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GENETIC CHARACTERIZATION OF SWAMP EEL OF BANGLADESH THROUGH DNA BARCODING AND RAPD TECHNIQUES

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Abstract

The freshwater air-breathing swamp eel *Monopterus* spp. are native to the freshwater of Bangladesh and throughout the Indian subcontinent. To identify the different swamp eel species and to check the genetic diversity among them, a total of twelve swamp eel specimens were collected from four districts (Tangail, Bogura, Bagerhat and Sylhet) representing the four division of Bangladesh. The extracted DNA from twelve fish samples was amplified by the PCR technique for DNA barcoding and RAPD analysis. Among 12 specimens, 8 specimens showed a 95-100% similarity with *M. cuchia* species published in the NCBI GenBank database and BOLD system. The studied mct3 (collected from Tangail region), mcs1, mcs2 and mcs3 (collected from Sylhet region) specimens showed about 83% homology with *Ophisternon* sp. MFIV306-10 as per BLAST search; whereas BOLD private database showed 99% similarity with *Ophisternon bengalense* (Bengal eel). From the phylogenetic tree analysis, 8 samples were clustered with *M. cuchia* and 4 samples showed similarity with *Ophisternon* sp. MFIV306-10 and *Ophisternon bengalense* _ANGBF45828-19. In RAPD-PCR based analysis, it was found that the maximum genetic distance (1.6094) was observed between mcba2 and mcs3, while between mct1 and mct2, the minimum genetic distance was 0.000. A total of 192 bands, of which 35 were polymorphic with 17.88% polymorphisms among swamp eel species and the size of the amplified DNA fragments ranged from 250 to 1800 bp. The information on DNA barcoding and RAPD analysis help measure the genetic diversity among swamp eel species, ensure the reliability of the published taxonomic information, and initiate proper management programs to conserve these vulnerable species to meet future export demand.

Key words: DNA barcoding, RAPD, *Monopterus* spp., *Ophisternon* spp.

Introduction

The freshwater air-breathing swamp/mud eel *Monopterus* spp. belong to the family synbranchidae of the order synbranchiformes (Rosen and Greenwood 1976, Shafi and Quddus 1982). It commonly inhabits in the freshwater of Bangladesh and throughout India (Talwar and Jhingran 1991). The freshwater mud eel, *Monopterus cuchia* is a delicious, tasty, nutritional and economically valuable fish. In Bangladesh, only the tribal

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people consume this fish but nowadays, it is a commercially exportable product because of its high demand worldwide (Begum *et al.* 2018). These hardy, pollution resistant species are carnivorous, nocturnal and like animal-based foods, such as small fishes, mollusks and worms (Nasar 1997). This species can also adapt to various adverse situations like low oxygen concentration, high temperature and shallow water (Rahman 2005). *Ophisternon* spp. is known as swamp eel belongs to the family of synbranchidae. Species of this family can breathe in the air, making them capable of surviving in low-oxygenated water and moving between ponds during the rainy season. Swamp eels are naturally distributed throughout India, Sri Lanka, Indonesia, Philippines and New Guinea (Roy *et al.* 2016). *Monopterus* are exported every year from Bangladesh to different countries like China, Japan, South Korea, Malaysia, Hong Kong, Thailand, Europe and earn 14 million US dollar in 2014 (DoF 2016). It is now over exploited from the natural water-bodies mainly for being exported (Begum *et al.* 2018). The swamp eel *viz. M. cuchia* and *O. bengalense* have been enlisted in the red list of threatened fishes of Bangladesh as a vulnerable (IUCN 2015). Therefore, it is very much important to identify the fish species accurately and unambiguously for conservation purposes. Genetic data are very important in assessing the gene flow between populations, which are also critical for maintaining genetic diversity. DNA based technologies have been recognized useful for their application in species identification (Palumbi and Cipriano 1998), monitoring fisheries (Menezes *et al.* 2006) and aquaculture (Liu *et al.* 1998). Genetic data can also be interpreted in such a way to set up conservation priorities. In many instances, genetics may be the best way to decide whether the species need conservation actions under the vulnerable category. Genetic data of threatened species can help to decide restocking of the species through translocation (Teske *et al.* 2003) by implementing a planned program based on quantitative genetics, life history data and DNA variation (Epifanio 2003). DNA barcoding is an identification tool in which a small fragment of mitochondrial genome acts as a 'DNA barcode' to identity an organism at its species level. DNA barcoding is suitable because the intra-species variations are lesser than inter-species variations (Peninal *et al.* 2017). DNA barcoding has the advantage of identifying the species from any sort of sample like whole fish, fillets, fins, juveniles, larvae, eggs, or tissue fragments. DNA barcoding can be a practical tool for the reliable identification of specimens for solving taxonomic problems and providing an in-depth analysis of gene flow (Smith *et al.* 2008).

Among DNA based systems, the RAPD-PCR procedure is simple and fast. Without prior information on DNA arrangements, it is conceivable to distinguish hereditary variety utilizing RAPD strategy. The technique, also known as arbitrary primer-polymerase chain

reaction (AP-PCR), has been widely used for revealing the intraspecific variation (Basagoudanavar *et al.* 1998). We studied the genetic diversity and relationship among the swamp eel specimens collected from the four natural habitats of Bangladesh (Tangail, Bagerhat, Bogura and Sylhet). The present study was aimed at the molecular identification and characterization of swamp eel species lives in the natural water bodies of different regions of Bangladesh based on DNA barcoding and RAPD techniques.

Materials and Methods

Twelve specimens of swamp eel were collected from four districts representing four divisions of Bangladesh *viz*., Bogura of Rajshahi division, Tangail of Dhaka division, Sylhet of Sylhet division, Bagerhat of Khulna division and were subjected to molecular study. Fishes were examined while still fresh. Total length (TL) and weight were measured. The primary identification was made according to Shafi and Quddus (1982) and Talwar and Jhingran (1991).

Fresh muscle samples were collected and preserved at -20^{0}C. DNA from the muscle of 12 swamp eel specimens was extracted using a commercial kit (Maxwell 16 MDx Research Instrument, Promega, USA) according to manufacturer's instruction. The absorbance of purified DNA by NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., USA) was checked at 260 nm. Extracted DNA used as a template for PCR amplification of a 650 bp fragment from the 5´ region of COI gene using the forward and reverse primer FishF1 (TCAACCAACCACAAAGACATTGGCAC) and FishR1 (TAGACTTCTGGGTGGCC AAAGAATCA) (Ward *et al.* 2005) and the PCR reaction mix for 25 µl contained GoTaq® G2 Hot Start Green Master Mix, 2X 12.5 µL, forward primer 1 µl, reverse primer 1 µl, DNA template 1 µl, nuclease free water 9.5 µl and thermal cycling conditions were followed as reported by Wong and Hanner (2008). Amplified DNA was then purified using Wizard PCR SV Gel and PCR Clean-Up System kit (Promega, USA) prior to sequencing. Sequencing of purified PCR products was performed through commercial service of the First Base laboratory, Malaysia.

COI sequences were identified by searching the GenBank database using the BLASTN algorithm (https://blast.ncbi.nlm.nih.gov) and by BOLD (http://www.boldsystems.org) identification engine to search DNA barcode records within BOLD (Ratnasingham and Herbert 2007). A Neighbor-Joining method (Saitou and Nei 1987) was used to generate tree in MEGA X (Kumar *et al*. 2018) software to observe the phylogeny among the studied specimens.

Among 10 primers tested for RAPD amplification, six primers [Operon Technologies Inc., USA (5) and University of British Columbia, Canada (1)] named OPA12 (TCGGCGATAG), OPC5 (GATGACCGCC), OPH4 (GGAAGTCGCC), OPG3 (AGTCGGCCCA), OPY7 (AGAGCCGTCA) and UBC4 (CCTGGGCTGG) exhibited good quality banding patterns and sufficient visibility. The bands of these six primers were further considered for the final RAPD analysis.

The PCR reaction mix for 25 µl contained Nuclease-Free Water 14, GoTaq® G2 Hot Start Green Master Mix 2X 7.5 µl, primer, 10 µM 1.5 µl, template DNA 2 µl. PCR amplification was done in an oil-free thermal cycle for 40 cycles after initial denaturation at 95^0C for 5 min, denaturation at 95^0C for 30 s, annealing at 34^0C for 30 s, extension at 72^0C for 1 min and a final extension at 72^0C for 5 min.

DNA bands were observed on UV-trans illuminator and photographed by a gel documentation system, after 1% agarose gel electrophoresis. The photographs were critically analyzed on the basis of presence (score 1) or absence (score 0) of the band, band size and overall polymorphisms of the bands (Kabir *et al.* 2017). The value of pairwise genetic distances was analyzed by using computer software "POPGENE 32" (version 1.31) among studied specimens of swamp eel of four geographically different regions from the data of six RAPD primers.

Results and Discussion

A total of twelve samples were collected from four different regions of Bangladesh, and the total length (TL) and the weight of the fish samples were measured (Table 1).

The extracted DNA from twelve fish samples was amplified by the PCR technique for Cytochrome c Oxidase subunit 1 (COI) gene through COI specific primers FishF1 and FishR1. All the samples showed a positive band at 650 bp (Fig 1).

The resulting PCR products were sequenced to generate full-length DNA barcodes of 678 bp in length, with no detectable insertions, deletions. Table 2 provides information about *viz.* maximum score, percentage of query coverage, E value, percentage identity, NCBI GenBank accession number, BOLD accession number of the matched sequences and the GenBank accession number of our submitted sequences of 12 swamp eel specimens.

Among 12 samples, 8 samples showed 95-100% homology with *M. cuchia* species that were already deposited in NCBI GenBank database. The studied mct3 (collected from Tangail region), mcs1, mcs2 and mcs3 (collected from Sylhet region) specimens showed 83% homology with *Ophisternon* spp. Four (mct3, mcs1, mcs2 and mcs3) were matched

with *Ophisternon bengalense* ANGBF45828-19 under BOLD private database whereas mct1, mct2, mcbo1, mcbo2, mcbo3, mcba1, mcba2 and mcba3 were matched with *M. cuchia* BOLD accession number AAF8878. Finally, among 12 specimens, eight samples showed their genetic identity with *M. cuchia* known as freshwater mud eel and four samples showed similarity with *Ophisternon bengalense* known as Bengal eel or Asian swamp eel.

Table 1. Length-Weight measurements of swamp eel specimens collected from four different regions.

SI	Collection place	T.L (cm)	Weight (gm)
1-3	Tangail	41	60
		66	250
		56.5	350
4-6	Bogura	58.5	510
		61	520
		60	350
7-9	Bagerhat	51	490
		25	170
		56	500
10-12	Sylhet	45	250
		56	430
		53	360

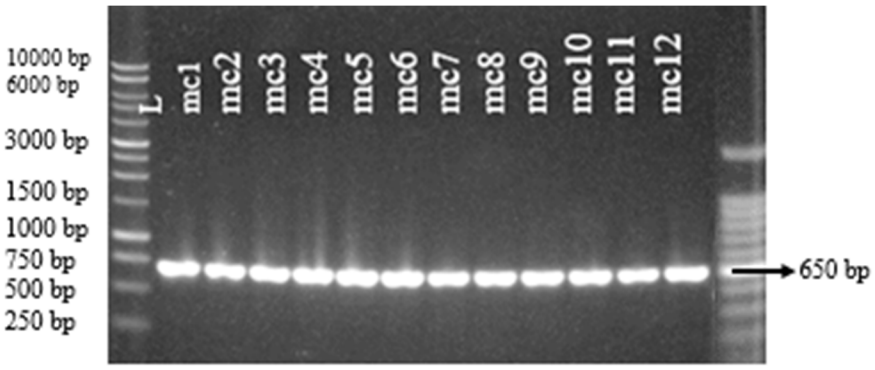

Fig. 1. PCR amplification of COI gene using FishF1 and FishR1 primers. L denotes 1 kb ladder.

COI gene sequence of mct1, mcbo1, mcba1 were compared with available sequences from the GenBank database and it has been found that these specimens were *M. cuchia.* After comparing these sequences with *M. cuchia* NBFGR: MC8069B_FJ4595, 37 (6.7%) out of 555 nucleotide bases found polymorphic (Fig. 2).

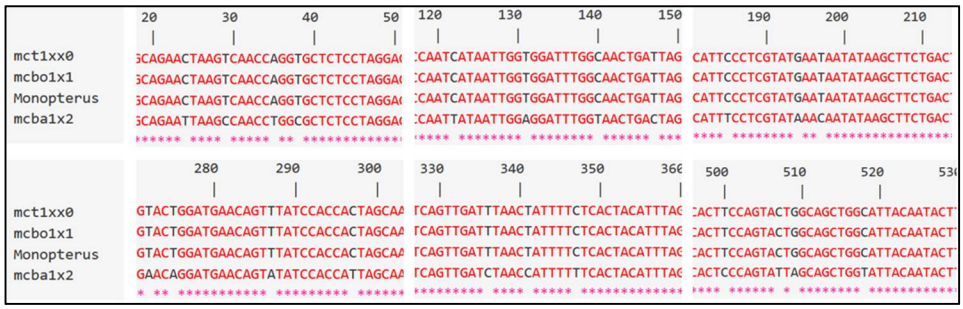

Fig. 2. Portion of Multiple Sequence Alignment (MSA) of COI gene fragment of swamp eel sample mct1, mcbo1, mcba1 and *M. cuchia*_NBFGR_MC8069B_FJ4595. Black nucleotides among the red indicate polymorphic sites.

A comparison of COI sequence of mct3, mcs1, mcs2 and mcs3 with available sequence from the GenBank database reflects that these specimens were under the genus *Ophisternon*. After comparing these sequences with *O. bengalense*_ANGBF45828-19, 74 (14.15%) out of 523 nucleotide bases found polymorphic (Fig. 3).

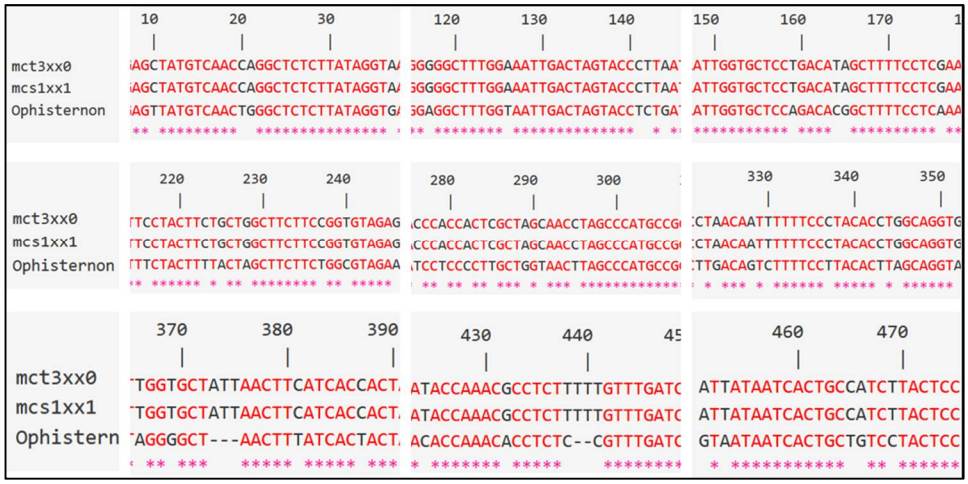

Fig. 3. Portion of Multiple Sequence Alignment (MSA) of COI gene fragment of swamp eel samples mct3, mcs1 and *O. bengalense*_ANGBF45828-19. Black nucleotides among the red indicate polymorphic sites.

Table 2. Identification of swamp eel specimens using the GenBank database and BOLD systems based on 678 base pairs of COI gene sequences.

Sl No.	Specimen ID	Study area	Description	Max Score	Query coverage (%)	E value	Identity (%)	GenBank Acc. no.	BOLD Accession	GenBank Acc. no. of studied sequence
1	mct1	Tangail	*M. cuchia* voucher M6	1026	100	0.0	100	MN520010.1	AAF8878	MT387298
2	mct2	Tangail	*M. cuchia* voucher M6	1026	100	0.0	100	MN520010.1	AAF8878	MT387299
3	mct3	Tangail	*Ophisternon bengalense* ANGBF45828-19	99.27% similarity as per private BOLD database				KP729589.1 unverified	ANGBF45828-19	MT387300
			Ophisternon sp. MFIV306-10	616	96	7e-172	83.72	JN313622.1	-	
4	mcbo1	Bogra	*M. cuchia* voucher M6	1026	100	0.0	100	MN520010.1	AAF8878	MT387301
5	mcbo2	Bogra	*M. cuchia* isolate B-3	1026	100	0.0	100	MN520004.1	AAF8878	MT387302
6	mcbo3	Bogra	*M. cuchia* isolate *B*-3	1026	100	0.0	100	MN520004.1	AAF8878	MT387303
7	mcba1	Bagerhat	*M. cuchia* IL15-0035	854	100	0.0	94.41	LC190133.1	AAF8878	MT387304
8	mcba2	Bagerhat	*M. cuchia* IL15-0035	854	100	0.0	94.59	LC190133.1	ADE0718	MT387305
9	mcba3	Bagerhat	*M. cuchia* NBFGR:MC8069	1026	100	0.0	100	FJ459510.1	AAF8878	MT387306
10	mcs1	Sylhet	*Ophisternon bengalense* ANGBF45828-19	99.45% similarity as per private BOLD database				KP729589.1 unverified	ANGBF45828-19	MT387307
			Ophisternon sp. MFIV306-10	621	96	2e-173	83.87	JN313622.1	-	
11	mcs2	Sylhet	*Ophisternon bengalense* ANGBF45828-19	99.45% similarity as per private BOLD database				KP729589.1 unverified	ANGBF45828-19	MT387308
			Ophisternon sp. MFIV306-10	621	96	2e-173	83.87	JN313622.1	-	
12	mcs3	Sylhet	*Ophisternon bengalense* ANGBF45828-19	99.27% similarity as per private BOLD database				KP729589.1 unverified	ANGBF45828-19	MT387309
			Ophisternon sp. MFIV306-10	616	96	7e-172	83.72	JN313622.1	-	

The phylogenetic tree (Fig. 4) indicates that mct1, mct2, mcbo1 are closely related to mcba3, mcbo2 and mcbo3 which confirming their similarity with the allocated species *M. cuchia.* On the other hand, the phylogenetic tree also supported the taxonomic position of mct3, mcs1, mcs2 and mcs3 as similar with the species under the genus *Ophisternon*; moreover, it can be said that these specimens are close to the species *Ophisternon bengalense*, but may be another species of the genus *Ophisternon* that is still not available in the GenBank or BOLD public database.

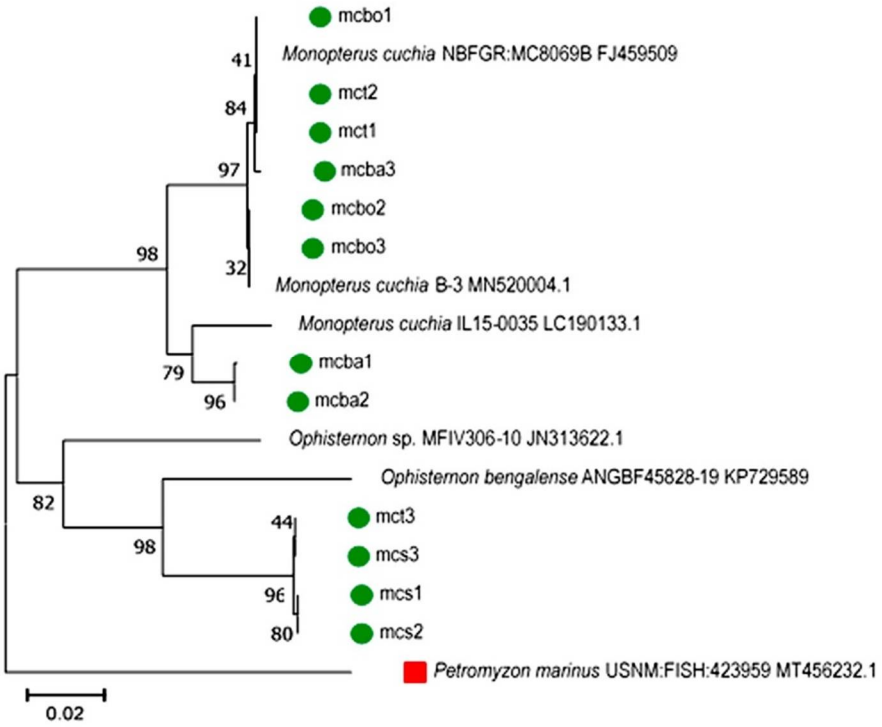

Fig. 4. Molecular Phylogenetic analysis by Neighbor-Joining method of studied 12 swamp eel sequences and downloaded 6 sequences from NCBI GenBank. *Petromyzon marinus* was used as an outgroup. The optimal tree with the sum of branch length =0.36912245 is shown. The analysis involved 18 nucleotide sequences. There were a total of 678 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al.* 2018).

RAPD based genetic diversity: DNA profiling and data scoring were studied separately for six primers that were used for RAPD analysis. The bands of six primers were seen in different levels of the length of DNA ranged from 250 to 1800bp. A total of 192 bands, of which 35 were polymorphic with 17.88 average percentage of polymorphisms among

species of swamp eel (Table 3). Primer OPH-04 gave the RAPD profile with the highest number of bands. The number and size of each unique band respective to each primer were shown in Table 3 and Fig. 5.

Table 3. Compilation of RAPD analysis data of swamp eel from four different locations of Bangladesh.

Primer code	Size ranges (bp)	Total bands	Number of polymorphic bands	Polymorphisms (%)	Average % polymorphism
OPA12	250-750	20	03	15.00	
OPY-07	375-1100	25	05	20.00	
OPG-03	300-1400	43	08	18.61	
OPH-04	250-1800	47	08	17.02	17.88
UBC-04	700-1000	12	02	16.67	
OPC-05	450-1100	45	09	20.00	
Grand total		192	35		17.88

The value of pair-wise genetic distances was analyzed by using computer software "POPGEME 32" (version 1.31). The genetic distances ranged between 0.0000 and 1.6094 (Below diagonal of Table 4) among 12 specimens of swamp eel. The maximum genetic distance (1.6094) was observed among mcs3 and mcba2, while between mct1 and mct2, the minimum genetic distance was 0.000.

After the analysis of RAPD fragments through computer software "POPGENE32" (version 1.31), the values of pair-wise genetic identity were ranged between 0.2000 and 1.000 (Above diagonal of Table 4). The highest genetic identity (1.000) was obtained between mct1 and mct2, while the lowest one (0.2000) was found between mcba2 and mcs3.

UPGMA based cluster analysis using linkage distance was done to reveal the phylogenetic relationships among twelve specimens of swamp eel genotypes examine under the current study (Fig. 6).

In the present study, UPGMA dendrogram showed that sample mct1 and mct2 were close to mcba3, whereas sample mcbo1, mcbo2 and mcbo3 were clustered together (Fig. 6). Specimen mcba1 and mcba2 were in the same cluster separated from other specimens. Sample mcs1, mct3, mcs2 and mcs3 formed a completely separate cluster away from

others that is also supported by the DNA barcode data as these four specimens identified as *Ophisternon* spp.

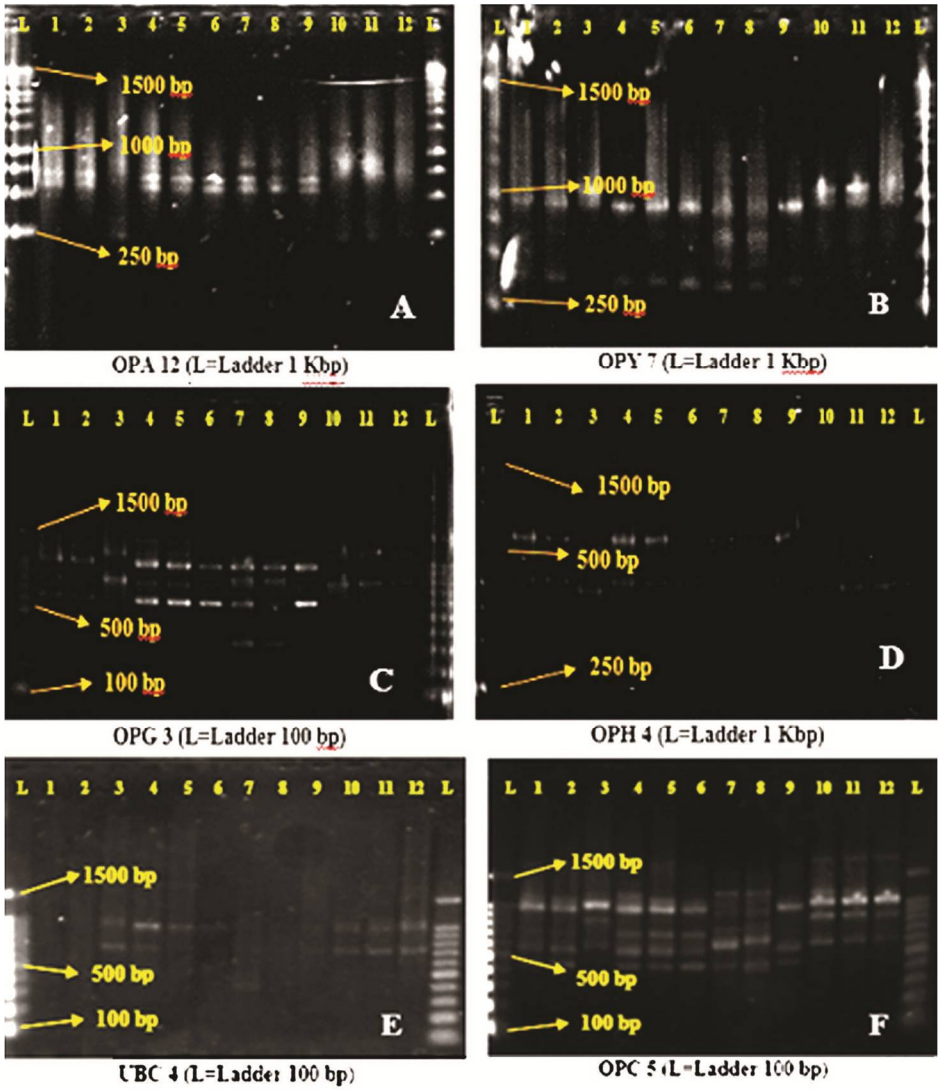

Fig. 5. RAPD analysis with six primers of twelve swamp eel samples. (Primers; A. OPA-12, B. OPY-07, C. OPG-03, D. OPH-04, E. UBC-04, F.OPC-05, where 1- 3=Tangail, 4-6=Bogura, 7- 9= Bagerhat and 10-12=Sylhet samples in order).

Table 4. Genetic distance and genetic identities among 12 samples of swamp eel collected from four different regions of Bangladesh.

POP ID	Sample ID	1	2	3	4	5	6	7	8	9	10	11	12
		mct1	mct2	mct3	mcbo1	mcbo2	mcbo3	mcba1	mcba2	mcba3	mcs1	mcs2	mcs3
1	mct1	***	1.0000	0.3429	0.7714	0.7714	0.8286	0.5143	0.5429	0.8286	0.3429	0.4000	0.3714
2	mct2	0.0000	***	0.3429	0.7714	0.7714	0.8286	0.5143	0.5429	0.8286	0.3429	0.4000	0.3714
3	mct3	1.0704	1.0704	***	0.4000	0.4000	0.4000	0.2571	0.2286	0.4000	0.8286	0.8857	0.9143
4	mcbo1	0.2595	0.2595	0.9163	***	0.9429	0.9429	0.5143	0.4857	0.7714	0.4000	0.4571	0.4286
5	mcbo2	0.2595	0.2595	0.9163	0.0588	***	0.9429	0.5143	0.4857	0.8286	0.4000	0.4571	0.4286
6	mcbo3	0.1881	0.1881	0.9163	0.0588	0.0588	***	0.5143	0.4857	0.8286	0.3429	0.4000	0.4286
7	mcba1	0.6650	0.6650	1.3581	0.6650	0.0588	0.6650	***	0.9714	0.5714	0.2571	0.2571	0.2286
8	mcba2	0.6109	0.6109	1.4759	0.7221	0.7221	0.7221	0.0290	***	0.6000	0.2286	0.2286	0.2000
9	mcba3	0.1881	0.1881	0.9163	0.2595	0.2595	0.1881	0.5596	0.5108	***	0.3429	0.4000	0.4286
10	mcs1	1.0704	1.0704	0.1881	0.9163	0.9163	1.0704	1.3581	1.4759	1.0704	***	0.8857	0.8571
11	mcs2	0.9163	0.9163	0.1214	0.7828	0.7828	0.9163	1.3581	1.4759	0.9163	0.1214	***	0.9143
12	mcs3	0.9904	0.9904	0.0896	0.8473	0.8473	0.8473	1.4759	1.6094	0.8473	0.1542	0.0896	***

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

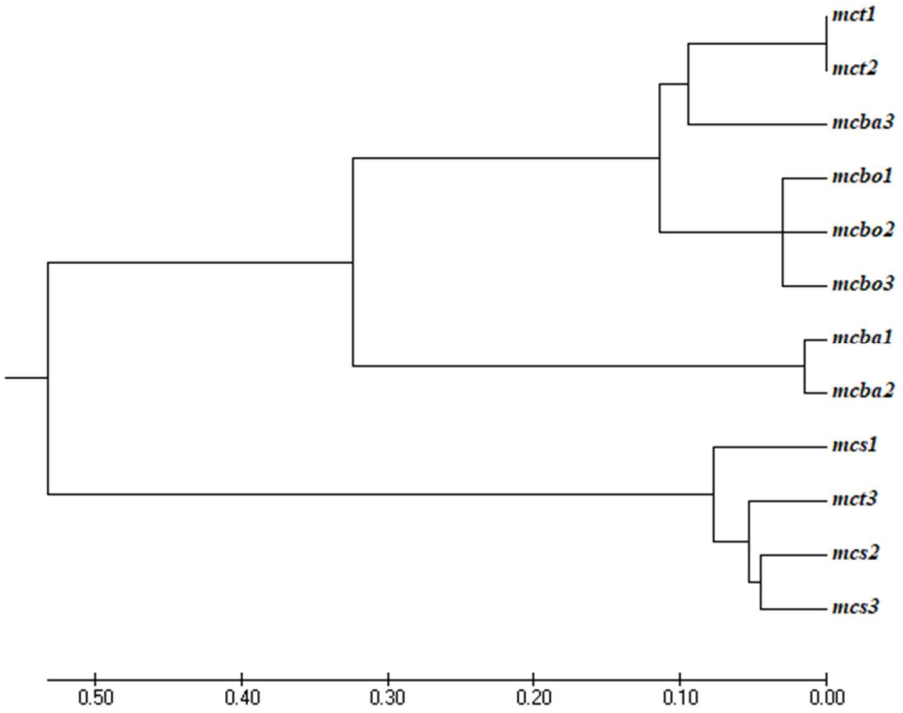

Fig. 6. UPGMA dendrogram based on RAPD analysis among 12 swamp eel samples. The dendrogram was constructed in MEGA7.

Bangladesh has vast water areas that are rich in fish and shellfish biodiversity (Rahman *et al*. 2016). Still many of our indigenous aquatic species are listed in the IUCN Red Book as endangered species. *Monopterus cuchia* and *Ophisternon bengalense* are red listed vulnerable species in Bangladesh (IUCN 2015). In the past, the identification of fish species was especially based on morpho-taxonomy (Kon *et al*. 2007). Expert taxonomists play an important role in the taxonomic classification of organisms and estimate species diversity, but this process is not enough to accurately identify the species. DNA barcoding provides rapid and accurate identification that can be used by the non-experts (Hebert *et al.* 2003). Lakra *et al*. (2016) worked on DNA barcoding of Indian freshwater fishes and observed the highest genetic distance (27.50%) between *Monopterus cuchia* and *Macrognathus pancalus*, which belongs to the order of Synbranchiformes. Java and Dasgupta (2007) worked on the morphometry of *M. cuchia* from the New Alluvial Zone of West Bengal. They concluded that the morphometric characters of *M. cuchia* showed a positive increase with the increase in the length of the fish. Matsumoto *et al*. (2010) used

mitochondrial DNA (mtDNA) sequencing to see the phylogenetic relationships and genetic diversity of *M. albus*. They found three genetically distinct clades based on geographical populations [China–Japan (Honshu + Kyushu), Ryukyu Islands, and Southeast Asia clades].

In this study, molecular identification of swamp eel species was performed by DNA barcoding. A total 12 samples were sequenced, among them eight samples were genetically identified as *M. cuchia* and four samples showed similarity with *Ophisternon bengalense* known as swamp mud eel. Four sequences *viz.* mct3, mcs1, mcs2 and mcs3 could not be identified in the public BOLD species reference database. Steinke *et al.* (2009) found that analysis of COI gene from 391 species from 8 coral reef locations revealed 98% of these species exhibit distinct barcode clusters, allowing accurate identification.

In RAPD analyses specimens of swamp eel produced different banding pattern with six primer combinations. The average polymorphism was about 17.88% revealing a low range of polymorphisms among the two populations. Miah *et al.* (2013, 2016) used three RAPD primers to see the genetic diversity of freshwater mud eel, *M. cuchia* and found 100% intra-specific polymorphism among swamp eel. Yin *et al.* (2005) evaluated the genetic variation of the wild and cultured swamp eels *M. albus* using RAPD technique and found 44.79% and 36.5% polymorphism, respectively. Studies on RAPD fingerprinting of two eel-loaches, *Pangio piperata* and *Pangio filinaris* estimated 83% and 60% polymorphism, respectively in 82 bands generated from five RAPD primers (Ruzainah *et al.* 2003). Wei *et al.* (2006) worked on five populations of rice field eel, *M. albus* in China mainland and found a low level of polymorphism (ranged from 29.51 to 66.39%). However, two other populations showed a high proportion of polymorphic loci (82.79%).

The study was conducted on the genetic characterization of swamp eel of Bangladesh through DNA barcoding and RAPD techniques. The swamp eel can meet the increasing demand for animal source protein in Bangladesh and earn foreign currencies that can help our national economy. The information on DNA barcoding to identify freshwater mud eel can help to know the genetic diversity of them, ensure the reliability of the published taxonomic information, and initiate proper management programs to conserve this vulnerable species. The diversity of mud eel of Bangladesh can expand our export limit, which may help in employment generation in the production and marketing of fisheries and aquaculture sectors.

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