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Phylogenetic analysis of the Red Sea tiger snake eel *Myrichthys maculosus* (Family Ophichthidae) by using 16S rRNA

Zeinab A. Mar'ie[1,2]

1- Zoology Department, Faculty of Science, South Valley University, Egypt.
2- Science Department, Faculty of Education, Hurghada, South Valley University, Egypt.

zainab.khalek@sci.svu.edu.eg

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ABSTRACT

The Red Sea tiger snake eel *Myrichthys maculosus* belongs to the family Ophichthidae, order Anguilliformes, represented with 11 relative species and two outgroups for phylogenetic analysis by mean of large subunit 16S rRNA sequence. *M. maculosus* contains a high concentration of AT (53.8%) more than GC (46.1%), higher also in other understudying Ophichthidae species. The genetic distance values among *M. maculosus* and other related species ranged from 0.0006 to 0.029, while among all species ranged from 0.006 to 0.037%. Overall the distance value was 0.167%. The smallest genetic distance (0.006) was between *M. maculosus* (MW435681) and the same one under the accession number of DQ645692, while the largest distance (0.036%) was between *Scolecenchelys breviceps* and *Ophichthus zophochir*&*O. apicalis*. The three phylogenetic trees Maximum likelihood (ML), Neighbour Joining (NJ), and Minimum Evolution (ME) applied here showed the same relations with slightly different in support values.

INTRODUCTION

Ophichthidae or snake eels is a family of order Anguilliformes, which comprise 19 families, 159 genera and nearly 938 species, most of them are marine, but some species entering rivers **(Nelson *et al*., 2016)**. The majority of Ophichthidae spends their time buried in sand and applies their sense of smell in hunting small fishes and crustaceans **(Nelson, 1994)**.Comparing with morays snake eels is much less seen. The tiger eel (spotted snake eel) may reach to 1 m long or more, generally lives at depths between 1 and 25 m or more down, mostly in sandy areas by reefs **(Debelius, 2011 ; Lieske and Myers, 2012)**.

Recently the phylogenetic relationship of eel species and revised taxonomic hierarchy, evaluated by short fragments of mitochondrial and also nuclear gene **(Jamandne *et al*., 2007; Tang and Fietitz 2012; Peninal *et al*., 2017)**.The variation of eel species and evidenced the major lineages originated between the end of Cretaceous

and Early Eocene revealed by molecular studies as demonstrated by **(Santini *et al.*, 2013)**. In **2018, Laskar *et al.*** confirmed that molecular data are very strong tool in species identification.

16S rRNA sequencing able to reclassifying the organism in to another new species **(Weisburg *et al.*, 1991)**. The large subunit 16S ribosomal RNA composed of 30 S small subunit of the mitochondrial ribosome in vertebrate has been reported 1640 bp long in fish (**Naock *et al.*, 1996)**. In **2005 Vences *et al.*** improved the role of 16S rRNA gene sequence in the bar coding of vertebrates. The mitochondrial (16S) gene extensively used in phylogenetic linkages of fishes at different taxonomic levels **(Faddagh *et al.*, 2012; Marʹie and Allam, 2019)**. Between species it is variable, but stable within same species **Yang *et al.* 2014**. The mitochondrial DNA has a circular structure thus allows more efficient amplification than the nuclear DNA **(Panday *et al.*, 2014).**

The point of this work is to confirm the strong role of large subunit "16S rRNA" sequencing in clarify the phylogenetic relationships of family Ophichthidae. Three phylogenetic methods; Maximum likelihood (ML), Neighbour Joining (NJ) and Minimum Evolution (ME) applied here to compare results with more widely used methods.

MATERIALS AND METHODS

Collection of the samples:

Tiger spotted eel collected by the helpful of fishermen in Hurghada , Red Sea in Egypt, then brought to the laboratory for the morphologically identification according to **Randall (1982)**. The muscles tissues were individually isolated and preserved in -20ºC until genomic DNA extraction.

DNA Extraction:

The genomic DNA 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 (16sar: 3'-CGCCTGTTTAACAAAAACAT-5') and reverse (16sbr: 5'-CGCCTGTTTAACAAAAACAT-3') primers according to **(Simon *et al.*, 1991)**. The PCR reactions were carried out with 10 pmoles of each primer, ~100 ng of genomic DNA and 12.5 µL PCR master mix (OnePCR™ ready-to-use, Catalogue Number: MB203-0100, GeneDireX, Miaoli County, Taiwan) in a final reaction volume of 25 µL. PCR reaction was carried out with, an initial denaturation for 2 minutes at 95ºC, followed by 35 cycles for 30s at 95ºC, annealing: for 30s at 50ºC 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:

PCR amplification and agarose gel electrophoresis resulted in a single band of (581 bp.). DNA sequencing was achieved by Macrogen (Seoul, South Korea). The sequenced region of 16S rRNA in *Myrichthys maculosus* was submitted in the (GenBank/NCBI) under accession number (MW435681). using the same primer used for amplification Sequence was subjected to BLAST/N at the National Centre for Biotechnology Information (NCBI). Eleven related Ophichthidae species were selected in additional to two species as out group (Table 1).

Table (1): details of the present study tiger eel *Myrichthys maculosus* and its related species (Ophichthidae) with two out group (Congridae) from the GenBank/ NCBI based on (16S) genes sequences

No.	Species	Accession umber	Size of the amplicons bp	Similarity	Query coverage (%)
1	*Myrichthys maculosus*	MW435681.1	581	100%	100%
2	*Myrichthys maculosus*	DQ645692.1	991	99.83%	100%
3	*Ophichthus celebicus*	KX426295.1	645	91.11%	100%
4	*Callechelys catostoma*	JX242987.1	645	90.61%	100%
5	*Brachysomophis crocodilinus*	DQ645689.1	994	90.12%	100%
6	*Myrichthys breviceps*	AF455777.1	549	91.47%	94%
7	*Ophichthus puncticeps*	AF455781.1	549	91.34%	94%
8	*Echelus myrus*	DQ645690.1	989	88.96%	100%
9	*Scolecenchelys breviceps*	DQ645691.1	996	87.61%	100%
10	*Ophichthus zophochir*	AY952487.1	528	88.18%	91%
11	*Ophichthus shaoi*	LC506439.1	511	89.19%	88%
12	*Ophichthus apicalis*	KX426283.1	638	89.59%	100%
13	*Gnathophis longicauda*	DQ645704.1	990	87.29%	100%
14	*Gnathophis bathytopos*	JX242952.1	641	86.82%	100%

Sequence Alignments:

Sequences of (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

Sequence variation using 16S:

Genetic distance:

It's pretty obvious from the data in table (2) that *Scolecenchelys breviceps*has the longest DNA sequences (994 pb), whereas *Ophichthus shaoi* has the smallest DNA sequences (511 pb). The A+T content is higher than C+G content in *Myrichthys*

maculosus (53.8% and 46.1%, respectively) and in all other species**.**The A nulceotide got the largest average with 34.9.

Table 2: Nucleotide frequencies and its average of 16S rRNA sequence in ***Myrichthys maculosus*** and its related family (Ophichthidae) species with two out groups (Congridae).

Species	Base pair length	Nucleotide Number %				A+T content %	C+G content %
		T %	C%	A%	G%		
Myrichthys maculosus	581	21.3	24.4	32.5	21.7	53.8	46.1
Myrichthys maculosus	991	19.6	23.6	37.3	19.4	56.9	43.0
Ophichthus celebicus	645	20.1	25.8	33.0	21.0	53.1	46.8
Callechelys catostoma	645	22.0	23.8	34.4	19.8	56.4	43.6
Brachysomophis crocodilinus	994	19.4	23.8	35.6	21.2	55.0	45.0
Myrichthys breviceps	549	22.8	22.8	31.3	23.1	54.1	45.9
Ophichthus puncticeps	549	22.4	22.8	33.5	21.3	55.9	44.1
Echelus myrus	989	19.8	21.7	38.6	20.0	58.4	41.7
Scolecenchelys breviceps	996	20.3	23.4	36.7	19.6	57.0	43.0
Ophichthus zophochir	528	22.2	22.3	35.6	19.9	57.8	42.2
Ophichthus shaoi	511	21.5	24.3	33.9	20.4	55.4	44.7
Ophichthus apicalis	638	20.7	24.6	33.6	21.2	54.3	23.8
Gnathophis longicauda	990	18.9	23.1	37.2	20.8	56.1	43.9
Gnathophis bathytopos	641	20.7	25.0	34.7	19.6	55.4	44.6
Avg.		20.8	23.7	34.9	20.6		

The genetic distance values among *Myrichthys maculosus* and its related species of the same family ranged from 0.0006 to 0.029%, while among all species ranged from 0.006 to 0.037%. Overall the distance value among all current fish species was 0.167% .The smallest genetic distance was between *M. maculosus* and the same one under accession number of DQ645692, while the largest distance (0.036%) was between *Scolecenchelys breviceps* and *Ophichthus zophochir* & *O. apicalis* (Table 3).

Phylogenetic inference:

The Red Sea tiger snake eel "*Myrichthys maculosus*" was submitted to phylogenetic analysis by (16S) sequence together with 11 of other related species from GenBank/NCBI, representing the same species under accession number DQ645692 , the same genera (*M. breviceps*), five species of the same genera *Ophichthus* (*O. celebicus, O. puncticeps, O. zophochir, O. shaoi, and O. apicalis*) and one species of genera; *Callechelys, Brachysomophis, Echelus,*and *Scolecenchelys*, in addition to 2 species as out group from family Congridae (*Gnathophis longicauda &G. bathytopos*).

Table 3: Pairwise distances based on (16S rRNA) sequence using Kimura 2- parameter among *Myrichthys maculosus*and its related family species additional to the out group.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	MW435681.1_Myrichthys maculosus		0.006	0.024	0.023	0.029	0.023	0.022	0.026	0.029	0.027	0.025	0.025	0.030	0.029
2	DQ645692.1_Myrichthys maculosus	0.009		0.025	0.023	0.029	0.024	0.023	0.028	0.031	0.027	0.027	0.025	0.031	0.030
3	KX426295.1_Ophichthus celebicus	0.118	0.129		0.027	0.034	0.034	0.027	0.031	0.034	0.030	0.014	0.026	0.029	0.030
4	JX242987.1_Callechelys catostoma	0.108	0.113	0.136		0.032	0.028	0.024	0.030	0.030	0.027	0.026	0.025	0.030	0.030
5	DQ645689.1_Brachysomophis crocodilinus	0.153	0.159	0.195	0.177		0.030	0.028	0.028	0.032	0.031	0.035	0.032	0.034	0.035
6	AF455777.1_Myrichthys breviceps	0.103	0.109	0.193	0.141	0.170		0.029	0.033	0.034	0.030	0.032	0.034	0.037	0.036
7	AF455781.1_Ophichthus puncticeps	0.097	0.108	0.141	0.120	0.141	0.146		0.025	0.025	0.027	0.026	0.031	0.029	0.028
8	DQ645690.1_Echelus myrus	0.133	0.144	0.185	0.168	0.145	0.179	0.117		0.026	0.032	0.032	0.030	0.029	0.029
9	DQ645691.1_Scolecenchelys breviceps	0.162	0.173	0.203	0.174	0.191	0.209	0.123	0.134		0.036	0.032	0.036	0.033	0.032
10	AY952487.1_Ophichthus zophochir	0.130	0.135	0.170	0.141	0.174	0.163	0.141	0.173	0.223		0.029	0.026	0.033	0.033
11	LC506439.1_Ophichthus shaoi	0.130	0.141	0.047	0.125	0.196	0.175	0.136	0.192	0.185	0.165		0.028	0.028	0.030
12	KX426283.1_Ophichthus apicalis	0.129	0.128	0.134	0.119	0.176	0.192	0.169	0.173	0.215	0.140	0.152		0.030	0.032
13	DQ645704.1_Gnathophis longicauda	0.178	0.184	0.166	0.173	0.203	0.233	0.161	0.161	0.201	0.196	0.166	0.178		0.015
14	JX242952.1_Gnathophis bathytopos	0.166	0.172	0.178	0.167	0.210	0.220	0.155	0.167	0.189	0.202	0.184	0.190	0.047	

Three phylogenetic methods; Maximum likelihood (ML), Neighbour Joining (NJ), and Minimum Evolution (ME) based on 16S rRNA gene were applied to confirm the relations among current Ophichthidae species. The three methods showed the same relations with slightly different in support values.

Myrichthys maculosus with the eleven related Ophichthyidae members showed distinct clustering, as follow: 1) *Myrichthys maculosus* with its identical DQ645692 clustering and cladded as sister species of *M. breviceps*. 2) *O.celebicus* & *O. shaoi* identical cladded together as a sister species of *C. catostoma* (single clad) and with *O. zophochir* and *O.apicalis* (sister clad). 3) *E. myrus* and *S. breviceps* clustering together as sister species of *B. crocodlinus* (single clad) and *O. puncticeps* (single clad). 4) The two out group family Congridae (formed a separate cluster from the rest (Figs. 1-3).

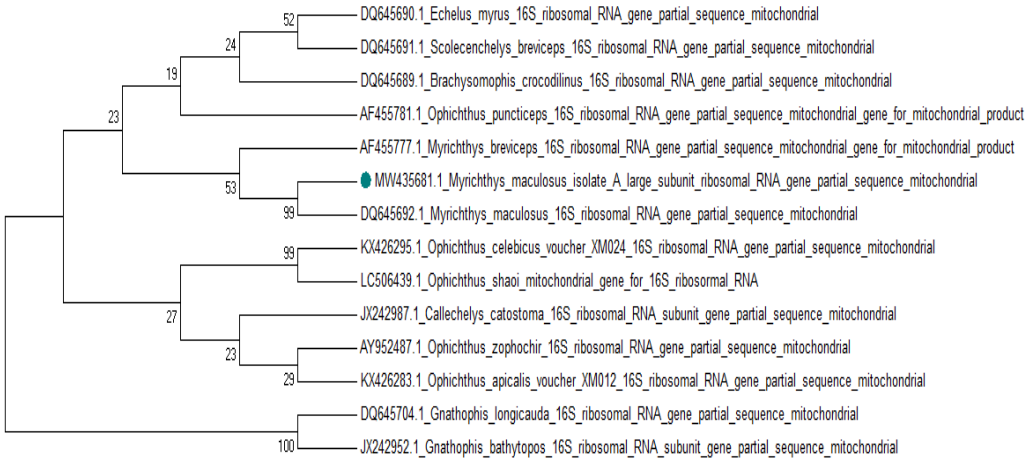

Fig. 1. Molecular phylogenetic tree by maximum likelihood method among tiger snake eel *Myrichthys maculosus* and related species of family Ophichthidae based on (16S) including the out group species.

Fig. 2. Molecular phylogenetic tree by minimum evolution method among tiger snake eel *Myrichthys maculosus and* related species of family Ophichthidae based on (16S) including the out group species.

Fig. 3. Molecular phylogenetic tree by neighbour Joining method among tiger snake eel *Myrichthys maculosus and* related species of family Ophichthidae based on (16S) including the out group species

DISCUSSION

In **2009, Rojas *et al*.** indicated that for species identification, the 16S rRNA gene which one of the mitochondrial gene considered a good marker . In the same vein, **Yang *et al*. (2014)** recorded that the genes of rRNA participate the homologous structures in species of organisms, from bacteria to humans due to similarity of several nucleotides, even though the genes exhibit inter and intra-specific nucleotide variations.

The present study employed the large subunit "16S rRNA" sequencing in clarify the phylogenetic relationships of family Ophichthidae, which utilized before by **Miglietta *et al*. (2009); Moura *et al*. (2011) and Saad (2019)** due to its facility to amplify in fish identification.

The concentration of A+T (53.8%) in *Myrichthys maculosus* was higher than the C+G (46.1%), like many pre-investment in fishes **(Perna and Kocher, 1995; Saccone *et al*., 1999 and 2000 and Marʹie and Allam 2019)**. The differences in CG amount among organism could affect reconstructions of evolutionary history because tree building techniques assigned unrelated species with similar GC content to the same group **(Mooers and Holmes, 2000)**. In **2019, Saha *et al*.** illustrated the polymorphic loci analysis of 16S rRNA of the Bombay duck (*Harpadon nehereus*) order Aulopiformes, Family Synodontidae, and found that GC more than AT.

The smallest genetic distance reported here (0.006) was between the current tiger snake eel *M. maculosus* and the same one with accession number (DQ645692), whereas the furthest was *Scolecenchelys breviceps* (0.029).By using 12S & 16S rRNA, and with no support to the lineage based on morphology, **Lo´pez*et al*. (2007)** proposed a close relation between Colocongridae and Derichthyidae (Order Anguilliformes). **Song and Tang (2017)** investigated the complete mitochondrial genome of *Ophichthus rotundus* and found the AT>GC, and the high relation to *M. maculosus* by using NJ tree.

CONCLUSION

The application of 16S rRNA sequence analysis helps to clarify the interrelationship among species of the same family. The author suggested more comprehensive studies of Red Sea eels by using different molecular data.

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