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Genetic Variations of *Epinephelus aeneus* Male and Female fishes from the Egyptian Mediterranean Sea by using Mitochondrial 16S rRNA Sequences

Sara Gamal A. Eisa[1]*, Mohamed K Hassan[1], Ali H. Abu Almaaty[1], Mohammad Allam[2]
[1]Zoology Department, Faculty of Science, Port Said University, Port Said, Egypt
[2]Zoology Department, Faculty of Science, Luxor University, Luxor, Egypt
***Corresponding author: saragamal2421@yahoo.com**

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ABSTRACT

Utilizing and conserving the genetic resources of grouper species depends on an understanding of fish genetic characterization. In this work, the genetic diversity and evolutionary relationships of several Serranidae species, which are significant both ecologically and economically, were evaluated using 16S rRNA gene. This study focused on the phylogeny relationships amongst two Serranidae species: *Epinephelus aeneus* (male), *Epinephelus aeneus* (female), and *Mycteroperca rubra* (male) collected from the Mediterranean Sea, Egypt, exactly from Port Said area. The average frequencies of adenine (A), thymine (T), cytosine (C), and guanine (G) were 29.7, 22.7, 24.1, and 23.7%, respectively. According to our results, the genetic relationships of two Serranidae species in the Mediterranean Sea are mostly related to each other in phylogeny analysis.

INTRODUCTION

The grouper (Serranidae) is an economically significant fish species worldwide **(He *et al.*, 2024)**. Groupers are species of fish that primarily inhabit rocky shallow waters and coral reef habitats. The habitat variety of groupers is highly varied, with several species occurring in shallow water having sandy-mud substrate. Groupers have a wide range of species because of the diversity of their habitat **(Allen & Erdmann, 2013)**. At least one-third of the genus in the subfamily Epinephelinae, particularly the *Epinephelus* and *Mycteroperca* genera, are categorized as endangered species due to the widespread human exploitation of groupers **(Morris *et al.*, 2000)**. Massive anthropogenic actions that negatively impact animal existence are what define the current era of history. Due to over-harvesting, habitat loss, species introduction, and co-extinction, human activities caused the extinction of numerous species **(Banks & Hochuli, 2017)**.

Groupers belong to the Serranidae family, specifically the Epinephelinae subfamily. Groupers are classified into 15 genera, with approximately 159 species **(Koedprang *et***

***al.*, 2007)**. They can be found in all oceans' tropical and subtropical waters **(Tupper & Sheriff, 2008)**. Notwithstanding their ecological and economic significance, groupers are still not well studied in Egypt **(El-Aiatt *et al.*, 2021)**.

Problems with groupers include the inability to precisely describe species when morphological characteristics of the species overlap, making species classification challenging **(Rimmer & Glamuzina, 2019)**. Furthermore, groupers are protogynous hermaphrodites, meaning they start out as females before changing to males. Grouper sex features make it difficult to catch mature males, which is the biggest obstacle to producing grouper artificially as larvae **(Oh *et al.*, 2013; An *et al.*, 2014)**.

Numerous hermaphrodite fish have the ability to change their sexual destiny from one sex to the other, such as serial sex change (bi-directional sex change), protandry (male-to-female sex change), and protogyny (female-to-male sex change). The process of determining a fish's sex is highly variable in evolutionary output across genera and families, and external factors can change it within individuals. Genetic, environmental (e.g., temperature), behavioral, and physiological factors are among the influences that can impact on the destiny of both somatic and germ cells within the primordial gonad. Exogenous sex steroids given during sex determination have the ability to significantly impact the development of sex differentiation in fish, indicating that they are essential for both gonad determination assignment and subsequent differentiation **(Devlin & Nagahama, 2002)**.

Endogenous estrogen levels have a significant impact on fish sex determination **(Guiguen *et al.*, 2010; Mei & Gui, 2015)**. An initial switch of either an ovary-differentiating or testis-differentiating molecular cascade is the exclusive cause of sex determination **(Devlin & Nagahama, 2002; Kim & Capel, 2006)**. In hermaphrodite fish, the choice of sexual phase for secondary sex determination depends on several characteristics, such as age, body size, and social considerations **(Devlin & Nagahama, 2002)**. The present study employed a comparative genetic analysis of 16S r-RNA sequences in several Serranidae fish genera, including *Mycteroperca rubra* (male), *Epinephelus aeneus* (female), and *Epinephelus aeneus* (male), to document molecular differences within and between a number of economically significant Serranidae fishes.

MATERIALS AND METHODS

Ethics statement

All studies were confirmed by the research animal care ethical committee of the Faculty of Science, Port Said University under protocol ERN: PSU.Sci. 86.

Samples collection

The Serranidae fishes were collected from the Egyptian Mediterranean Sea, specifically in Port-Said area, where two species of family Serranidae (*Epinephelus*

aeneus "male and female", *Mycteroperca rubra* "male") were grouped and identified, the muscle tissues of the samples were obtained from the dorsal muscles of the fish and stored at 20°C. The muscle tissue was utilized for DNA extraction using QIAamp DNA Mini kit (QIAGEN, Hilden, Germany).

Fig. 1. *Epinephelus aeneus* "male and female"

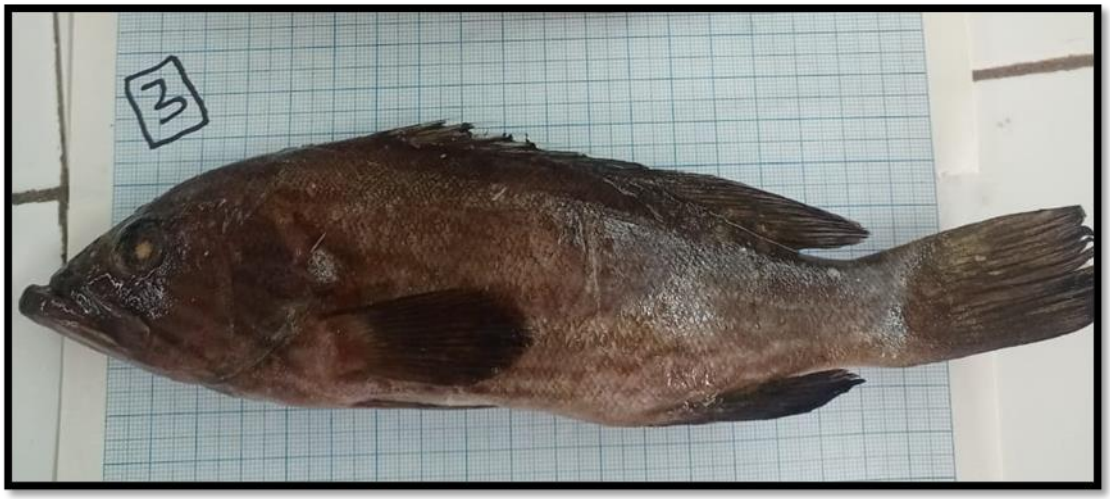

Fig. 2. *Mycteroperca rubra* "male"

Fig. 3. *Epinephelus aeneus* male and female showing sexual polymorphism

Sexual polymorphism is observed in *Epinephelus aeneus*, where the female has a darker, more uniform skin appearance compared to the male, whose skin displays spotted vertical lines. This can lead to mistaken identification as two separate species rather than one.

Utilizing PCR amplification and DNA extraction

We employed primers in accordance with the guidelines to amplify the mitochondrial ribosomal 16S rRNA gene in the Serranidae fish species, the sequence of forward primer was 5' CGCCTGTTTAACAAAAACAT 3' and the sequence of reverse primer was 5' CCGGTCTGAACTCAGATCACGT 3' **(Simon *et al.*, 1991)**. In a final reaction volume of 50µL, the PCR reactions included 1µL of genomic DNA, 25µL of PCR master mix, and 1µL of forward and reverse primers. The PCR program included an initial denaturation for 5 minutes at 94ºC. This was followed by 30 cycles of denaturation for 60 seconds at 94ºC, annealing for 60 seconds at 49°C, and extension at 72°C for 60 seconds, with post-cycling extension at 72ºC for 5 minutes. The PCR products were separated using a 1.5% agarose gel stained with ethidium bromide.

PCR product sequencing and phylogenetic tree construction

Using the same forward primer used for amplification, the samples were sent to Macrogen (Seoul, South Korea) that performed all DNA sequencing. The 16S rRNA mitochondrial rRNA gene sequences were submitted to the National Center for Biotechnology Information (GenBank/NCBI). The sequences were aligned using Clustal W algorithm integrated to MEGA software version 11 **(Tamura *et al.*, 2021)** using default settings to implement the phylogenetic tree analyses using three phylogenetic methods: Minimum evolution (ME), neighbor joining (NJ), and maximum likelihood (ML). Additionally, 1000 bootstrap were used as replicates in each tree constructed **(Felsenstein, 1985)**.

RESULTS

The sequences' lengths of 16S rRNA gene partial fragments in species of family Serranidae (*Epinephelus aneus* "male and female", *Mycteroperca rubra* "male") expanded from 558 to 564bp. The nucleotide sequences were inserted into the GenBank/NCBI with accession numbers (PP377881.1 - PP377883.1). Our results showed that the longest nucleotide sequence (564bp) was found in *Epinephelus aneus* "male and female" species, while *Mycteroperca rubra* showed the shortest sequence (558bp). The average frequencies of nucleotides were 29.7, 22.7, 24.1 and 23.7% for adenine (A), thymine (T), cytosine (C) and guanine (G), respectively. The *16S rRNA* gene displayed A+T ratio bigger than the C+G ratio in all species (Table 1). The average content of C+G ranged from 46.8 to 48.4%, which was lower than the A+T in all species.

Table 1. Accession number, nucleotide frequencies, A+T contents and their averages of *16S rRNA* gene sequences in three species of family Serranidae

N	Species	Accession Number	Base pair length	Nucleotide Number %				A+T Content (%)	C+G Content (%)
				A%	T%	C %	G%		
1	*Epinephelus aneus* (Male)	PP377881.1	564	29.8	23.4	23.8	23	53.2	46.8
2	*Epinephelus aneus* (Female)	PP377882.1	564	29.6	22	23.9	24.5	51.6	48.4
3	*Mycteroperca rubra* (Male)	PP377883.1	558	28.7	22.9	24.6	23.8	51.6	48.4
	Average	-------	562	29.7	22.7	24.1	23.7	52.1	47.9

The sequences of 16S rRNA gene obtained from the three Serranidae species collected from the Egyptian Mediterranean Sea in Egypt, revealed final alignments of 566bp. Among these, there were 519 conserved sites, 44 variable sites and 44 singleton sites (Fig. 4).

Fig. 4. Alignment of (16S rRNA) sequences in three Serranidae species. Dots show similar nucleotides while A, C, G, and T display the variance nucleotides

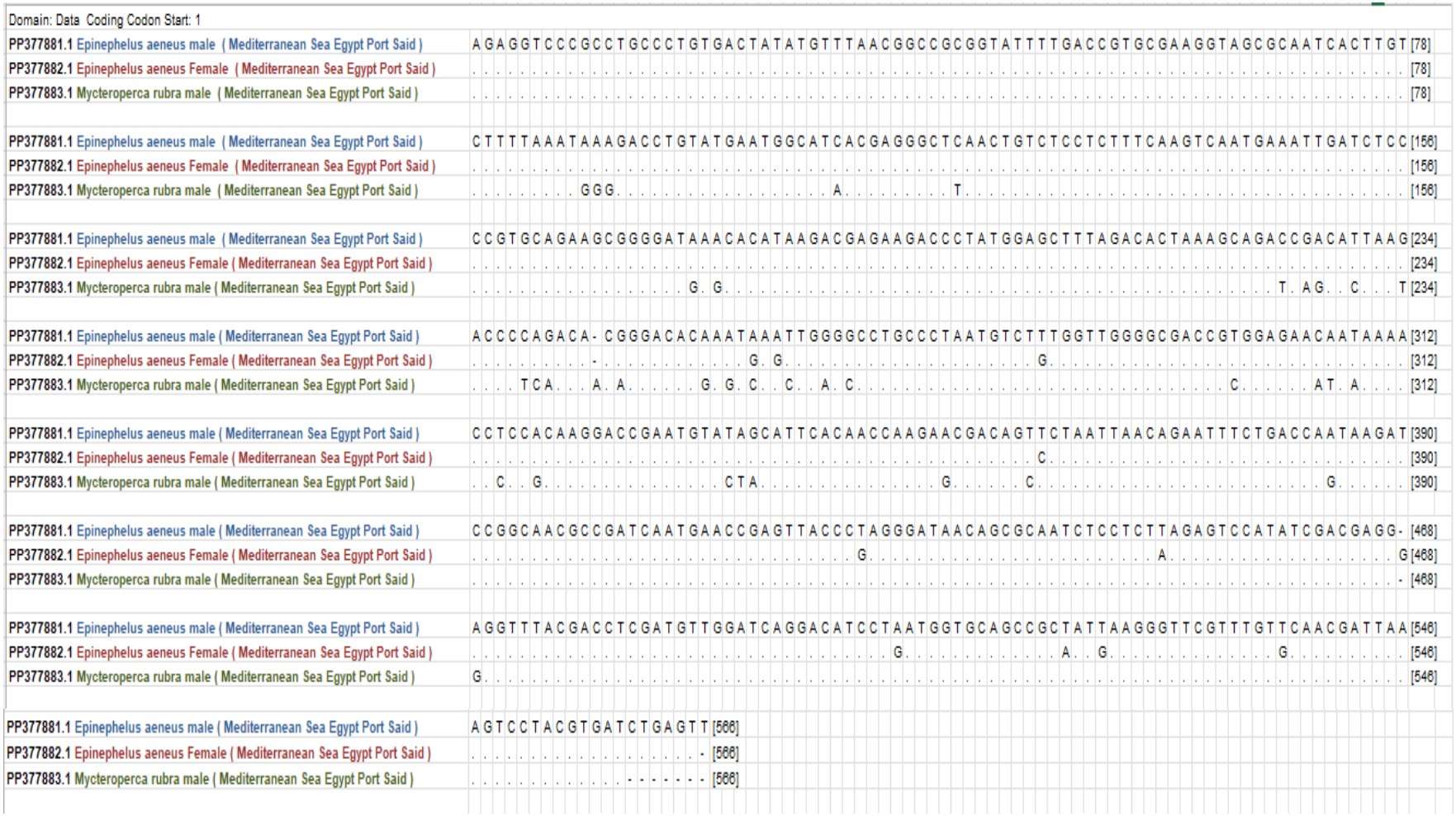

Table 2. Pairwise distances by the mean of 16S rRNA gene amongst three species of Serranidae family with their linkage species from the GenBank/NCBI

1		2	3	4	5	6	7	8	9	10	11	12	13	14
2	PP377881.1_Epinephelus_aeneus_male_16SrRNA_gene_mitochondrial_(_Mediterranean_Sea_Egypt_Port_Said)		0.0122763013	0.0793487005	0.0000000000	0.0042373072	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0020202020	0.0793487005	0.0793487005	5.4411062225
3	PP377882.1_Epinephelus_aeneus_Female_16SrRNA_gene_mitochondrial_(_Mediterranean_Sea_Egypt_Port_Said)	0.0122763013		0.0919390687	0.0122763013	0.0172297924	0.0122763013	0.0123776028	0.0122763013	0.0122763013	0.0143718622	0.0919390687	0.0919390687	5.5947725459
4	PP377883.1_Mycteroperca_rubra_male_16SrRNA_gene_mitochondrial_(_Mediterranean_Sea_Egypt_Port_Said)	0.0793487005	0.0919390687		0.0793487005	0.0885975910	0.0793487005	0.0800755361	0.0793487005	0.0793487005	0.0819752172	0.0020161290	0.0020161290	6.7575487013
5	AY947593.1_Epinephelus_aeneus_16SrRNA_gene_mitochondrial_(USA)	0.0000000000	0.0122763013	0.0793487005		0.0042373072	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0020202020	0.0793487005	0.0793487005	5.4411062225
6	AY731178.1_Epinephelus_aeneus_16SrRNA_gene_mitochondrial_(Spain)	0.0042373072	0.0172297924	0.0885975910	0.0042373072		0.0042373072	0.0042373072	0.0042373072	0.0042373072	0.0063559322	0.0885975910	0.0885975910	5.6388888889
7	LC545417.1_Epinephelus_aeneus_16SrRNA_gene_(Korea)	0.0000000000	0.0122763013	0.0793487005	0.0000000000	0.0042373072		0.0000000000	0.0000000000	0.0000000000	0.0020202020	0.0793487005	0.0793487005	5.4411062225
8	GQ485302.1_Epinephelus_aeneus_16SrRNA_gene_mitochondrial_(_Turkey_Istanbul_)	0.0000000000	0.0123776028	0.0800755361	0.0000000000	0.0042373072	0.0000000000		0.0000000000	0.0000000000	0.0020366599	0.0800755361	0.0800755361	5.1447447447
9	OR091278.1_Epinephelus_aeneus_16SrRNA_gene_mitochondrial_(Greece)	0.0000000000	0.0122763013	0.0793487005	0.0000000000	0.0042373072	0.0000000000	0.0000000000		0.0000000000	0.0020202020	0.0793487005	0.0793487005	5.4411062225
10	KM077971.1_Epinephelus_aeneus_16SrRNA_gene_mitochondrial_(France)	0.0000000000	0.0122763013	0.0793487005	0.0000000000	0.0042373072	0.0000000000	0.0000000000	0.0000000000		0.0020202020	0.0793487005	0.0793487005	5.4411062225
11	AY731177.1_Epinephelus_marginatus_16SrRNA_gene_mitochondrial_(Spain)	0.0020202020	0.0143718622	0.0819752172	0.0020202020	0.0063559322	0.0020202020	0.0020366599	0.0020202020	0.0020202020		0.0819752172	0.0819752172	5.4411062225
12	AY947587.1_Mycteroperca_rubra_16SrRNA_gene_mitochondrial_(USA)	0.0793487005	0.0919390687	0.0020161290	0.0793487005	0.0885975910	0.0793487005	0.0800755361	0.0793487005	0.0793487005	0.0819752172		0.0000000000	6.7575487013
13	KM077991.1_Mycteroperca_sp._16SrRNA_gene_mitochondrial_(France)	0.0793487005	0.0919390687	0.0020161290	0.0793487005	0.0885975910	0.0793487005	0.0800755361	0.0793487005	0.0793487005	0.0819752172	0.0000000000		6.7575487013
14	NC_021405.1_Plectropomus_areolatus_complete_genome_(out_group)	5.4411062225	5.5947725459	6.7575487013	5.4411062225	5.6388888889	5.4411062225	5.1447447447	5.4411062225	5.4411062225	5.4411062225	6.7575487013	6.7575487013	

Pairwise genetic distances among the two Serranidae species and the out group revealed the highest value (6.757548) was observed between *Mycteroperca_rubra*_male' Egypt' (PP377883.1) and *Plectropomus areolatus* 'out group' (NC_021405.1). While 0.000 value was observed between *Epinephelus_aeneus*_male 'Egypt' (PP377881.1) and *Epinephelus_aeneus* 'USA' (AY947593.1), *Epinephelus_aeneus* 'Korea' (LC545417.1), *Epinephelus_aeneus* ' Turkey_Istanbul' (GQ485302.1), *Epinephelus_aeneus* 'Greece' (OR091278.1) and between *Epinephelus_aeneus* ' France' (KM077971.1). The *P*-distances among the understudied Egyptian Serranidae fishes expanded from 0.0122 to 0.09193%. The highest value (0.09193) was found for *Mycteroperca_rubra*_male' Egypt' (PP377883.1) and *Epinephelus_aeneus*_female 'Egypt' (PP377882.1), while the lowest *P*-distance (0.0122) was found between *Epinephelus_aeneus*_female 'Egypt' (PP377882.1), and *Epinephelus_aeneus*_male 'Egypt' (PP377881.1) (Table 2). To complete the phylogenetic tree investigation by the dint of *16S rRNA* sequence, the sequences acquired from two Serranid species were exercised in this work for a widely combination phylogenetic investigation. For widely illustrative phylogenetic investigation by using *16S rRNA* gene, neighbor joining, minimum evolution and maximum likelihood phylogenetic methods were used. With some variation in the support rate (Figs. 5,6 and 7).

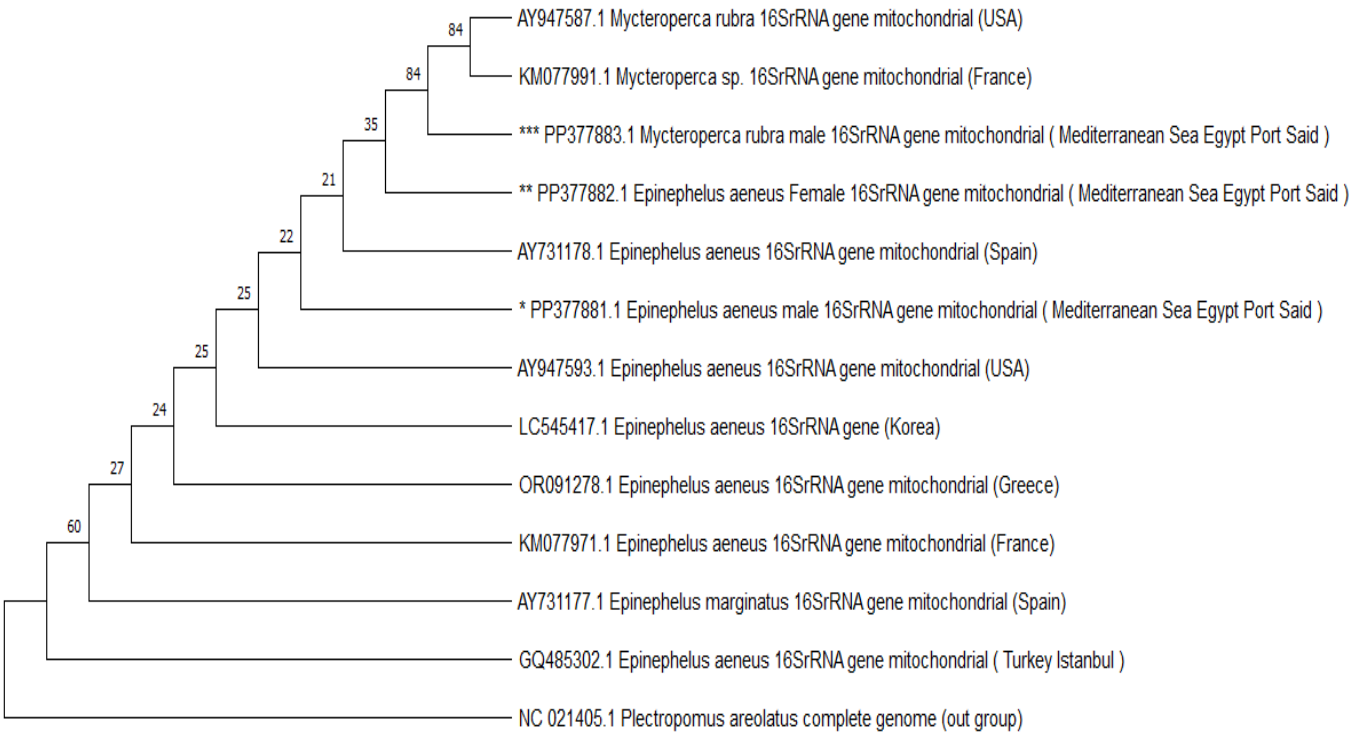

Fig. 5. Neighbor joining phylogenetic tree in the three Serranidae species and their linked Serranidae species with the outgroup by employing the 16s rRNA gene

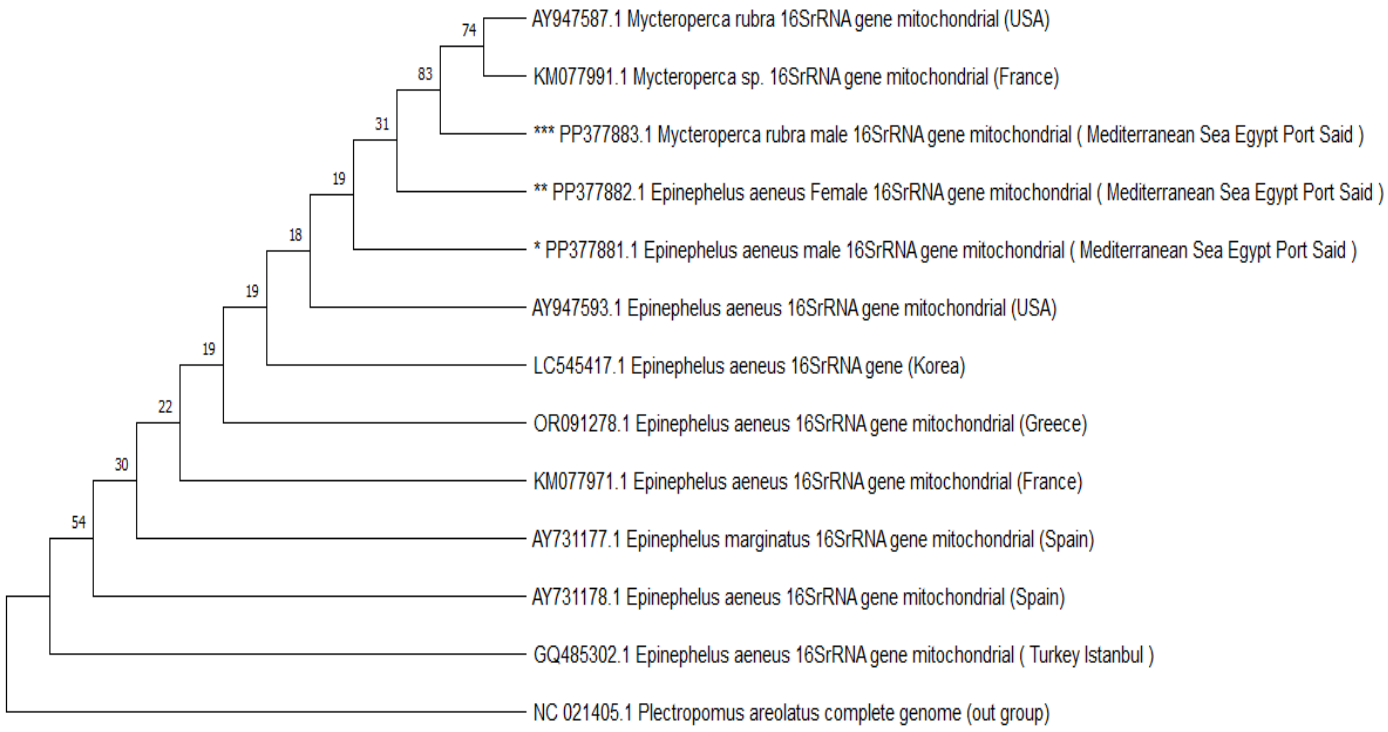

Fig. 6. Minimum evolution phylogenetic tree in the three Serranidae species and their linked Serranidae species with the outgroup by employing the 16s rRNA gene

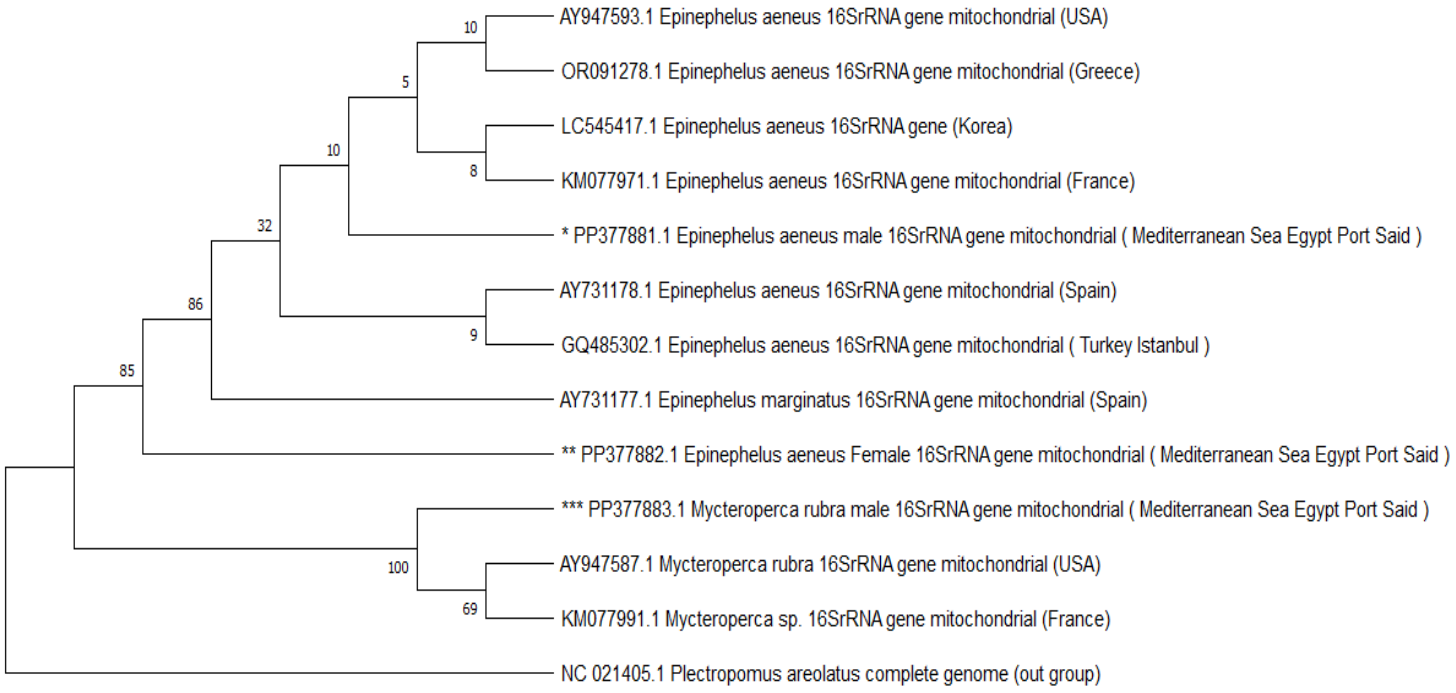

Fig. 7. Maximum likelihood phylogenetic tree in the three Serranidae species and their linked Serranidae species with the outgroup by employing the 12s rRNA gene

DISCUSSION

There are over 15,000 aquatic species in marine life **(Zemlak *et al.*, 2009)**, including grouper fish **(Noikotr *et al.*, 2013)**. Fish genera belonging to the Serranidae family, which includes Plectropomus, Cephalopholis, and Epinephelus live in tropical and subtropical seas worldwide **(Craig & Hastings, 2007)**. Approximately, there are 300 species in this family, which is significant to the marine environment **(Smith, 1971)**. It is essential to understand the characterization and evolution in order to protect the Serranidae family of fishes, as the true evolutionary relationships and phylogenetic relationships within this family are yet unknown.

Sometimes inaccurate identification results from morphological characterization **(Hubert *et al.*, 2008)**, for instance in sister taxa of the Serranidae family. Molecular marker-based fish characterization has been effectively used to investigate fish biodiversity and evolution and to improve fisheries conservation **(Craig & Hastings, 2007; Zhu & Yue, 2008; Zhang & Hanner, 2012)**. Natural and sexual selection are examples of evolutionary mechanisms that can contribute to the emergence of new species and accelerate speciation **(Civetta & Singh, 1999; Schluter, 2001)**. Examining evolutionary differences **(Saad *et al.*, 2012)**. Fish DNA sequence polymorphism, especially that of the Serranidae family, would aid in the definition of appropriate fish conservation units. Identification of taxa and molecular variants is necessary to preserve Serranidae fish resources **(Rashed *et al.*, 2008; Saad *et al.*, 2011)**.

Mitochondrial DNA markers **(Ward *et al.*, 2008; Zhang & Hanner, 2012)** provide precise species identification systems, therefore the aquaculture and fisheries industries should use them for the protection of marine genetic resources **(Saad *et al.*, 2011)**. The sensitivity of the 16S mitochondrial ribosomal DNA system makes it the best method for examining the evolutionary differences in aquatic creatures **(Craig *et al.*, 2001; Pondella *et al.*, 2003)**.

The average content of C+G sequence length of 16S rRNA gene in two Serranid species (*Epinephelus aneus* "male and female", *Mycteroperca rubra* "male") ranged from 46.8 to 48.4%, which was lower than the A+T in all species. This finding is consistent with several previous studies. **Bo *et al.* (2013)** reported that the entire 16S rRNA gene exhibits A+T affluence, compared to C+G. **Basheer *et al.* (2015)** observed a small C+G value of 16S rRNA, compared to A+T through the study on Rastrelliger species. Moreover, **Mar'ie and Allam (2019)** found in two puffer fishes a bigger A+T ratio compared to C+G. Our results of the 16S rRNA gene displayed C+G content ranging from 48.52 to 50.08. The GC diversity among the four species of family Lutjanidae may be considered as a notation of adaptation **(Ali *et al.*, 2021)**.

The final alignments of incomplete 16S rRNA sequences in the two species of family Serranidae illustrated highly conserved sites. Our result agrees with **Basheer *et al*. (2015)**, who found 575 consistent locations of 590bp in three Rastrelliger species by using 16S rRNA aligned sequences. **Ramadan *et al*. (2023)** employed the 16S gene in Snappers phylogenetic analysis and found 535 conserved regions of 575.

The low genetic distance between *Epinephelus aeneus* female 'Egypt' (PP377882.1), and *Epinephelus marginatus* "Spain" (AY731177.1) with maximum likelihood phylogenetic method is attributed to the close linkage between them. While *Epinephelus aeneus* male 'Egypt' (PP377881.1) show low genetic distance with *Epinephelus aeneus* 'USA' (AY947593.1), with neighbor joining and minimum evolution phylogenetic methods used, indicating strongly related species between them. Besides, *Mycteroperca_rubra*_male' Egypt' (PP377883.1) show low genetic distance with *Mycteroperca_rubra* 'USA' (AY947587.1), *Mycteroperca* sp. 'France' (KM077991.1) and *Epinephelus aeneus* female 'Egypt' (PP377882.1) with all phylogenetic methos. However, *Epinephelus aeneus* male 'Egypt' (PP377881.1), *Epinephelus aeneus* female 'Egypt' (PP377882.1) and *Mycteroperca_rubra*_male' Egypt' (PP377883.1) show highly genetic distance with *Plectropomus areolatus* 'out group' (NC_021405.1). This result concurs with the data reported in the study of **Kaleshkumar *et al*. (2015)**, who stated that low genetic distance values are found in closely related species.

Heino (2014) stated that a living organism's morphological qualities can vary according to the ecological conditions it lives in. Fish exhibit a great deal of variation in their physical attributes, both within and between groups **(Brraich & Akhter, 2015)**. Morphological changes of fishes represent a type of environmental adaptation **(Hossain *et al*., 2010)**. Physical features can be affected by ecological and genetic factors **(Sala *et al*., 2022)**. These may reflect the non-monophyly of some genera in family Serranidae.

CONCLUSION

Biodiversity required to preserve these biological resources could be found by reconstructing the evolutionary relationships among certain Serranidae fish. Using a comparative genetic analysis of some 16S r-RNA sequences in several Serranidae fish genera (*Epinephelus aeneus* (male), *Epinephelus aeneus* (female), and *Mycteroperca rubra* (male), this study aimed to give details on the molecular differences between and within a variety of economically significant fish species. According to the findings, there is no monophyletic grouping of fishes in the Mediterranean Sea Serranidae.

Epinephelus marginatus from "Spain" that has accession number (AY731177.1) is closely related to *Epinephelus aeneus* female from 'Egypt' that has accession number

(PP377882.1) and is always located between species of *Epinephelus aeneus* in all phylogenetic tree, hence more studies are required to clear its position.

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