pairwise genetic distances among understudied *Arothron hispidus* and other species of genus *Arothron* ranged from 0.014 to 0.038, and among other genera from 0.035 to 0.056. Based on (cyt-b) gene results, *Arothron meleagris* and *Arothron nigropunctatus* were closely related to *Arothron hispidus* where the genetic distance value was (0.028), but *Arothron mappa* was the highest genetic distance to *Arothron hispidus* (0.038) comparing to other species of genus *Arothron*. The results illustrated that, *Lagocephalus lunaris* was the highest genetic distance (0.056) to *Arothron hispidus* among all puffer fish species.

Phylogenetic inference:

To carry out the phylogenetic analysis, Nile puffer fish *Tetraodon lineatus* and marine puffer fish *Arothron hispidus* were submitted to analysis together with 13 of other marine puffer fishes sequences from GenBank/NCBI, representing four genera of family Tetraodontidae (Genus *Arothron* includes nine species, genus *Sphoeroides* contains two species and one species of both genera; *Canthigaster* and *Lagocephalus*), in addition to 3 species as outgroup (*Lutjanus*, Family Lutjanidae, Order Perciformes) (Table 1).

To confirm the phylogenetic relations among puffer fishes we used three phylogenetic methods; Maximum likelihood (ML), Neighbour Joining (NJ) and Minimum Evolution (ME) based on cytochrome b gene. All methods showed the same relations among puffer fishes with some differents in support values, and revealed 5 main features: (1) species of the outgroup formed a separate cluster, (2) *Lagocephalus lunaris* formed a separate cluster from the rest of puffer fishes, (3) all species under genus *Arothron* found in one clad and formed some sister clads with each others, (4) all trees illustrated that, the under study Nile puffer fishes *Tetraodon lineatus* was closely related to genus *Arothron* than other marine puffer fishes genera, (5) The under study puffer fishes Nile *Tetraodon lineatus* and marine puffer fish *Arothron hispidus* (MN186251 and FJ434546.1) were found as a sister clad, which indicated the related linkage between them (Figs. 1-3).

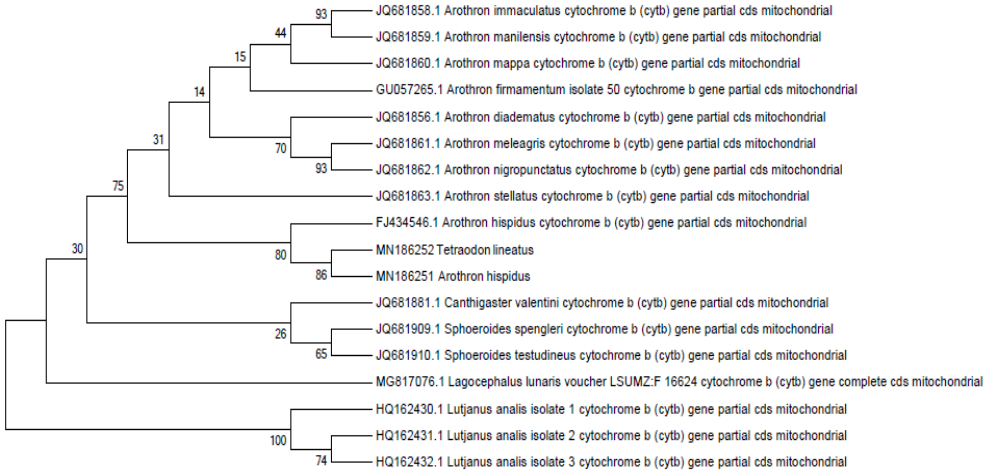

Fig. 1: Phylogenetic tree using the Maximum Likelihood method among Nile puffer fish *Tetraodon lineatus* and marine puffer fishes based on (cyt-b) including the outgroup.

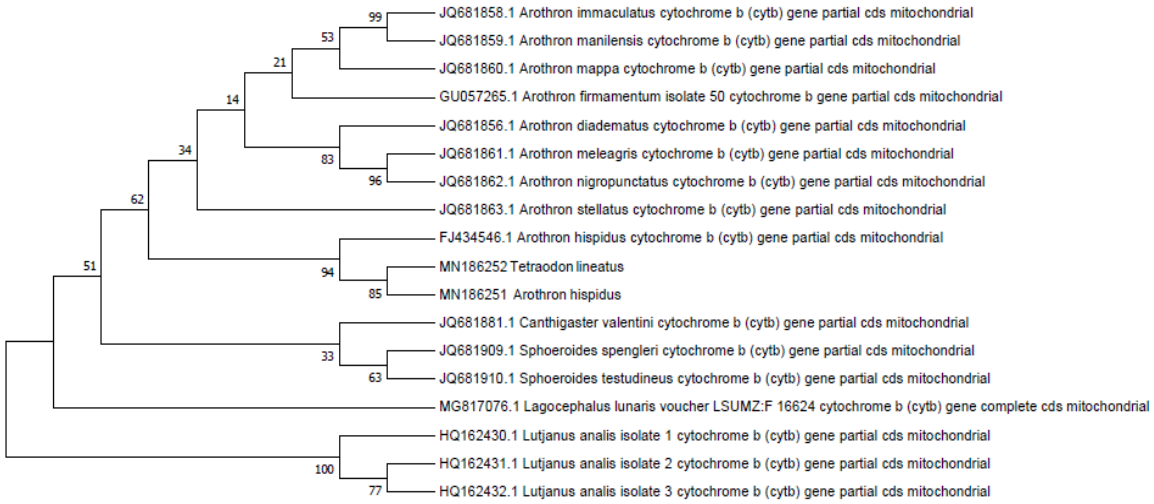

Fig. 2: Phylogenetic tree using the Neighbor Joining method among Nile puffer fish *Tetraodon lineatus* and marine puffer fishes based on (cyt-b) including the outgroup.

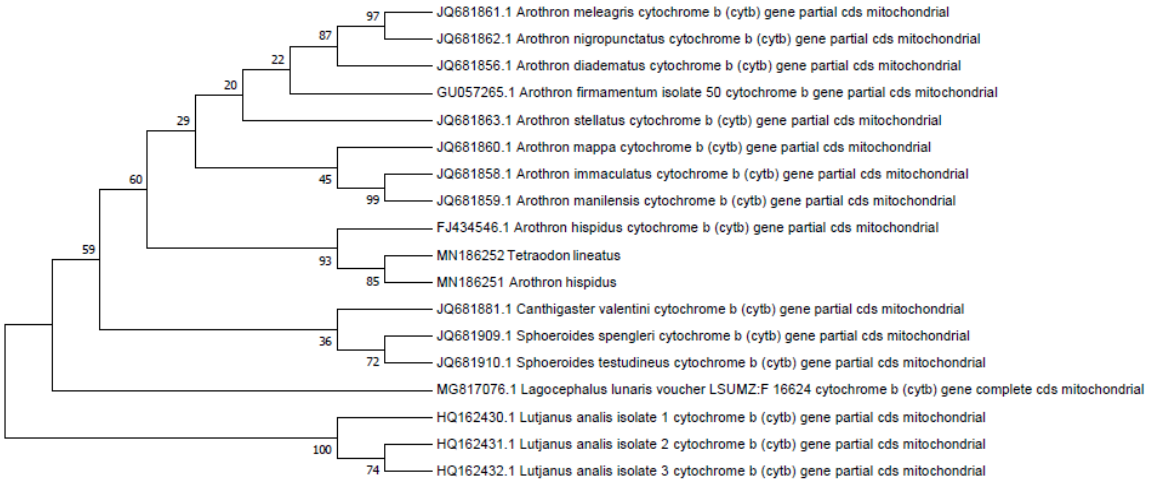

Fig. 3: Phylogenetic tree using the Minimum Evolution method among Nile puffer fish *Tetraodon lineatus* and marine puffer fishes based on (cyt-b) including the outgroup.

Sequence variation using 16S:

Genetic distance:

In additional to Nile puffer fish *Tetraodon lineatus* has longer DNA sequencing than marine puffer fish *Arothron hispidus*, the results also illustrated that, C+G contents of *Tetraodon lineatus* (47.3) were higher than that of *Arothron hispidus* (40.3), but A+T contents of *Arothron hispidus* were higher than that of *Tetraodon lineatus* (Table 4).

Table 4: Nucleotide frequencies and its average of 16S sequence in River Nile *Tetraodon lineatus* and marine *Arothron hispidus*.

Species	Base pair length	Nucleotide Number %				A+T Content (%)	G+C Content (%)
		A%	T%	C %	G%		
Arothron hispidus	186	33.3	26.4	17.2	23.1	59.7	40.3
Tetraodon lineatus	237	32	20.7	23.2	24.1	52.7	47.3
Average *%*	-	32.7	23.5	20.2	23.6	56.2	43.8

The percentage of genetic distance values among puffer fishes species ranged from 0.000 to 0.105, while among all species ranged from 0.00 to 0.149 %. Overall the distance value among all fish species was 0.161 % (Table 5).

The genetic distance values among Nile puffer fish *Tetraodon lineatus* and marine puffer fish species of genus *Arothron* ranged from (0.016 to 0.083). While the values of genetic distance among Nile puffer fish *Tetraodon lineatus* and marine species; *Canthigaster valentine*, *Lagocephalus lunaris*, *Lagocephalus gloveri*, *Acanthostracion polygonius*, *Acanthostracion quadricornis*, *Ostracion cubicus* and *Lactophrys bicaudalis* ranged from (0.030 to 0.043).

Table 5: Pairwise distances based on (16S) sequence using Kimura 2-parameter among River Nile *Tetraodon lineatus* and marine puffer fishes additional to the outgroup.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
MN175976.1 *Tetraodon lineatus*	1		0.083	0.016	0.022	0.028	0.027	0.027	0.031	0.030	0.039	0.039	0.041	0.043	0.041	0.043	0.042	0.043	1
MN186287.1 *Arothron hispidus*	2	0.346		0.062	0.076	0.085	0.080	0.081	0.081	0.100	0.105	0.105	0.142	0.149	0.142	0.130	0.110	0.111	2
AB742006.1 *Arothron hispidus*	3	0.041	0.268		0.011	0.018	0.017	0.017	0.020	0.023	0.027	0.027	0.033	0.034	0.033	0.034	0.035	0.033	3
AB742005.1 *Arothron firmamentum*	4	0.068	0.321	0.023		0.012	0.013	0.010	0.017	0.024	0.024	0.024	0.033	0.037	0.032	0.033	0.040	0.032	4
AB742013.1 *Arothron stellatus*	5	0.097	0.351	0.048	0.023		0.017	0.017	0.018	0.027	0.023	0.023	0.037	0.040	0.038	0.035	0.041	0.032	5
AB742009.1 *Arothron mappa*	6	0.089	0.336	0.041	0.029	0.041		0.012	0.016	0.022	0.033	0.033	0.042	0.044	0.041	0.039	0.048	0.039	6
AB742004.1 *Arothron diadematus*	7	0.089	0.336	0.041	0.017	0.041	0.023		0.016	0.022	0.029	0.029	0.035	0.040	0.037	0.032	0.042	0.033	7
AB742012.1 *Arothron reticularis*	8	0.112	0.346	0.061	0.047	0.047	0.041	0.041		0.026	0.033	0.033	0.040	0.042	0.042	0.038	0.042	0.034	8
AY679666.1 *Canthigaster valentini*	9	0.105	0.400	0.068	0.068	0.083	0.061	0.061	0.082		0.030	0.030	0.035	0.040	0.036	0.037	0.041	0.038	9
KT718807.1 *Lagocephalus lunaris*	10	0.153	0.423	0.096	0.081	0.074	0.120	0.104	0.127	0.104		0.000	0.042	0.045	0.038	0.040	0.044	0.046	10
KT718806.1 *Lagocephalus gloveri*	11	0.153	0.423	0.096	0.081	0.074	0.120	0.104	0.127	0.104	0.000		0.042	0.045	0.038	0.040	0.044	0.046	11
KT600989.1 *Acanthostracion polygonius*	12	0.174	0.559	0.132	0.132	0.157	0.176	0.140	0.174	0.149	0.185	0.185		0.016	0.024	0.018	0.046	0.039	12
AY679659.1 *Acanthostracion quadricornis*	13	0.176	0.567	0.133	0.150	0.168	0.177	0.159	0.176	0.168	0.194	0.194	0.041		0.028	0.016	0.042	0.039	13
DQ532926.1 *Ostracion cubicus*	14	0.173	0.574	0.131	0.131	0.165	0.174	0.156	0.182	0.147	0.165	0.165	0.093	0.108		0.025	0.051	0.050	14
AY679656.1 *Lactophrys bicaudalis*	15	0.174	0.515	0.132	0.132	0.140	0.157	0.124	0.156	0.149	0.166	0.166	0.048	0.041	0.093		0.040	0.036	15
AY279696.1 *Cymolutes praetextatus*	16	0.178	0.483	0.152	0.179	0.188	0.217	0.188	0.196	0.179	0.197	0.197	0.197	0.185	0.236	0.168		0.021	16
AY279697.1 *Cymolutes torquatus*	17	0.180	0.475	0.137	0.129	0.129	0.163	0.137	0.145	0.163	0.207	0.207	0.168	0.160	0.229	0.141	0.060		17
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	

The genetic distance value (0.030) between Nile puffer fish *Tetraodon lineatus* and marine *Canthigaster valentine* was little than the value (0.031) between Nile puffer fish *Tetraodon lineatus* and marine *Arothron reticularis*. That indicated the closely genetic relation among *Canthigaster valentine* and marine species of genus

Arothron in one side and Nile puffer fish *Tetraodon lineatus* in the other side. The results of (16S) sequencing revealed that, the pairwise genetic distances among understudied *Arothron hispidus* and other species of genus *Arothron* ranged from 0.062 to 0.085, and among other genera ranged from 0.100 to 0.149 (Table 5).

The four species of Boxfishes, Family Ostraciidae, Order Tetraodontiformes were genetically distanced from the understudied species compared to species of family Tetraodontidae.

In general, the Nile puffer fish *Tetraodon lineatus* was distantly related to marine puffer fishes of genera *Arothron* and *Canthigaster* more than the rest of marine genera; *Lagocephalus*, *Acanthostracion*, *Ostracion* and *Lactophrys*. Among all samples *Arothron hispidus* (AB742006.1) and *Arothron firmamentum* (AB742005.1) were related to the Nile puffer fish *Tetraodon lineatus*, where the genetic distance values were 0.016 and 0.022, respectively.

Phylogenetic inference:

To carry out the phylogenetic analysis using (16S) sequencing, Nile puffer fish *Tetraodon lineatus* and marine puffer fish *Arothron hispidus* were submitted to analysis together with 13 of other marine fishes sequences from GenBank/NCBI, representing nine species of Family Tetraodontidae, Order Tetraodontiformes, found in three genera (*Arothron* includes six species, *Lagocephalus* contains two species and one species of genus *Canthigaster*). Also, the blastn results of GenBank/NCBI showed similarity with four species of Boxfishes, Family Ostraciidae, Order Tetraodontiformes, which were (*Ostracion cubicus*, *Acanthostracion polygonius*, *Acanthostracion quadricornis* and *Lactophrys bicaudalis*). In addition to the outgroup sequences (*Cymolutes*, family Labridae, Order Perciformes) (Table 1).

For more illustrative phylogenetic relations, we used more than one phylogenetic method; Maximum likelihood, Neighbour Joining and Minimum Evolution based on 16S gene. The methods revealed 3 main features: (1) species of the outgroup formed a separate cluster, (2) species of Boxfishes formed a separate cluster from the rest fishes, (3) the results illustrated that, the under study marine puffer fish *Arothron hispidus* found in a sister clad with *Arothron hispidus* from GenBank, and the Nile puffer fish *Tetraodon lineatus* was very related to this clad, which indicated the related linkage between *Tetraodon lineatus* and genus *Arothron*, specially species *Arothron hispidus* (Figs. 4-6).

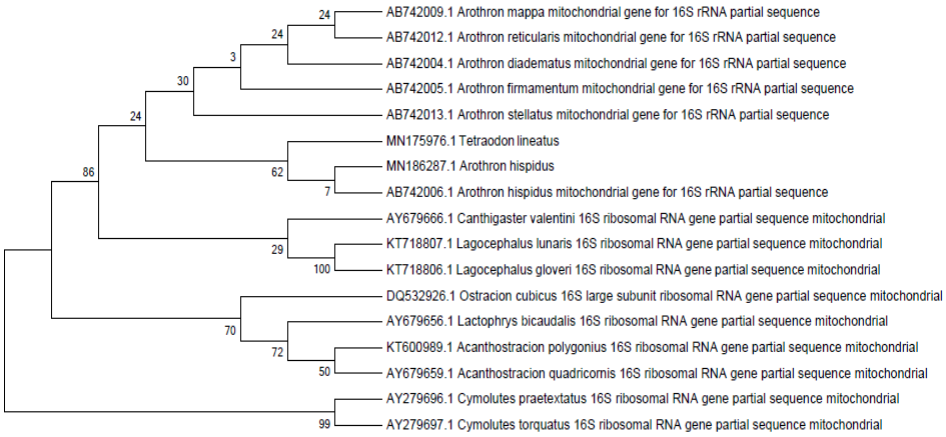

Fig. 4. Phylogenetic tree using the Maximum Likelihood method among Nile puffer fish *Tetraodon lineatus* and marine puffer fishes based on (16S) including the outgroup species.

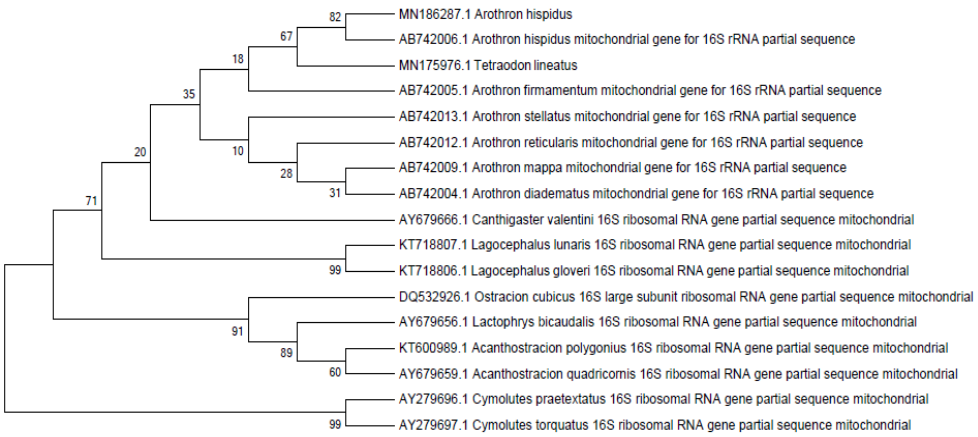

Fig. 5: Phylogenetic tree using the Neighbor Joining method among Nile puffer fish *Tetraodon lineatus* and marine puffer fishes based on (16S) including the outgroup species.

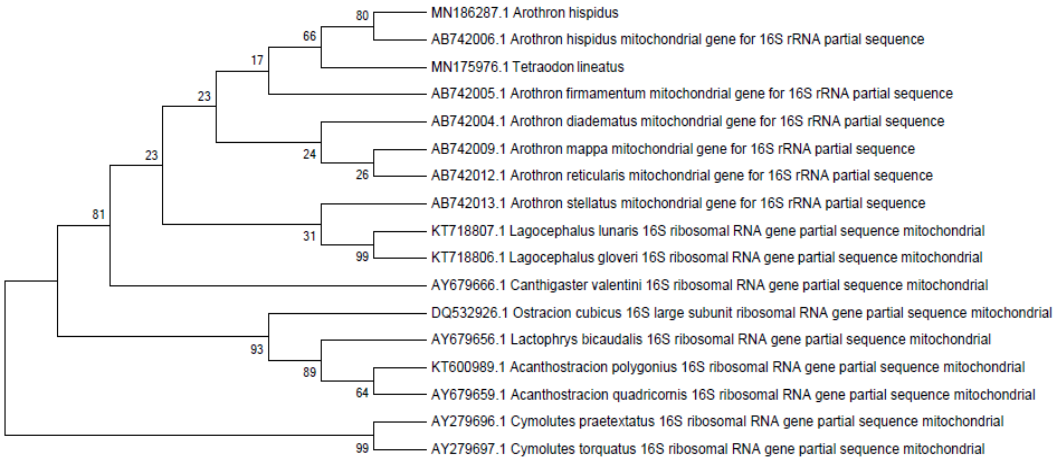

Fig. 6. Phylogenetic tree using the Minimum Evolution method among Nile puffer fish *Tetraodon lineatus* and marine puffer fishes based on (16S) including the outgroup species.

DISCUSSION

The study of nucleotide variations among fishes is the main principle for exploring biodiversity via molecular markers (Noikotr *et al*., 2013; Saad and Abd El-Sadek, 2017 and Saad, 2019). Puffer fishes are suitable group to study the genome size evolution, specially, family Tetraodontidae which has a haploid genome size of ~400 million bp. (Hinegardner and Rosen, 1972 and Neafsey and Palumbi, 2003).

In Egypt, *Tetraodon lineatus* is the single freshwater puffer fish species (Geba *et al*., 2016). Our phylogenetic reconstruction was carried out to provide a comprehensive analysis of the evolutionary relationships among Nile *Tetraodon lineatus* and marine puffer fishes, in additional to the phylogenetic linkage among Egyptian Red Sea *Arothron hispidus* and its related marine puffer fishes species from GenBank/NCBI using cytochrome b and 16S genes.

The cytochrome b characterized by its sequence variability which makes it very useful in the phylogenetic relationships of species within genera and families (Castresana, 2001). So we used the cytochrome b gene to give a general view about the molecular linkages of the Nile puffer fish *Tetraodon lineatus* and other marine puffer fishes specially *Arothron hispidus* using the available data on GenBank/NCBI.

Kaleshkumar *et al*. (2015) reported that, closely related species have the lowest genetic distance, while the highest genetic distance refers to highly diverged case. Based on Sequence variation using cytochrome b, the understudied *Arothron hispidus*

was closely related to *Arothron stellatus* (0.029) than *Arothron immaculatus* (0.30) this was agree with the results of (Kaleshkumar *et al*., 2015), who found *Arothron hispidus* was closely related to *Arothron stellatus* than *Arothron immaculatus* based on (mt COI). The same author showed that, *Arothron hispidus* was closely related to *Arothron stellatus* than *Arothron nigropunctatus* based on (mt COI), but our results based on (cyt-b) revealed lower genetic distance between *Arothron hispidus* and *Arothron nigropunctatus* than *Arothron hispidus* and *Arothron stellatus*.

The studies of (Cantatore *et al*., 1994; Almodovar *et al*., 2000; Duokakis, 2000 and Çiftci *et al*., 2013) on the cytochrome b gene illustrated that, the cyt-b gene tend to be rich in C, A, and T nucleotide ratios, but low in G content, this was agree with our results, where the nucleotide ratios of C, A and T respectively was more than G ratios (Table 2).

In many fishes studies, 16S rRNA gene has been the system of choice for barcoding and identification of fishes because of it is easier to amplify and sequence (Miglietta *et al*., 2009; Moura *et al*., 2011; Rosas, *et al*., 2018 and Saad, 2019). There for, we used the (16S) sequencing to reveal the molecular phylogenetics relationships of Nile puffer fish *Tetraodon lineatus* and *Arothron hispidus* to marine puffer fishes using the available data on GenBank/NCBI.

The results of 16S rRNA gene sequence illustrated that, A+T contents of both *Tetraodon lineatus* and *Arothron hispidus* were higher than C+G contents (Table 4), this was in corroboration with (Lakra *et al*., 2009 and Singh *et al*., 2015) who found high A+T contents in their study on fishes using 16S rRNA .

The high genetic distance values of 16S rRNA gene among *Arothron hispidus* and other species of puffer fishes (0.062 to 0.149) indicating its efficiency to reveal the phylogenetic relationships among the puffer fishes, this was in agreement with (Vinson *et al*., 2004, Chakraborty *et al*., 2006 a and b and Singh *et al*., 2015) who reported high nucleotide divergence among their under studied fishes using 16S gene, indicating the usefulness of this gene sequence for accurate identification of species.

The results indicated that the 16S rRNA system was efficient (Quraishia *et al*., 2015) in detecting genetic variations among these fishes. The blastn results of (16S) sequencing in the GenBank/NCBI showed similarity with four species of Boxfishes, family Ostraciidae, Order Tetraodontiformes which were not appeared with (cyt-b). We suggested that, this may be due to the highly conserved of (16S rRNA) gene, which was in agreement with (Singh *et al*., 2015).

The results of (16S rRNA) gene were in similarity with that of (cyt-b), where both genes revealed that, Nile puffer fish *Tetraodon lineatus* was closely related to genus *Arothron* than other marine puffer fishes genera. Also, low genetic distance value among *Canthigaster valentine* and both of Nile puffer fish *Tetraodon lineatus* and species of genus *Arothron* reflects the genetic relationships among them. These similarity results between (16S rRNA) and (cyt-b) genes indicate to high efficiency of both genes to investigate the genetic linkages among various species and genera as were observed in many studies like (Kartavtsev *et al*., 2007; Teletchea, 2009; Singh *et al*., 2015 and Saad, 2019).

CONCLUSION

Tetraodon lineatus is the single freshwater puffer fish in Egypt, so our study was performed to examine the phylogenetic relationships among the River Nile *Tetraodon lineatus* and the Red Sea *Arothron hispidus* to other marine puffer fishes using the available data on GenBank/NCBI, based on Mitochondrial DNA (mtDNA)

sequences of cytochrome b and 16S rRNA genes. The results of both (16S) and (cyt-b) genes were very similar to each others and illustrated closely genetic linkage among Nile puffer fishes *Tetraodon lineatus* and marine species of genus *Arothron* as well as *Canthigaster valentine* species. These similarity results of (16S rRNA) and (cyt-b) genes proved the usefulness of them for phylogenetic analysis of puffer fishes.

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ARABIC SUMMARY

العلاقات الوراثية الجزيئية لأسماك الفهقة النيلية والبحرية باستخدام تتابع الحمض النووي للميتوكوندريا Cytochrome b and 16S rRNA

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أجريت هذه الدراسة علي أسماك الفهقة النيلية *Tetraodon lineatus* - النوع الوحيد في نهر النيل بمصر- والفهقة البحرية *Arothron hispidus* بهدف دراسة العلاقات الوراثية الجزيئية بين أسماك الفهقة النيلية والبحرية بمصر وذلك باستخدام اثنين من تتابع الحمض النووي للميتوكوندريا (Cytochrome b و 16S rRNA) وقد أظهرت النتائج من خلال البيانات المتوفرة في بنك الجينات GenBank أن طول تتابع القواعد النيتروجينية في كل من الفهقة البحرية والنيلية باستخدام جين cyt-b هو (٣٣٧ و ٣٦٧ علي الترتيب) وباستخدام جين 16S rRNA هو (١٨٦ و ٢٣٧ علي الترتيب). تم استخدام ثلاث طرق مختلفة لدراسة العلاقات الوراثية الجزيئية هي (Maximum Likelihood و Neighbour Joining و Minimum Evolution) لمقارنة النتائج علي نطاق واسع.

بشكل عام أوضح التحليل الإحصائي الدقيق للمسافات الوراثية بين الأنواع محل الدراسة باستحدام جيني (cyt-b و 16S rRNA) أن التقارب الوراثي بين الفقهة النيلية *Tetraodon lineatus* وأسماك الفهقة البحرية من جنسي *Arothron* و *Canthigaster* أكبر من التقارب الوراثي بين الفقهة النيلية *Tetraodon lineatus* وأسماك الفهقة البحرية الاخري. كما أن التشابه في نتائج جيني (cyt-b و 16S rRNA) يؤكد مدي فاعليتهما في دراسة العلاقات التصنيفة الوراثية المرتبطة بهذا النوع من الأسماك.