

Research Article

MOLECULAR IDENTIFICATION AND PHYLOGENETIC ASSESSMENT OF SOME MARINE CATFISHES OF THE BAY OF BENGAL

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ABSTRACT

The present study was designed to analyze partial sequence of Cytochrome Oxidase subunit I (COI) gene for species identification and phylogenetic relationships among 6 species of catfish sampled from Cox's Bazar. The nucleotide sequences of COI consisted of about 652 nucleotide base pairs. The mitochondrial COI region of all samples was successfully amplified using PCR. The comprehensive barcoding identification results were based on GenBank databases. Database revealed definitive identity in the range of 96%-98% for consensus sequences of six species namely *Osteogeneiosus militaris*, *Netuma thalassinus*, *Plicofollis tenuispinis*, *Plicofollis polystaphylodon*, *Hemiaris sona*, and *Arius venosus*. Multiple sequence alignment was done using examined sequences and phylogenetic tree was constructed where species in the present study were clustered independently within their corresponding genera. Among the 10 fish samples, morphologically 5 different species were identified while using DNA barcoding of 10 samples, 6 different species were found. Moreover, among the 10 samples which were sequenced using COI gene specific primer, 3 samples were different in morphological and molecular level. This research demonstrates that partial sequences of the COI gene can efficiently identify the six species of marine catfish in Bangladesh, indicating the usefulness of COI-based approach in species identification.

Keywords: DNA barcoding, COI Gene, Multiple sequence alignment, Phylogenetic tree.

INTRODUCTION

Catfish (Order: Siluriformes) are diverse groups of fish with constitution of more than 3,000 species, 478 genera and 36 families (Ferraris and De Pinna, 1999). So far 21 species of catfishes under 9 genera of 2 families have been reported from the region of the Bay of Bengal belongs to Bangladesh (Rahman *et al.*, 2009). All of these species are identified by the study of traditional taxonomy ignoring the molecular characterization particularly the DNA barcoding.

Fish species identification is traditionally based on external morphological features, including body shape, pattern of colors, scale size and count, number relative position of and type of fin rays, or various relative measurements of body parts (Strauss and Bond, 1990). Yet, in some cases morphological features are of limited value for identification and differentiation purposes, even with

whole specimens, because they can show either considerable intraspecific variations or small differences between species. Furthermore, the identification of early life stages (egg and larvae) is even more complicated than adult identification (Strauss and Bond, 1990). Taken all together, these difficulties explained why researchers have attempted to develop new methods for identifying fish species without relying on morphological features.

The Fish Barcode of Life Initiative (Ward *et al.*, 2009; FISH-BOL, 2010) is a concerted global effort to aid assembly of a standardized reference sequence library for all fish species; one that is derived from voucher specimens with authoritative taxonomic identifications. FISH-BOL has the primary goal of gathering DNA barcode records for all the world's fishes, about 31,000 species (Ward *et al.*, 2009). DNA-based identification methods offer an analytically

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powerful addition or even an alternative. This work intends to provide an updated and extensive overview on the PCR-methods for fish species identification. Besides, approach of molecular tools can provide valuable information for species identification to complement the taxonomic data and validation of systematic positions and phylogeny. This study was thus aimed to identify marine catfish collected from Bay of Bengal of Bangladesh genetically along with their morphometric traits. The specific objectives were to characterize 10 species of marine catfishes genetically through DNA barcodes of a mitochondrial gene COI (Cytochrome Oxidase Subunit I), to investigate genetic relationship among those catfishes of the Order Siluriformes collected from Bay of Bengal, to confirm or establish a method for molecular identification of current taxa of the order siluriformes by the phylogenetic analysis using the DNA sequences.

MATERIALS AND METHODS

Collection of samples and morphological identification

A total of 10 samples of marine catfishes were collected from the fishermen catch at BFDC fish landing station of Cox's Bazar which were caught from the Bay of Bengal (Geographical coordinates: 21°26'22.07"N; 92°0'27.84"E). All the samples were identified morphologically according to Day (1878), Muntro (1955) and Rahman *et al.* (2009). According to the morphological identification, these species belonged to the genera *Arius*, *Plicofollis* and *Osteogeneiosus* under the order Siluriformes. Digital photographs of all the fishes were taken immediately and fish samples were stored at -20 °C, after taking the digital images, white muscle samples were collected and preserved at -20 °C. The muscle samples also preserved with absolute alcohol.

DNA extraction

Tissue samples from the region of pectoral fin were collected and preserved at -20 °C. The tissue samples were preserved with absolute alcohol in cryovials. Genomic DNA from each specimen was extracted using the DNeasy Tissue Kit (QIAGEN, Valencia, CA) following the manufacturer's protocol for animal tissue.

PCR Amplification

In order to amplify 652 bp fragment from the 5' end of mitochondrial COI gene. The primers used to amplify 5' end of mitochondrial COI gene were, fishf1 and fishr1

(Ward *et al.*, 2005). The amplification reactions were performed in a total volume of 25 µl and included 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 primers, 100 ng of genomic DNA.). The reactions were conducted using a PTC-200 DNA Engine Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) 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 (Ivanova *et al.*, 2007). To ensure that the reactions yielded adequate amplicon sizes, PCR products were electrophoresed and visualized on 1.0 % agarose gels containing ethidium bromide (10%).

Mitochondrial COI Region Sequencing

Amplified PCR products were subsequently cleaned by the Exo-SAP method (Dugan *et al.*, 2002). Five µl of PCR product, 0.7µl of Exonuclease I 10x Buffer (New England Biolabs, MA, USA), 0.5 µl of Exonuclease I and 5.3 µl of nanopure water were incubated at 37°C for 30 min before being denatured at 80°C for 20 min. The purified products were labeled using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Sanger Sequencer) in a total reaction mixture of 10 µl containing 4.94 µl of nanopure water, 1.94 µl of 5x BigDye Buffer (400 mM Tris-HCl pH 9.0 and 10 mM MgCl₂), 2 µl of 10 pmol of M13F or M13R, 0.12 µl of BigDye Terminator, and 1 µl ExoSAP products. Sequence-PCR products were cleaned up using the ethanol/EDTA precipitation method and sequenced bi-directionally on an ABI 3130 x 1.

Data Analysis

Nucleotide sequences were manually assembled using DNASTAR Lasergene SeqMan v 7.0 software. Assembled contigs were end-trimmed to a homologous region using the SeqMan program (DNASTAR, WI, USA). Sequences were identified using BLAST search within nucleotide database to determine the highest homolog. To test the efficiency of DNA barcoding as a species identification tool, a blind sampling test was conducted, in which samples, identity unknown except to the submitting individual, were selected and sequenced. Phylogenetic tree was constructed using MEGA, version 5.0 (Tamura *et al.*, 2011). The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000). Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

Sequences of the species of *Labeo rohita*, obtained from NCBI database was used as out group and the species of present study.

RESULTS

Morphological identification of catfishes

Marine catfishes were morphologically identified. A total of 10 samples of marine catfishes were collected from Bay of Bengal of Bangladesh. These species are belonging to the genera *Arius*, *Netuma*, *Hemirius* and *Osteogeneiosus* (Table 1).

Sequence output

From the chromatogram, the sequence data were transferred to FASTA format and blasted within nucleotide database for the authentication of the morphological identification. Ten samples were sequenced and blasted. The nucleotide sequences of these samples matched to 6 different species (Table 2). Most of the identifications were similar to morphological identification. Among the 10 fish samples, 5 different species were identified

morphologically while using DNA barcoding of this 10 samples, 6 different species were identified.

Moreover, among the 10 samples which were sequenced using COI gene specific primer, 3 samples were in different identification in morphological and molecular level. Similar identification was observed in 5 catfish samples. Two samples were detected as *Arius tenuispinis* by morphological study which was detected as *Plicofollis tenuispinis* by DNA barcoding.

Among the species identified, four species belonged to genus *Arius* and *Osteogeneiosus* each and two to *Hemirius* and one to *Netuma*.

PCR amplification

The extracted DNA from 10 marine catfish of Bay of Bengal was amplified by PCR amplification for Cytochrome Oxidase subunit 1 (COI) gene using COI specific primer which has product size of around 652 bp. All the samples showed bright band at 750 bp except sample 5 which showed light band (Figure 1).

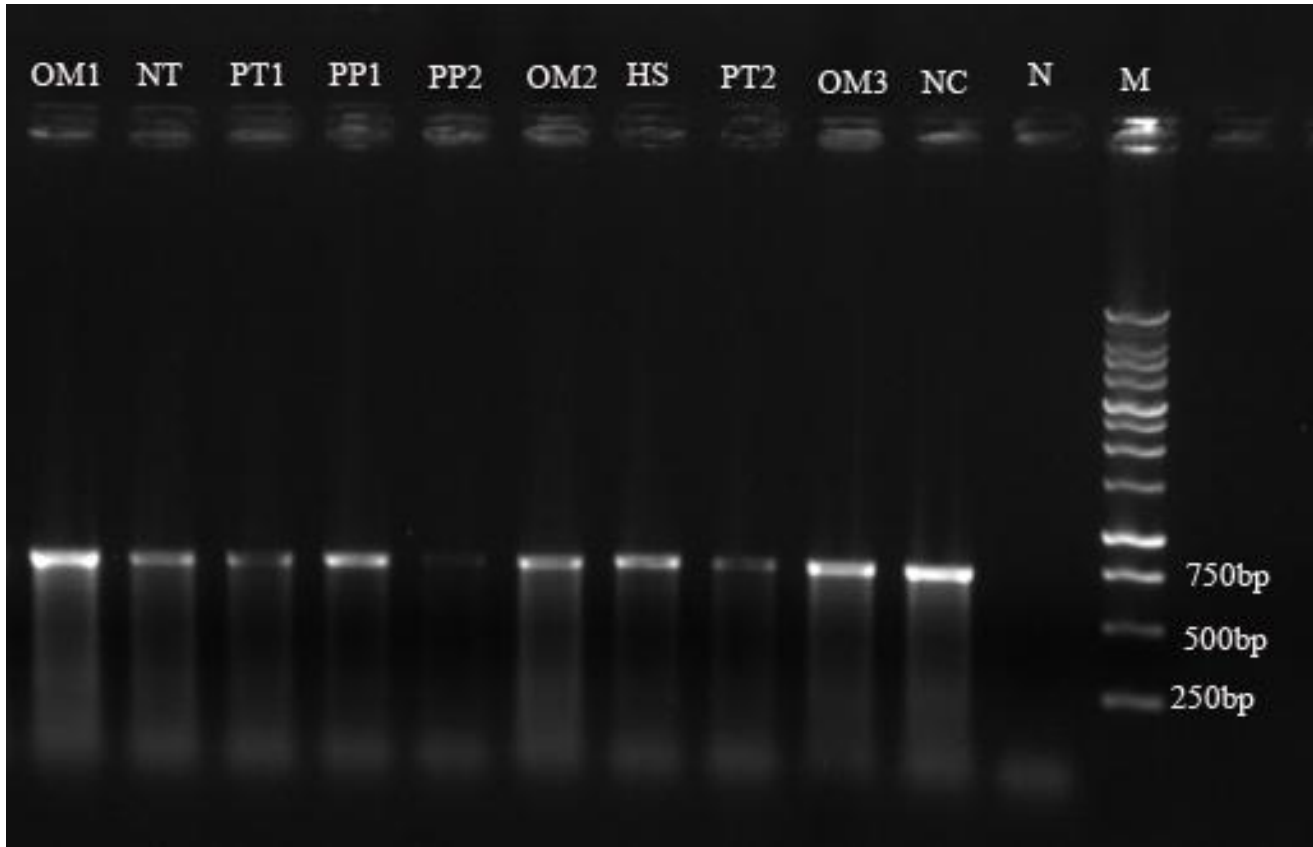


Figure 1. PCR amplification of 10 fish samples PCR. Lane 1-10: Sample 1-10, Lane N: Negative control, Lane M: 100 bp Lader.

Table 1. Morphological identification of collected samples of marine catfish.

Sl no.	Barbel pair	Number of Fin Rays / Spines					Body Measurement (cm)						Head Measurement (cm)				Fin Base Length (cm)				Name of Species		
		DFR	P ₁ FR	P ₂ FR	AFR	CFR	TL	FL	SL	PDL	BD	PL	PD	HL	ED	Pr.OL	Po.OL	IoL	DFB	PFB		P ₁ FB	AFB
1	1	I/7	I/10	7	20	19	42.7	11	35.7	15	5.6	2.4	1.6	9.8	0.8	3.8	5.2	7	3.7	2.2	1.7	5.7	<i>Osteogeneiosus militaris</i>
2	3	I/7	I/11	7	15	25	33	8.2	27.3	10.8	4.6	2.5	1.2	8.5	1.4	3.9	3.7	5.4	2.7	1.6	1.1	3	<i>Netuma thalassinus</i>
3	3	I/7	I/11	6	17	22	44.4	11.6	35	13.4	5.3	3.3	1.9	10.7	1.6	4.4	4.7	6.1	4.6	1.7	1.4	4	<i>Arius tenuispinis</i>
4	3	I/7	I/11	7	13	19	33.2	8	28	11.9	4.5	1.4	1.1	8.3	1.2	3.5	3.6	5.5	1.9	1.4	1	3.2	<i>Arius dussumieri</i>
5	1	I/7	I/10	7	20	19	31	27.5	26	9.8	4.2	2.2	0.8	6.8	0.4	2.5	4	4.9	3.1	0.9	0.8	3.7	<i>Osteogeneiosus militaris</i>
7	1	I/7	I/10	7	20	19	40	36.8	34.8	14	5.3	3.7	1.4	10.3	1.4	3.7	5.2	6.2	3.3	1.8	1.9	5.4	<i>Hemiaris sona</i>
8	3	I/7	I/11	7	13	20	37.3	31.2	29.6	13.1	4.8	2	1.2	9.3	1.4	4.4	3.9	5.8	3	1.4	1	3	<i>Arius tenuispinis</i>
9	3	I/7	I/9	14	25	21	45.5	40.6	39	14.1	5.6	3	1.8	12.4	1.2	5	6.2	7	5.5	1.7	1.3	6	<i>Osteogeneiosus militaris</i>
10	3	I/7	I/11	7	13	19	35.2	8.6	30	12.9	4.7	1.6	1.3	8.6	1.2	3.9	3.5	5.6	2.8	1.6	1.1	3.4	<i>Arius dussumieri</i>

N.B:TL = Total Length
FL = Fork Length
SL = Standard
PDL= Pre-dorsal
PL = Peduncle
P₁FR = Pectoral Fin rays
P₂FR = Pelvic Fin rays
AFR = Anal Fin rays
PD = Peduncle Depth
HL = Head
ED = Eye Diameter
DFR = Dorsal Fin rays /spines
DFB = Dorsal Fin base
PFB = Pectoral Fin base
P₁FB = Pelvic Fin base
AFB = Anal Fin Base
IOL = Inter-orbital length
BD = Body Depth
CFR = Caudal Fin rays
Pr, OL= Pre-orbital
Po. OL = Post-orbital

The mitochondrial cytochrome oxidase I (COI) region of all samples was successfully amplified using PCR. The comprehensive barcoding identification results were based on GenBank databases. Database revealed definitive identity matches in the range of 96%-98% for consensus sequences of six species (*Osteogeneiosus militaris*, *Netuma thalassinus*, *Plicofollis tenuispinis*, *Plicofollis polystaphylodon*, *Hemiaris sona*, and *Arius venosus*). GenBank-based identification for all species yielded an alignment E-value of 0.0 (Table 2).

Multiple sequence alignment was done using examined sequences and downloaded sequences and phylogenetic tree was constructed (Figure 2). According to the Phylogenetic tree the species in the present study were clustered independently with related species. In the present study, the sequences of three samples matched to *Osteogeneiosus militaris* in the gene bank database with more than 80% bootstrap value, but they were different in nucleotide sequence. Moreover, two samples each matched to *Plicofollis tenuispinis* and *Plicofollis polystaphylodon*

with more than 90% bootstrap value. Moreover, *Arius venosus* formed cluster to *Arius venosus* with 99.8% bootstrap value. Similarly, *Hemiaris sona* created subclade to *Arius subrostratus* with bootstrap value of 99.9%. *Netuma thalassinus* former cluster with *Netuma thalassinus* with 100% bootstrap value.

Pair-wise genetic distance

Pair-wise genetic distance values (Kimura 2 parameter) based on *cyt b* using MEGA 5 are given in Table 3. The average genetic distances of individuals among catfish species was estimated as 0.140 ± 0.000 . Congeneric interspecies distances ranged from 0.031 ± 0.006 to 0.053 ± 0.008 and the intergeneric distances ranged from 0.890 ± 0.010 to 0.175 ± 0.014 . The highest congeneric interspecies genetic distance (0.053 ± 0.008) was between OM1 and OM2 and the lowest (0.031 ± 0.006) was between PP1 and PP2. The highest intergeneric distance (0.175 ± 0.014) was between HS and PT-2 while the lowest (0.0989 ± 0.010) was between PP2 and PT1.

Table 2. Molecular identification catfish samples using DNA barcoding with conventional morphological identification.

Sample ID	Morphological identification	Match to the NCBI database	Identity (%)
1	<i>Osteogeneiosus militaris</i>	<i>Osteogeneiosus militaris</i>	97
2	<i>Netuma thalassinus</i>	<i>Netuma thalassinus</i>	96
3	<i>Arius tenuispinis</i>	<i>Plicofollis tenuispinis</i>	98
4	<i>Arius dussumieri</i>	<i>Plicofollis polystaphylodon</i>	97
5	<i>Osteogeneiosus militaris</i>	<i>Plicofollis polystaphylodon</i>	97
7	<i>Osteogeneiosus militaris</i>	<i>Osteogeneiosus militaris</i>	98
8	<i>Hemiaris sona</i>	<i>Hemiaris sona</i>	97
9	<i>Arius tenuispinis</i>	<i>Plicofollis tenuispinis</i>	97
10	<i>Osteogeneiosus militaris</i>	<i>Osteogeneiosus militaris</i>	97
11	<i>Arius dussumieri</i>	<i>Arius venosus</i>	98

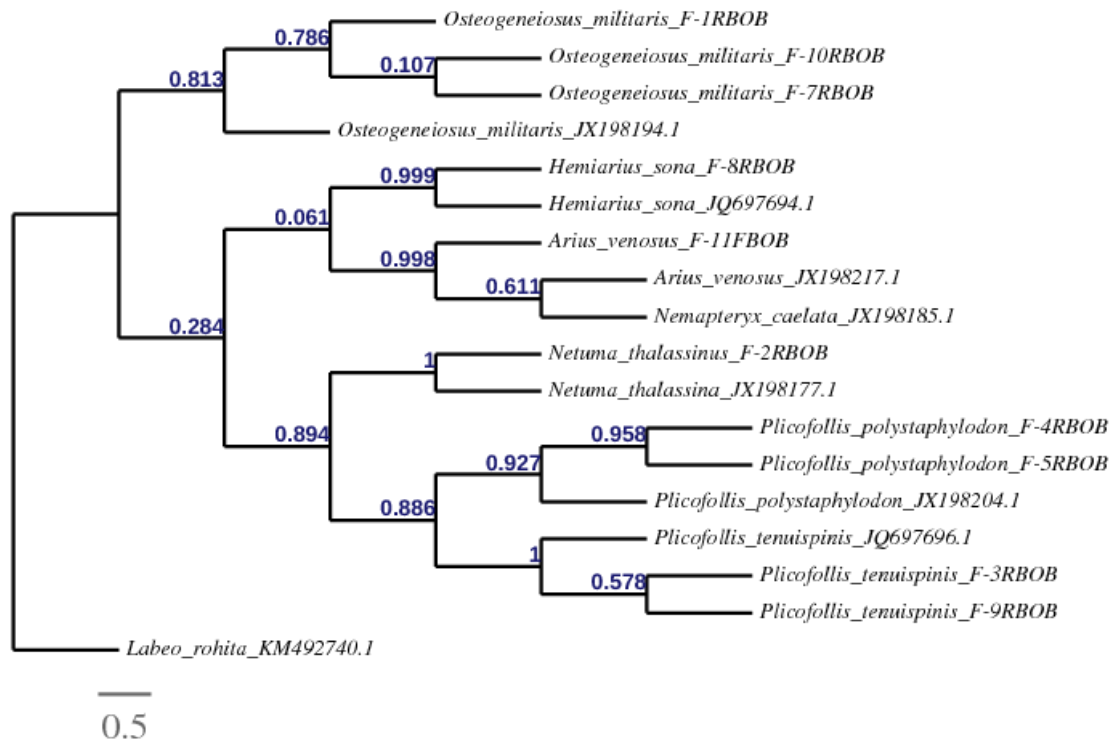


Figure 2. Phylogenetic tree reconstructed with COI nucleotide sequences from different marine catfish of the Siluriform species examined. Sequence of the species of *Labeo rohita* was used as outgroup. Suffix with BOB indicates samples from Bay of Bengal.

Table 3. Estimates of Evolutionary Divergence between Sequences. The number of base substitutions per site between sequences is shown. Standard error estimate(s) are shown above the diagonal. Analyses were conducted using the Kimura 2-parameter model. The analysis involved 10 nucleotide sequences. Evolutionary analyses were conducted in MEGA5.

	OM1	NT	PT1	PP1	PP2	OM2	HS	PT2	OM3	NC
OM1		0.016	0.014	0.014	0.014	0.008	0.014	0.014	0.007	0.014
NT	0.170		0.013	0.013	0.013	0.014	0.014	0.013	0.015	0.014
PT1	0.151	0.137		0.010	0.010	0.013	0.014	0.007	0.013	0.013
PP1	0.156	0.147	0.092		0.006	0.014	0.012	0.012	0.014	0.013
PP2	0.155	0.140	0.089	0.031		0.013	0.012	0.012	0.014	0.013
OM2	0.053	0.168	0.153	0.159	0.160		0.012	0.014	0.009	0.014
HS	0.158	0.163	0.164	0.154	0.156	0.151		0.014	0.014	0.013
PT2	0.159	0.148	0.038	0.112	0.114	0.173	0.175		0.014	0.013
OM3	0.036	0.171	0.143	0.161	0.160	0.053	0.150	0.163		0.014
NC	0.160	0.166	0.152	0.153	0.159	0.167	0.157	0.165	0.161	

OM: *Osteogeneiosus militaris*, NT: *Netuma thalassinus*, PT: *Plicofollis tenuispinis*, PP: *Plicofollis polystaphylodon*, HS: *Hemiarius sona*, NC: *Nemapteryx caelata*.

DISCUSSION

In the past, mainly morphological characters were used for inferring fish phylogenetic relationships to understand their speciation. In the case of marine catfishes, it is difficult to differentiate the species because of the similarity in external morphology. Therefore, the reconstructed phylogenetic trees based on morphology were controversial due to the complex evolutionary changes in either morphological or physiological characters. Recent advances in molecular biology have changed this situation. Recently, the genetic analysis of mtDNA has conducted to resolve the controversial taxonomic problem (Ruber *et al.*, 2006; Erguden *et al.*, 2010) and have proved that the molecular markers can facilitate the discrimination of morphologically similar species. Many researchers have studied about the catfishes (Siluriformes) and reported that they are monophyletic (Kartavtsev *et al.*, 2007). Present study showed that each genus belonging to the order Siluriformes formed a monophyletic group.

Molecular species identification using DNA barcoding has been applied successfully elsewhere but techniques and consensus barcodes had not been developed and validated in commercial catfish species. In this study, it has been sequenced the COI region of the mitochondrial DNA to create a set of barcode sequences used to identify 10 catfish from five genera. In the present study, seven species were identified morphologically but six species were detected by DNA barcoding. Moreover, among the 10 samples, only 4 samples were correctly identified using morphological characteristics which demonstrate the necessity of molecular identification.

Species identification search only if the species in the reference database has at least three barcoded specimens and identifies the query sequences if it matches the reference sequence within the conspecific distance of less than 2% (Tamura *et al.*, 2007) or not exceeding 3% as suggested by Wong and Hanner (2008). Therefore, correct species labeling, morphological taxonomy and voucher documentation should be prioritized in case that reassessment of spurious data is necessary (Ward *et al.*, 2005). Mislabeling is not unexpected since both of these species are genetically homologous (Na-Nakorn *et al.*, 2002) and morphologically similar.

One crucial barcoding criteria is that congeneric divergence should be higher than conspecific divergence (Hubert *et al.*, 2008). While the sequence variation between five genera observed in this study was atypically high, averaging 18.3%, other studies showed a lower congeneric variance such as 7.48% in shark and rays (Ward *et al.*, 2008), 8.37% in Canadian freshwater fishes (Hubert *et al.*, 2008), and 9.93% in Australian marine fishes (Ward *et al.*, 2005). In view of this, population genetic and taxonomic analysis will be able to provide a clearer picture of the evolutionary history of catfish in this study. A maximum genetic distance of 3% is sufficient to distinguish all the catfish in this study

CONCLUSION

Six species of marine catfishes found in the Bay of Bengal were clearly classified into five genera by determining and analyzing the partial sequences of mitochondrial cytochrome oxidase subunit I gene. Partial sequence information of mitochondrial cytochrome oxidase subunit I gene can be used as a diagnostic molecular marker in identification and resolution of taxonomic ambiguity of marine catfishes found in Bay of Bengal.

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