Genetic Characterization of Some Marine Catfishes in Bangladesh using DNA Barcodes

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science of Fisheries.

By

Examination Roll Curzon-609

Session 2012-2013

Registration No. HA-1197 (2008-09)

Department of Fisheries

University of Dhaka

March, 2014

Acknowledgement

All admirations belong to the Almighty holy ISHWAR, who is the most congenial and the most merciful, for flourishing completion of the work.

I convey my deepest appreciation to my reverent teacher and supervisor Mrs Wahida Haque, Associate Professor and Chairperson, Department of Fisheries, University of Dhaka, for her continual supervision, designing the study, guidance and inspiration throughout the work. I am also indebted to her for her ceaseless endurance for careful reading and reviewing the write-up. Without her constant encouragement and continuous help it would not have been possible to complete the study.

I am also grateful to Mr. Md. Mizanur Rahman, Assistant Professor, Department of Zoology, University of Dhaka, under whose constant guidance and supervision this research was carried out. I am also indebted to him for his ceaseless endurance for care full reading and reviewing the write-up.

I am also grateful to Dr. A. M. A. M Zonaed Siddiki, Professor and Director, Department of Pathology & Parasitology, Chittagong Veterinary and Animal Sciences University, Chittagong, for permitting me to work his laboratory and his support.

I am very grateful to the Department of Fisheries, University of Dhaka.

Last but not least, My Mother; her kind cooperation in every step helps me to fulfill this noble work successfully.

March, 2014 The Author

Abstract

Marine catfish (Siluriformes) found in Bay of Bengal are economically important resources for Bangladesh. However, there are no studies on phylogenetic analysis of catfish species in Bangladesh at a molecular level. The species level analysis of catfish species is usually carried out through morphological characters and controversial due to phenotypic variation. In this study, the partial sequence of COI (Cytochrome Oxidase subunit I) gene was analyzed for species identification and phylogenetic relationships among 6 species of catfish sampled from Cox's Bazar. The nucleotide sequences of COI consisted of about 750 nucleotide base pairs. 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 namely *Osteogeneiosus militaris*, *Netuma thalassinus*, *Plicofollis tenuispinis*, *Plicofollis polystaphylodon*, *Hemiarius sona*, and *Nemapteryx caelata*. GenBank-based identification for all species yielded an alignment E-value of 0.0. Multiple sequence alignment was done using examined sequences and downloaded sequences and phylogenetic tree was constructed where species in the present study were clustered independently within their corresponding genera. In the present study the sequence of three samples matched to *Osteogeneiosus militaris* in the gene bank database, but they were different in nucleotide sequence. Moreover, two samples each matched to *Plicofollis tenuispinis* and *Plicofollis polystaphylodon* with little nucleotide sequence difference. Among the 11 fish samples, morphologically identified 5 different species while using DNA barcoding of 10 samples, 6 different species were found. Moreover, among the 10 samples which where sequenced using COI gene specific primer, 3 samples showed were different identification 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.

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Chapter 1
Introduction

1.1 General background

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 fin number and type of finrays, or various relative measurements of body parts (Strauss and Bond 1990). Gill rakers are sometimes counted to differentiate very similar species. 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. For instance it is very difficult to differentiate, based on morphological features only. Besides, once most morphological features have been removed during either digestion (e.g., in stomach contents) or various processing (e.g., canning, filleting), the identification becomes difficult or even impossible. 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.

DNA barcoding is a molecular method for species-level identification of eukaryotic organisms based on the analysis of short, standardized gene sequences. In most animals, the 5' region of the cytochrome *c* oxidase subunit I (COI) in the mitochondrial genome has been used as the target sequence, which encodes part of the terminal enzyme of the respiratory chain in mitochondria. Barcoding provides a rapid and cost-effective method for the identification of eukaryotes and is revolutionizing the application of taxonomy for taxa

with validated data sets (e.g. fishes). DNA barcodes have provided new perspectives in ecology, diversity, and the taxonomy of fishes from many geographic regions; for example, Canada (Steinke *et al.* 2009a), Central America (Valdez-Moreno *et al.* 2009), and Australia (Ward *et al.* 2005, 2008). Fishes constitute a morphologically diverse group of vertebrates that exhibit deep phenotypic changes during development. In this context, the identification of all fish species is challenging and practically impossible, when based on morphology alone.

DNA barcoding can be used to identify fish species from whole fish, fillets, fins, fragments, juveniles, larvae, eggs, or any properly preserved tissue available. The ability of barcoding to provide species assignments also has important implications for the discovery of cryptic species (Hebert *et al.* 2004, Sriwattanarothai *et al.* 2010). Applications of DNA barcoding are emerging in the fields of fish conservation (e.g. Holmes *et al.* 2009, Steinke *et al.* 2009b), and management aspects such as quota, by-catch monitoring and sustainable fisheries (Rasmussen *et al.* 2009). In the fields of food safety and consumer fraud, DNA barcoding has demonstrated that 25% offish samples from markets and restaurants in New York (USA) and Toronto (Canada) were mislabeled or substituted (Wong and Hanner 2008). DNA barcoding can also be applied successfully to cooked or processed fish (Smith *et al.* 2008), grilled or deep-fried fillets (Wong and Hanner 2008), and boiled samples (Cohen *et al.* 2009). Samples with degraded DNA, due to a combination of high pressure and temperature as used in canning, require the use of shorter fragments, the so-called mini-barcodes (Meusnier *et al.* 2008, Rasmussen *et al.* 2009, Ward *et al.* 2009). Given its utility, barcoding is being used by the US Food and Drug Administration as a replacement for the time-consuming technique of protein iso-electric focusing for fish and fish product identification.

The Fish Barcode of Life Initiative (FISH-BOL; http://www.fishbol.org; Ward *et al.* 2009) 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. The benefits of barcoding fishes include facilitating species identification for all potential users, including taxonomists; highlighting specimens that represent a range expansion of known species; flagging previously unrecognized species; and, perhaps most importantly, enabling identifications where traditional methods are

not applicable. 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, Eschmeyer 2010).

The present brief status report provides a taxonomic and geographic overview of the FISH-BOL progress since its inception in 2005, and it aims to direct ongoing or future sampling campaigns toward neglected or underrepresented taxa or geographic regions. The overview also discusses current laboratory protocols and aims to provide an outline for the concerted curation of barcode data pertaining to fishes. The data presented in this article were taken from the FISH-BOL web site (http://www.fishbol. org), version July 2010. Additional data from the taxonomy browser of the Barcode of Life Data Systems (BOLD; http://www.boldsystems.org) (Ratnasingham and Hebert 2007) were used to calculate success rates.

DNA barcoding involves the amplification and sequencing of a short universal molecular tag of approximately 650 bp from the 5 region of the mitochondrial cytochro oxidase I (COI) gene (Hebert *et al.* 2003, Tavares and Baker 2008). DNA barcoding using COI has been widely employed in various biological fields with proven ability to differentiate closely related species in studies molecular systematics (Hardman 2005). Importantly, community-based efforts to develop extensive DNA barcode libraries, most notably the Barcode of Life Data Systems (BOLD), has 24 led to the adoption of DNA barcoding technology as the gold standard for species identification and has greatly expanded the power of the technique. The BOLD database provides detailed information of COI-sequenced species including the origin and current location of voucher specimens (Ratnasingham and Hebert 2007). Out of almost 30,000 fish species estimated in the world, barcodes for more than 10,000 fish species are currently recorded in the BOLD database. These COI barcodes are gathered from several sources including the Fish Barcode of Life Initiative (FISH-BOL) (FISH-BOL 2010; Ward *et al.* 2009).

1.2 Problem statement

Identification and characterization of catfishes are usually based on morphological, meristic characters (Kim 1997). However, considerable ambiguity exists due to morphological similarity and they have led to some controversial hypotheses on species identification (Uchida, 1939; Jayaram, 1968; Jeon, 1984; Lee and Kim, 1990; Kim *et al.*

2003). Synonym citations in FishBase indicate the possibility of ambiguous identification with respect to some catfish species (Froese and Pauly 2006). Accurate identification of morphologically similar species is essential for population dynamic assessment and fisheries management.

1.3 Rationale

Fish species identification is traditionally based on external morphological features. Yet, in many catfishes and especially their diverse developmental stages are difficult to identify by morphological characters. DNA-based identification methods offer an analytically 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.

1.4 Research needs

Very few literatures are available on molecular characterization of marine water catfish, but not yet evaluated properly. Literature on molecular characterization and phylogenetic assessment of marine catfishes in Bay of Bengal was not found. This study was thus aimed to characterize some marine catfishes by DNA Barcoding with the phylogenic relationship to distinguish the species of marine catfish which are available on fishermen from the Bay of Bengal.

1.5 Socio-economic importance

In the proposed study we will develop and evaluate DNA barcodes for use in differentiating domestic and imported marine catfish species. These alignments allowed the development and analyses of consensus barcode sequences for each species and comparison with limited sequences in public databases (Gen Bank and Barcode of Life Data Systems). Validation tests carried out in blinded studies and with commercially purchased catfish samples (both frozen and fresh) revealed the reliability of DNA barcoding for differentiating between these catfish species. The developed protocols and consensus barcodes are valuable resources as increasing market and governmental

scrutiny is placed on catfish and other fisheries and aquaculture products labeling in Bangladesh.

1.6 Objectives

The overall objective of the study was the molecular characterization of marine catfish collected from Bay of Bengal of Bangladesh to identify genetically along with their morphometric traits. The specific objectives were:

1. To characterize 11 species of marine catfishes genetically through DNA barcodes of a mitochondrial gene COI (Cytochrome Oxidase Subunit I).
2. To investigate genetic relationship among those catfishes of the Order Siluriformes collected from Bay of Bengal.
3. To confirm or establish a method for molecular identification of current taxa of the Order Siluriformes by the phylogenetic analysis using the DNA sequences.

Chapter 2
Materials and Methods

2.1 Collection of samples

A total of 11 samples of marine catfishes were collected from the Bay of Bengal of Bangladesh. These species belonging to the genera *Arius, Plicofollis* and *Osteogeneiosus* under the order Siluriformes (Table 1) were collected from the fishermen catch at BFDC fish landing station of Cox'sBazar (Figure 1). Digital photographs of all the fishes were taken immediately and fish samples were stored at -20^{0}C, after taking the digital images, white muscle samples were collected and also preserved at -20^{0}C. The muscle samples also preserved with absolute alcohol.

Table 1 Collection data for samples used in the study

Species of fish	Number of samples	Collection locality	Geographical coordinates
Osteogeneiosus militaris	3	BFDC Fishery Ghat, Cox's Bazar	21°26'22.07"N; 92° 0'27.84"E
Arius tenuispinus	3	,,	21°26'22.07"N; 92° 0'27.84"E
Arius dussumieri	2	,,	21°26'22.07"N; 92° 0'27.84"E
Hemiarius sona	2	,,	21°26'22.07"N; 92° 0'27.84"E
Netuma thalassinus	1	,,	21°26'22.07"N; 92° 0'27.84"E

2.2 Transportation and preservation of samples and laboratory of investigation

Fish samples were kept in ice box and transported in the laboratory of the Department of Fisheries; University of Dhaka and stored at -20ºC. Morphological studies and identification was carried out in the Aquatic Laboratory of the Department of Fisheries, University of Dhaka. Tissue sample collection was done in the same Department. The tissue was preserved in absolute alcohol. Molecular activities (DNA extraction, DNA amplification by PCR, DNA visualization by Gel electrophoresis, Purification of PCR products) for species identification by DNA barcoding were carried out in the Department of Pathology & Parasitology, Chittagong Veterinary and Animal Sciences

University, Chittagong. DNA sequencing was done in the Center of Advanced Research in Sciences (CARS), University of Dhaka.

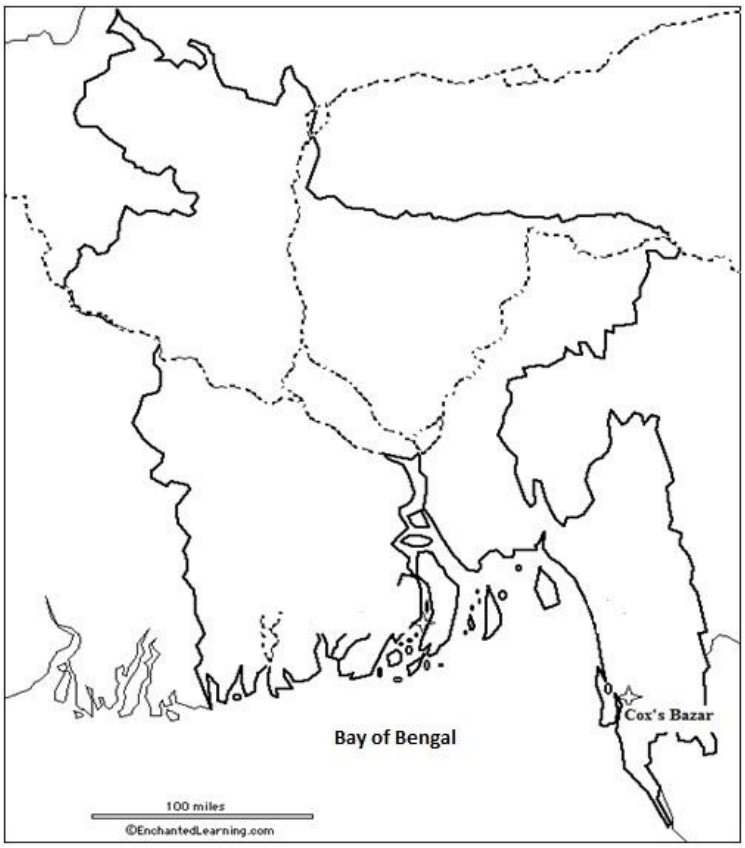


Figure 1 Collection sites of specimens used in this study

2.3 Morphological Identification

All the fishes were identified morphometrically, with the help of Day (1878), Muntro (1955) and Rahman *et.al*. (2009). Besides, identification was based on the following diagnostic characteristics.

2.3.1 Characteristics of Siluriformes

- Body elongate and compressed, either naked or covered with bony plates
- Eyes usually small
- Barbel present
- Spines present at the front of dorsal and pectoral fins. No pelvic spine
- Adipose dorsal fin usually present

2.3.2 Characteristics of Osteogeneiosus militaris (Plate 1)

- A single pair of semiosseous and stiff maxillary barbel.
- Elongated body, head strongly depressed, head smooth without granules.
- Eyes fairly small, in the anterior half of the head above the angle of the mouth.

2.3.3 Characteristics of Arius tenuispinus (Plate 4)

- Head shield striated, finely granulated
- 3 pairs of barbels , maxillary barbel reaching the pectorals, mandibullar much shorter
- Dorsal and caudal fins with dark margins.

2.3.4 Characteristics of Arius dussumieri (Plate 3)

- Head shield rugose, dorsomedian head groove shallow and narrow
- Dorsal fin with strong and sharp keel
- Basal bone of dorsal is narrow and S- shaped

2.3.5 Characteristics of Hemiarius sona (Plate 6)

- Body elongate, head broad and depressed, head shield with strong , radiating granules.
- Dorsal spine granulated anteriorly, serrated posteriorly as long as the head excluding the snout, but not so high as the rays
- Fins including dorsal adipose with a bluish tint at outer margins.

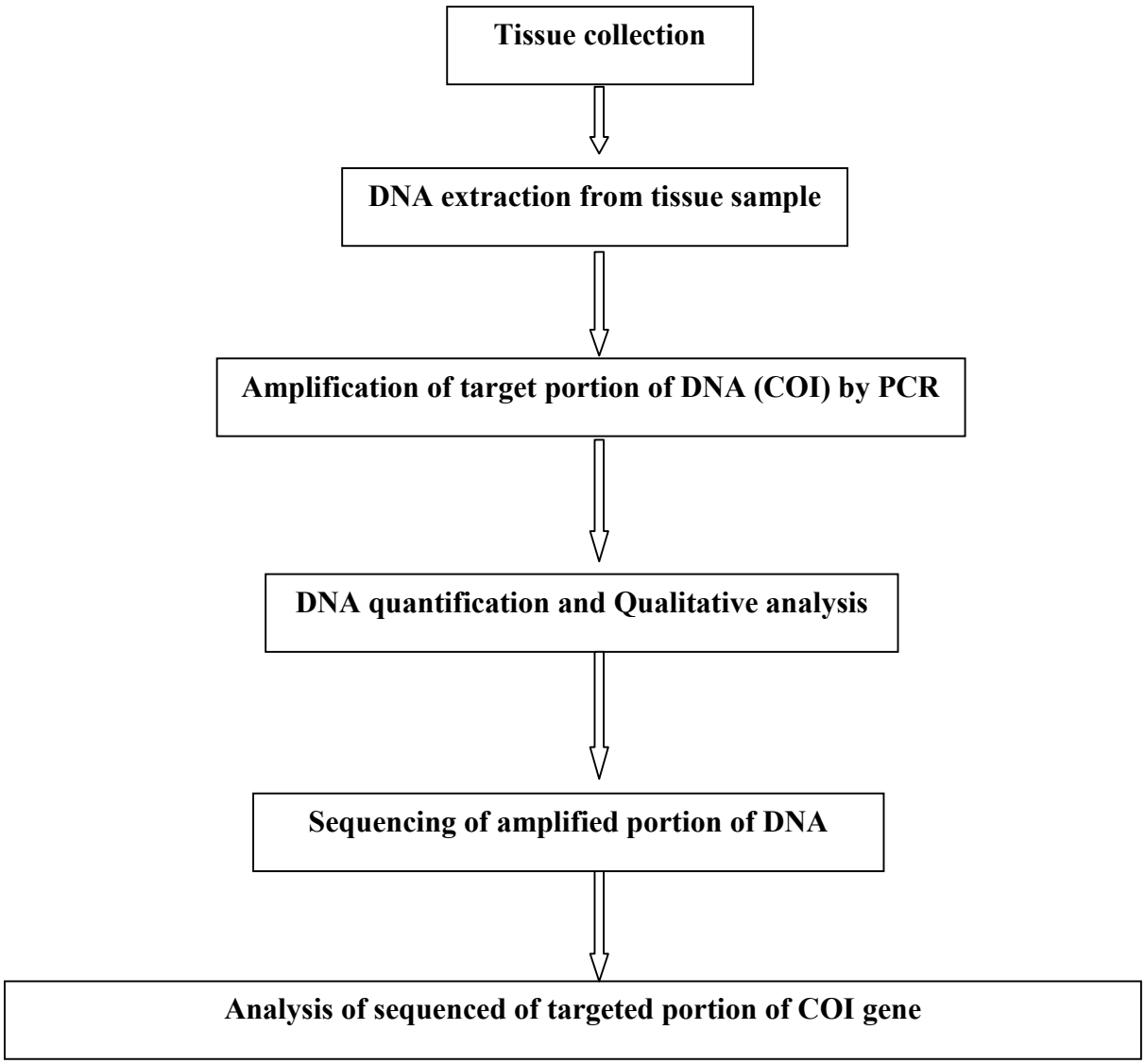
2.3.6 Characteristics of *Netuma thalassinus* (Plate 2 and 5)

- Head shield rugose, dorsomedian head groove lancoelate
- Mouth terminal
- Dorsal fin spine thick, compressed, with internal transverse partitions
- Body reddish brown to charcoal-blue above

2.4 Molecular identification

Molecular identification basing on DNA barcoding is widely accepted method and is being applied in species identification. For species identification, it requires some steps. The steps have been described below.

Figure Flow chart of different steps of DNA barcoding



2.4.1Collection of tissue

Tissue samples from the region of pectoral fin were collected (Plate 9) and preserved at -20 ºC. The tissue samples were preserved with absolute alcohol in cryovials. The following precautions were followed:

a) Sterile forceps, scissors, scalpel and knife were used and they were made sterile prior to move o to next fish specimen
b) Instruments exposed to the previous sample of tissue were rinsed with high concentration of EtOH (i.e. alcohol swab) and ignited with a lighter.
c) Left side of the fish was not damaged which was used for morphological studies.

2.4.2 *DNA extraction*

The extraction of DNA is an important preliminary step in which purified DNA is obtained from cellular components. There are a number of procedures for the isolation of genomic DNA. They all start with some form of cell lysis followed by DNA separation and recovery of DNA.

2.4.3 Cell lysis

Cell lysis or cell disruption is the phenomenon of rupture of the cell and the nuclear DNA. This is usually done by Lysis Buffer

2.4.4 DNA separation:

- Treated with Proteinase K, to digest the contaminating proteins.
- Mixing with Phenol: Chloroform: Isoamylalcohol (25:24:1), Chloroform: Isoamylalcohol (24:1) to remove non polar proteins and lipids from the solution.
- Centrifugation:
    - Centrifugation is a technique by which the gravitational force on a particle is increased to affect its sedimentation.
    - Centrifugation of the emulsion formed by the mixing produces a lower organic phase, separated from the upper aqueous phase by an inter phase of denatured proteins.
    - The aqueous solution is recovered and deproteinised repeatedly through several times centrifugations.

- Once the nucleic acid complex (DNA) has been purified, precipitation has accomplished.

2.4.5 DNA Recovery

- DNA are precipitated as pellet using chilled 70% and absolute EtOH which is simple , rapid, and quantitative and precipitates every nanogram quantities of DNA in few minutes.
- Precipitated DNA pellet is stored in TE buffer or Nuclease free water or Deionized water at -20 ^{o}C for longtime preservation to use in further steps

Protocol for Isolation of DNA from Tissue Sample

i. Approximately, 20 mg (5 mm) of tissue sample was taken.
ii. Tissue was cut into smaller pieces as small as possible.
iii. Then the cutted pieces were homozniged gently in 2 volume (w/v) cold TES buffer/Lysis buffer and the volume adjusted with Lysis buffer/TES buffer.
iv. 5-10µl of 20mg/ml of Proteinase K, was added, then incubated it in 56 C 1-18 hours until the tissue was totally dissolved.
v. Equal volume of Phenol : Chloroform: Isoamylalcohol Cl (25:24:1) was added and mixed thoroughly for few minutes
vi. After that the samples were centrifuged for 10 minutes with 12000 rpm.
vii. After centrifugation upper phase was transferred to new 1.5 ml tube and equal volume of Chloroform: Isoamylalcohol were added and centrifuged with 12000 rpm for 10 minutes.
viii. Then the upper aqueous layer was transferred in a fresh sterilized micro-centrifuge tube and then double volume of chilled absolute ethanol was added
ix. The above sample was kept at -200C for overnight for precipitation
x. The above sample was centrifuged at 10000 rpm for 10 minutes
xi. The supernatant was decant and the pellet was retained
xii. 500µL of 70% ethanol was added to the pellet and centrifuged at 7000 rpm for 10 minutes again and the supernatant was decant
xiii. The pellet was kept for air dry under laminar air flow

2.5 PCR Amplification

In order to amplify 651 bp fragment from the 5 end of mitochondrial COI gene, PCl reactions were conducted using universal primer (Table 2) (Ivanova *et al.* 2007). 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 $MgCl_2$, 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%).

Table 2 Primer used for PCR and sequencing

Primer name	Primer Sequence (5'-3')	Reference
fwd_name: fishf1	TCAACCAACCACAAAGACATTGGCAC	Ward *et al.* 2005
rev_name: fishr1	TAGACTTCTGGGTGGCCAAAGAATCA	Ward *et al.* 2005

2.6 DNA Purification

DNA Purification by Centrifugation

I. PCR product was placed in a Collection.

II. PCR product was then transferred to the SV Minicolumn assembly and incubate for 1 minute at room temperature.

III. SV Minicolumn assembly was centrifuged in a microcentrifuge at 16,000 ^{x} *g* (14,000rpm) for 1 minute. Ten SV Minicolumn was removed from the Spin Column assembly and discard the liquid in the Collection Tube.

IV. SV Minicolumn was returned to the Collection Tube.

V. Te column was washed by adding 700 µL of Membrane Wash Solution, previously diluted with 95% ethanol, to the SV Minicolumn. Centrifuge the SV Minicolumn assembly for 1 minute at 16,000 x *g* (14,000rpm). Empty the Collection Tube as before and place the SV Minicolumn back in the Collection Tube.

VI. Washing was repeated with 500 µL of Membrane Wash Solution and centrifuge the SV Minicolumn assembly for 5 minutes at 16,000 x *g*.

VII. SV Minicolumn assembly was then removed from the centrifuge, being careful not to wet the bottom of the column with the flowthrough.

VIII. The Collection tube was made empty and the column assembly was recentrifuged for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.

IX. SV Minicolumn was carefully transferred to a clean 1.5ml microcentrifuge tube. Apply 50 µL of Nuclease-Free Water directly to the center of the column without touching the membrane with the pipette tip. Incubate at room temperature for 1 minute.

X. It was ten centrifuged for 1 minute at 16,000 x *g* (14,000rpm).

XI. SV Minicolumn was discarded the microcentrifuge tube containing the eluted DNA was stored at 4°C or -20°C.

2.7 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 wate 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 l.

2.8 Data Analysis

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. Phlogenetic tree was constructed using MEGA, version 5.0 (Tamura *et al.* 2007). Sequence of different species of the order siluriformes were downloaded from the Gene bank database for the reconstruction of phylogenetic tree. Four species of insects were used as outgroup

List of Plates

Plate 1: *Osteogeneiosus militaris*

Plate 2: *Netuma thalassinus*

Plate 3: ***Arius dussumieri***

Plate 4: ***Arius tenuispinus***

Plate 5: ***Netuma thalassinus***

Plate 6: ***Hemiarius sona***

Plate: 7 Tissue collection for DNA isolation

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PCR and other apparatus

Plate: 8 Vortex and Centrfuge machine

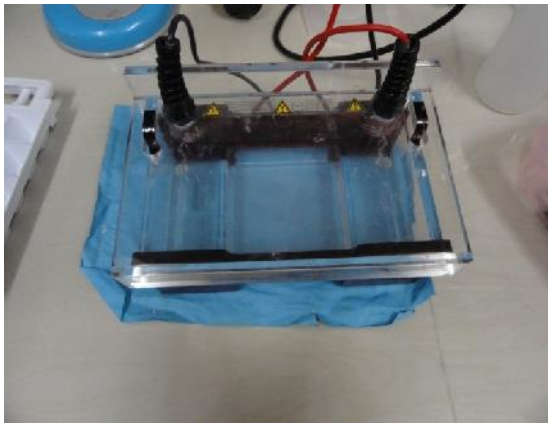

Plate: 9 Gel Electrophoresis system

Plate: 10 Water bath

Plate: 11 PCR Machine

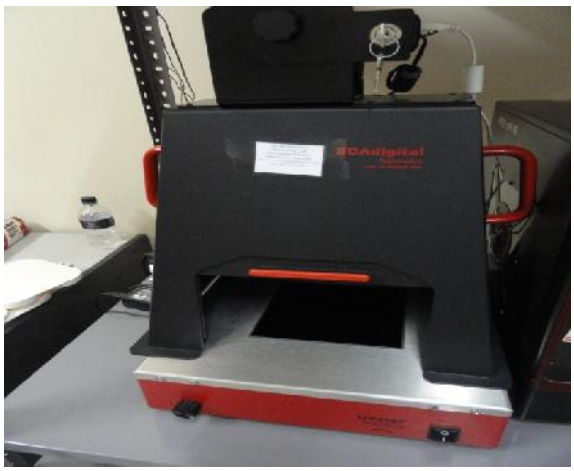

Plate: 12 Gel Documentation system

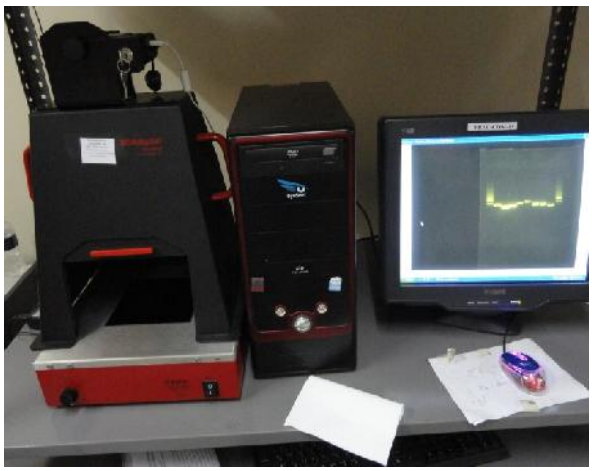

Plate: 13 Gel Documentation system with computer

Chapter 3
Results

3.1 Morphological identification of catfishes

Marine catfishes were morphologically identified. A total of 11 samples of marine catfishes were collected from Bay of Bengal of Bangladesh. Among the samples seven different species were identified morphologically under three different genera. These species belonging to the genera *Arius, Netuma, Hemiarius* and *Osteogeneiosus* (Table 3 and 4). Among the species identified, four species belonged to genus *Arius* and *Osteogeneiosus* each and two to *Hemirius* and one to *Netuma*.

Table 3. Morphological identification of collected samples of marine catfish

Sl no.	Barbel pair	Number of Fin Rays / Spines					Body Measurement							Head Measurement					Fin Base Length				Name of Species
		DFR	P_1FR	P2FR	AFR	CFR	TL	FL	SL	PDL	BD	PL	PD	HL	ED	Pr.OL	Po.OL	IoL	DFB	PFB	P1FB	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	*Osteogeneios us militaris*
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	*Netuma thalassinus*
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	*Ariuss tenuispinis*
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	*Arius dussumieri*
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	*Osteogeneios us militaris*
6.	3	I/7	I/11	8	13	17	26.6	23.6	22.5	8.9	3.5	1.3	0.5	6.9	0.7	2.6	3.6	3.6	2.4	1.2	0.8	2.7	*Hemiarius sona*

Table 3 cont.

7.	3	I/7	I/11	11	18	20	42.5	37	35.5	14	5.4	3.5	1.6	10.8	1.2	4.7	4.9	6.2	3	1.8	1.2	4.2	*Osteogeneios us militaris*
8.	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	*Hemiarius sona*
9.	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	*Arius tenuispinis*
10.	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	*Osteogeneios us militaris*
11.	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	*Arius dussumieri*

N.B:TL = Total Length
FL = Fork Length
SL = Standard
PDL= Pre-dorsal
PL = Peduncle
P_1FR = Pectoral Fin rays
P_2FR = 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
P_1FB = 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

Table 4 Taxonomic position of the experimental fish

Sample No	Phylum	Class	Order	Family	Species	Reference
1	Chordate	Osteichthyes	Siluriformes	Aridae	*Osteogeneiosus militaris*	Linnaeus 1757
2	Chordate	Osteichthyes	Siluriformes	Aridae	*Netuma thalassinus*	Ruppel 1837
3	Chordate	Osteichthyes	Siluriformes	Aridae	*Ariuss tenuispinis*	Day, 1878
4	Chordate	Osteichthyes	Siluriformes	Aridae	*Arius dussumieri*	Valenciennes 1840
5	Chordate	Osteichthyes	Siluriformes	Aridae	*Osteogeneiosus militaris*	Linnaeus 1757
6	Chordate	Osteichthyes	Siluriformes	Aridae	*Hemiarius sona*	Hamilton 1822
7	Chordate	Osteichthyes	Siluriformes	Aridae	*Osteogeneiosus militaris*	Linnaeus 1757
8	Chordate	Osteichthyes	Siluriformes	Aridae	*Hemiarius sona*	Hamilton 1822
9	Chordate	Osteichthyes	Siluriformes	Aridae	*Arius tenuispinis*	Day, 1878
10	Chordate	Osteichthyes	Siluriformes	Aridae	*Osteogeneiosus militaris*	Linnaeus 1757
11	Chordate	Osteichthyes	Siluriformes	Aridae	*Arius dussumieri*	Valenciennes 1840

3.2 Molecular identification and PCR amplification

3.2.1 PCR amplification

The extracted DNA from 11 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 6 which showed light band (Figure 2).

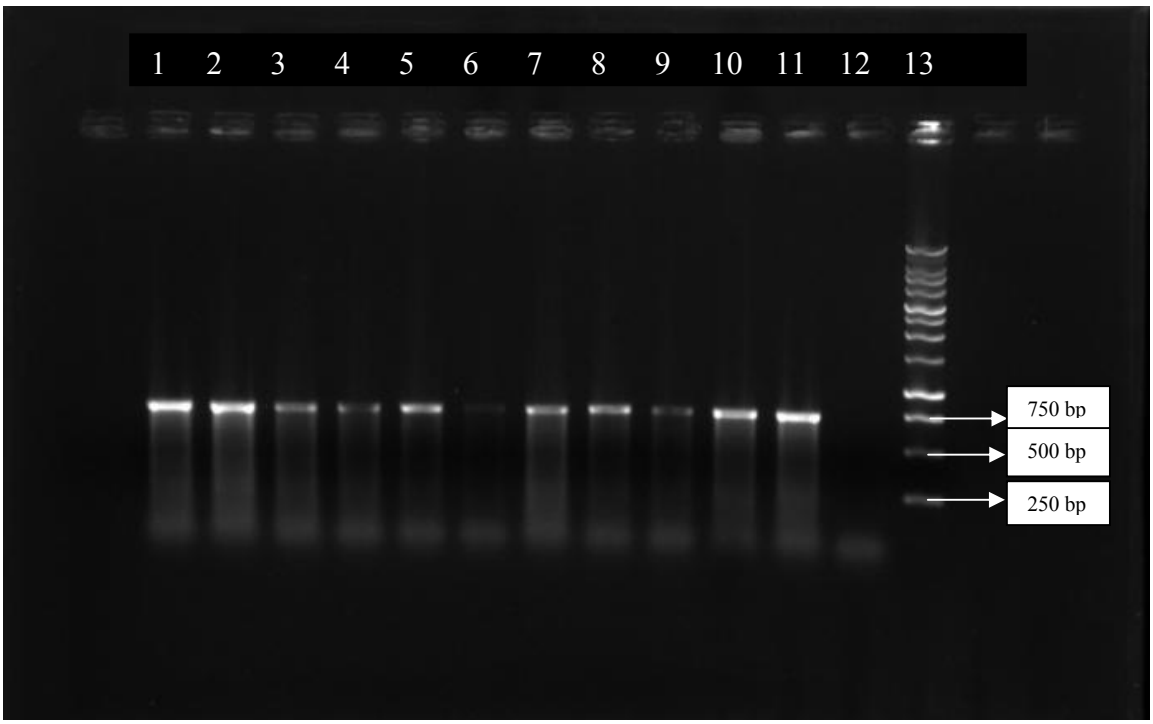

Figure 2 PCR amplification of 11 fish samples PCR. Lane 1-11: Sample 1-11, Lane 12: Negative control, Lane 13: 100 bp Lader.

3.2.2 Sequence output

From the chromatogram, the sequence data were transferred to FASTA format and blasted within nucleotide database to identify species (Appendix 1 and 2). Ten samples were sequenced and blasted. The nucleotide sequences of these samples matched to 6 different species (Table 5). Most of the identifications were similar to morphological identification. Among the 11 fish samples, morphologically identified 5 different species while using DNA barcoding of 10 samples, 6 different species were found.

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

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*, *Hemiarius sona*, and *Nemapteryx caelata*). GenBank-based identification for all species yielded an alignment E-value of 0.0 (Table 5).

Table 5 Molecular identification catfish samples using DNA barcoding with conventional morphological identification

Sample ID	Morphological identification	Molecular identification	Identity (%)
1	*Osteogeneiosus militaris*	*Osteogeneiosus militaris*	97
2	*Netuma thalassinus*	*Netuma thalassinus*	96
3	*Ariuss tenuispinis*	*Plicofollis tenuispinis*	98
4	*Arius dussumieri*	*Plicofollis polystaphylodon*	97
5	*Osteogeneiosus militaris*	*Plicofollis polystaphylodon*	97
6	*Hemiarius sona*	**Not sequenced**	-
7	*Osteogeneiosus militaris*	*Osteogeneiosus militaris*	98
8	*Hemiarius sona*	*Hemiarius sona*	97
9	*Arius tenuispinis*	*Plicofollis tenuispinis*	97
10	*Osteogeneiosus militaris*	*Osteogeneiosus militaris*	97
11	*Arius dussumieri*	*Nemapteryx caelata*	98

Multiple sequence alignment was done using examined sequences and downloaded sequences and phylogenetic tree was constructed (Appendix 3). According to the Phylogenetic tree (Figure3) the species in the present study were clustered independently within their corresponding genera. In the present study the sequence of three samples matched to *Osteogeneiosus militaris* in the gene bank database, but they were different in nucleotide sequence. Moreover, two samples each matched to *Plicofollis tenuispinis* and *Plicofollis polystaphylodon* with little nucleotide sequence difference.

In the present study, *Nemapteryx caelata* formed cluster to *Arius venosus* with 97% bootstrap value. Similarly, *Hemiarius sona* created subclade to *Arius subroastratus* with bootstrap value.

Sequence of four species of insects, obtained from NCBI database, were used as outgroups and the species of present study were discrete from outgroups.

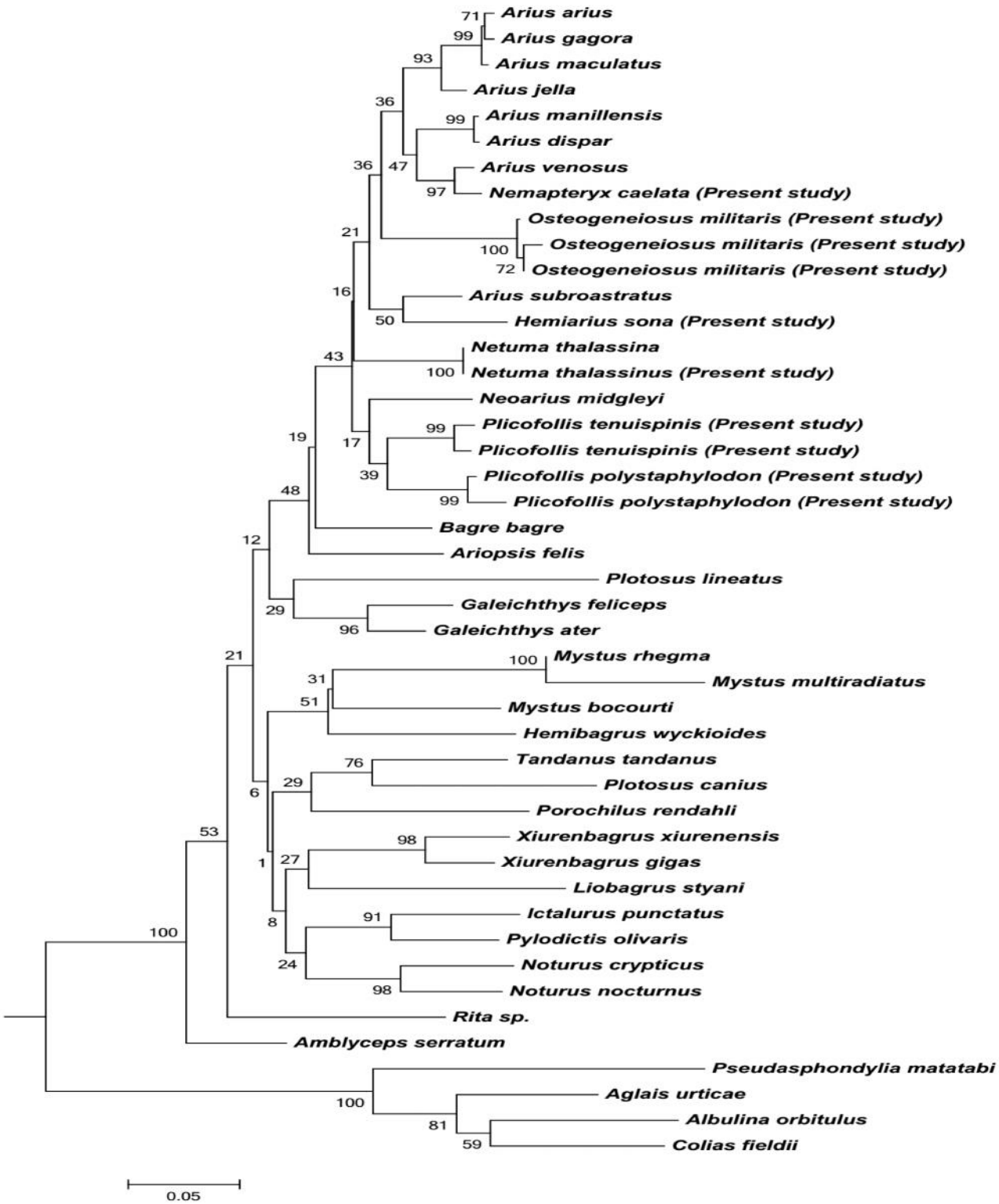

Figure 3 Phylogenetic tree reconstructed with COI nucleotide sequences from different marine catfish of the Siluriform species examined in this study and other related species of the same order. Four species of insects were used as outgroup

Chapter 4
Discussion

Species Identification Based on Morphology and BLAST

Regulatory scrutiny of seafood and their labeling has lagged behind a surge in availability of imported wild-caught and aquaculture species. The particularly dramatic growth in catfish imports, their impact on the domestic catfish industry, and widespread questions regarding transparency in imported catfish origins and culture conditions, have combined to place catfish at the fore of emerging efforts to heighten fish product inspections in different developed countries. A critical component of seafood inspections is determination of accuracy in species labeling. 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.

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.

Sequence Divergence and Phylogenetic Analysis

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. As expected, species from the same genera were clustered tightly into a single clade with well supported bootstrap proportion (Steinke *et al.* 2009).

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 (Suzuki *et al.* 2005; Rüber *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 (Okazaki *et al.* 1999; Peng *et al.* 2004; Kartavtsev *et al.* 2007). Present study showed that each genus belonging to the order Siluriformes formed a monophyletic group.

Chapter 5
Conclusion and Recommendation

5.1 Conclusion

DNA barcoding is emerging as an invaluable tool to regulatory agencies and fisheries managers for species authentication, food safety, conservation management as wellas consumer health and support. Here, Developed and validated DNA barcoding techniques and consensus sequences for important aquaculture and wild species of catfish. The findings indicate that DNA barcoding is a powerful technique, accurately identifying samples regardless of sample source. The barcodes will be deposited in a searchable catfish barcoding database that will be updated as additional samples and species are sequenced. Consensus barcodes from these species will also speed the development of fast-turnaround/high-throughput array or SNP-based assays based on informative COI polymorphic sites.

5.2 Recommendations

Probably this is the first attempt to study the taxonomy of marine catfish of Bangladesh at molecular level. There are several species of marine catfish in the Bay of Bengal all of which could not be possible to take under the present study. So further study is needed to take as many species as possible under investigation.

Moreover, only COI gene was amplified in the present study and species identification was done accordingly. Species identification may be based on other genes like 16S rRNA, Cytochrome *b*.

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Appendices

Appendix 1: FASTA format sequences of the studied fish species

> *Osteogeneiosus militaris* sequence exported from chromatogram file

CAGAAAACCAGCAGAGGTGTTGGTATAGGATTGGGTCTCCCCCTCCTGCGGGGTCAAAGAAGTG
GTATTAAGGTTTCGGTCTGTTAGTAGTATTGTGATACCAGCAGCAAGGACTGGGAGGGAAAGAA
GTAGGAGCACGGCATTGATTAAAATAGCCCAAACAAATAAGGGCGTTTGGTATTGTGAAATAGC
TGGAGGTTTCATGTTAATGATGGTTGTGATAAAGTTAATGGCTCCCAGAATTGATGATACTCCTG
CTAGATGGAGGGAAAAAATAGTAAGATCTACAGAAGCTCCTGCGTGTGCGAGGTTCCCAGCGAG
GGGGGGATACACGGTCCATCCTGTTCCTGCCCCTGCCTCGACTCCTGATGAAGCAAGGAGAAGT
AGGAAGGATGGGGGAAGGAGTCAGAAGCTCATGTTGTTTATTCGGGGGAATGCCATGTCGGGG
GCTCCGATTATTAGGGGGACAAGTCAGTTTCCAAAGCCTCCAATTATGATGGGCATTACTATAAA
GAAAATTATTACGAAAGCATGGGCGGTAACGATAACGTTATAGATTTGGTCATCGCCTAGAATG
CGCCTGGTTGGCTAATTCTGCTCGAATTACATGCTAAGGCGTTCGACTAGTCCGCCAGCACAACA
CTAGTAATGTGCCATGTCTTGTGTGATGAAACTGGCGCATAA

> *Netuma thalassinus* sequence exported from chromatogram file

AACTAGCATGTTGATAAGATTGGATCTCCTCCTCCGGCGGGGTCAAAGAAAGTATTATTA
AGGTTTCGGTCTGTTAGCAGCATATTGATGCCGGCAGCTAGAACTGGGAGGGAAAGAAGT
AAGAGTACAGCATTAATTAAAATGGCTCAAACAAATAAAGGTGTTTGATATTGTGAAATA
GCTGGAGGTTTCATGTTAATAATAGTTGTGATAAAGTTGATGGCTCCTAGAATTGATGAT
ACCCCTGCCAGGTGAAGAGAGAAGATGGTAAGGTCTACGGAAGCTCCTGCGTGTGCAAGG
TTTCCAGCAAGGGGTGGGTATACAGTTCACCCTGTTCCCGCCCCTGCTTCAACTCCTGAT
GAAGCAAGTAGGAGTAGGAAGGAGGGGGGAAGGAGTCAGAAGCTTATATTATTTATTCGG
GGGAATGCCATGTCAGGGGCTCCGATTATTAGGGGGACGAGTCAGTTCCCCAAGCCTCCA
TTTATGATTGGTATTTACTATAAAAGAAAATTTATAACAAAAGGCGTGGGCCAGTAACGA
TAACATTATAAATTTTGGGTCATCGCCTAAAAGAAGCACCGGGTTGGGCTAATTCTGCCC
GAAATTATTAGGCTAAGGGCGGTTCCAACTATTCCCGGCTCAGGCCCTAACACTATATAT
GAGGGGGCCCAATGTCCTTTGTGGTTGGTTGAAACACGGGCCTATAAATA

> *Plicofollis tenuispinis* sequence exported from chromatogram file

CCCTCCCACGAGGTGTTGATAAGGATTGGGTCTCCTCCTCCTGCGGGGTCAAAGAAAGTA
GTATTGAGGTTTCGGTCTGTTAATAGCATAGTAATACCAGCAGCCAGAACTGGGAGAGAA
AGGAGTAAGAGTACGGCAGTAATTAAAATAGCTCAAACAAATAGAGGTGTTTGATATTGG
GAGATTGCTGGAGGTTTTATGTTAATGATAGTTGTGATGAAATTAATAGCCCCTAGAATT
GATGATACCCCTGCTAGGTGAAGAGAAAAAATAGTAAGGTCTACAGAGGCTCCTGCATGT
GCGAGGTTTCCAGCAAGGGGTGGGTATACAGTTCATCCTGTTCCTGCCCCTGCTTCAACT
CCTGATGAAGCAAGAAGAAGTAGGAAAGATGGAGGGAGAACTCAGAAGCTTATGTTATTT
ATTCGGGGGAATGCCATGTCAGGGGCTCCGATTATTAGGGGAACAAGTCAGTTTCCAAAG
CCTCCAATTATGATTGGTATTACTATAAAGAAAATTATCACAAAAGCGTGGGCGGTGACG
ATAACATTATAAATTTGATCGTCGCCTAGAAAGGGCACCGGGTTGGCTAATTCTGCCCGA
ATTAGCATGCTAAGGCCGGTTCCAACTATTCCGGCTCAGCACCAAACACTACGTAGAGGG

TGCCATGTCTTCGTGGATGGTTGAAAACCTGGGCGCGAAAAAAA

> *Plicofollis polystaphylodon* sequence exported from chromatogram file
CACGTACTGTTGGTAAGGATGGGTCTCCCCCTCCTGCGGGATCAAAGAAAGTAGTGTTAA
GGTTTCGATCTGTTAGTAGTATAGTGATGCCGGCAGCTAAAACTGGGAGGGAAAGAAGTA
AGAGTACGGCAGTAATTAAAATGGCTCAAACAAATAGAGGTGTTTGATATTGTGAAATAG
CTGGGGGTTTCATGTTAATGATAGTTGTGATGAAATTGATAGCCCCTAGAATTGATGATA
CCCCTGCTAGGTGGAGAGAAAAAATATTGAGGTCTACGGAGGCTCCTGCGTGTGCGAGGT
TTCCAGCAAGGGGGGGATATACAGTTCACCCTGTTCCTGCTCCTGCTTCAACTCCTGATG
AAGCAAGAAGAAGTAGGAAAGATGGATGATGGACTCACAAGCTTATGTTATTTATTCGGG
GGAGTGCCATGTCAGGGGCTCCAATTATTAGGGGAACAAGTCACTTTCCAAAGCCTCCAA
TTATGATTGGTATTACTATAAAGAAAATTATTACAAAAGCATGGGCGGTAACGATAACAT
TGTAAATTTGATCGTCGCCTAGAAGGGCGCCGGGTTGGGCTAATTCTGCCCGAATTATCA
TGCTAAGGGCGGTTCCAACTATTCCCGGCTCATGCACCGAACACTAGGTAGAGGGTTACC
AATGTCTCTGGTGGTTGGTTGAAAACTGGGTCTCAAAAAAAA

> *Plicofollis polystaphylodon* sequence exported from chromatogram file
TCCCCTCCAGAGTTGGTAAGGATGGGTCTCCCCCTCCTGCGGGATCAAAGAAAGTAGTGT
TAAGGTTTCGATCTGTTAGTAGTATAGTGATGCCGGCAGCTAAAACTGGGAGGGAAAGAA
GTAAGAGTACGGCAGTAATTAAAATGGCTCAAACAAATAGAGGTGTTTGATATTGTGAAA
TAGCTGGGGGTTTCATGTTAATGATAGTTGTGATGAAATTGATAGCCCCTAGAATTGATG
ATACCCCTGCTAGGTGGAGAGAAAAAATAGTGAGGTCTACGGAGGCTCCTGCGTGTGCGA
GGTTTCCAGCAAGGGGGGGATATACAGTTCACCCTGTTCCTGCTCCTGCTTCAACTCCTG
ATGAAGCAAGAAGAAGTAGGAAAGATGGATTAAGGAGTCAGAAGCTTATGTTATTTATTC
GGGGGAATGCCATGTCAGGGGCTCCAATTATTAGGGGAACAAGTCACTTTCCAAAGCCTC
CAATTATGATTGGTATTACTATAAAGAAAATTATTACAAAAGCATGGGCGGTAACGATAA
CATTGTAAATTTGATCGTCGCCTAGAAAGGGCGCCGGGTTGGGCTAATTCTGCCCGAATT
ATCATGTCTAAGGGCGGTTCCAACTATTCCAGGCTCAGGCACCGAAACACTAGGTAGAGG
GGTACCTATGGTCCTTCGTGGTTGGTTTGAAAACTGGGCGCCAAAAA

> *Osteogeneiosus militaris* sequence exported from chromatogram file
CCCCCCACAGCAGAGGTGTTGGTATAGGATTGGGTCTCCCCCTCCTGCGGGGTCAAAGAA
AGTGGTATTAAGGTTTCGGTCTGTTAGTAGTATTGTAATACCAGCACCAAGGACTGGGAG
GGAAAGAAGTAGGACCACGGCATTGATTAAAATACCCCAAACAAATAAGGGCGTTTGGTA
TTGTGAAATAGCTGGAGGTTTCATGTTAATGATGGTTGTGATAAAGTTAATGGCTCCCAA
AATTGATGATACTCCTGCTAGATGGAGGGAAAAAATAGTAAGATCTACAGAAGCTCCTGC
GTGTGCGAGGTTCCCAGCGAGGGGGGGATACACGGTCCATCCTGTTCCTGCCCCTGCCTC
GACTCCTGATGAAGCAAGGAGAAGTAGGAAGGATGGGGGAAGGACTCAGAAGCTCATGTT
GTTTATTCGGGGGAATGCCATGTCGGGGGCTCCGATTATTAGGGGGACAAGTCAGTTTCC
AAAGCCTCCAATTATGATGGGCATTACTATAAAGAAAATTATTACGAAAGCATGGGCGGT
AACGATAACGTTATAGATTTGGTCATCGCCTAAAGGGCGCCGGGTTGGGCTAATTCTGCT
CGAAATTACCAGGCTAAGGGCGGTTCCAACTGATTCCCGGCCCAAGCCCCAAACACTAGG
TAAAAGGGGGGCCAATGTCCTTTGTGGGTGGGTTGAAAACTGGGGGCCTAAAAAAA

> *Hemiarius sona* sequence exported from chromatogram file
GGTAGAGAGCTGATAAGGATGGGTCTCCCCCTCCTGCGGGGTCAAAGAAGTGGTATTGAG
GTTTCGGTCTGTTAATAGCATATTGATGCCGGCACCCAGAACTGGGAGGGAGAGAAGTAC
TACTACGGCAGTAATTAGGATGGCTCAAACAAATAAAGGTGTCTGATATTGTGAAATAGC
TGGGGGCTTCATGTTAATAATAGTTGTGATGAAGTTGATGGCCCCTACAATTGACAAAAC
TCCTGCTAGGTGGAGAGAAAAGATAGTAAGGTCTACGGAAGCCCCTGCGTGTGCAAGGTT
TCCAGCAAGCGGGGGGTATACAGTTCACCCTGTTCCTGCTCCTGCTTCAACTCCTGATGA
AGCAAGGAGAAGTAGGAAAGATGGGGGGAGGAGTCAAAAGCTTATGTTGTTTATTCGGGG
GAATGCTATGTCGGGGGCTCCGATTATTAGGGGGACAAGTCAATTCCCAAAGCCCCCGAT
TATGATTGGCTTTACTATAAAGAAAATTATTACGAAAGCATGGGCGGTAACGACACGTTA
TAGATCTGGGTCATCGCCTAAAAAAGCGCCGGGTTGGGCTAATTCTGCTCAATTTATTAT

TGCTGAGGGCCGGTTCCAACTATCCCCGCCCCAGGCACCAAAACACTAGGTAAAGGGGTG
CCCAATTGTCCTTGTGGTTTGGTTGAAAAACTTGGTCTTAAAAAAAA

> *Plicofollis tenuispinis* sequence exported from chromatogram file
CCACCCCCACGAGGTGTTGATAAGGATTGGGTCTCCTCCTCCTGCGGGGTCAAAGAAAGT
AGTATTGAGGTTTCGGTCTGTTAATAGCATAGTAATACCAGCAGCCAGAACTGGGAGAGA
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> *Osteogeneiosus militaris* sequence exported from chromatogram file
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> *Nemapteryx caelata* sequence exported from chromatogram file
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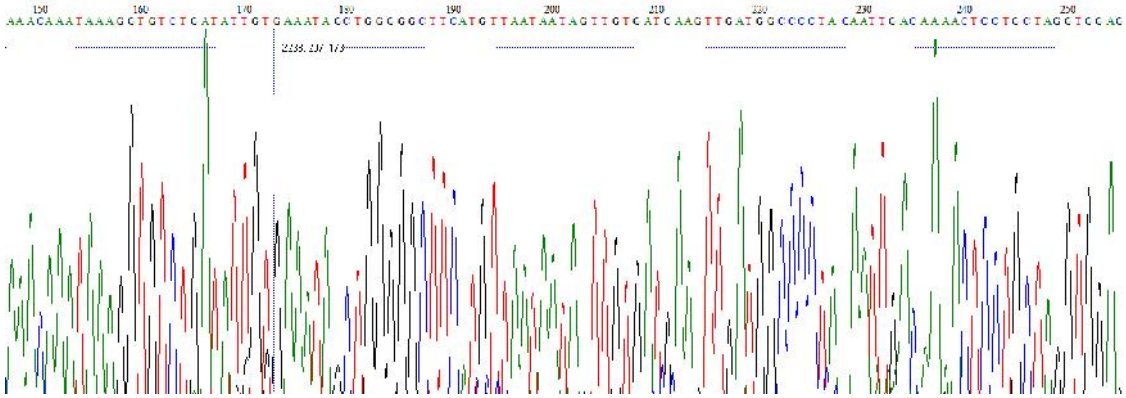
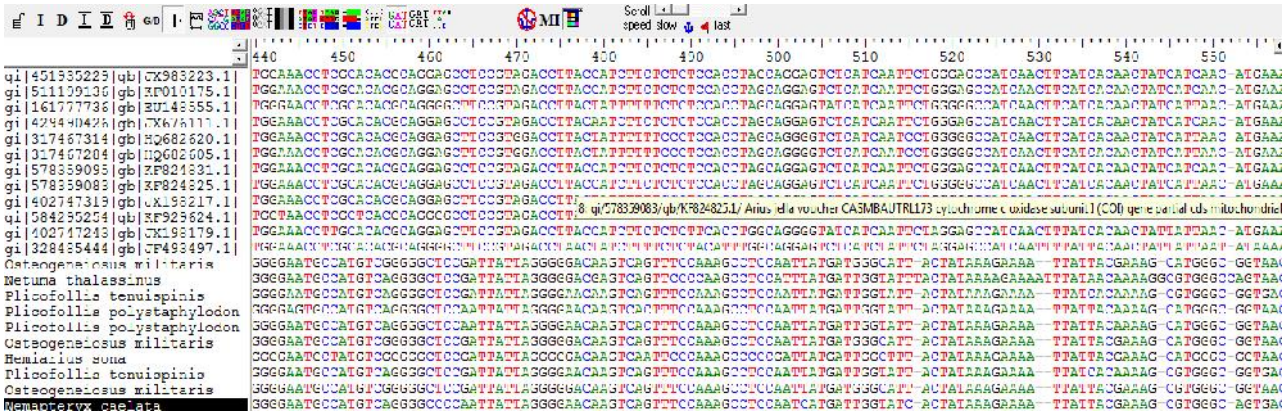
Appendix 2 Sample chromatogram

Osteogeneiosus militaris



Appendix 3 Multiple Sequence Alignment



Appendix 4: Composition of Buffers and Solutions

A. Membrane Wash Solution

(after ethanol addition)

10mM potassium acetate (pH 5.0)

80% ethanol

16.7µM EDTA (pH 8.0)

To prepare this solution, add 95% ethanol to the supplied Membrane Wash Solution (concentrated) as described in Table 2 in Section IV.A.

1X TE buffer

10mM Tris-HCl (pH 7.5)

1mM EDTA (pH 8.0)

1X TBE buffer

89mM Tris base

89mM boric acid

2mM EDTA (pH 8.0)

1X TAE buffer

40mM Tris base

5mM sodium acetate

1mM EDTA (pH 8.0)

B. Membrane Binding Solution

4.5M guanidine isothiocyanate

0.5M potassium acetate (pH 5.0)