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Phylogenetic Inference of Some Species of the Family Apogonidae Using *12S rRNA* Sequence

Maryham M. Aziz[1*], Ali H. Abu Almaaty[1],Mohammad Allam[2]

[1]Zoology Department, Faculty of Science, Port Said University, Port Said 42526, Egypt
[2]Zoology Department, Faculty of Science, Luxor University, Luxor 1369919, Egypt

[*]**Corresponding Author: maryham_magdy@hotmail.com**

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ABSTRACT

This work used small (*12S rRNA*) mitochondrial rRNA gene to assess the evolutionary connections of some species of the cardinal fishes belonging to order Kurtiformes. Small mitochondrial rRNA sequences varied in length from 920 to 944 base pairs. Of the 947 characters in the aligned *12S rRNA* data set, 834 were consistent sites, 19 were parsimony informative, and 111 were variable. Small mitochondrial rRNA sequences' nucleotides were uploaded to GenBank with accession numbers (PP830791-PP830794). The *P*-distances among the understudied cardinalfish species, expanded from 0.0060 to 0.0130. The highest value (0.0130) was found between *Cheilodipterus novemstriatus* and *Ostorhinchus fasciatus*. While, the lowest *P*-distance (0.0060) was found between *Cheilodipterus quinquelineatus* and *Cheilodipterus macrodon*. Our results demonstrated the utility of *12S rRNA* in the phylogenetic analysis of the cardinalfish species.

INTRODUCTION

Worldwide, cardinalfishes (Apogonidae) are found on coral reefs and can represent the most numerous families of fish in a community **(Bellwood, 1996)**. Paternal mouthbrooders, cardinalfishes are active nighttime feeders and seek protection during the day, sometimes in large groups with other reef structures, including reef-dwelling invertebrates **(Vagelli, 2011; Marnane & Bellwood, 2002; Gardiner & Jones, 2005, 2010)**. Cardinalfishes are small bodied, highly numerous fish that frequently show loyalty to a reef site, they likely have a significant role in the food chain as prey for larger, predatory fish and as planktivores that aid in the recycling of nutrients within the reef community **(Marnane, 2000; Marnane & Bellwood, 2002)**.

Cardinalfishes are diverse and important to the ecosystem, but our understanding of their life histories is still incomplete, the life-history characteristics that are vital to the

survival of a population, such growth and reproduction rates, are also poorly documented **(Gould *et al*., 2016)**.

The family has traditionally been divided into two subfamilies: Pseudamiinae, which contains only 21 species, and Apogoninae, which includes the majority of the species (327 species) **(Eschmeyer & Fong, 2014)**. Within the Pseudamiinae subfamily, **Baldwin and Johnson (1999)** recognized four genera: *Pseudamia Bleeker* 1865, *Gymnapogon Regan* 1905, *Pseudamiops Smith* 1954, and *Gymnapogon Paxton* Baldwin and Johnson 1999. However, according to two recent molecular studies, the genus *Gymnapogon* is nested inside the Apogoninae clade **(Thacker & Roje, 2009; Cowman & Bellwood, 2011)**.

Determining and classifying the various species is thought to be one of the first fundamental stages in the monitoring and protection of biodiversity **(Dayrat, 2005)**. Morphological characteristics are the conventional basis for fish identification. However, fish and their many developmental phases are frequently difficult to identify by utilizing physical traits alone due to considerable diversity and morphological plasticity **(Rasmussen *et al*., 2009)**. The identification techniques based upon DNA have been progressed and proven to be analytically powerful **(Zhang *et al*., 2004; Comi *et al*., 2005; Teletchea, 2009)**.

Molecular phylogenies were based on information from one or a few genes for several years. These data were usually obtained using Sanger sequencing and PCR amplification **(Field *et al*., 1988; Aguinaldo *et al*., 1997)**. The advent of novel sequencing technology has led to the creation of enormous databases with orders of magnitude more genes **(Telford *et al*., 2015)**. The number of taxa that can be taken into consideration is also greatly increasing due to the simplicity and low cost of genome and transcriptome sequencing, as evidenced by recent proposals for sequencing the genomes of every species on Earth **(Lewin *et al*., 2018)**. Although there is a growing amount of material available, accurately rebuilding the tree of life is not always simple **(Kapli *et al*., 2020)**.

In almost all eukaryotes, the mitochondrial (mt) genome is necessary for living. Vertebrates have compact mt genomes with a highly conserved repertoire of encoded genes. For 37 genes and two noncoding regions, the mt genome's gene order also tends to be preserved among vertebrates; these are essentially ordered in the same order from hagfish to eutherian mammals **(Anderson *et al*., 1981; Roe *et al*., 1985; Tzeng *et al*., 1992; Chang *et al*., 1994)**.One of the most often targeted genes for the phylogenetic study of various taxa, such as genera, is the mtDNA *12S rRNA* gene **(Murphy & Collier, 1996; Gatesy *et al*., 1997)**.

The main goal of this study was to understand the phylogenetic relationship of family Apogonidae using partial sequence of *12S rRNA* gene.

MATERIALS AND METHODS

Sample preparation and DNA extraction

The fish were caught from the Red Sea and classified according to **Randall (1982)** and **Akel and Karachle (2017)**. The muscle tissues were taken from the caudal peduncle and stored at -20°C. 15-25 milligrams of muscle tissue were used to extract DNA using the QIAamp DNA Mini kit (Qiagen, Germany).

Polymerase chain reaction (PCR) amplification

We utilized primers in accordance with **Jin *et al*. (2013)** to amplify the small mitochondrial rRNA (*12S rRNA*) gene in the four cardinalfish species. The PCR reactions comprised of a final reaction volume of 50, 1µL of genomic DNA, 1µL of each forward and reverse primers, and 25µL of PCR master mix. The settings for the PCR cycling were as follows: a five-minute initial denaturation at 94^{o}C; thirty cycles of denaturation for sixty seconds at 94^{o}C, annealing for sixty seconds at 50^{o}C, and an extension at 72°C for sixty seconds, with a post-cycling extension at 72^{o}C for five minutes. The PCR products were separated using a 1.5% agarose gel that had been stained with ethidium bromide.

The sequencing of PCR products and phylogenetic tree construction

Macrogen (Seoul, South Korea) performed the DNA sequencing. The *12S rRNA* gene sequences were uploaded to GenBank/NCBI in order to receive accession numbers. MUSCLE **(Edgar, 2004)** was used for sequence alignment, with the default settings. We used molecular evolutionary genetics analysis (MEGA) version 11.0.11 **(Tamura *et al*., 2021)** to execute the phylogenetic trees analyses by the two phylogenetic methods: neighbor joining (NJ) and UPGMA. Bootstrap analysis was determined with 1000 replicates **(Felsenstein, 1985)**. The sequence divergences were computed using Kimura 2-parameter distances in order to produce a graphical depiction of the divergence between cardinalfish species **(Kimura, 1980)**.

RESULTS AND DISCUSSION

Small mitochondrial rRNA sequences' nucleotide were uploaded to GenBank with accession numbers (PP830791 - PP830794). Small mitochondrial rRNA sequences varied in length, with *Cheilodipterus quinquelineatus* having 920bp and *Cheilodipterus novemstriatus* having 944bp. Of the 947 characters in the aligned *12S rRNA* data set, 834 were consistent sites, 19 were parsimony informative, and 111 were variable.

The A+T ratio of the *12S rRNA* is more concentrated than the C+G. Additional information regarding the averages of the *12S rRNA* sequences in cardinalfish, as well as the nucleotide frequencies, A+T contents, and pyrimidine contents found in Table (1).

Table 1. Nucleotide frequencies, A+T contents, pyrimidines contents and their averages of *12S rRNA* sequence in cardinalfish

No.	**Species**	**Length**	**A**	**T**	**C**	**G**	**A+T**	Pyrimidines **C+T**
1	*Cheilodipterusquinquelineatus*	920	31.20	19.89	27.28	21.63	51.09	47.17
2	*Cheilodipterusmacrodon*	941	31.88	19.66	26.99	21.47	51.54	46.65
3	*Cheilodipterusnovemstriatus*	944	30.72	20.87	26.06	22.35	51.59	46.93
4	*Ostorhinchusfasciatus*	939	30.14	21.30	26.41	22.15	51.44	47.71
Avg.		936	30.98	20.44	26.68	21.90	51.42	47.12

The *P*-distances among the understudied cardinalfish, expanded from 0.0060 to 0.0130. The highest value (0.0130) was found between *Cheilodipterus novemstriatus* and *Ostorhinchus fasciatus*. While the lowest *P*-distance (0.0060) was found between *Cheilodipterus quinquelineatus* and *Cheilodipterus macrodon* (Table 2). Additionally, *Cheilodipterus macrodon* revealed low *P*-distance (0.0108) to *Ostorhinchus fasciatus* compared to other understudied two species;*C. quinquelineatus*, and *C. novemstriatus*, which reflect a closely genetic relationship between *Cheilodipterus macrodon* and *Ostorhinchus fasciatus* (Table 2).

Phylogenetic analysis

Cardinalfish sequencing was submitted for study together with 23 similar cardinalfishesin addition, three outgroup species (order, Scorpaeniformes)from GenBank/NCBI in order to perform phylogenetic analysis utilizing the *12S rRNA* gene.Using *12S rRNA* data for the phylogenetic tree analysisrevealed species of the outgroup formed a separate cluster. *Cheilodipterus quinquelineatus, Cheilodipterus macrodon* and *Cheilodipterus novemstriatus* formed sister clad with their related GenBank cardinalfish species, while *Ostorhinchus fasciatus* was found near to *Ostorhinchus fasciatus*NC 058293.1 from GenBank (Fig.1a, b).

Table 2. Pairwise distances by the mean of *12S rRNA* gene amongst cardinalfish with their linked species from the GenBank/NCBI

		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
1	PP830791.1_Cheilodipterus_quinquelineatus		0.0060	0.0095	0.0122	0.0148	0.0144	0.0098	0.0111	0.0130	0.0156	0.0053	0.0147	0.0128	2.4450	0.0124	0.0104	0.0140	0.0122	0.0124	7.4275	0.0117	0.0137	0.0103	0.0134	0.0120	0.3687	0.0148	0.0203	0.0202	0.0200
2	PP830792.1_Cheilodipterus_macrodon	0.0320		0.0101	0.0108	0.0142	0.0139	0.0092	0.0087	0.0036	0.0170	0.0079	0.0128	0.0119	2.6404	0.0119	0.0097	0.0125	0.0120	0.0119	6.3199	0.0104	0.0133	0.0099	0.0124	0.0114	0.3864	0.0141	0.0191	0.0197	0.0195
3	PP830793.1_Cheilodipterus_novemstriatus	0.0672	0.0744		0.0130	0.0154	0.0149	0.0125	0.0132	0.0167	0.0047	0.0090	0.0161	0.0147	3.7789	0.0138	0.0127	0.0149	0.0138	0.0138	6.0473	0.0130	0.0164	0.0124	0.0159	0.0144	0.3833	0.0150	0.0225	0.0224	0.0221
4	PP830794.1_Ostorhinchus_fasciatus	0.0983	0.0769	0.1045		0.0150	0.0144	0.0105	0.0122	0.0139	0.0174	0.0122	0.0154	0.0132	1.3605	0.0044	0.0111	0.0092	0.0052	0.0044	4.7385	0.0132	0.0147	0.0119	0.0135	0.0129	0.4269	0.0143	0.0208	0.0206	0.0204
5	OP056953.2_Apogon_binotatus	0.1222	0.1175	0.1297	0.1229		0.0058	0.0135	0.0151	0.0177	0.0191	0.0142	0.0177	0.0141	2.6953	0.0143	0.0130	0.0167	0.0135	0.0143	4.7613	0.0150	0.0149	0.0111	0.0142	0.0142	0.6201	0.0107	0.0187	0.0190	0.0188
6	OP056855.1_Apogon_pseudomaculatus	0.1217	0.1156	0.1276	0.1164	0.0263		0.0131	0.0148	0.0167	0.0190	0.0140	0.0175	0.0138	3.1723	0.0138	0.0128	0.0159	0.0130	0.0138	6.1136	0.0147	0.0152	0.0109	0.0127	0.0140	0.5443	0.0102	0.0201	0.0202	0.0199
7	NC_022510.1_Apogon_semilineatus	0.0670	0.0602	0.0975	0.0729	0.1098	0.1035		0.0111	0.0111	0.0183	0.0100	0.0134	0.0110	2.9577	0.0104	0.0062	0.0122	0.0102	0.0104	4.8138	0.0109	0.0128	0.0099	0.0114	0.0112	0.4422	0.0128	0.0196	0.0201	0.0200
8	LC277940.2_Cheilodipterus_intermedius	0.0825	0.0552	0.1125	0.0932	0.1277	0.1230	0.0788		0.0108	0.0178	0.0119	0.0147	0.0142	2.5232	0.0139	0.0112	0.0136	0.0137	0.0139	3.6631	0.0120	0.0152	0.0116	0.0151	0.0130	0.3469	0.0154	0.0210	0.0215	0.0213
9	ON888954.1_Cheilodipterus_macrodon	0.0643	0.0063	0.0980	0.0687	0.0991	0.0885	0.0468	0.0468		0.0186	0.0144	0.0140	0.0132	4.3409	0.0162	0.0123	0.0146	0.0170	0.0162	4.2216	0.0141	0.0207	0.0124	0.0149	0.0150	0.6891	0.0176	0.0324	0.0336	0.0330
10	MH248244.1_Cheilodipterus_novemstriatus	0.1145	0.1286	0.0137	0.1264	0.1400	0.1393	0.1265	0.1366	0.1125		0.0140	0.0192	0.0193	6.7729	0.0179	0.0181	0.0187	0.0183	0.0179	8.3780	0.0180	0.0236	0.0168	0.0212	0.0205	0.4858	0.0197	0.0336	0.0332	0.0328
11	NC_040863.1_Cheilodipterus_quinquelineatus	0.0237	0.0480	0.0589	0.0933	0.1111	0.1116	0.0731	0.0878	0.0658	0.0921		0.0155	0.0125	3.1817	0.0127	0.0104	0.0137	0.0122	0.0127	6.3978	0.0111	0.0131	0.0101	0.0131	0.0121	0.3576	0.0144	0.0201	0.0199	0.0197
12	LC519395.2_Fibramia_amboinensis	0.1249	0.1052	0.1420	0.1338	0.1562	0.1498	0.0999	0.1247	0.0786	0.1522	0.1247		0.0149	3.3226	0.0149	0.0141	0.0143	0.0152	0.0149	5.3711	0.0143	0.0179	0.0145	0.0153	0.0163	0.5973	0.0172	0.0252	0.0256	0.0254
13	MN937193.1_Jaydia_carinatus	0.1029	0.0909	0.1194	0.1030	0.1128	0.1124	0.0855	0.1140	0.0625	0.1402	0.0994	0.1222		2.9236	0.0127	0.0104	0.0134	0.0123	0.0127	6.1542	0.0110	0.0134	0.0108	0.0137	0.0127	0.5309	0.0140	0.0220	0.0228	0.0226
14	MK537324.1_Jaydia_lineata	5.9132	5.8028	6.5722	4.7486	5.7877	6.1412	5.8764	5.8573	5.7701	8.4086	6.2373	6.3979	5.7962		1.4682	2.6978	1.6432	1.2052	1.4682	11.8909	4.0714	2.0010	2.5122	4.4838	3.4144	3.0473	3.4649	3.8539	3.5870	3.5870
15	NC_058293.1_Ostorhinchus_fasciatus	0.0941	0.0868	0.1084	0.0165	0.1164	0.1111	0.0702	0.1063	0.0821	0.1241	0.0997	0.1195	0.0980	4.7588		0.0106	0.0074	0.0031	0.0000	3.4252	0.0143	0.0147	0.0120	0.0135	0.0128	0.4410	0.0135	0.0216	0.0214	0.0212
16	NC_056170.1_Ostorhinchus_fleurieu	0.0767	0.0669	0.1048	0.0800	0.1030	0.0997	0.0313	0.0804	0.0546	0.1305	0.0771	0.1096	0.0780	5.6400	0.0729		0.0118	0.0104	0.0106	5.1680	0.0109	0.0123	0.0097	0.0119	0.0114	0.3719	0.0127	0.0197	0.0204	0.0202
17	LC791740.1_Ostorhinchus_kiensis	0.1179	0.0962	0.1232	0.0597	0.1357	0.1280	0.0873	0.1135	0.0816	0.1407	0.1031	0.1206	0.1023	4.8689	0.0366	0.0821		0.0070	0.0074	2.1043	0.0134	0.0163	0.0133	0.0151	0.0143	0.3280	0.0158	0.0240	0.0231	0.0229
18	NC_053536.1_Ostorhinchus_novemfasciatus	0.0909	0.0866	0.1094	0.0226	0.1080	0.1018	0.0703	0.1047	0.0870	0.1295	0.0922	0.1224	0.0935	4.4826	0.0082	0.0716	0.0338		0.0031	3.4521	0.0139	0.0146	0.0115	0.0128	0.0123	0.4115	0.0131	0.0211	0.0209	0.0207
19	NC_063691.1_Ostorhinchus_pleuron	0.0941	0.0868	0.1084	0.0165	0.1164	0.1111	0.0702	0.1063	0.0821	0.1241	0.0997	0.1195	0.0980	4.7588	0.0000	0.0729	0.0366	0.0082		3.4252	0.0143	0.0147	0.0120	0.0135	0.0128	0.4410	0.0135	0.0216	0.0214	0.0212
20	OP056957.2_Phaeoptyx_xenus	10.6593	9.0550	8.7156	7.7414	7.5173	8.2960	7.9007	6.7743	5.5309	9.3508	9.6898	7.6722	8.5477	19.7449	6.5487	8.3889	5.7690	6.7672	6.5487		5.5133	4.0411	4.3382	4.7230	4.3738	0.8235	3.0423	8.8349	8.2994	8.2994
21	LC499255.2_Pristiapogon_exostigma	0.0869	0.0746	0.1067	0.1055	0.1164	0.1141	0.0773	0.0901	0.0741	0.1367	0.0788	0.1200	0.0769	6.8382	0.1113	0.0755	0.1073	0.1058	0.1113	8.2741		0.0136	0.0101	0.0144	0.0136	0.3924	0.0134	0.0217	0.0219	0.0217
22	OP035185.1_Pristiapogon_kallopterus	0.1131	0.1105	0.1421	0.1263	0.1268	0.1303	0.1044	0.1247	0.1213	0.1816	0.1076	0.1545	0.1126	5.1645	0.1297	0.1011	0.1418	0.1270	0.1297	7.1625	0.1046		0.0124	0.0148	0.0149	0.3981	0.0161	0.0203	0.0208	0.0206
23	AP018928.1_Pristicon_trimaculatus	0.0749	0.0709	0.0981	0.0885	0.0777	0.0761	0.0695	0.0860	0.0577	0.1165	0.0753	0.1177	0.0800	5.8962	0.0886	0.0693	0.0994	0.0830	0.0886	7.4434	0.0680	0.1027		0.0124	0.0115	0.4352	0.0102	0.0177	0.0182	0.0180
24	NC_022511.1_Pterapogon_kauderni	0.1023	0.0932	0.1335	0.1033	0.1195	0.1021	0.0847	0.1192	0.0749	0.1539	0.1062	0.1285	0.1155	7.1398	0.1036	0.0935	0.1231	0.0977	0.1036	7.1552	0.1173	0.1259	0.1005		0.0131	0.4319	0.0138	0.0205	0.0206	0.0204
25	AP018927.1_Sphaeramia_orbicularis	0.0907	0.0834	0.1211	0.1014	0.1170	0.1137	0.0798	0.0992	0.0741	0.1513	0.0888	0.1365	0.0921	6.3164	0.0998	0.0829	0.1129	0.0910	0.0998	7.7050	0.0993	0.1255	0.0840	0.1062		0.5039	0.0136	0.0209	0.0217	0.0215
26	MN066612.1_Taeniamia_macroptera	2.5711	2.6513	2.6056	2.7365	3.1931	3.0354	2.8255	2.4686	2.8745	2.7545	2.5075	3.2325	3.0262	6.4784	2.7446	2.5980	2.3276	2.6632	2.7446	3.8535	2.6392	2.6862	2.8386	2.7508	2.9972		0.4968	0.4737	0.4136	0.4136
27	OP035214.1_Zapogon_evermanni	0.1201	0.1106	0.1272	0.1107	0.0744	0.0689	0.0989	0.1248	0.0949	0.1418	0.1170	0.1435	0.1075	6.1005	0.1026	0.0998	0.1236	0.0968	0.1026	6.6184	0.0988	0.1360	0.0689	0.1160	0.1118	2.9371		0.0204	0.0207	0.0205
28	NC_082787.1_Gymnocanthus_detrisus	0.1978	0.1866	0.2209	0.2012	0.1765	0.1919	0.1865	0.2004	0.2312	0.2835	0.1932	0.2430	0.2120	7.2121	0.2015	0.1848	0.2318	0.2018	0.2015	12.1768	0.2045	0.1959	0.1654	0.1982	0.1986	2.9795	0.1987		0.0054	0.0052
29	NC_034650.1_Gymnocanthus_intermedius	0.2032	0.1975	0.2191	0.2007	0.1804	0.1939	0.1977	0.2125	0.2432	0.2811	0.1962	0.2537	0.2193	7.0472	0.2025	0.1959	0.2265	0.2008	0.2025	11.5244	0.2122	0.2055	0.1725	0.2015	0.2114	2.7872	0.2048	0.0226		0.0011
30	NC_082786.1_Gymnocanthus_pistilliger	0.2012	0.1955	0.2170	0.1987	0.1785	0.1919	0.1957	0.2103	0.2388	0.2777	0.1942	0.2512	0.2171	7.0472	0.2004	0.1939	0.2242	0.1988	0.2004	11.5244	0.2100	0.2035	0.1706	0.1994	0.2094	2.7872	0.2028	0.0214	0.0011	

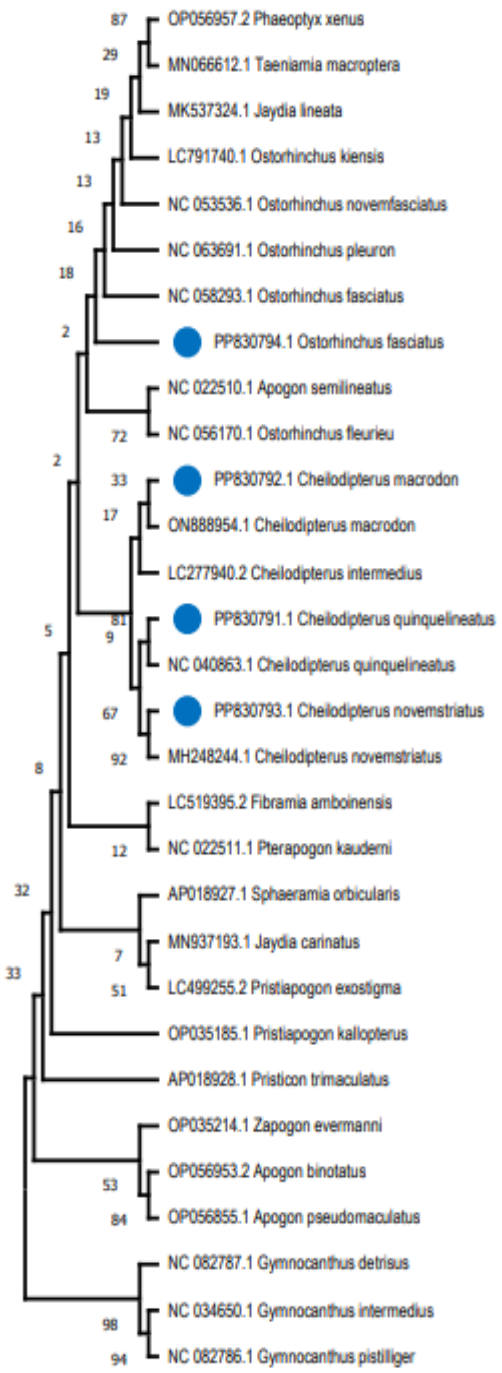

Fig.1a. Neighbor joining phylogenetic tree amongstcardinalfish and their related species, in addition, the outgroup using *12S rRNA* gene

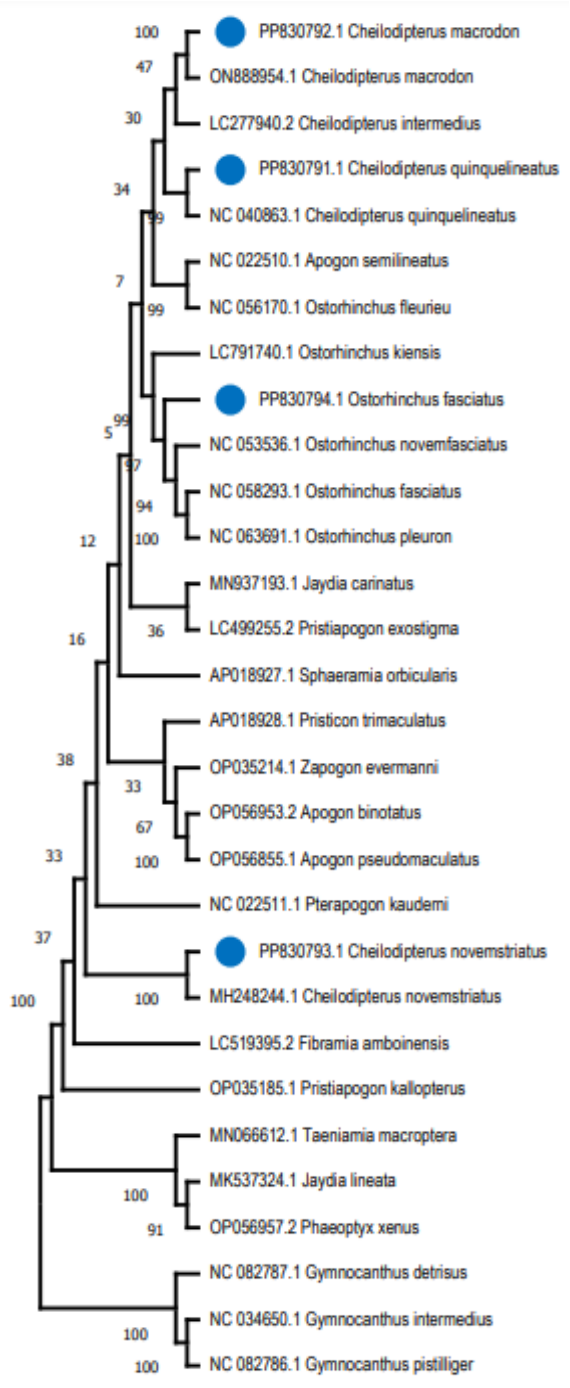

Fig.1b.UPGMAphylogenetic tree amongst cardinalfish and their related species, in addition, the outgroup using *12S rRNA* gene

In all understudied species, our analysis of the *12S rRNA* gene indicated a higher A+T composition than the C+G. This is consistent with several studies. **Norazila and Ismail (2002)** applied the *12S rRNA*/tRNA-Val gene on three varieties (normal, green, and yellow) of the tiger barb (*Puntius tetrazona*). **Sivaraman *et al.* (2009)** characterized the *12S rRNA* gene in four Cyprinid species. **Widayanti *et al.* (2021)** studied the genetic variation and phylogenetic analysis of the Indonesian indigenous catfish (baung fish) based on the mitochondrial *12S rRNA* gene. Similarly, **Mahrous and Allam (2022)** found similar findings during their study on eleven catfish species using the *12S rRNA* gene.

Low *P*-distance between *Cheilodipterus quinquelineatus* and *Cheilodipterus macrodon* indicated a close relation between them. Additionally, *Cheilodipterus macrodon* revealed low *P*-distance to *Ostorhinchus fasciatus* compared to other under studied two species of genus *Cheilodipterus*, which reflect a closely genetic relationship between *Cheilodipterus macrodon* and *Ostorhinchus fasciatus*. This is in line with the findings of **Kaleshkumar *et al.* (2015)**, who found that closely related species have low genetic distance values, but situations with a significant genetic divergence are caused by the maximum genetic distance.

CONCLUSION

Using small rRNA sequences, this study was able to assess the phylogenetic connections of cardinalfish. The *12S rRNA* gene appear to be helpful in revealing the phylogenetic relationship of Apogonidae family.

ETHICS STATEMENT

All animal experimental procedures were approved by the Ethics of Animal Experiments Committee of Port Said University, Faculty of Science (ERN: PSU. Sci.52.).

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