

IDENTIFICATION OF *AWAOUS GUAMENSIS* AND *A. GRAMMEPOMUS* BASED ON MORPHOLOGY AND MOLECULAR ANALYSIS

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Abstract

Work on two freshwater Gobi fish species such as *Awaous guamensis* and *A. grammepomus* was carried out on morphometric and meristic study for their morphological identification. Sequence data of mitochondrial cytochrome oxidase subunit I (COI) gene was used for this purpose. Around 620 bp sequences were obtained when universal primers were used. An alignment was performed between the sequences which showed some inter-specific variation. An entropy plot was generated for all the aligned positions which showed that the entropy rarely touches a scale of one, which is a sign of better alignment in the region. This work can be utilized for species identification, phylogenetic analysis, to study molecular diversity and evolution, and most importantly for the conservation of these fauna in near future.

Introduction

Bangladesh enjoys enough advantages being situated at the foot of the Himalayas, influenced by the monsoons and inundated by numerous rivers and streams. The hilly areas with diverse topographic relief and cool waters and remarkable areas of hill streams are predicted to be biodiversity hotspots in Bangladesh. The country has vast fishery potentials within her boundaries and the territorial and economic zones in the sea which offer excellent scope for pisciculture. Thus the nation's total area of waters having fish production potential is very great. The wetlands of Bangladesh are interplay of social, environmental, resource management and developmental concerns⁽¹⁾. All the identifications, description, and taxonomic or systematic literature exist on Bangladesh fishes based on the morphometric characteristics and most of the information is based on the non-vouchered information. Most of the freshwater fishes have not been identified or characterized based on molecular data. The molecular characterization of fishes is urgently needed for their proper identification and conservation. Therefore, the present study is aimed primarily at the molecular identification of Gobi fishes of Bangladesh.

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DNA barcoding is a systematic tool for species identification which provides an accurate information and fills the conceptual gap between traditional taxonomy and different fields of molecular systematics. The rationale behind DNA barcoding originates from the accumulation of evidence during the last two decades, as a consequence of an increasing access to genomic data, that the characterization and documentation of biodiversity using phenotypes are currently bridled by several limits inherent to morphological characters. The application of cytochrome c oxidase I (COI) gene for species identification in fish triggered the international initiative for barcoding all fishes⁽²⁾. The encouraging results have prompted international efforts to standardize screening of species diversity and to accelerate the process of cryptic species identification. Gobiidae is one of the largest families of teleost fishes, with nearly 2000 extant species currently recognized, distributed all over the world, consists of poorly explored freshwater fishes of Bangladesh. In the case of Perciformes fishes, it is difficult to differentiate the species because of the similarity in external morphology. The current study highlighted the use of COI gene as a genetic marker for the identification of morphologically similar species. This study will be useful for *Awaous* sp. identification, and most importantly for the conservation of these species in near future.

Materials and Methods

Two specimens of two freshwater Gobi fish species (Fig. 1) were collected from different regions of Bangladesh. Tissues from these samples were preserved in 95% ethanol for molecular study and stored at -20°C . Apart from this, the whole samples were also preserved in formalin as voucher specimen for further study.

The isolation of genomic DNA from the fish samples was carried out by CTAB method⁽³⁾. For this purpose, the muscle tissues were homogenized in 500 μl extraction buffer containing 100 mM NaCl, 50 mM Tris-HCl (pH 8.0), and 10 mM EDTA, followed by digestion in 10 μl of proteinase K to a final concentration of 0.4 mg/ml at 55°C for one day. By the phenol: chloroform extraction method, DNA was purified. Ethanol was precipitated with 0.1 vol of 3M sodium acetate and 2 vol of absolute ethanol at -20°C for one day. The DNA pellets were washed with 70% ethanol and allowed to dry at 37°C for 6 to 10 min. Thus the prepared DNA was dissolved in 100 μl TE buffer and stored at -20°C until used. Finally, the extracted DNAs were electrophoresed on a 1.0% low-melting-temperature agarose gel and stained with ethidium bromide for band characterization using ultraviolet transillumination⁽⁴⁾.

Extracted DNAs of the samples were amplified using PCR. DNA amplification of MT-COI gene was carried out in Thermo Cycler by using the modified universal forward⁽⁵⁾ and reverse⁽⁶⁾ primers (Table 1) of freshwater fish. Around 650 bp of the COI gene was amplified by using these primers. A mixture of 25 μl solution containing master mix (1x Invitrogen Platinum Taq Buffer, 0.25 mM each of deoxynucleotide

triphosphate (dNTPs), 2.0 mM MgCl₂, and 0.5 units of Taq DNA polymerase), 10 pmol of each primer, 100 ng of genomic DNA). The reactions were conducted using a Thermal Cycler under the following conditions: an initial denaturation at 95°C for 6 min; 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 40 s; and concluded with a final elongation step of 72°C for 10 min followed by a hold at 4°C. For PCR, a negative control containing double distilled water instead of DNA was included. Electrophoresis of amplified DNA products was conducted with 1.0% agarose gel in 1X electrophoresis buffer and visualized under ultraviolet (UV) light after staining with 5 µl ethidium bromide. A 100 bp DNA ladder (Bioron, Germany) was used to estimate sizes of the products.



Fig. 1. Gobi fishes (a) *Awaous guamensis* (Valenciennes, 1837) (Scribbled Goby) and (b) *Awaous grammepomus* (Bleeker, 1849) (Streaked River Goby).

Table 1. Primers used in this study.

Primer name	Sequence (5 → 3)	Reference
CO1 F	GGTCAACAAATCATAAAGATATTGG	Folmer <i>et al.</i> 1994 ⁽⁵⁾
CO1 R	TAGACTTCTGGGTGGCCAAAGAATCA	Ward <i>et al.</i> 2005 ⁽⁶⁾

PCR products were purified using the PCR Cleanup kit. After purification they were sent to Centre for Advanced Research in Science (CARS), University of Dhaka for sequencing. The sequencing was carried out by Sanger Sequencing method.

To determine the highest homolog, sequences were identified by using BLAST search at NCBI. SEQUENCHER⁽⁷⁾ and BIOEDIT⁽⁸⁾ were used for sequence analysis. Sequences were aligned using CLUSTAL W⁽⁹⁾. This alignment shows both similarity and dissimilarity between the sequences. An Entropy Hx plot was drawn by BioEdit software to show variability between the sequences.

Results and Discussion

Two species of Gobi fish were identified morphologically by using taxonomic keys⁽¹⁰⁾. A morphometric and meristic study was performed comprised of average body, head measurement and average number of fin/spines measurements (Table 2). However, sometimes morphological study is not enough for identification. Species identification by morphological features is sometime ineffective and misleading, because, larval stages of some species groups often cannot be assigned to the correct species⁽¹¹⁾. Besides, the morphological identification is more complicated when the species are damaged due to rough handling, and there may have chances for fish fraud⁽¹²⁾.

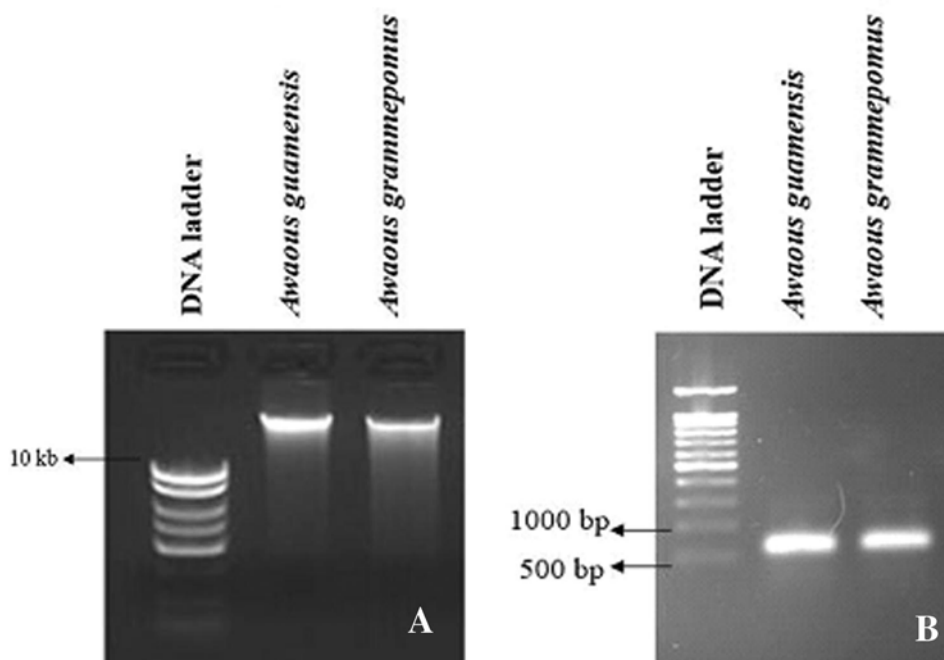


Fig. 2. Images of agarose gel electrophoresis for extracted DNA of *A. guamensis* and *A. grammepomus* (A), COI primer based PCR (B).

Total genomic DNA of both the species were isolated (A of Fig. 2) and were amplified successfully through polymerase chain reaction (PCR). One per cent AGE showed PCR products of COI gene of about 650 bp for *A. guamensis* and *A. grammepomus* (B of Fig. 2). In the present study, for both the species, universal primers for partial COI gene showed satisfactory results whereas in some cases universal primers are not enough for species identification and species-specific COI primer is required as a study showed that the success of the amphibian species-specific primers (78%) had an increase of 46% over the universal primers⁽¹³⁾.

Table 2. Morphological characteristics of Gobi fishes.

Species	Body measurements (average)					Head measurements (average)				
	TL (cm)	SL (cm)	PDL (cm)	BD (cm)	PD (cm)	HL (cm)	ED (mm)	Pr.OL (mm)	Po.OL (mm)	IOL (mm)
<i>Awaous guamensis</i>	12.5	8.7	1.4	1.9	1.2	3.2	0.4	8.7	1.4	0.5
<i>Awaous grammepomus</i>	11	8.2	1.3	1.5	1.2	3.0	0.3	7.8	1.4	0.4

Table contd. (right side).

Average fin base length (mm)					Average number of fin rays/spines					
DFB		P1FB	P2FB	AFB	DFR			P1FR	AFR	CFR
1st	2nd				D1 FS	D2FS	D2FR			
0.3	3.3	0.7	2.4	1.4	7	1	10	16	10	13
0.3	3.2	0.6	2.2	1.4	6	1	10	15	10	13

For the above mentioned COI primer, both the samples were sequenced in both directions, with an average DNA quality of 98% (after trimming) and an average sequence length of 620 bp. Values of overall sequence quality were calculated by the Sequencher 4.10.1 program.

Blast identified both the species accurately to species level and showed 99% of similarity to the existing sequences of particular species in the NCBI Genbank database. A BLAST pairwise circular tree was drawn (Fig. 3) by fast minimum evolution tree method which showed the existence of same ancestry for both the species as taxonomy refers.

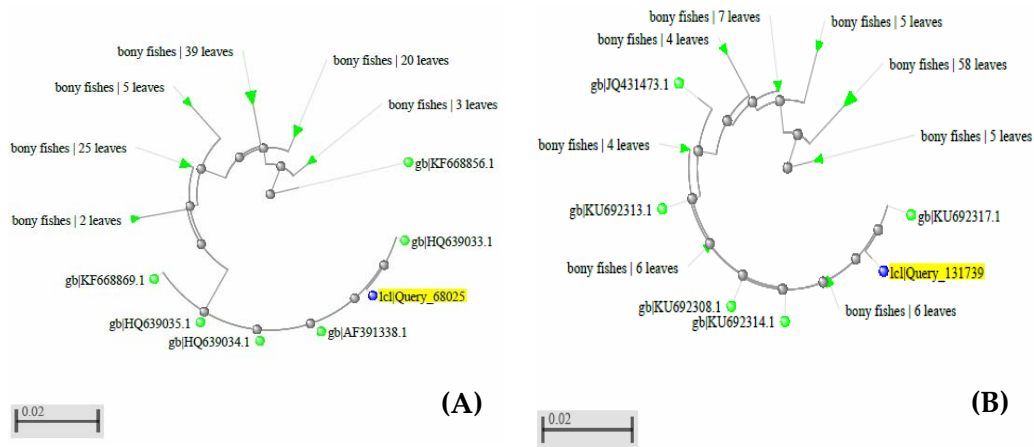


Fig. 3. BLAST search result showing BLAST pairwise tree (Fast minimum evolution tree method) for *A. guamensis* (A) and *A. grammepomus* (B).

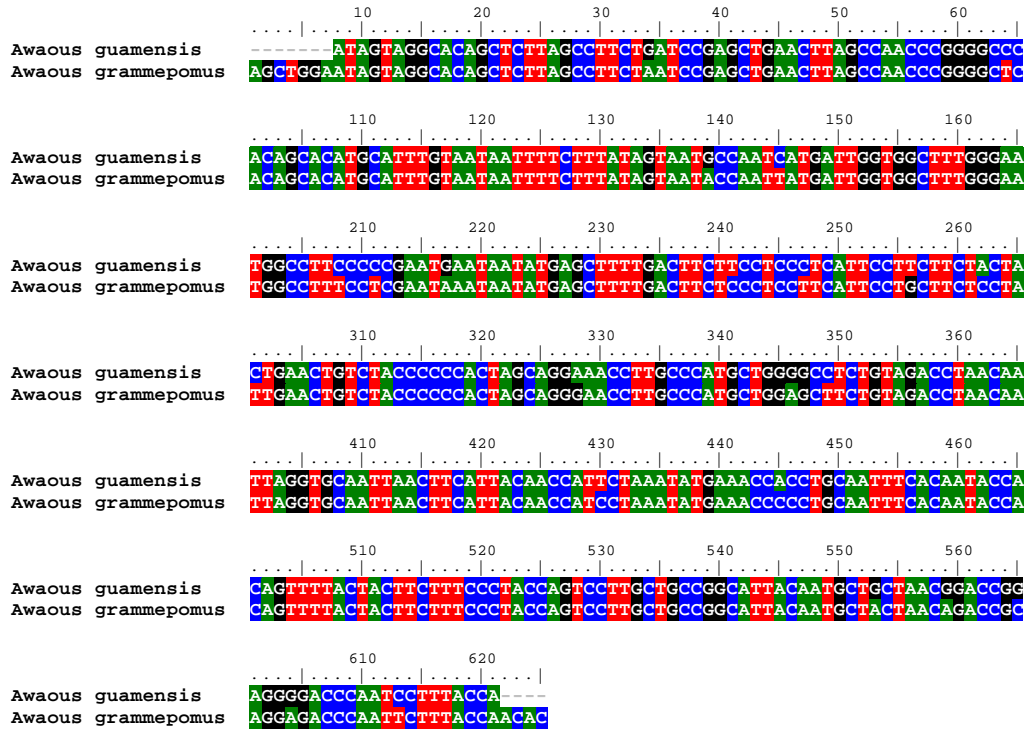


Fig. 4. Interspecific alignment pattern between *A. guamensis* and *A. grammepomus*.

Interspecific comparison can classically be defined as the comparison of gene sequences between different organisms of the same genus to identify the regions of similarities and dissimilarities⁽¹⁴⁾. In this study, the interspecific variation of partial

regions of mitochondrial COI gene between *A. guamensis* and *A. grammepomus* from Bangladesh was studied. Sequences were aligned using Bioedit and 19 polymorphic sites were observed (Fig. 4) within a total of 614 base pairs. The percentage of polymorphic site is 3% which shows the genetic diversity between two closely related species. Genetic diversity in the wild is crucial for the culture of farmed populations.

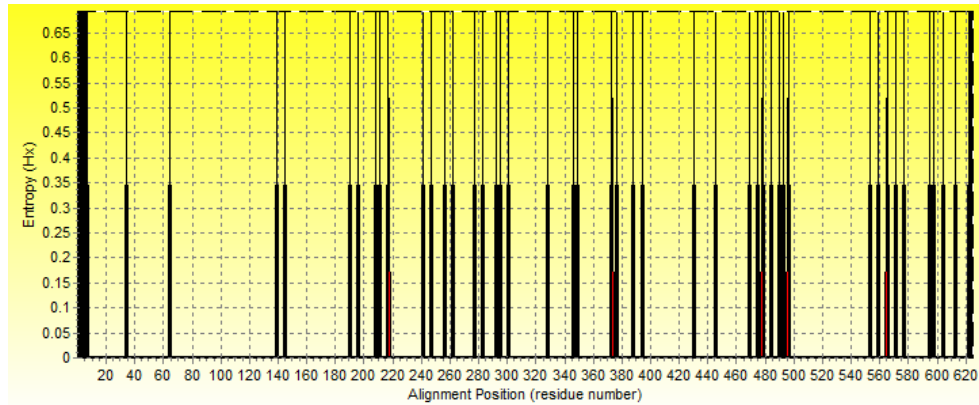


Fig. 5. Entropy Hx plot.

An entropy plot can give an idea of the amount of variability through a column in an alignment⁽⁴⁵⁾. In this study, an entropy plot (Fig. 5) was presented for all the aligned positions which shows that the entropy barely touches a scale of one and in most of the positions it is below 0.35, which is a sign of better alignment in the region.

Conclusion

Gobies are cryptic bottom-dwelling carnivores and poorly explored in the freshwater of Bangladesh. Despite the spectacular variation and commercial importance of Gobi fishes, morphological and molecular data of this group from Bangladesh is still insufficient. These cryptic species can not be identified accurately by morphology based taxonomy. So, the use of COI gene as a genetic marker could be a better choice for the morphologically similar species identification. Besides, in recent years, species have been declining rapidly due to overfishing, pollution and other environmental disturbances. Therefore, proper emphasis should be given to conserve these species for a better understanding of the genetic diversity of wild populations.

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