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The Biometric and Cytochrome Oxidase sub unit I (COI) Gene sequence Analysis of *Syngnathus abaster* (Teleostei: Syngnathidae) in Caspian Sea

Hamed Kolangi-Miandare[1], Ghasem Askari[1,*], Davoud Fadakar[1], Mostafa Aghilnegad[1], Siti Azizah[2]

1) Faculty of Fisheries and Environmental Sciences, Gorgan University of Agricultural Sciences and Natural Resources, Iran
2) School of Biological Sciences, University Sains Malaysia, Pulau Pinang, Malaysia

A B S T R A C T

The black-striped pipefish, *Syngnathus abaster*, is a species of the Syngnathidae family. This study provides data on the morphometric, meristic and genetic structuring of the Caspian Sea pipefish, *Syngnathus abaster*. Eight morphometric characteristics based on total length and five morphometric characteristics based on standard length were analyzed. A total of 50 specimens were collected in brackish-water biotopes. The average of total length (L_T) and standard length (L_S) were 102.37 mm and 98.68 mm, respectively. Also in this study, DNA barcodes were expanded for Caspian Sea Black-striped pipefish, hence 652 base-pair of the cytochrome oxidase I (COI) gene was sequenced in accordance with standard DNA barcoding protocols. Since Pipefish (*S. abaster*) is one of the most endangered fishes in the Caspian Sea, information about its phylogenetic relationships are very rare; therefore, DNA barcoding will give a more accurate picture of the future persistence of Black-striped pipefish populations.

Key words: Black-striped pipefish, morphometric and meristic, DNA barcoding, Caspian Sea.

INTRODUCTION

Pipefish has 200 species while sea horses have 25, all of which can be found in open seas, in brackish waters and fresh water [1]. In the Syngnathidae, one of the most specialized forms of parental care can be seen. In some species, parental care is provided by males while in other species, females provide the care [2]. In Syngnathidae, parental care is the responsibility of males [3]. In the Caspian Sea, there is only one species; the

* Address for correspondence: Faculty of Fisheries & Environmental Sciences, Gorgan University of Agricultural Sciences & Natural Resources, Iran
Tel: +98-936-314-8045
E-mail: Askarighasem82@gmail.com

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Syngnathus genus [4]. The black-striped pipefish, *Syngnathus abaster* (Risso, 1827) is a small pipefish belonging to this family, also found in the open sea, brackish waters, and fresh water [5, 6]. The usual habitat of this species is in estuaries and coastal areas [6, 7]. It has wide distribution in the Mediterranean, Black and Caspian Seas [7-9].

S. abaster of the genus *Syngnathus*, lack economic importance; however, due to their reversed sex roles, short life-cycles with limited reproduction and different food habitats they can be considered as appropriate models for ecological studies [9-12]. These fish can be found in coastal areas with sandy and mud bed grasslands [8, 13]. Although significant differences in their meristic characters have been found in populations of *S. abaster* from the Danube River, the Black and the Azov Seas [6, 9], no congruent information about the morphological differentiation of the *S. abaster* populations from the Caspian Sea has been reported.

DNA barcoding as a new approach to taxonomy seeks to identify species using standardized DNA regions. It is becoming an increasingly acceptable tool for identifying species that have received more attention recently. DNA studies can be divided into two stages, the first being the foundation of an archive for resource DNA barcodes based on DNA sequences from taxonomically documented and verified voucher specimens. The second stage is the use of this archive as a fast method to verify new, unknown specimens to known species [14]. For animals, the gene region proposed for the standard barcode is a 658 base pair region in the gene encoding the mitochondrial cytochrome c oxidase 1 (COI) [15] which can identify a large variety of species [16-18]. Pipefish (*S. abaster*) is one of the most endangered fishes in the Caspian Sea. This fact highlights the importance of improving knowledge regarding *S. abaster*, which despite its insignificant economic importance, is one of the few *Syngnathus* included in the red lists of the Conservation of Natural Habitat and Wild Fauna and Flora (1992). It is also listed as “Least Concern”, according to the IUCN Red List of Threatened Species [19].

The aim of this study was thus to identify *S. abaster* using morphometric, meristic characteristics and preparing primary DNA barcoding documents as well as comparing the documented DNA sequences of the Syngnathidae family, despite the fact that the information about phylogenetic relationships and genetic markers of Pipefish is very rare.

MATERIALS AND METHODS

Sample Collections and Morphometric and Meristic Analysis: Fifty samples of *S.abaster*, migrating to the coasts of the Gorgan golf for reproduction in May to July 2012, were collected from Gorgan golf (Caspian Sea) using nets. All specimens were fixed in 96% ethanol and transferred to the lab. Total length, standard length and weight were measured. A total of 15 morphometric and 5 meristic characters were measured [9, 20, 21]. Eight morphometric characteristics based on total length and five morphometric characteristics based on standard length were analyzed. The morphometric parameters included total length, standard length, maximum body height and width, length of dorsal and anal fin basis, height of dorsal, anal and pectoral fin, head length, snout length, head width, interorbital distance, eye diameter, and mouth width. The five meristic

characteristics included the number of rays in dorsal, anal, pectoral and caudal fins, and the number of preanal rings. Bowel and ovary samples were examined and feeding type and number of eggs within the ovary were determined. The morphometric and meristic characteristics measured are shown in Table 1. These characteristics were analyzed using SPSS (version 18) software. The number of eggs in the ovaries was counted using a Stereo Microscope. After opening the bowel, the contents were examined.

DNA extraction and PCR amplification: Total DNA was extracted from pectoral and pelvic fins using the traditional proteinase-K digestion and standard phenol/chloroform protocol after being stored at -20°C [22]. In order to amplify fragments of the mitochondrial COI gene, PCR reactions were conducted using primer cocktails of Fish F2-5′ TCG ACT AAT CAT AAA GAT ATC GGC AC3′ and FishR2-5′ACT TCA GGG TGA CCG AAG AAT CAG AA3′ [17]. The 25 µl PCR reaction mixes included 18.75 µl ultrapure water, 2.25 µl 10 × PCR buffer, 1.25 µl $MgCl_2$ (50 mM), 0.25 µl of each primer (0.01 mM), 0.125 µl of each dNTP (0.05mM), 0.625 U *Taq* polymerase, and 0.5–2.0 µl of the DNA template. Amplifications were performed using a Mastercycler® Eppendorf gradient thermal cycler (Brinkmann Instruments, Inc.). The thermal regime consisted of an initial step of 2 min at 95˚C followed by 35 cycles of 0.5 min at 94˚C, 0.5 min at 54˚C, and 1 min at 72˚C, followed in turn by 10 min at 72˚C and then held at 4˚C. PCR products (25 µl) were visualized on 1.2% agarose gels containing ethidium bromide (10 mg/ml) and the most intense products were selected for sequencing. Products were labeled using a BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.) and sequenced using an ABI3730 (Applied Biosystems 3730/ DNA Analyzers) capillary sequencer, following the manufacturer's instructions.

Data Analysis: Sequences were edited for correction with the SeqScape version 2.6 software (Applied Biosystems). Sequences were submitted to the GenBank Barcode database with accession numbers KF280320-KF280323. Sequences from GenBank and BOLD databases together with those from our dataset were aligned with Mega 5.0 [23], and adjusted by eye. For sequence comparisons, pairwise genetic distances were quantified based on the Kimura 2-parameter (K2P) distance model [24], using MEGA, version 5.0 [23]. Neighbor-joining (NJ) trees of K2P distances were created to provide a graphic representation of the patterning of divergence between species [25]. The robustness of the NJ tree was assessed by performing bootstrapping analysis with 1000 replicates [26].

RESULTS

Kolmogorov Simonov test did not reveal any statistically significant difference between individuals in the meristic characters ($p>0.05$). The average total length and standard length ± S.D were 102.37±2.38 and 98.68±2.70 (mm), respectively. Maximum and minimum body height were 4.19 and 2.05, respectively. The average morphometric and meristic characteristics are presented in table 1. The average number of eggs in each ovary was 100.1±20.2 (Table 1). In most cases the intestines were found to be empty of food and only in a limited number of cases there was a little sand in the gut. The samples

included all ages of fish, immature to mature. It was observed that the adult fish can care for 30 eggs under their belly. The egg color was yellow (Fig. 2, part D & E).

Table 1: Morphological characters of *Syngnathus abaster* from Gorgan Gulf (Caspian Sea)

	Syngnathus abaster		***Syngnathus acus* (Gürkan, 2008)**	
	Min-Max	Mean± S.D.	Min-Max	**Mean± S.D.**
L_T (mm)	73.6-148.33	102.37±22.38	65-245	112.05±1.7
L_S (mm)	71.43-144.65	98.68±21.70	-	-
% L_S				
Maximum body height	2.05-4.19	2.81±0.42	1.97-6.73	3.07±0.11
Maximum body width	1.90-3.55	2.53±0.37	1.35-7.78	3.00±0.11
Length of dorsal fin basis	9.28-12.87	11.15±0.97	0.94-25.72	11.01±0.3
Height of dorsal fin	1.04-3.39	2.19±0.69	-	-
Length of anal fin basis	0.42-0.86	0.57±0.11	-	-
Height of anal fin	0.56-1.58	1.10±0.21	-	-
Length of pectoral fin	1.60-2.81	2.12±0.33	0.44-3.45	1.58±0.06
Head length	12.92-16.09	14.44±0.81	0.43-32.31	12.27±0.33
$\%L_H$				
Snout length	43.91-55.91	49.95±3.38	-	-
Head width	10.43-22.24	18.33±2.75	-	-
Interorbital distance	7.25-11.90	9.29±1.19	-	-
Eye diameter	7.25-17.76	12.87±2.36	0.37-3.18	1.24±0.05
Mouth width	5.54-14.44	8.61±2.22	0.51-2.07	2.01±0.03
Meristic				
Number of rays in dorsal	33-36	33.92±0.899	25-33	29.96±0.67
Number of rays in anal	3-3	3±0	-	-
Number of rays in pectoral	12-13	12.6±0.49	9-10	9.75±0.47
Rays in caudal fin	7-10	9.25±0.79	4-10	7.63±1.08
Number of preanal rings	14-16	14.71±0.71	14-19	16.87±0.07
Number of egg	73.31	100.1±20.2	-	-

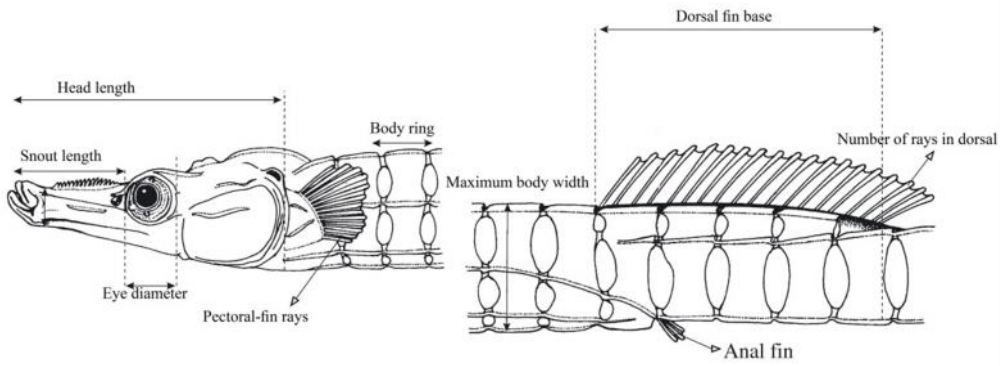

Figure 1: Some of the morphometric and meristic characters described in Table 1 [27].

Figure 2: (A) *Syngnathus abaster*, (B) Brood pouch, (C) Embryo, (D) Eggs, (E) Ovary, (F) Bowel.

The mitochondrial cytochrome oxidase I (COI) region of 4 samples was successfully amplified using PCR, but 1 sample failed. We did not find any sequences of *S. abaster* in GenBank and BOLD, which reveals a short coming in these databases for *S. abaster* species. As shown in Table 2, 652 bp of COI consensus barcodes for each species were treated as discrete units to estimate the pairwise level of genetic divergence using the Kimura 2-parameter (K2P) correction model [28].

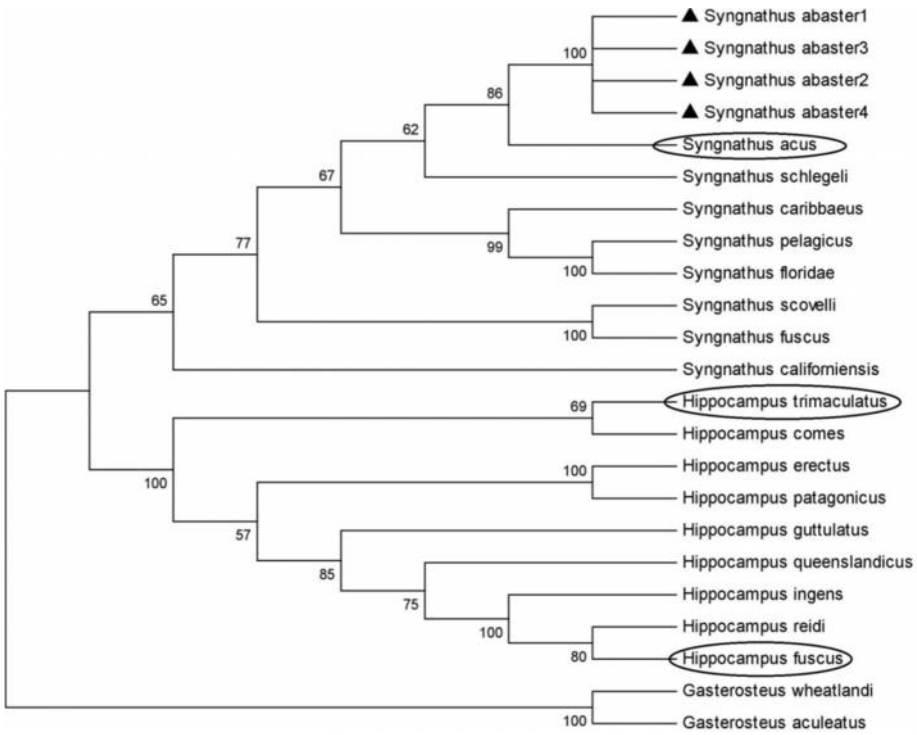

Figure 3: Phylogenetic consensus tree of Syngnathidae species constructed with 652 nucleotide of COI gene using NJ Method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [26]. *Gasterosteus wheatlandi* and *G. aculeatus* were used as out group [28].

The K2P distance between species ranged from a low 0.21% (*H. reidi* and *H. fuscus*) to a maximum value of 26.4% (*H. guttulatus and S. pelagicus*). Maximum and minimum K2P distance was found between *S. abaster* with *H. erectus* (26%) and *S. acus* (11.5%), respectively. According to the Neighbour-joining (NJ) tree (Fig. 3), the species in the present study were clustered independently. The NJ tree (Fig. 3), was consistent in defining the separation between S. *abaster* and other species, whose clusters were supported by the high bootstrap values.

Table 2: Estimates of Pairwise Genetic Distances between Syngnathidae Species under Kimura 2-Parameter Model [24].

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	*S. abaster*	0.000																	
2	*S. pelagicus*	0.172																	
3	*S. scovelli*	0.183	0.177																
4	*S. fuscus*	0.187	0.189	0.092															
5	*S. schlegeli*	0.143	0.141	0.153	0.165														
6	*S. floridae*	0.168	0.024	0.171	0.187	0.145													
7	*S. acus*	0.115	0.136	0.173	0.187	0.117	0.132												
8	*S. californiensis*	0.191	0.208	0.194	0.208	0.173	0.196	0.185											
9	*S. caribbaeus*	0.162	0.102	0.144	0.167	0.123	0.096	0.140	0.181										
10	*H. trimaculatus*	0.242	0.233	0.221	0.245	0.200	0.218	0.211	0.211	0.204									
11	*H. erectus*	0.260	0.246	0.239	0.241	0.228	0.239	0.239	0.230	0.224	0.138								
12	*H. reidi*	0.237	0.244	0.232	0.257	0.215	0.244	0.242	0.221	0.213	0.129	0.128							
13	*H. queenslandicus*	0.236	0.238	0.247	0.261	0.216	0.234	0.220	0.214	0.206	0.137	0.109	0.076						
14	*H. patagonicus*	0.249	0.228	0.235	0.219	0.207	0.226	0.220	0.228	0.211	0.145	0.069	0.132	0.122					
15	*H. ingens*	0.242	0.241	0.228	0.246	0.212	0.239	0.239	0.214	0.204	0.127	0.124	0.030	0.076	0.138				
16	*H. guttulatus*	0.248	0.264	0.254	0.250	0.229	0.258	0.259	0.218	0.228	0.125	0.139	0.101	0.097	0.143	0.097			
17	*H. comes*	0.246	0.230	0.225	0.230	0.228	0.223	0.221	0.239	0.215	0.107	0.151	0.124	0.126	0.136	0.120	0.125		
18	*H. fuscus*	0.237	0.244	0.230	0.250	0.219	0.237	0.240	0.219	0.215	0.131	0.126	0.021	0.082	0.136	0.025	0.101	0.122	0.000

DISCUSSION

So far, only few studies have concerned themselves with morphometric and meristic analyses of *S. abaster* populations. Pipefish morphometric and meristic studies focused on head features, except for Cakić et al (2002) who studied morphometric characteristics mainly related to head and mouth features. Although *S. abaster* is not economically important, it is significant from the aspect of ichthyofuna conservation and overall fish diversity. Information about *S. abaster* is very rare [29], and further and more complex investigations combining its morphological and genetic research are required. The average total length and standard length ± S.D were 102.37±2.38 and 98.68±2.70 (mm), respectively. Cakic et al (2002) reported average total length and standard length ± S.D to be 107.1±2.00 and 103.8±1.91 (mm), respectively. In the present study, morphometric and meristic variations between *S. abaster* and *S. acus* are demonstrated. The mean mouth and head width values of *S. abaster* (mean mouth width: 8.61, mean head width: 18.33) were found to be greater than *S. acus* (mean mouth width: 1 .04, mean head width: 9.51), *Hippocampus trimaculatus* (mean head width: 28.42) and *Hippocampuc fuscus* (mean hHead width: 33.38). The differences in the morphological and meristic characteristicss of specimens is related to the aquatic ecosystems from which they originated [9, 30]. The present results showed that the Gorgan Gulf population of *S. abaster* is not significantly different in morphometric and meristic characters from other studies on *S. abaster* [9, 31]. The results showed that the *S. abaster* was significantly different in some morphometric characters and meristic characters from *S. acus*, *H. trimaculatus* and *H. fuscus* [21, 32].

Fast access to biodiversity information is critical [33] and DNA barcoding can help us make informed decisions about the management of their remaining populations. The black-striped pipefish is one of the rarest fish in Iran. Habitat degradation and decline of populations has caused the risk of extinction in the Caspian Sea, requiring accurate, up-to-date information to deliver appropriate conservation actions [33, 34]. Molecular species identification using DNA barcoding has been applied successfully elsewhere, but techniques and consensus barcodes had not been developed and validated in Black-striped

pipefish. In this study, we have sequenced the COI region of the mitochondrial DNA to create a set of barcode sequences used to identify Black-striped pipefish. We compared our results to other species in BOLD and NCBI GenBank databases records, where no records regarding *S. abaster* were found. In this study, the first sequences of COI gene were identified for the Black-striped pipefish. Since the Black-striped pipefish is endangered in the Caspian Sea, genetic and morphometric analyses will give us a more accurate picture of the future persistence of its populations.

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