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Phylogenetic Relationships Among Some Carangid Species Based on Analysis of Mitochondrial *16S rRNA* Sequences

Najiah M. Alyamani[1], Eman Fayad[2], Ali H. Abu Almaaty [3], Abeer Ramadan[3,4] , Mohammad Allam[5,*]

[1]Department of Biology, College of Science, University of Jeddah, Jeddah 21493, Saudi Arabia
[2]Department of Biotechnology, College of Sciences, Taif University, Taif 21944, Saudi Arabia
[3]Zoology Department, Faculty of Science, Port Said University, Port Said, Egypt
[4]Department of Basic Sciences, Deanship of Preparatory Year and Supporting Studies, Imam Abdulrahman Bin Faisal University, Dammam 34212, Saudi Arabia
[5]Zoology Department, Faculty of Science, South Valley University, Qena, Egypt

[*]**Corresponding Author: mohammad_allam10@sci.svu.edu.eg**

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ABSTRACT

Carangid fishes are abundant and highly lucrative in the commercial world. Since the evolutionary lineages of this family are still unknown, this work was completed to provide a thorough picture of the phylogenetic relationships among several species of the Carangidae family based on large subunit ribosomal RNA. For *16S rRNA*, the understudied species' sequences ranged in length from 588 to 606 base pairs (bp). The accession numbers for the nucleotide sequences were gained from Gen Bank (MW165070.1 - MW165077.1). All species had A+T values that were greater than C+G, and the average A+T amount was 51.33%. The pairwise genetic distances amongst our characterized Carangid fish ranged from 0.0037 to 0.0322%. The lowest P-distance (0.0037) was existent between *Carangoides bajad* and *Caranx sexfasciatus*. While the highest value (0.0322) was present between *Carangoides chrysophrys* and *Scomberoides lysan*. Maximum Likelihood, Maximum Parsimony, and Neighbor-Joining were the three phylogenetic methodologies used to analyze the evolutionary relationships among the Carangid fishes. The results of the phylogenetic study were generally relatively comparable, supporting the sturdy relationship between the genera *Caranx* and *Carangoides*.

INTRODUCTION

Molecular identification methods supply when there are several ambiguities in traditional methods, molecular approaches are a helpful option for species identification; in this situation, molecular techniques should be used for the delicate identification **(Basheer *et al.*, 2015; Saha *et al.*, 2019)**. Mitochondrial DNA plays a significant role in

the evolutionary relation study and species identification. Among mitochondrial DNA genes, *16S rRNA* gene has been exceedingly utilized in phylogenetic research **(Bej *et al.*, 2012; Patwardhan *et al.*, 2014)**. Variation of the *16S rRNA* gene is stable within species and between species **(Yang *et al.*, 2014)**. Given that the mitochondrial *16S rRNA* gene has a slow evolution and is highly conserved **(Page and Holmes, 1998)**, it has been extensively used in fish phylogenetic research **(Ortí *et al.*, 1996; Moyer, *et al.*, 2004; Feng *et al.*, 2005; Li *et al.*, 2008; Sokefun, 2017)**.

Due to inter- and intra-specific variation, morphological classification has mainly some confusion, although molecular techniques can unquestionably identify species, as well as variations and cryptic taxa, carefully and swiftly. **(Holland *et al.*, 2004; Le Roux & Wieczorek, 2009; García-Morales & Elías-Gutiérrez, 2013)**. Carangid fish are widely distributed and found in all tropical and subtropical seas, as well as being among the world's economically very important coastal pelagic fish. **(Lin & Shao, 1999)**. The name of Carangidae family is referred to the genus *Caranx* and includes a various group of fishes **(Honebrink, 2000)**. During the growth of Carangids, many significant changes occur in pigmentation and morphology, leading to the misclassification of samples and contributing to general taxonomic confusion **(Bohlke & Chaplin, 1993; Mat Jaafar *et al.*, 2012)**.

In the light of the afore- mentioned data, this study was executed to appreciate the phylogenetic linkages amongst some Carangid species by employing mitochondrial *16S rRNA* sequence.

MATERIALS AND METHODS

Collection of samples and DNA extraction

The Red Sea is the study sampling area where the fish were gathered **(Randall, 1982)**. The samples' muscle tissues were dissected from the caudal peduncle and were kept at -20°C for storage. DNA was extracted from 15–25 milligrams of muscle tissue using the QIAamp DNA Mini kit (Qiagen, Hidden, Germany).

DNA sequencing with polymerase chain reaction (PCR) amplification

Using the primers specified in the study of **Simon *et al.* (1991)**, the mitochondrial *16S rRNA* region was amplified. A final reaction volume of 50µL was used for the polymerase chain reaction. This volume included 25µL of a 2X master mix (OnePCR^{TM}), 1µL of genomic DNA, 1 µL of each primer (10 mmol/L), and 22 µL of nuclease-free water.

The PCR plan was conducted as follows: denaturation at 95°C for 4min, followed by 35 cycles of denaturation, annealing and extensions at 94, 48 and 72°C, respectively, for 60s, with an extension at 72°C for 7min as the last step. To see the PCR results, a 1.8% agarose gel containing ethidium bromide was used. 100 V was used to run a 40-

minute 100bp DNA Ladder RTU (Ready-to-Use) GeneDireX gel electrophoresis. The sequences were carried out by Macrogen (Seoul, South Korea).

Phylogenetic study of sequences

To receive the accession numbers, the sequences were uploaded to (GenBank/NCBI). With the default settings, CLUSTAL W **(Thompson *et al.*, 1994)** was used to align the sequences. Three methodologies; Maximum Likelihood, Maximum Parsimony, and Neighbour Joining performed in MEGA software version 7.0 18 **(Kumar *et al.*, 2016)** were utilized for phylogenetic reconstructions in order to compare the harmony of the results. Kimura two-parameter distances **(Kimura, 1980)** were used to implement sequence divergences, and 1000 bootstrap iterations were performed **(Felsenstein, 1985)**.

RESULTS

Using large subunit ribosomal RNA (*16S rRNA*), this study evaluated the phylogenetic significance of eight Carangid species from the Red Sea (*Carangoides bajad, Carangoides chrysophrys, Carangoides malabaricus, Caranx melampygus, Caranx sexfasciatus, Elagatis bipinnulata, Scomberoides lysan,* and *Trachinotus ovatus*.

The lengths of *16S rRNA* sequence in eight Carangid fishes extend from 588 to 606 bp. All sequences were inserted into the GenBank with accession numbers (MW165070.1 - MW165077.1) (Table 1). The results indicate *Carangoides chrysophrys* has the longest (606 bp.) sequence, while *Elagatis bipinnulata* has the shortest sequence (588 bp.). The average nucleotide frequencies of adenine (A), thymine (T), cytosine (C) and guanine (G) were 27.67, 23.67, 26.15 and 22.52% respectively. The average content of A+T was higher than the C+G in all species. The nucleotide frequencies, A+T contents and their averages were given in (Table 1). The sequences determined from eight Carangid species from the Red Sea, as well as 12 related sequences from GenBank, were applied in this study, for a more compound phylogenetic analysis as shown in (Table 2). The final alignments are composed of 623 bp. Of them 423, 182 were conserved and variable sites respectively, and 59 were Parsimony informative sites (Fig. 1).

Among all fish species, the P-distances extend from 0.0000 to 0.0404%. Overall, the distance value among all species was 0.12%. Amongst the Carangid species, the P-distances ranged from 0.000 to 0.0322%. The highest value (0.0322) was found between *Carangoides chrysophrys* and *Scomberoides lysan*. While (0.000) value was found between *Alepes kleinii* and *Alepes vari* (Table 3). Among understudied Carangid fish, the P-distances expanded from 0.0037 to 0.0322%. The highest value (0.0322) was found between *Carangoides chrysophrys* and *Scomberoides lysan*. While the lowest P-distance (0.0037) was found between *Carangoides bajad* and *Caranx sexfasciatus*. The lowest genetic distance among all species under the genus *Carangoides* was (0.0055). The

lowest P-distance among all species under the genus *Caranx* was (0.0043). While the lowest genetic distance among all species under the two genera *Carangoides* and *Caranx* was (0.0037) between *Carangoides bajad* and *Caranx sexfasciatus* (Table 3).

Table 1: Accession number, nucleotide frequencies, A+T contents and their averages of (*16S rRNA*) sequence in eight Carangid fishes.

No.	Species	Accession number	Base pair length	Nucleotide frequencies %				A+T Content (%)
				A%	T%	C %	G%	
1	*Carangoides bajad*	MW165070.1	597	27.30	23.28	26.97	22.45	50.59
2	*Carangoides chrysophrys*	MW165071.1	606	27.06	23.76	26.90	22.28	50.83
3	*Carangoides malabaricus*	MW165072.1	600	27.67	23.50	26.33	22.50	51.17
4	*Caranx melapygus*	MW165073.1	605	27.93	23.31	26.45	22.31	51.24
5	*Caranx sexfasciatus*	MW165074.1	602	27.41	23.09	26.91	22.59	50.50
6	*Elagatis bipinnulata*	MW165075.1	588	27.89	23.81	25.34	22.96	51.70
7	*Scomberoides lysan*	MW165076.1	600	28.33	23.50	25.83	22.33	51.83
8	*Trachinotus ovatus*	MW165077.1	598	27.76	25.08	24.41	22.74	52.84
	Average %	-	599.5	27.67	23.67	26.15	22.52	51.33

Table 2: The understudied eight Carangid fishes with their related Carangid species, with out-group species from the GenBank/NCBI utilizing the *16S rRNA* sequences.

No.	Species	Accession number
1	*Carangoides bajad*	MW165070.1
2	*Carangoides chrysophrys*	MW165071.1
3	*Carangoides malabaricus*	MW165072.1
4	*Caranx melapygus*	MW165073.1
5	*Caranx sexfasciatus*	MW165074.1
6	*Elagatis bipinnulata*	MW165075.1
7	*Scomberoides lysan*	MW165076.1
8	*Trachinotus ovatus*	MW165077.1
9	*Alectis indica*	EF613265.1
10	*Caranx lugubris*	JQ396646.1
11	*Caranx heberi*	MK335844.1
12	*Trachurus environmenta*	KC603533.1
13	*Caranx ignobilis*	DQ427054.1
14	*Alepes kleinii*	MK561615.1
15	*Caranx hippos*	MW630345.1
16	*Selaroides leptolepis*	EF613270.1
17	*Caranx crysos*	KP273458.1
18	*Alepes djedaba*	EF613269.1
19	*Caranx latus*	AF055611.1
20	*Alepes vari*	MT123333.1
Out-group	*Onigocia macrolepis*	KT862622.1
	Onigocia spinosa	KT862633.1
	Onigocia sibogae	KT862626.1

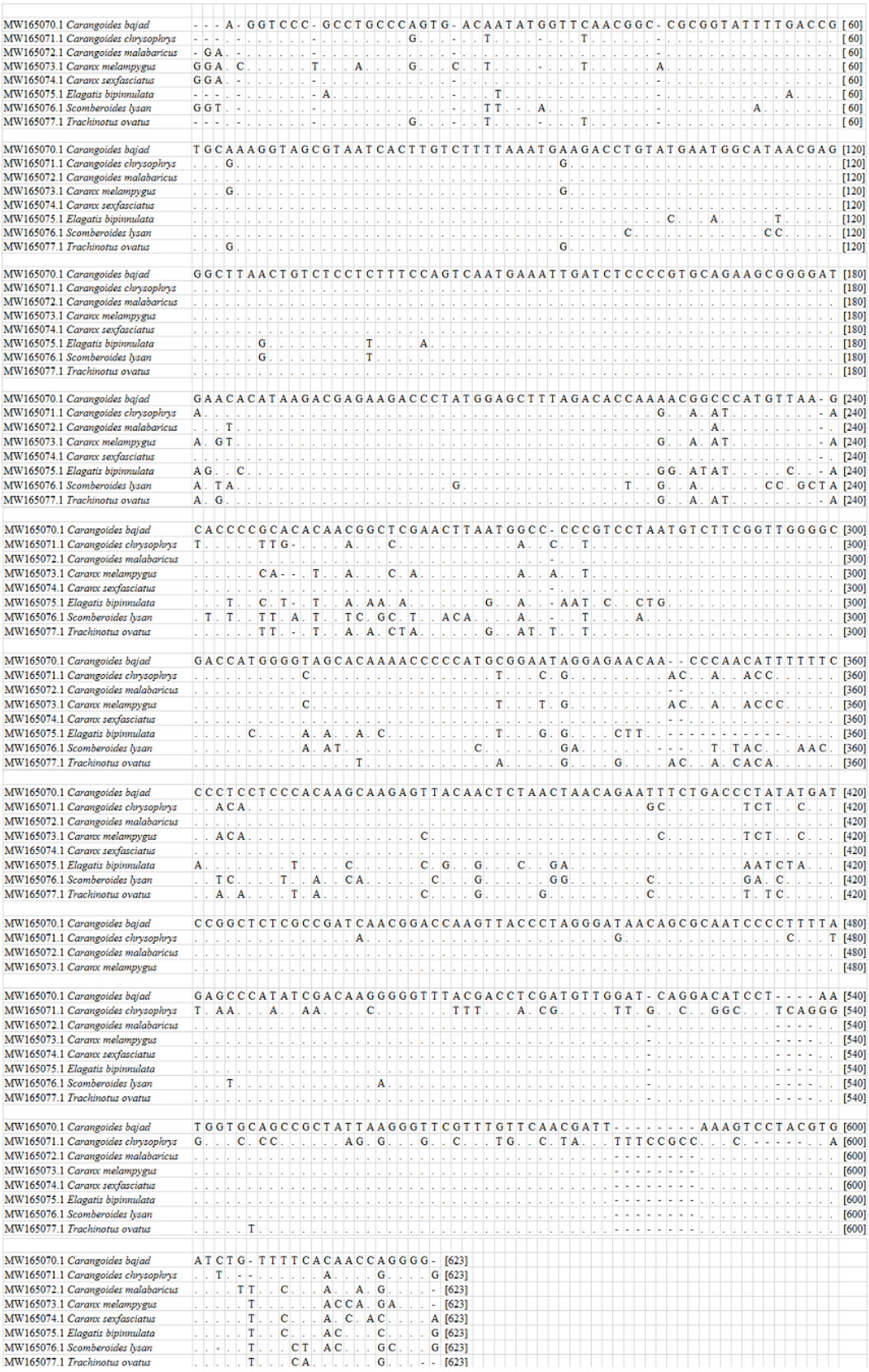

Fig.1: Alignment of *16S rRNA* partial sequences in eight Carangid fishes. Dots show similar nucleotides while A, C, G, and T display the variance nucleotides.

Table 3: Pairwise distances using *16S rRNA* gene among eight Carangid fishes with their related Carangid species, 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
1	MW165070.1 *Carangoides bajad*		0.0222	0.0055	0.0151	0.0037	0.0201	0.0226	0.0143	0.0100	0.0120	0.0125	0.0130	0.0126	0.0138	0.0134	0.0129	0.0143	0.0136	0.0130	0.0153	0.0275	0.0285	0.0309
2	MW165071.1 *Carangoides chrysophrys*	0.1771		0.0221	0.0179	0.0223	0.0310	0.0322	0.0209	0.0210	0.0152	0.0151	0.0249	0.0148	0.0186	0.0147	0.0202	0.0168	0.0186	0.0155	0.0210	0.0383	0.0382	0.0404
3	MW165072.1 *Carangoides malabaricus*	0.0171	0.1745		0.0141	0.0046	0.0192	0.0227	0.0147	0.0102	0.0124	0.0125	0.0140	0.0127	0.0141	0.0134	0.0132	0.0147	0.0141	0.0130	0.0162	0.0271	0.0286	0.0310
4	MW165073.1 *Caranx melampygus*	0.0941	0.1314	0.0895		0.0154	0.0206	0.0251	0.0123	0.0143	0.0086	0.0073	0.0152	0.0083	0.0111	0.0069	0.0128	0.0102	0.0125	0.0050	0.0126	0.0249	0.0258	0.0286
5	MW165074.1 *Caranx sexfasciatus*	0.0085	0.1769	0.0119	0.0979		0.0188	0.0221	0.0151	0.0105	0.0122	0.0125	0.0137	0.0133	0.0146	0.0141	0.0137	0.0150	0.0144	0.0138	0.0163	0.0275	0.0285	0.0309
6	MW165075.1 *Elagatis bipinnulata*	0.1530	0.2739	0.1429	0.1559	0.1427		0.0268	0.0192	0.0179	0.0191	0.0197	0.0215	0.0209	0.0200	0.0197	0.0197	0.0204	0.0198	0.0199	0.0225	0.0292	0.0318	0.0326
7	MW165076.1 *Scomberoides lysan*	0.1746	0.2815	0.1751	0.2020	0.1677	0.2160		0.0222	0.0218	0.0222	0.0203	0.0220	0.0219	0.0215	0.0228	0.0236	0.0214	0.0222	0.0231	0.0232	0.0317	0.0328	0.0308
8	MW165077.1 *Trachinotus ovatus*	0.0921	0.1681	0.0968	0.0744	0.0987	0.1432	0.1739		0.0113	0.0102	0.0104	0.0145	0.0114	0.0100	0.0115	0.0114	0.0119	0.0100	0.0107	0.0124	0.0244	0.0271	0.0289
9	EF613265.1 *Alectis indica*	0.0475	0.1675	0.0517	0.0868	0.0535	0.1231	0.1588	0.0592		0.0113	0.0118	0.0104	0.0121	0.0113	0.0122	0.0141	0.0125	0.0113	0.0125	0.0109	0.0243	0.0262	0.0297
10	JQ396646.1 *Caranx lugubris*	0.0669	0.1049	0.0711	0.0365	0.0689	0.1359	0.1661	0.0502	0.0614		0.0044	0.0136	0.0052	0.0105	0.0048	0.0100	0.0088	0.0109	0.0073	0.0114	0.0240	0.0274	0.0284
11	MK335844.1 *Caranx heberi*	0.0695	0.1010	0.0696	0.0271	0.0695	0.1394	0.1467	0.0505	0.0637	0.0106		0.0144	0.0043	0.0095	0.0047	0.0106	0.0083	0.0105	0.0059	0.0103	0.0248	0.0270	0.0282
12	KC603533.1 *Trachurus environmental*	0.0821	0.2075	0.0905	0.1025	0.0887	0.1651	0.1759	0.0913	0.0515	0.0837	0.0888		0.0143	0.0125	0.0138	0.0158	0.0136	0.0129	0.0133	0.0135	0.0252	0.0259	0.0279
13	DQ427054.1 *Caranx ignobilis*	0.0704	0.0974	0.0703	0.0368	0.0767	0.1523	0.1611	0.0596	0.0674	0.0144	0.0107	0.0877		0.0097	0.0045	0.0108	0.0076	0.0106	0.0064	0.0103	0.0248	0.0276	0.0295
14	MK561615.1 *Alepes kleinii*	0.0814	0.1418	0.0856	0.0634	0.0897	0.1474	0.1692	0.0481	0.0568	0.0524	0.0446	0.0700	0.0469		0.0093	0.0120	0.0100	0.0054	0.0100	0.0000	0.0244	0.0283	0.0293
15	MW630345.1 *Caranx hippos*	0.0750	0.0974	0.0749	0.0253	0.0814	0.1391	0.1698	0.0597	0.0677	0.0125	0.0126	0.0832	0.0125	0.0449		0.0105	0.0076	0.0100	0.0047	0.0099	0.0244	0.0268	0.0286
16	EF613270.1 *Selaroides leptolepis*	0.0744	0.1534	0.0765	0.0780	0.0807	0.1462	0.1777	0.0595	0.0870	0.0530	0.0573	0.1041	0.0589	0.0698	0.0570		0.0119	0.0122	0.0111	0.0128	0.0248	0.0277	0.0319
17	KP273458.1 *Caranx crysos*	0.0815	0.1183	0.0861	0.0505	0.0880	0.1441	0.1586	0.0616	0.0694	0.0392	0.0352	0.0810	0.0310	0.0509	0.0292	0.0633		0.0103	0.0084	0.0106	0.0238	0.0251	0.0282
18	EF613269.1 *Alepes djedaba*	0.0815	0.1404	0.0861	0.0713	0.0880	0.1386	0.1675	0.0452	0.0587	0.0553	0.0512	0.0742	0.0548	0.0160	0.0488	0.0715	0.0506		0.0108	0.0056	0.0248	0.0276	0.0293
19	AF055611.1 *Caranx latus*	0.0736	0.1030	0.0735	0.0143	0.0800	0.1410	0.1722	0.0539	0.0705	0.0260	0.0183	0.0774	0.0219	0.0496	0.0126	0.0620	0.0353	0.0555		0.0106	0.0256	0.0255	0.0294
20	MT123333.1 *Alepes vari*	0.0922	0.1605	0.1013	0.0713	0.1011	0.1683	0.1846	0.0670	0.0528	0.0566	0.0480	0.0777	0.0487	0.0000	0.0466	0.0736	0.0530	0.0153	0.0516		0.0255	0.0305	0.0306
21	KT862622.1 *Onigocia macrolepis*	0.2188	0.3368	0.2127	0.1904	0.2188	0.2367	0.2690	0.1857	0.1823	0.1834	0.1899	0.1915	0.1868	0.1864	0.1841	0.1954	0.1837	0.1903	0.1912	0.1916		0.0092	0.0216
22	KT862633.1 *Onigocia spinosa*	0.2202	0.3251	0.2206	0.1951	0.2202	0.2491	0.2744	0.2040	0.1942	0.2049	0.2016	0.1912	0.2083	0.2152	0.2016	0.2167	0.1917	0.2088	0.1894	0.2247	0.0379		0.0228
23	KT862626.1 *Onigocia sibogae*	0.2495	0.3497	0.2500	0.2253	0.2495	0.2608	0.2519	0.2257	0.2302	0.2163	0.2168	0.2178	0.2271	0.2295	0.2176	0.2572	0.2171	0.2295	0.2276	0.2305	0.1588	0.1679	

To implement the phylogenetic tree analysis using *16S rRNA* sequence, eight Carangid species were analyzed together with the 12 related Carangid fishes and the outgroup species from GenBank/NCBI as exhibited in (Table 2). More than one phylogenetic method was used for more interpretative phylogenetic relationships by employing *16S rRNA* gene. These techniques were Neighbour Joining, Maximum Parsimony, and Maximum Likelihood. With some modifications in the support parameters, the approaches produced findings that were essentially identical and displayed three basic lineaments.: (1) species of the outgroup were found in a separate cluster. (2) *Caranx sexfasciatus* formed one clade with *Carangoides malabaricus* or *Carangoides bajad*. (3) *Carangoides chrysophrys* formed one clade with *Caranx ignobilis* (Figs. 2-4).

Fig.2. Maximum Likelihood phylogenetic tree among eight Carangid fishes and their linked Carangid fishes with the outgroup by employing the *16S rRNA* gene.

Fig.3. Maximum parsimony phylogenetic tree among eight Carangid fishes and their linked Carangid fishes with the outgroup by employing the *16S rRNA* gene.

Fig.4. Neighbour Joining phylogenetic tree among eight Carangid fishes and their linked Carangid fishes with the outgroup by employed the *16S rRNA* gene.

DISCUSSION

Because there are no standards for character selection or coding in classical taxonomy, the morphomeristic data sets can occasionally be rather random, making it challenging to identify species. In these circumstances, genetic analysis might be used as an additional technique to establish taxonomic identity **(Basheer *et al.*, 2015)**. The earlier investigations found that the evolutionary relationships in the genera of the subfamily Caranginae were quite complex. **(Smith-Vaniz, 1984; Kijima *et al.*, 1986 and Gushiken, 1988)** the number, connection, and each genus from each branch were agreed upon. **(Thu *et al.*, 2019)**.

In comparison to other mtDNA genes, *16S rRNA* has lower substitution rates and a slower rate of mutation, making it useful in the research of species, populations, and families. **(Garland and Zimmer, 2002)**. Moreover, the *16S rRNA* gene can be used to determine fish phylogenetic relationships at both the species and general levels. **(Moyer, *et al.*, 2004 and Chakraborty & Iwatsuki, 2006)**. In general, the molecular identification method should be a trustworthy, affordable, and accessible way to distinguish between distinct genera and the species that make up those genera. These advantages are preserved by the identifying mechanism (*16S rRNA*). Therefore, it is

advised for the reconstruction of useful phylogenetic links and appropriate identification methods in studies of the evolution of fish **(Saad *et al*., 2019)**.

All understudied fishes displayed (A+T) content higher than the (C+G). The whole *16S rRNA* gene displays A+T affluence, compared to C+G **(Bo *et al.*, 2013)**. **Basheer *et al.* (2015)** during the study on *Rastrelliger* species found the C+G content of *16S rRNA* was shorter than the A+T. Also, **Mar'ie and Allam (2019)** found a high A+T proportion compared to C+G in two puffer fish. C+G content of the *16S rRNA* gene ranged from 47.15 to 49.42. The variation in GC content among Carangid species may consider a sign of adaptation **(Ali *et al*., 2021)**.

The alignment of eight Carangid fishes' incomplete *16S rRNA* sequences reveals many highly conserved sites. The final alignments' total length was 623 bp, of which 423 were conserved sites. **Basheer *et al.* (2015)** reported that the three *Rastrelliger* species have 575 consistent locations with a total length of 590 bp in their *16S* rRNA aligned sequences. **Sokefun (2017)** Cichlid phylogenetic analysis employing the *16S* gene revealed (463) bp of alignment with 72.7% (337) conserved regions.

Caranx resembles members of the Carangoides morphologically. Several scholars taxonomize them into the same genus due to their similarities **(Smith-Vaniz, 1984 and Reed *et al*., 2002)**. This relationship between the genera *Caranx* and *Carangoides* is supported genetically by the findings of our investigation. Since the results of the sequencing of the large subunit ribosomal RNA (*16S rRNA*) gene showed little genetic distance between the species of both the genera *Carangoides* and *Caranx*. As a result, the genera *Carangoides* and *Caranx* had a greater degree of a sturdy relationship than the other genera *Elagatis*, *Scomberoides*, and *Trachinotus*. This was based on **(Kaleshkumar *et al.*, 2015)** who said that strongly related species had low genetic distance values, whereas cases with great genetic divergence are caused by the highest genetic distance.

Using *16S rRNA* sequences, three phylogenetic approaches were utilized to assess the evolutionary relationships among eight Carangid taxa. The three phylogenetic approaches produced nearly identical findings, confirming the close relationship between the species belonging to the genera *Carangoides* and *Caranx*. **Jacobina *et al.* (2014).** The shared karyotypes of *Carangoides bartholomaei* and *Caranx latus*, as well as the presence of 18S rDNA sites that appear at equilocal positions on the first chromosome pair at the short arm in three species, support the phylogenetic correlation between *Carangoides* and *Caranx*. This conclusion was consistent with that of molecular genetic research on a few species of the Carangidae family that was reported by **(Mar'ie and Allam, 2017)** and **(Allam and Mar'ie, 2021)**, where it was determined that the genera *Caranx* and *Carangoides* were related robustly.

The position and phylogenetic association of suspicious taxa must be clarified because the family Carangidae's phylogenetic relationships are still unclear **(Damerau *et al*., 2018)**. As well as **Thu *et al.* (2019)** established a complex systematic phylogenetic relationship between the genera in the subfamily Caranginae. As a result, multiple

investigations were conducted using different molecular markers to evaluate the evolutionary link between species and genera of the family Carangidae **(Thu *et al.*, 2019; Torres and Santos, 2019; Li *et al.*, 2020 and Allam and Mar'ie, 2021)**.

CONCLUSION

This study was achieved to estimate the phylogenetic relationships of eight Carangid species using large mitochondrial rRNA sequences. The data of *16S rRNA* sequences showed that *Caranx* and *Carangoides* genera were assigned to be distantly related to each other. *16S rRNA* gene seems to be useful in exposing the phylogenetic of Carangid species.

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