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Mitochondrial *16S rRNA* gene as a molecular marker in the phylogenetic relationships of some Rabbitfishes species (Siganidae: Perciformes)

Najiah M. Alyamani*

Department of Biology, College of Science, University of Jeddah, Jeddah, Saudi Arabia

ABSTRACT

Background: Siganidae is a marine teleost family consisting of a single extant genus, *Siganus* Forsskål, 1775, which included 29 recognized species of rabbitfish.

Aim: The main goal of this study was the use of the mitochondrial *16S rRNA* gene as a potential molecular marker in the phylogenetic relationships study of some rabbitfishes species (Siganidae: Perciformes).

Methods: The samples were gathered from the Red Sea. The sequences of four rabbitfishes (*Siganus argenteus, Siganus luridus, Siganus rivulatus,* and *Siganus stellatus*) were deposited into NCBI to gain the accession numbers (PP488874–PP488877) and then analyzed with their related rabbitfishes depending on available sequence data of the mitochondrial *16S rRNA* gene.

Results: The results of *16S rRNA* sequences illustrated that the average A+T values were greater than C+G.

Conclusion: The low genetic distance between *S. luridus* and *Siganus rivulatus* indicated a close linkage between them.

Keywords: Mitochondrial *16S rRNA* gene, Molecular marker, Rabbitfishes.

Introduction

With 27 species, siganids, or "rabbit fishes," are a small family of marine herbivorous fish known as is widely spread throughout the tropical waters of the Indian Ocean, Red Sea, and Indo-Pacific (Woodland, 1983; Saoud *et al*., 2008). Moreover, subtropical Mediterranean locations have been reported to harbor these fish (Saoud *et al*., 2008; Insacco and Zava, 2016). A large range of salinity and temperature were tolerable to siganidas (Woodland, 1983; Saoud *et al*., 2007). In terms of growth, siganida grows similarly to other marine organisms that are cultivated. Its maximum weight and length are 318.2 g and 32 cm, respectively (Bariche, 2005).

It is challenging to accurately identify fish and infer the evolutionary relationships among species based on their morphology in many taxonomic groups that are distributed around the world. This is because species that are descended from convergent evolution share comparable morphological traits, and the pattern of speciation is highly complex (Rice and Westneat, 2005; Duftner *et al.,* 2007).

Nowadays, it is thought that molecular marker-derived genetic information is crucial for the sustainable management, exploitation, and conservation of fisheries and animals as well as for promoting sustainable aquaculture (Casey *et al*., 2016; Lind *et al*., 2016).

Species characterization using morphology and anatomical characters causes sometimes errors in the proper identification of closely related species. Because of these issues, molecular markers have been used as a complementary tool for taxonomic identification (AL-Qurashi and Saad, 2022).

To comprehend biodiversity assessments, conservation management, evolutionary patterns, and processes, accurate species delimitation, and phylogenetic reconstruction are essential (Traldi *et al*., 2020; McCord *et al*., 2021).

Fish species identification, fish resource management, and seafood monitoring are all performed achievable by mitochondrial DNA (Teletchea, 2009; Rubinoff *et al*., 2006).

The mitochondrial *16S rRNA* gene was used for molecular phylogenetic research in several fish species (Li *et al*., 2013). Because these genes are preserved and non-coding, they were crucial in establishing phylogenetic relationships (Rathipriya *et al*., 2022).

The basic goal of this work was to evaluate the phylogenetic linkages of some species of rabbitfishes belonging to the family Siganidae by the mean of large mitochondrial rRNA (*16S rRNA*) gene.

***Corresponding Author:** Najiah M. Alyamani. Department of Biology, College of Science, University of Jeddah, Jeddah, Saudi Arabia. Email: *nmalyamani@uj.edu.sa*

Materials And Methods

Samples collection and species identification

The study sampling site was the Red Sea, where four species of family Siganidae (*Siganus argenteus*, *Siganus luridus*, *Siganus stellatus,* and *Siganus rivulatus*) were compiled and identified. In order to isolate DNA, the sample muscles were taken out and preserved at –20°C

DNA isolation, and PCR amplification

Using the DNA Mini kit (Qiagen, Germany) according to the manufacturer's instructions, the genomic DNA was extracted from the conserved muscles. Using previously published primers, PCR was utilized to amplify a partial sequence of the mitochondrial *16S rRNA* (Simon *et al*., 1991). Using 23 µL of 2X master mix, 1 µL of genomic DNA, 1 µL of each primer, and 20 µL of nuclease-free water, the PCR was finished in 46 µL. The amplification conditions included five minutes of denaturation at 95°C, thirty cycles of denaturation, annealing, and extension at 94°C, 48°C, and 72°C, respectively, for sixty seconds, and a final extension at 72°C for seven minutes. On a 1.5% agarose gel containing ethidium bromide and a 100 bp DNA ladder, the PCR results were electrophoresed.

Sequences and phylogenetic analysis

The final sequences were finished by Macrogen (South Korea, Seoul). In order to obtain accession numbers, the *16S rRNA* sequences were deposited into GenBank/NCBI. The sequences were aligned using CLUSTAL W (Thompson *et al*., 1994), using the default parameters. Using MEGA software version 7.0 (Kumar *et al*., 2016), two approaches were used for phylogenetic reconstructions: neighbor joining and minimum evolution. We employed 1,000 bootstrap iterations of Kimura two-parameter distances (Kimura, 1980) to finalize the sequence divergences (Felsenstein, 1985).

Results

This work establishes the evolutionary lineages of four species of the family Siganidae: *S. argenteus*, *S. luridus*, *S. stellatus,* and *S. rivulatus*. This was achieved by employing large subunit ribosomal RNA (*16S rRNA*) sequences.

In all four species, the *16S rRNA*-produced bands range in length from 521 to 570 bp. The *16S rRNA* sequences were shown in GenBank/NCBI to obtain the accession numbers (PP488874—PP488877). The findings show that *S. argenteus* and *S. rivulatus* possess the shortest sequence (521 bp.) while *S. stellatus* possesses the sequence with the greatest length (570 bp.). Adenine (A), thymine (T), cytosine (C), and guanine (G) exhibited average frequencies of 28,63, 22.72, 25, and 23.65%, respectively. As was shown in Table 1, the average attribution for A+T was more significant compared to that of C+G. The final alignments comprised 589 base pairs. The sites that were variable, and conserved were 17 and 534, respectively.

The P-distances across the entire fish fluctuated between 0.0000 and 0.0197%. The distance value was 0.05% overall. The P-distances among the *Siganus* species ranged from 0.0000 to 0.0082%. The largest value (0.0082) was found between *_Siganus_javus* and both *Siganus canaliculatus* and *S. rivulatus* (DQ898115.1). The smallest value (0.0000) was found between *Siganus_canaliculatus* and *S. rivulatus* (DQ898115.1) as well as understudied *S. stellatus* and both *S. stellatus* (KT952627.1) and *Siganus punctatus*. The P-distances among the studied species of *Siganus* spanned the range from 0.0035 to 0.0070%. The largest difference (0.0070) was observed between *S. stellatus*, and *S. rivulatus*. Conversely, *S. luridus* and *S. rivulatus* had the smallest P-distance (0.0000) (Table 2 and Fig. 1).

The sequences obtained from four fish in the Siganidae family, along with 24 linked sequences and the three outgroup species from GenBank, were used in this work for widely combination phylogenetic investigation in order to finish the phylogenetic tree investigation using the sequence of *16S rRNA* sequence. More than one phylogenetic technique was employed for the very illustrative phylogenetic analysis utilizing the *16S rRNA* gene: Neighbor Joining and Minimum Evolution. Although the support rate varied slightly, the methods yielded results that were essentially comparable and highlighted two main points: (1) The outgroup species creating a distinct cluster. (2) Each species of the studied species creating a distinct cluster with the comparable species from GenBank (Figs. 2 and 3).

Table 1. Accession number, nucleotide frequencies, A+T contents, and their averages of (*16S rRNA*) sequence in four species of the family Siganidae.

No.	Species	Accession number	Base pair length	Nucleotide frequencies %				A+T Content (%)
				A%	T%	C %	G%	
1	*S. argenteus*	PP488874.1	521	29.37	22.46	24.56	23.61	51.83
2	*S. luridus*	PP488875.1	540	27.78	22.41	25.74	24.07	50.19
3	*S. stellatus*	PP488876.1	570	28.95	23.51	24.21	23.33	52.46
4	*S. rivulatus*	PP488877.1	521	28.41	22.46	25.52	23.61	50.87
	Average %	-	538	28.63	22.72	25	23.65	51.35

Table 2. Pairwise distances using 16S rRNA gene among four species of the family Siganidae, and the outgroup.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1	PP488874.1_Siganus_argenteus		0.0067	0.0064	0.0058	0.0058	0.0058	0.0058	0.0062	0.0067	0.0052	0.0060	0.0034	0.0064	0.0064	0.0070	0.0075	0.0055	0.0054	0.0069	0.0069	0.0104	0.0104	0.0105	0.0116	0.0101	0.0112	0.0114	0.0122	0.0180	0.0183	0.0190
2	PP488875.1_Siganus_luridus	0.0222		0.0068	0.0035	0.0039	0.0042	0.0036	0.0053	0.0000	0.0067	0.0065	0.0075	0.0068	0.0068	0.0075	0.0077	0.0071	0.0075	0.0072	0.0075	0.0086	0.0098	0.0097	0.0102	0.0102	0.0099	0.0103	0.0103	0.0189	0.0171	0.0179
3	PP488876.1_Siganus_stellatus	0.0197	0.0229		0.0070	0.0069	0.0070	0.0067	0.0068	0.0068	0.0065	0.0028	0.0072	0.0000	0.0000	0.0028	0.0047	0.0040	0.0046	0.0039	0.0045	0.0093	0.0104	0.0104	0.0107	0.0106	0.0108	0.0105	0.0112	0.0187	0.0185	0.0176
4	PP488877.1_Siganus_rivulatus	0.0177	0.0060	0.0238		0.0021	0.0021	0.0029	0.0048	0.0035	0.0061	0.0067	0.0067	0.0070	0.0071	0.0076	0.0079	0.0073	0.0076	0.0060	0.0077	0.0094	0.0100	0.0101	0.0108	0.0108	0.0105	0.0115	0.0116	0.0188	0.0177	0.0185
5	KR824528.1_Siganus_canaliculatus	0.0177	0.0075	0.0239	0.0019		0.0000	0.0020	0.0048	0.0039	0.0061	0.0067	0.0067	0.0070	0.0068	0.0076	0.0082	0.0073	0.0076	0.0079	0.0077	0.0089	0.0099	0.0092	0.0109	0.0101	0.0098	0.0104	0.0109	0.0158	0.0181	0.0189
6	OQ858115.1_Siganus_rivulatus	0.0177	0.0079	0.0238	0.0019	0.0000		0.0020	0.0048	0.0042	0.0061	0.0067	0.0067	0.0070	0.0070	0.0076	0.0082	0.0073	0.0076	0.0079	0.0077	0.0094	0.0100	0.0098	0.0111	0.0105	0.0103	0.0112	0.0116	0.0184	0.0173	0.0181
7	OQ858100.1_Siganus_fuscescens	0.0177	0.0059	0.0217	0.0039	0.0019	0.0059		0.0043	0.0036	0.0065	0.0064	0.0067	0.0067	0.0067	0.0073	0.0079	0.0070	0.0073	0.0076	0.0074	0.0090	0.0098	0.0094	0.0107	0.0101	0.0098	0.0107	0.0111	0.0188	0.0169	0.0177
8	OQ858117.1_Siganus_spinus	0.0157	0.0141	0.0218	0.0117	0.0117	0.0117	0.0097		0.0053	0.0061	0.0065	0.0062	0.0068	0.0068	0.0075	0.0074	0.0071	0.0075	0.0077	0.0068	0.0086	0.0093	0.0101	0.0110	0.0107	0.0107	0.0113	0.0109	0.0188	0.0169	0.0178
9	OQ532959.1_Siganus_luridus	0.0222	0.0000	0.0225	0.0060	0.0075	0.0059	0.0059	0.0141		0.0067	0.0065	0.0075	0.0068	0.0068	0.0075	0.0077	0.0071	0.0075	0.0072	0.0075	0.0086	0.0098	0.0096	0.0102	0.0102	0.0099	0.0103	0.0103	0.0188	0.0170	0.0178
10	OQ858120.1_Siganus_woodlandi	0.0137	0.0202	0.0198	0.0177	0.0177	0.0177	0.0197	0.0176	0.0202		0.0062	0.0043	0.0065	0.0065	0.0071	0.0061	0.0061	0.0065	0.0063	0.0063	0.0100	0.0105	0.0112	0.0122	0.0113	0.0110	0.0115	0.0114	0.0178	0.0185	0.0196
11	OQ858108.1_Siganus_guttatus	0.0177	0.0202	0.0039	0.0218	0.0217	0.0217	0.0197	0.0197	0.0202	0.0178		0.0068	0.0025	0.0028	0.0040	0.0056	0.0027	0.0035	0.0049	0.0034	0.0094	0.0100	0.0104	0.0108	0.0107	0.0109	0.0108	0.0111	0.0181	0.0183	0.0191
12	EF210163.1_Siganus_argenteus	0.0058	0.0265	0.0239	0.0218	0.0218	0.0218	0.0218	0.0198	0.0265	0.0065	0.0218		0.0072	0.0072	0.0077	0.0075	0.0061	0.0063	0.0077	0.0071	0.0105	0.0102	0.0110	0.0121	0.0106	0.0111	0.0119	0.0122	0.0185	0.0187	0.0194
13	OQ858113.1_Siganus_punctatus	0.0197	0.0222	0.0000	0.0238	0.0238	0.0238	0.0217	0.0218	0.0222	0.0198	0.0039	0.0239		0.0000	0.0028	0.0047	0.0040	0.0046	0.0039	0.0045	0.0098	0.0103	0.0107	0.0112	0.0110	0.0113	0.0111	0.0114	0.0178	0.0180	0.0187
14	KT852627.1_Siganus_stellatus	0.0198	0.0230	0.0000	0.0239	0.0231	0.0238	0.0218	0.0218	0.0229	0.0199	0.0039	0.0240	0.0000		0.0028	0.0047	0.0040	0.0046	0.0040	0.0045	0.0089	0.0104	0.0101	0.0106	0.0106	0.0109	0.0105	0.0112	0.0188	0.0186	0.0173
15	OQ858119.1_Siganus_vulpinus	0.0239	0.0265	0.0039	0.0280	0.0279	0.0279	0.0259	0.0259	0.0265	0.0240	0.0078	0.0281	0.0039	0.0039		0.0054	0.0049	0.0055	0.0049	0.0054	0.0102	0.0108	0.0112	0.0118	0.0115	0.0117	0.0116	0.0120	0.0179	0.0182	0.0189
16	OQ858109.1_Siganus_javus	0.0259	0.0265	0.0098	0.0280	0.0300	0.0300	0.0279	0.0259	0.0265	0.0178	0.0138	0.0280	0.0098	0.0098	0.0138		0.0055	0.0059	0.0045	0.0059	0.0103	0.0111	0.0119	0.0122	0.0116	0.0115	0.0116	0.0128	0.0177	0.0178	0.0186
17	OQ858107.1_Siganus_doliatus	0.0157	0.0243	0.0078	0.0259	0.0259	0.0259	0.0238	0.0238	0.0243	0.0178	0.0039	0.0177	0.0078	0.0078	0.0118	0.0138		0.0034	0.0048	0.0043	0.0099	0.0106	0.0109	0.0114	0.0106	0.0114	0.0107	0.0115	0.0186	0.0185	0.0197
18	OQ858118.1_Siganus_vermiculatus	0.0157	0.0265	0.0098	0.0280	0.0279	0.0279	0.0259	0.0259	0.0265	0.0198	0.0058	0.0198	0.0098	0.0098	0.0138	0.0158	0.0058		0.0053	0.0040	0.0098	0.0103	0.0107	0.0111	0.0104	0.0119	0.0110	0.0119	0.0187	0.0184	0.0192
19	OQ858112.1_Siganus_puellus	0.0239	0.0243	0.0078	0.0301	0.0300	0.0300	0.0279	0.0280	0.0243	0.0198	0.0118	0.0281	0.0078	0.0078	0.0118	0.0098	0.0118	0.0138		0.0060	0.0107	0.0109	0.0119	0.0119	0.0113	0.0120	0.0112	0.0119	0.0183	0.0184	0.0193
20	OQ858105.1_Siganus_corallinus	0.0239	0.0265	0.0098	0.0280	0.0279	0.0279	0.0259	0.0218	0.0265	0.0198	0.0058	0.0239	0.0098	0.0098	0.0138	0.0158	0.0098	0.0078	0.0179		0.0084	0.0091	0.0100	0.0112	0.0109	0.0113	0.0110	0.0114	0.0178	0.0180	0.0188
21	AB8422 76.1_Platigeneion_rubigenium	0.0471	0.0373	0.0399	0.0408	0.0385	0.0407	0.0385	0.0342	0.0373	0.0428	0.0385	0.0472	0.0407	0.0379	0.0450	0.0450	0.0428	0.0407	0.0495	0.0321		0.0051	0.0058	0.0080	0.0085	0.0091	0.0084	0.0084	0.0152	0.0155	0.0162
22	AB8422 79.1_Emmelichthys_nitidus	0.0471	0.0434	0.0459	0.0449	0.0445	0.0450	0.0428	0.0385	0.0434	0.0450	0.0428	0.0449	0.0450	0.0480	0.0494	0.0494	0.0472	0.0450	0.0494	0.0363	0.0134		0.0085	0.0094	0.0084	0.0090	0.0091	0.0093	0.0181	0.0184	0.0171
23	OQ532874.1_Erythrocles_monodi	0.0480	0.0433	0.0452	0.0438	0.0393	0.0416	0.0394	0.0481	0.0431	0.0507	0.0438	0.0508	0.0481	0.0432	0.0507	0.0553	0.0484	0.0481	0.0553	0.0416	0.0174	0.0203		0.0091	0.0084	0.0082	0.0091	0.0083	0.0155	0.0162	0.0185
24	MH633938.1_Kyphosus_sydneyanus	0.0580	0.0494	0.0548	0.0538	0.0506	0.0558	0.0536	0.0558	0.0492	0.0627	0.0515	0.0626	0.0537	0.0580	0.0582	0.0605	0.0580	0.0537	0.0605	0.0537	0.0319	0.0416	0.0410		0.0078	0.0105	0.0074	0.0095	0.0151	0.0148	0.0152
25	OQ345333.1_Sinipercia_roulei	0.0470	0.0516	0.0529	0.0538	0.0516	0.0515	0.0492	0.0537	0.0514	0.0560	0.0493	0.0514	0.0516	0.0530	0.0561	0.0561	0.0493	0.0471	0.0538	0.0516	0.0380	0.0334	0.0350	0.0313		0.0095	0.0051	0.0103	0.0147	0.0148	0.0153
26	EF120866.1_Omopercia_pekensi	0.0484	0.0435	0.0475	0.0440	0.0391	0.0418	0.0395	0.0483	0.0433	0.0482	0.0482	0.0484	0.0485	0.0476	0.0531	0.0508	0.0508	0.0531	0.0554	0.0485	0.0377	0.0350	0.0331	0.0495	0.0433		0.0095	0.0078	0.0162	0.0165	0.0172
27	AY838947.1_Sinipercia_chuatsi	0.0558	0.0516	0.0494	0.0562	0.0522	0.0560	0.0537	0.0562	0.0514	0.0560	0.0493	0.0604	0.0516	0.0495	0.0561	0.0561	0.0493	0.0516	0.0538	0.0516	0.0380	0.0376	0.0332	0.0277	0.0148	0.0433		0.0057	0.0152	0.0154	0.0160
28	EF120865.1_Centrarchops_chapini	0.0598	0.0478	0.0538	0.0530	0.0517	0.0529	0.0506	0.0485	0.0474	0.0530	0.0507	0.0569	0.0530	0.0539	0.0577	0.0624	0.0553	0.0577	0.0577	0.0530	0.0334	0.0393	0.0350	0.0432	0.0517	0.0310	0.0474		0.0159	0.0167	0.0171
29	MW633045.1_Canarx_hippox	0.1214	0.1112	0.1086	0.1085	0.1036	0.1080	0.1086	0.1060	0.1107	0.1163	0.1166	0.1242	0.1139	0.1089	0.1166	0.1139	0.1220	0.1220	0.1193	0.1139	0.0934	0.1006	0.0990	0.0943	0.0920	0.1068	0.0991	0.1043		0.0050	0.0052
30	OQ427053.1_Canarx_mitempygus	0.1218	0.1137	0.1141	0.1162	0.1129	0.1137	0.1110	0.1084	0.1132	0.1241	0.1190	0.1239	0.1163	0.1132	0.1190	0.1163	0.1245	0.1190	0.1218	0.1163	0.0956	0.1029	0.1065	0.0968	0.0930	0.1094	0.1036	0.1119	0.1130		0.0052
31	OQ427054.1_Canarx_ignobilis	0.1265	0.1188	0.1191	0.1216	0.1177	0.1190	0.1163	0.1137	0.1183	0.1256	0.1245	0.1294	0.1218	0.1181	0.1245	0.1218	0.1301	0.1245	0.1273	0.1218	0.1005	0.1082	0.1065	0.0991	0.0954	0.1145	0.1069	0.1119	0.1130	0.0143	

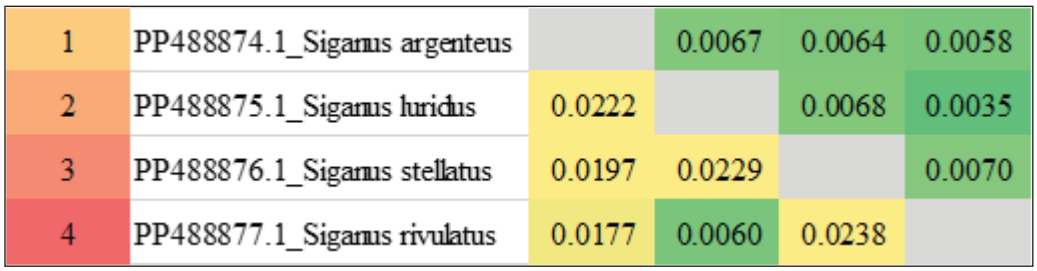

Fig. 1. Heatmap visualization of The P-distances among four species of the family Siganidae by employed the 16S rRNA gene.

Discussion

It can be difficult to identify species in traditional taxonomy since there are often arbitrary morpho meristic data sets and a lack of guidelines for character selection or coding. Under certain circumstances, genetic analysis can be employed as a further way of establishing taxonomic identity (Basheer *et al.*, 2015). Due to its slower mutation rate and lower substitution rates than other mtDNA genes, mitochondrial *16S rRNA* has been found to be valuable for studying species, populations, and families (Garland and Zimmer, 2002). Moreover, fish phylogenetic relationships can be estimated at both the species and generic levels using the *16S rRNA* gene (Moyer, *et al.*, 2004; Chakraborty and Iwatsuki, 2006). Therefore, in fish evolutionary studies, *16S rRNA* is advised for the reconstruction of informative phylogenetic links and a proper identification system (Saad *et al.*, 2019).

This study showed that the average bidder for the fish that were understudied was (A+T) rather than (C+G). This aligned with multiple research investigations. In contrast to C+G, the entire *16S rRNA* gene displays A+T affluence, according to Bo *et al.* (2013). Basheer *et al.* (2015) found that *16S rRNA* had a lower C+G value than A+T in their investigation of *Rastrelliger* species. Additionally, Mar'ie and Allam (2019) discovered a greater A+T ratio than C+G in two puffer fish. In some species of catfish, Mahrous and Allam (2022) the proportion of A+T was greater than that of C+G.

The C+G concentration of the *16S rRNA* gene varied between 48.52 and 50.09 in our data. The four species in the family Siganidae's GC variety may indicate adaptation (Ali *et al*., 2021).

High levels of conservation were found in the final alignments of partial *16S rRNA* sequences in the four species belonging to the Siganidae family. Using *16S rRNA* aligned sequences, Basheer *et al.* (2015) discovered 575 consistent sites of 590 bp in three *Rastrelliger* species. Using a phylogenetic analysis of Cichlids and the 16S gene, Sokefun (2017) discovered 337 conserved sites comprising 463 bp of alignment. Numerous highly conserved regions are revealed by aligning the partial *16S rRNA* sequences of eight Carangid fishes (Alyamani *et al*., 2023). The research done by Ramadan *et al*., (2023) showed that the four species of Lutjanus fish have an average (A+T) that is higher than the average (C+G).

According to (Kaleshkumar *et al.*, 2015), strongly related species had low genetic distance values, whereas cases with great genetic divergence are caused by the

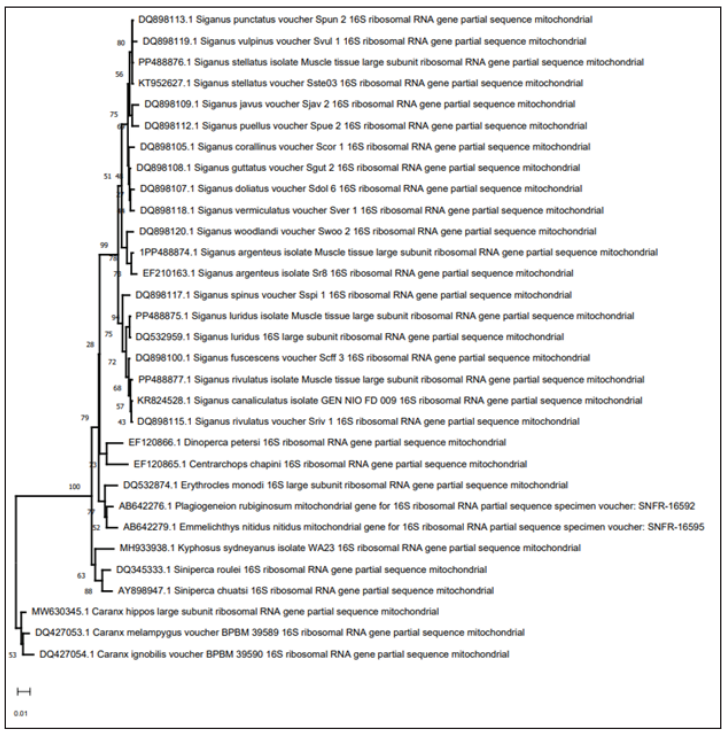

Fig. 2. Neighbour joining phylogenetic tree among four species of the family Siganidae, and the outgroup with the outgroup by employed the *16S rRNA* gene.

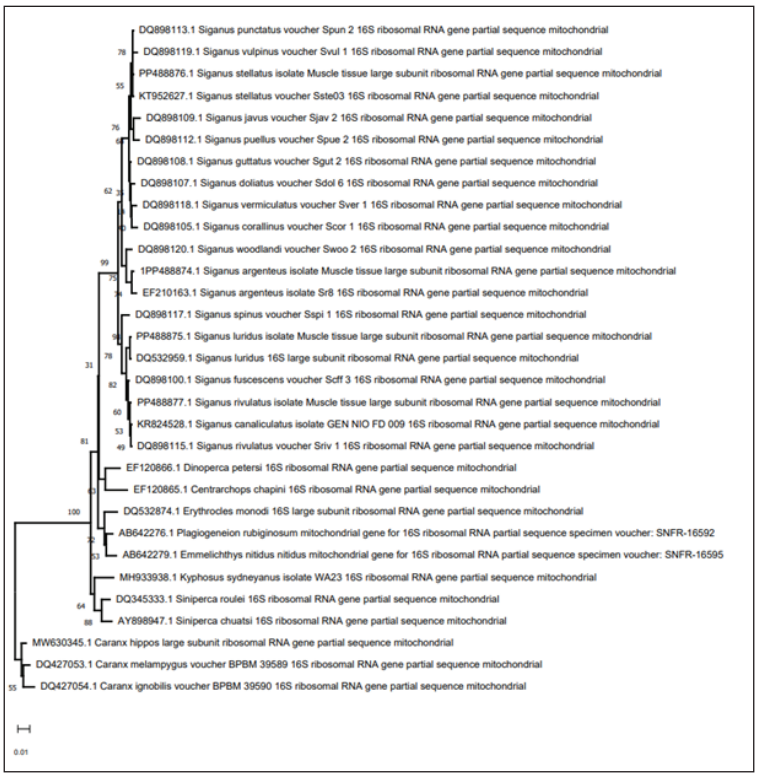

Fig. 3. Minimum evolution phylogenetic tree among four species of the family Siganidae, and the outgroup with the outgroup by employed the *16S rRNA* gene.

highest genetic distance. The low genetic distance between *S. luridus* and *S. rivulatus* indicated a close linkage between them.

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Authors' contributions

There is one author for this manuscript.

Conflict of interest

The author declares that there is no conflict of interest.

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Data availability

All data are provided in the manuscript.

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