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Evaluation of Genetic Relationship of some Squirrelfishes through DNA Barcode

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HIGHLIGHTS

- Confusion in the taxonomy of squirrelfishes was caused by the numerous nominal species.
- Our phylogeny provides a basis for evolutionary analyses of the Holocentridae.
- Silverspot squirrelfish was clustered as closest taxa to sabre squirrelfish.

Abstract: Species of the subfamily Holocentrinae, family Holocentidae, commonly called, squirrelfishes, are widely distributed from tropical to warm temperate waters. In Egypt, no data are available on genetic and evolutionary relationships of the family Holocentridae. Therefore, the study of the genetic relationship among Holocentrids species is crucial for proper management and convenient strategies. The purpose of this study was to evaluate the genetic relationship among eight species belonging to the family Holocentridae from the Mediterranean Sea and the Red Sea in Egypt using DNA barcoding. Based on this molecular marker, a phylogenetic tree was constructed for the studied Holocentrids species. 12S rRNA sequences discovered that *Sargocentron caudimaculatum* was clustered as closest taxa to *Sargocentron spiniferum*, being a sister group to each other. Also, *Sargocentron punctatissimum* and *Sargocentron macrosquamis* were more related to each other and formed a sister group. Moreover, this study discusses the building of genetic relationship among *Sargocentron spinosissimum* and *Sargocentron macrosquamis* for the first time to the other studied *Sargocentrons*. DNA barcoding using 12S rRNA gene provided efficient DNA barcodes for all of the studied species. The constructed phylogenetic tree based on the employed molecular marker provided the update for the barcoded Holocentridae species evolution.

Keywords: crowned; Holocentrinae; homocolour; nocturnal; *Sargocentron*

INTRODUCTION

The squirrelfishes (Holocentridae; Holocentrinae) are abundant members of tropical water assemblages [1], residing in caves and reef cracks during the day and foraging on the reef flat or in the water column at night [2, 3]). Genus *Sargocentron* has a great diversity among its species, which belongs to the Holocentridae family known as squirrelfish. *Sargocentron* is a pantropical genus includes 33 species [4], six species of Holocentrins are widespread in Egyptian cost [5]. Recently, *Sargocentron spinosissimum* and *Sargocentron tiereoides* are recorded for the first time in Egypt [6]. The novel findings of *Sargocentron spinosissimum* species that is distributed Northwest Pacific: southern Japan to Taiwan; also reported from Hawaii and

Sargocentron tiereoides is distributed in Indo-Pacific regions and East Africa showed the success of the migration of these species to the Mediterranean water with a good adaptation to the new habitats [6].

Sargocentrons have compressed and elongated body with thin caudal peduncle; ridges and mucous channels dorsally on the head and have very large eyes. It has a single spine in pelvic fin with soft rays 5-8 (mode 7). Although dorsal fin long with 10-13 spines but, a notched soft-rayed part is found with 11-17 rays. Anal fin has four spines and 7-16 soft rays. Forked caudal fin has 18 or 19 rays. Scales are very rough ctenoid and large. Most *Sargocentrons* are brilliant reddish in color. Usually are nocturnal. Usually cryptic during the day in beneath ledges of reefs or crevices. *Sargocentrons* feed on worms and small fishes [7].

Due to features such as increased level of nucleotide sequence difference, rapid rate of evolution, compact genome and insufficiency of recombination, its maternal inheritance and higher mutation rates compared to those of nuclear genes, the mitochondrial DNA (mtDNA) has demonstrated to be valuable in molecular phylogenetic studies [8]. The mitochondrial 16S rRNA gene was used to explore the phylogenetic relationships of fishes at various taxonomic levels [9**–**11], mainly due to the fact that it is highly preserved and has a slow evolution [12]. Moreover, the 12S rRNA gene is considered a promising tool for tracing the history of more recent evolutionary events [13], and it has been widely used to study the phylogenetic relationships among different levels of taxa such as families [14**–**16], genera [17**–**20], and species [21, 22].

The confusion in the taxonomy of this subfamily has been caused by the numerous nominal species, their incomplete descriptions, loss of holotypes, and arbitrary synonymies [3]. The colour pattern and external morphology of *Sargocentrons* can be very similar among the different species, and this makes the recognition of members of this genus mostly difficult, there is a need to apply modern molecular techniques for more checking and confirmation in recognition and resolving the phylogenetic relationship among the studied squirrelfishes species.

MATERIAL AND METHODS

Collection of Samples and laboratory procedures

Thirty specimens of holocentrins were collected from different localities of the Egyptian coast. These specimens are: four samples from sabre squirrelfish (*Sargocentron spiniferum*), six specimens of the silverspot squirrelfish (*Sargocentron caudimaculatum*), two specimens of the Speckled squirrelfish (*Sargocentron punctatissimum*), two samples of the bigscale squirrelfish (*Sargocentron macrosquamis*) and three specimens of the crowned squirrelfish (*Sargocentron diadema*) were collected from Marsa Abu Dabab coast in the Red Sea. Six samples of the redcoat squirrelfish (*Sargocentron rubrum*) were captured from the Alexandria coasts. Additionally, four samples of the North Pacific squirrelfish (*Sargocentron spinosissimum*) and three samples of the pink squirrelfish (*Sargocentron tiereoides*) were collected from the Damietta coast of the north of Egypt, in the south-eastern part of the Mediterranean Sea. All locations of the study area (Figure 1) were visited throughout three years from October 2016 to September 2019.

The first member of the studied Holocentrins, sabre squirrelfish *S. spiniferum* (Forsskål, 1775), is widely distributed in Indo-Pacific [23] where it lives in a variety of coral reefs to a depth of at least 122 m [24]. This nocturnal species is the largest squirrelfish which feeds on crabs, shrimps and small fishes [25]. The second one, *S. caudimaculatum* (Rüppell, 1838), the silverspot squirrelfish, is native to the Indian and Pacific Oceans from East Africa to Japan and northern Australia and as far east as the Marshall Islands. It habitats near reefs, but can also be found at depths between 2 and 40 m [24].

The redcoat squirrelfish, *S. rubrum* (Forsskal, 1775), the third species, is among members of the family; Holocentrinae, is the first migrant Holocentrid to the Mediterranean Sea via Suez Canal and became the common immigrant *Sargocentron* species. It is the wide distribution from the Red Sea to the Indo- Pacific waters, [26], while the fourth member, *S. diadema* (Lacepède, 1802), known commonly as the crowned squirrelfish, is found in or near cracks and caves of coral reefs from 3-77 m, often in lagoons or bays at < 20 m [27]. It is the most common species of *Sargocentron* wherever it is found.

S. macrosquamis (Golani, 1984), bigscale squirrelfish, the fifth studied Holocentrid species, lives in the western Indian Ocean; the Red Sea to Mozambique [7]. Moreover, the sixth member, *S. punctatissimum* (Cuvier, 1829), known commonly as the Speckled squirrelfish, lives in the Indo-Pacific; the Red Sea and Algoa Bay, South Africa [7]. *S. spinosissimum* (Temminck and Schlegel, 1843), North Pacific squirrelfish is the seventh member of the studied species which lives in Northwest Pacific, southern Japan to Taiwan; also reported from Hawaii [7], but recently, finds in the Mediterranean Sea [6], and the last one, *S. tiereoides* (Bleeker, 1853), pink squirrelfish, is habitats in Indo-Pacific [7], and lives in the Mediterranean Sea [6].

DNA extraction PCR amplification

Liver tissue was obtained from each *Sargocentron* specimen, then preserved in 95% alcohol and stored in deep freezer at –4°C, where the *Sargocentron* species' DNA was extracted using a GeneJET™ kit Genomic DNA Kit#K0721 following manufacturer's protocol.

12S rRNA gene was amplified using primers L1091-5' AAA AAG CTT CAA ACT GGG ATT AGA TAC CCC ACT AT-3' and H1478-5' TGA CTG CAG AGG GTG ACG GGC GGT GTG T-3' [28]. The polymerase chain reactions (PCR) consisting of approximately 50 ng of template DNA were carried out in volumes of 15 µl with 1× PCR Buffer, 2 mM MgCl, 0.5 µM of each FF and FR, 0.2 mM of dNTP, and 0.6 U of Taq DNA Polymerase. The thermal cycler started with an initial denaturation at 94°C for 4 min, 53°C for 2 min, and72°C for 1 min (one cycle); 94°C for 1 min, 53°C for 2min, and 72°C for 1 min (30 cycles); and 94°C for 1 min,53°C for 2 min, and 72°C for 5 min (one cycle).

PCR products were checked by running in 1.5% agarose gels and stained with ethidium bromide. Successful PCR bands were cut out and purified using the QIAquick PCR purification kit from Quiagen®. The clean PCR products were sequenced using an automated sequencer following the manufacturer's protocols.

Alignment and sequence properties

All rRNA gene nucleotide sequences were aligned by using the Clustal W software and identical sequences were considered as the same haplotype. Using MEGA X software [29], Kimura 2-parameter distance matrix of all studied species was calculated to construct a Maximum Likelihood phylogenetic tree.

Basic Local Alignment Search Tool (BLAST)

To investigate and recognize created sequences, each was blast searched as a request through NCBI (National Center for Biotechnology Information) Blastn tool (www.ncbi.nlm.nih.gov/BLAST/). Sequences with better hits were recovered and used for further comparison to 12S rRNA gene sequences from the current study. On the other hand, there is a lack of sequences for *S. tiereoides*, *S. spinosissimum* and *S. macrosquamis*.

RESULTS

The resulted 12S rRNA gene sequences of Holocentrids were submitted to the GenBank (NCBI) and the accession numbers were represented in table 1. Eleven sequences collected from NCBI belonging to various studied taxa were used for constriction of phylogram and genetic distance detection.

The average nucleotide frequencies are 36.1% (A), 18.8% (T/U), 23.4% (C) and 21.7% (G). The percent composition of nucleotide varied from 34. 7 to 37.2% (A), 17.4 to 19.7% (T), 21.8 to 24.3% (C), and 21.2 to 22.1% (G), which indicate that 12S rRNA gene sequences of these species are A rich and poor in T, C and G (Table 2).

The transition/transversion rate ratios are k1 = 16.475 (purines) and k2 = 34.049 (pyrimidines). The content of pyrimidine was higher than that of purine. These values showed a strong A + T (54.8%) to G + C (45.1%) asymmetry in nucleotide composition. The maximum AT content was found in *S. diadema* (57.1%) and the minimum in *S. spiniferum* and *S. spinosissimum* (54. 0%). The maximum and minimum GC contents were observed in *S. spiniferum* (46.0%) and *S. diadema* (43.0%) respectively (Table 2).

The genetic distance was calculated between the species included in the same genus of Holocentride. Distances calculated between species pairs showed that the smallest differences (0.220) existed between *S. punctatissimum* and *S. macrosquamis* whereas the highest genetic distance detected between *S. caudimaculatum* and *S. punctatissimum* amounted to 0.271 (Table 3).

The topological structures of the tree illustrated that there are two mainly separate genetic branches, one branch for *S. spiniferum*, *S. caudimaculatum*, *S. rubrum*, *S. spinosissimum*, *S. diadema* and *S. tiereoides* and the other branch for *S. punctatissimum* and *S. macrosquamis*. The first branch divided into two separated groups, *S. spiniferum*, *S. caudimaculatum*, *S. spinosissimum* and *S. tiereoides* were included in group one while group two contained *S. rubrum*. In addition, *S. caudimaculatum* was more related to *S. spiniferum* and formed a sister group. Instead, *S. diadema* was lying into a separate group. The second branch consists of *S. punctatissimum* and *S. macrosquamis* which were more related to each other and formed a sister group (Figure 2).

In case of the mitochondrial tree, a total of eleven unique haplotypes were identified in sequences from 12S rRNA gene of thirty specimens of Holocentridae. All haplotypes of *S. tiereoides* formed the monophyletic

cluster with the clade included *S. caudimaculatum* and *S. spiniferum*. Moreover, this tree was splitting into two main clades, in the first clade, *S. caudimaculatum* was clustered as closest taxa to *S. spiniferum*, being a sister group to each other. Also, *S. spinosissimum* and *S. tiereoides* were placed in two different branches. Moreover, *S. diadema* was monophyletic clustered to *S. spinosissimum* and *S. rubrum* in this mitochondrial tree. In the second clade, the haplotypes of *S. punctatissimum* and *S. macrosquamis* were deposited together in the phylogram and appeared as a sister group (Figure 3).

Table 1. List of studied *Sargocentrons* members sequenced at mitochondrial DNA loci (12S rRNA gene) with a lack of sequences for *S. tiereoides*, *S. spinosissimum* and *S. macrosquamis*.

Species	No. sequences	Accession number
Sargocentron caudimaculatum	1	LC021252
Sargocentron spiniferum	1	LC492341
Sargocentron tiereoides	0	———
Sargocentron spinosissimum	0	———
Sargocentron rubrum	6	AB974507, LC458088, LC474169, LC474170, LC474171, LC578992
Sargocentron diadema	1	LC021238
Sargocentron macrosquamis	0	———
Sargocentron punctatissimum	2	LC069511, LC492342

Table 2. Percentage composition of nucleotides A, T, G, C, AT and GC in studied Holocentinae species.

Species	A%	T%	G%	C%	AT%	CG%
S. caudimaculatum	36.6	18.0	22.1	23.3	54.6	45.4
S. spiniferum	36.0	18.0	22.7	23.3	54.0	46.0
S. tiereoides	37.2	18.0	21.5	23.3	55.2	44.8
S. spinosissimum	36.6	17.4	22.1	23.8	54.0	45.9
S. rubrum	37.8	17.4	21.5	23.3	55.2	44.8
S. diadema	35.3	21.8	21.2	21.8	57.1	43.0
S. macrosquamis	34.7	19.7	21.4	24.3	54.4	45.7
S. punctatissimum	34.7	19.7	21.4	24.3	54.4	45.7
Mean	36.1	18.8	21. 7	23.4	54.8	45.1

Table 3. Total genetic distance between the studied species.

	S. caudimaculatum	*S. spiniferum*	*S. tiereoides*	*S. spinosissimum*	*S. rubrum*	*S. diadema*	*S. macrosquamis*	*S. punctatissimum*
S. caudimaculatum	0							
S. spiniferum	0.222	0						
S. tiereoides	0.227	0.233	0					
S. spinosissimum	0.235	0.235	0.233	0				
S. rubrum	0.247	0.242	0.238	0.236	0			
S. diadema	0.259	0.255	0.251	0.250	0.250	0		
S. macrosquamis	0.265	0.261	0.257	0.255	0.253	0.230	0	
S. punctatissimum	0.271	0.268	0.262	0.258	0.255	0.239	0.220	0

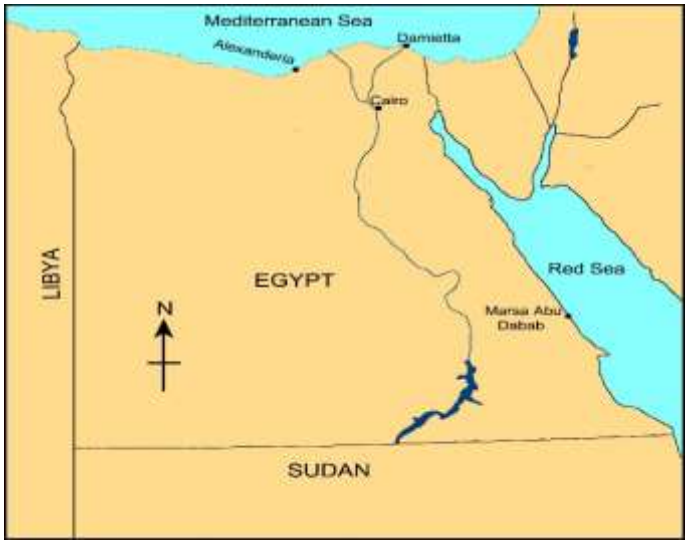

Figure 1. Map of Egypt showing localities of *S. caudimaculatum*, *S. punctatissimum*, *S. macrosquamis* and *S. diadema* were collected from Marsa Abu Dabab coast in the Red Sea. *S. rubrum* was captured from the Alexandria coasts. *S. spinosissimum* and *S. tiereoides* were collected from the Damietta coast in the Mediterranean Sea.

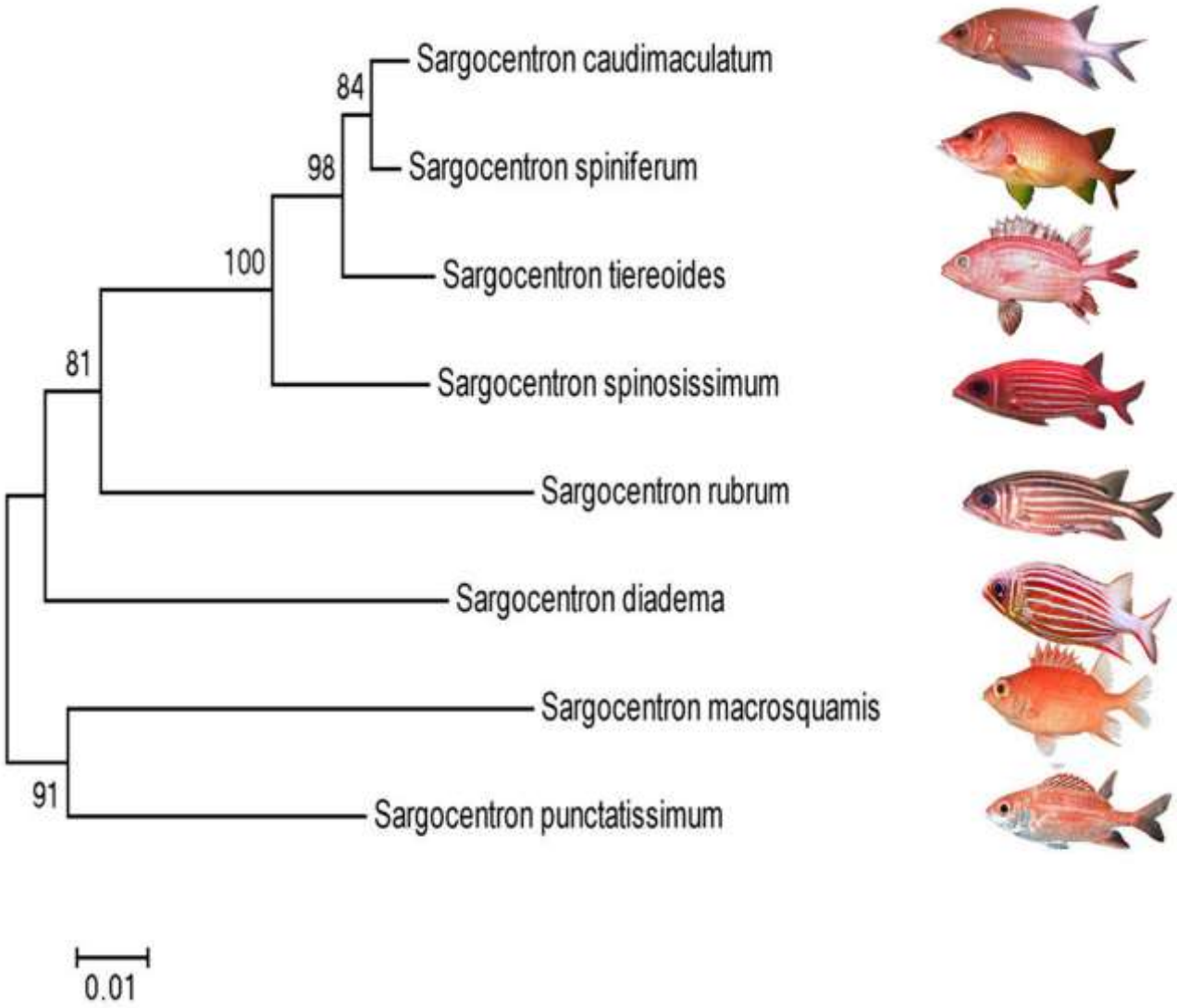

Figure 2. Maximum Likelihood phylogenetic tree based on 12S rRNA gene sequences of eight barcode sequences from the studied *Sargocentron* species, using Kimura 2-Parameter distances and values at nodes represent bootstrap confidence level (1000 replicates).

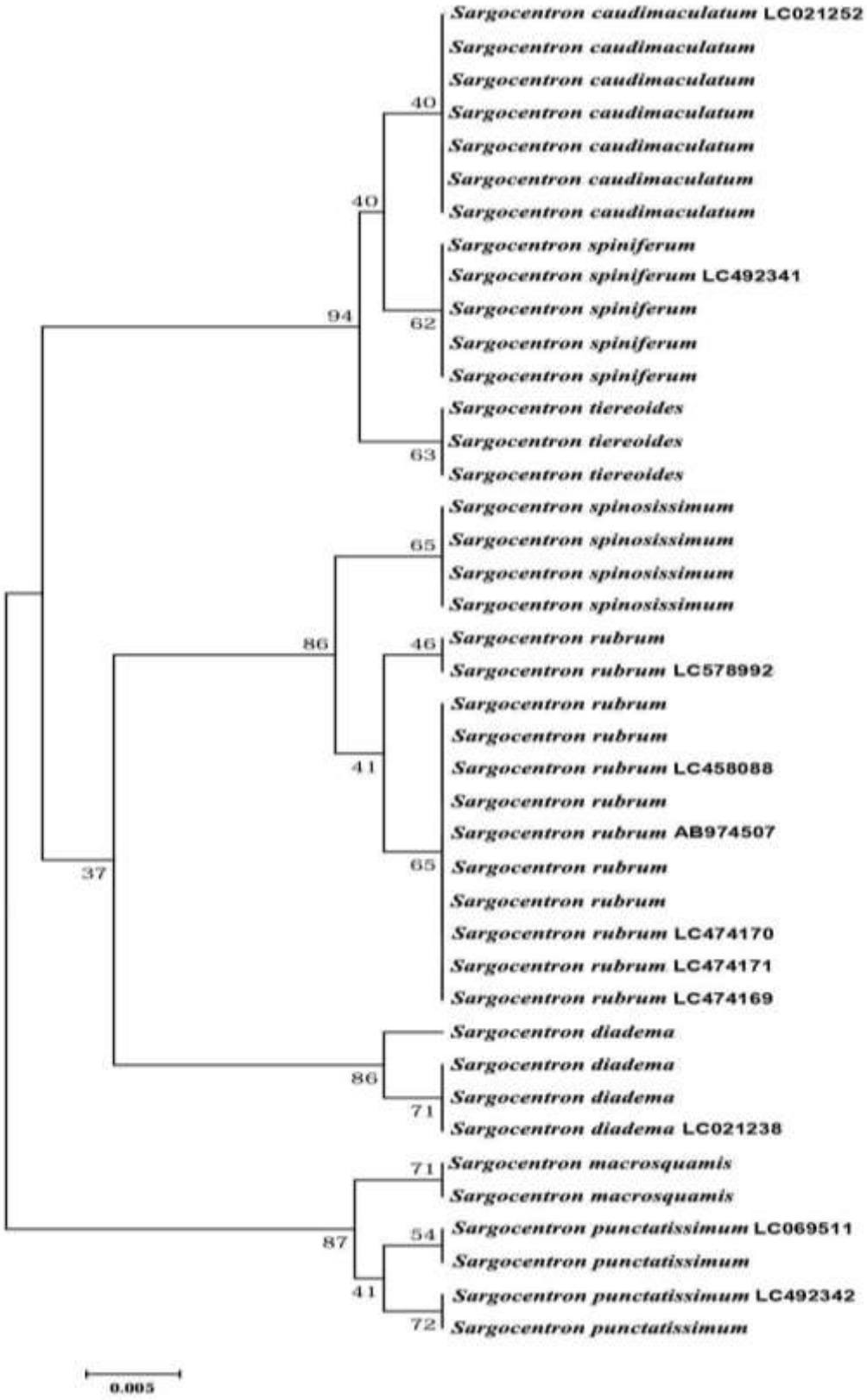

Figure 3. Kimura 2-parameter distance Maximum Likelihood tree analyses based on 12S rRNA gene from 41 barcode sequences from 8 species belonging to the family Holocentridae. The values at nodes represent bootstrap confidence level (1000 replicates). Specimen's number denotes the accession number of GenBank.

DISCUSSION

Using DNA barcoding and molecular markers is nowadays essential since it represents a useful method for studying taxonomy and phylogenetic relationships among species. This technology has been taken as good tools for identifying species as well as in archaeological remains and museum samples because of the degradation nature and fragmentation of ancient DNA [30].

Previous studies were limited and insufficient to assess genetic relationships among Holocentridae species. Chen and coauthors [31] reported that *S. spiniferumis* sister to *S. rubrum* that belongs to the same genus. On the contrary, this study results that *S. caudimaculatum* was clustered as closest taxa to *S. spiniferum* in agreement with Dornburg and coauthors [32] and *S. rubrum* was laying into another clade.

The species of *Sargocentron* (*S. spiniferum*) is located within the first major clade of the Holocentrinae that includes several of the species identified by Woods [33] as the deep-bodied, more uniformly-colored, larger-sized species that he placed in *Sargocentron* (e.g., *S. spiniferum, S. tieroides*, and *S. praslin*).

The phylogenetic trees generated in this work, regardless of inference method, resolve the Holocentrinae into two main clades that are *S. spiniferum, S. caudimaculatum, S. rubrum, S. spinosissimum* and *S. tiereoides* which forms the first clade and the second clade for *S. punctatissimum, S. macrosquamis* and *S. diadema*. This genetic divergence may be related to spatial and temporal variations in recruitment [34], habitat effects [35] and other factors. Habitat availability [36] and habitat preferences [37, 38] also play a role.

The presence of *S. spinosissimum* and *S. tiereoides* in the Mediterranean Sea may be attributed to various studies that have to infer that physical variables, in particular substrate, depth, and currents, have a great influence on the distribution of fish and other aquatic organisms [39].

12S rRNA gene sequences discovered that *S. caudimaculatum* was clustered as closest taxa to *S. spiniferum*, being a sister group to each other. They are greatly look alike to each other and characterized by the absence of white stripes. Also, *S. punctatissimum* and *S. macrosquamis* which were more related to each other, formed a sister group, resemble each other and have homocolour without white stripes. Additionally, the study discusses the building of genetic relationships among *S. spinosissimum* and *S. macrosquamis* for the first time to the other studied *Sargocentrons*.

CONCLUSION

With the Holocentrids phylogenetic relationships resulted in this study, this aim to prompt a reexamination of genetic markers that have been used to diagnose and delimit taxonomic groups within the clade traditionally. This phylogeny provides a basis for subsequent comparative evolutionary analyses of the Holocentridae. The employed molecular marker in this study was efficient in species genetic diversification. 12S rRNA was successfully utilized in species barcoding.

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