Genetic diversity of Hilsa and Chapila in Bangladesh usingDNA barcodingand RAPD techniques

A thesis submitted to the Department of Fisheries, University of Dhaka
in partial fulfillment of the requirements for the degree of
Masters of Science (MS) in Fisheries

Submitted By

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Declaration

I hereby declare that the dissertation entitled **"Genetic diversity of Hilsa and Chapilain Bangladesh using DNA Barcoding andRAPD techniques"**submitted to the Department of Fisheries, University of Dhaka for the degree of Masters of Science (MS) is based on self-investigation, carried out under the supervision of**Md. Alamgir Kabir**and**Dr Mohammad Shamsur Rahman**, Department of Fisheries, University of Dhaka, Dhaka-1000, Bangladesh.

I also declare that this or any part of this work has not been submitted for any other degree anywhere. All sources of knowledge used have been duly acknowledged.

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Certificate

This is to certify that the research work embodying the results reported in this thesis entitled **"Genetic diversity of Hilsa and Chapilain Bangladesh using DNA Barcoding and RAPD techniques"** submitted by**Utpal Chandra Ray,** Roll: Curzon 819, Session: 2015-16, Registration No: 2011-112-778, has been carried out under our supervision in the Aquatic Laboratory of Department of Fisheries, University of Dhaka.

This is further to certify that it is an original work and suitable for the partial fulfillment of the Degree of Master of Science (MS) in Fisheries from the Department of Fisheries, University of Dhaka, Bangladesh.

We wish his every success in life.

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Abstract

Fisheries is one of the most important sectors in the socio-economic context of our country. Fishes are the major source of animal protein and it contributes about 60% of the animal protein intake. *Tenualosa ilisha* is the national fish of Bangladesh. “Jatka” are captured in a large quantity by artisanal fishers and sold on local markets as “chapila”. The main purposes of the study were morphological identification and genetic characterization of “chapila” and different species of “hilsa”.

Morphometric study and DNA barcoding were used for species identification. Species identification based on the DNA sequence of a fragment of the cytochrome c oxidase subunit I gene in the mitochondrial genome, DNA barcoding, is widely applied to assist in sustainable exploitation of fish resources. RAPD (Randomly Amplified Polymorphic DNA) analyses were used to find out the genetic distances by ten arbitrary oligonucleotide RAPD primers.

Four different species of chapila (*Gudusia chapra*), hilsa (*Tenualosa ilisha*), toli shad (*Tenualosa toli*) and big eye ilish (*Ilisha megaloptera*) were identified by morphometric study. But DNA barcoding shows there were two different species of Indian oil sardine (*Sardinella longiceps)* and kelee shad (*Hilsa kelee)*. A total of 134 bands were produced in RAPD analysis among four species where 23 bands were polymorphic indicating 18.48% polymorphisms. The molecular size of the amplified DNA fragments ranged between 200 to 1480bp and 83unique RAPD bands were observed among four populations.The values of pair-wise genetic distances ranged between 0.5077 and 0.9933with some degrees of genetic variation among the populations.The highest genetic distance (0.9933) was found between *Gudusia chapra* and *Sardinella longiceps*. While the lowest genetic distance (0.5077) was found between *Sardinella longiceps* and *Hilsa kelee.*The UPGMA dendrogramsegregated the four populations in three major clusters *viz.* C1, C2 and C3. Clusters C1 and C2 comprised *Sardinella longiceps and Hilsa kelee.* On the other hand, *Gudusia chapra* created the new distant cluster C3.

Results show that DNA barcoding is a reliable tool for species identification. Genetic diversity information may be used for improved breeding programme and conservation of those four populations.

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Chapter 1

Introduction

1.1 Background

Fisheries is one of the most important sectors in the socio-economic context of our country. Its production contributes to the livelihood and employment of millions of people. Fisheries have a significant contribution in our Gross Domestic Product (GDP). Around 3.69% contribution comes from fisheries sector. Fisheries sector also contribute about 2.01% of our annual export earnings. Bangladesh ranks 4th in inland culture fisheries and 5th in capture fisheries among the world's largest fish producing countries. About 11% of our total population leads their livelihood from fisheries sector. Fishes are the major source of animal protein. Fish provides about 60% of the animal protein intake. Per capita annual fish consumption in Bangladesh is about 19.30kg/year against a minimum requirement of 21.90kg/year (DoF, 2015).

Genetic distance is that the total range of genetic characteristics within the genetic makeup of a species. It's distinguished from genetic variability; that describes the tendency of genetic characteristics to vary. Genetic diversity is how for populations to adapt to dynamic environments. Those people square measure additional doubtless to survive to supply offspring bearing that gene. The population can continue for additional generations due to the success of those people. Mostafa et al. (2009)applied RAPD method into two river and one hatchery population of *L. calbasu*to assess intraspecific genetic variation and relatedness among the populations. Kabir et al. (2012)tried to find thegenetic diversity in three forms of *Anabas testudineus*Bloch wild (native, non-spotted), Thai (introduced from Thailand, spotted) and Thai (a spotted- released form from local hatcheries. Maximum intraspecific divergence for widespread species *Gammaruslacustris* was 3.5% and mean interspecific divergence reached 21.9% (Zhong-e HOU et al.,2009).Hubert et al. (2008) applied DNA barcoding to identify Canadian freshwater fishes. The results shows that average genetic distance was 27 fold higher between species and K2P distance was 8.3% among congeners & 0.3% among conspecifics. The distribution of K2P distance between individuals and species overlapped and identifications were only possibleto species group using DNA barcodes in these cases. Chandra et al. (2012) worked on snow trout of Indian Coldwater Fishes

from Western Himalaya. They found mean intra-specific divergence was 1.75% (range 0.00-3.50%) and inter-specific divergence of *S. richardsonii*is 0.00% (range 0.00040-0.00080%) &*S. progastus* is 0.00% (range 0.00036-0.000206).

1.1.1 Studied Species

Tenualosa ilisha the national fish of Bangladesh is a species of Clupeidae family. It is also called ilish, hilsa, hilsa herring or hilsa shad. Ilish is a popular food fish in South Asia. It can grow up to 60cm in length with a weight up to 3kg. The young fish returning to the sea are known in Bangladesh as "jatka", which includes any ilish fish up to 23cm long. Hilsa is found in rivers and estuaries in our country. It contributes about 1% of GDP in our country and it also contributes about 12% of our total fish production. About 450,000 people are directly involved in catching of hilsa for their livelihood and around four to five million people are indirectly involved with hilsa fisheries (DoF, 2015).

Fig. 1.1. Photograph showing the national fish of Bangladesh, *Tenualosa ilisha*

(Source:http://d1iraxgbwuhpbw.cloudfront.net/tools/uploadphoto/uploads/tenualosa_ilisha.jpg)

Gudusia chapra is also a fish species of Clupeidae family. It is also called "chapila" in Bangladesh. This fish species is found in the rivers of Bangladesh and India. Nowadays it is also found in Pakistan and Nepal. Besides the rivers it also found in the ponds, beels, ditches and inundated fields.

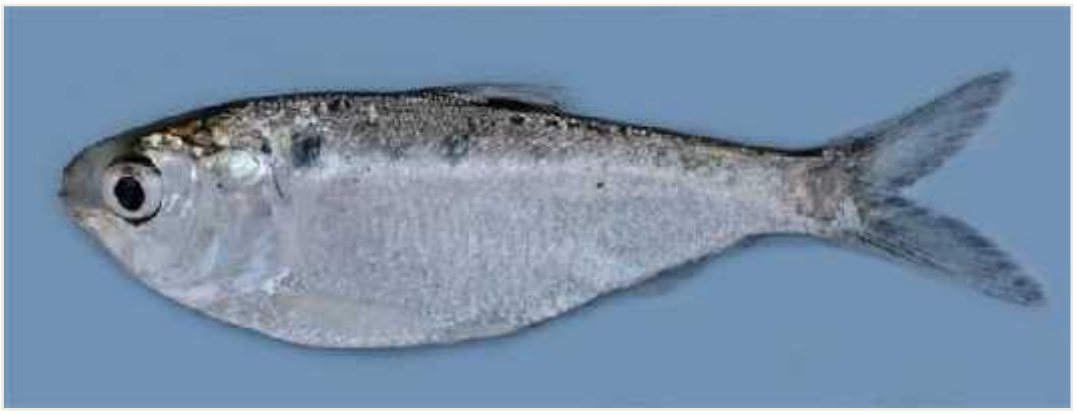


Fig. 1.2. Photograph showing chapila, *Gudusia chapra*

(Source:http://www.fishbase.org/tools/UploadPhoto/uploads/gudusia_chapra_2.jpg)

1.1.2 Taxonomic Study

The word morphology refers both to the branch of biology dealing with the form and structure of organs or other parts of organisms, and with the form and structure of organism as a whole. In fish, the major characters used for description and identification are descriptive, referring to distinguishable characters (e.g., shape of caudal fin), morphometric, referring to continuous variables (e.g., head length as a fraction of body length) or meristic, referring to discontinuous variables (e.g., the number of rays and spines in a dorsal fin).The morphological data incorporates descriptive characters in multiple choice fields and morphometric and meristic characters in numeric fields. It is mainly the meristic characters that are used for quick identification, following the database identification scheme (Froese and Papasissi, 1990). Choice fields present the user with preprogrammed choices of descriptions for a body part or feature (e.g., Cross section - circular; oval; compressed; flattened; angular; others). The choices included were kept to a minimum, including only general descriptions covering the most common shapes or forms. In most cases, another choice is included for those species which might have aberrant features or shape of a body part. When other is chosen for a field, a detailed description of the particular body part is included in Remarks field.

Numeric fields on the other hand, were used for morphometric and meristic. In most cases, ranges were entered in separate lower and upper limit fields. When a range or several values are given in the literature, but the field allows only a single number to be entered (as in the fields for body proportions), the mean of the available values was

entered. The remarks field accommodates characters that are either not included in the choice fields or require more detailed descriptions. In these fields, distinctive features, and how these features might be found in closely related species, are highlighted. Notes on color variations (ontogenetic, sexual and geographic) are also entered in this field, when available(http://www.fishbase.org/manual/fishbasethe_morphology_table.htm, accessed on 02 Feb 2017).

1.1.3 DNA Barcoding

Paul Hebert's research group at the University of Guelph published a paper titled "Biological identification through DNA barcodes" in 2003, than DNA barcodes first came to the attention of the scientific community. This is a new system of species identification and discovering by using a short section of DNA from a standardized region of the genome. Different species can be identified by that DNA sequence, as like as a supermarket scanner uses the familiar black stripes of the UPC barcode to identify purchases product.

DNA barcoding seeks to advance each species identification and discovery through the study patterns of sequence divergence during a standardized cistron region. For the barcode of animal kingdom a segment near the 5′-terminus of the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene has been selected (Hebert et al., 2003). Its effectiveness has been validated for various animal groups and most investigated species (>94%) possess distinct barcode arrays, with low intraspecific variation and high divergences from closely allied taxa (Ward et al.,2005; Hajbabaei et al.,2006). DNA barcode sharing has been found between a few congeneric species, largely among taxa that are known to hybridize. According to Hebert et al. (2004) most prior barcode studies have generated hypothesis concerning overlooked (cryptic) species. Many of which have subsequently been recognized as having morphological and ecological differences (Ward et al.,2005; Hajbabaei et al., 2006).

According to Hogg and Hebert (2004); Hebert et al. (2003) DNA barcode studies revealed of sequence variation in local faunas. But these are now leading to continental or global barcode campaigns for a few groups such as birds, fish. According to Nelson et al.(2007) DNA barcoding efficacy has gained validation; prior work on mammals has been restricted to two studies of primate species, most represented by a single individual. For seafood identification to the species level, DNA barcoding is already a powerful tool.

Its helps to identify the phylogeographic patterning of genetic diversity and can also inform aspects of traceability.

According to Namree et al.(2015) DNA barcoding aims to provide an efficient method for species-level identifications using an array of species specific molecular tags derived from the 5′ region of the mitochondrial cytochrome c oxidase I (COI) gene. Fishes constitute a highly diverse group of vertebrate development. In this context, the identification of fish species is challenging and DNA barcoding provide new perspective in ecology and systematics of fishes. It is designed to provide accurate and automated identification of the species through the use of molecular species tags based on short, standardized gene regions.
The primary goal of DNA barcoding is focusing on the assembly of reference libraries of barcode sequences for known species in order to develop reliable, molecular tools for species identification in nature. At present the result suggest that, in a large array of organisms, species are generally well delineated by a particular sequence or by a tight cluster of very similar sequences that allow unambiguous identifications.

A 648-bp segment of the 5′ region of mitochondrial cytochrome c oxidase (COI) gene forms the library of primary barcodes for the animal kingdom. Large scale molecular tagging is possible by the mitochondrial DNA (mtDNA). This genome is present in a large number of copies yielding substantial amounts of genomic DNA from a variety of extraction methods. Small effective population size and high mutation rate make it often an informative genome about evolutionary processes and patterns. For a barcoding approach to species identification to succeed, however, within species DNA sequences need to be more similar to one another than to sequences in different species. Detection of mixed genealogy between closely related species has been previously estimated to occur nearly 20% of the cases in the wild. Recent barcoding studies emphasized that this percent can vary widely among phyla, yet species assignment failures typically do not exceed 5% to 10% in a large array of organisms.

Sequencing and comparison of a standardized portion of the genome to aid in specimen identification and species discovery is the main theme of DNA barcoding. The largest effort to catalogue biodiversity using molecular approaches is represented by DNA barcoding method. Although initially regarded as controversial, numerous cases have been reported where the analysis of DNA sequence variation in the cytochrome c oxidase

subunit 1 (CO1) region of mtDNA has proven highly effective for the delineation and identification of animal species in general and fish in particular.

DNA barcoding, the analysis of sequence diversity in a standardized gene region, has gained considerable validation as a tool for species identification and discovery. DNA barcoding is effective for identifying both fresh and marine water fishes. It also helps to build a barcode library for all the fish species. At present, records are available for 41771 fishes, which represent 6566 species of fish on the Barcode of Life Data System (BOLD, http://www.boldsystems.org/). Barcoding also provided an independent means of testing the validity of existing taxonomic system, revealing cases of inappropriate synonym of overlooked taxa. Results suggest that for the heavily exploited populations, species boundaries need to be examined, to properly inform conservation strategies and planning.

The primary goal of barcoding focuses on the assembly of reference sequence libraries derived from expert identified voucher specimens in order to develop reliable molecular tools for species identification in nature. Barcoding has been mischaracterized as molecular taxonomy, although it is not intended to replace classical taxonomy. The main purpose of DNA barcoding is to facilitate species identification by non-expert and to do so in a rapid and cost effective manner. According to Khallaf et al.(2014) the effectiveness of barcoding has been demonstrated in diverse taxa, including spiders, flies, fishes, mammals and birds. Barcoding system now also established for plants, macro algae and bacteria.

The Fish Barcode of Life Initiative (FISH-BOL)is an international effort to coordinate an assembly of a standardized reference collection library for all fish species, one this is derived from voucher specimens with authoritative taxonomic identifications. the blessings of barcoding fishes encompass facilitating species identity for all potential customers, which include taxonomists; highlighting specimens that represent a selection growth of regarded species; flagging formerly unrecognized species; and possibly most importantly, allowing identifications where traditional techniques aren't applicable (http://www.fishbol.org/).

DNA barcoding has been adopted in numerous studies illustrating its speed, reliability and accessibility. It exhibits a sufficient level of variation to discriminate among species. The main advantage of DNA barcoding is that once a solid reference database has been

established, the method does not require expert taxonomic knowledge in order to identify specific sample (Lockley and Bardsey, 2000). Further identification can be done with small tissue samples from any part of the organism and the identification process is generally fast and reproducible, but does not require reproductive material. A limitation of the method is that no single universal DNA regions that can be used across all taxonomic group have been identified. Many DNA barcoding study of animal have achieved high rates of species discrimination using a single regions to obtain sufficient discrimination success. According to Hebert et al.(2003) barcoding technology is an emerging molecular based authenticity technology that uses variation within a single genetic marker region (CO1 region of mtDNA) to dentify plants and animal species through DNA sequencing.

Mini-barcodes (e.g., 100–300 bp) have been found effective for species-level identification in DNA-damaged samples and in situations, where it is difficult to obtain a full-length barcode. Additionally, components, such as average nucleotide composition, patterns of strand asymmetry, and a high frequency of hydrophobic amino acid encoding codons can be accurately predicted from a short barcode sequence (Min and Hickey, 2007). Furthermore, it has been shown that mini-barcodes may provide measures at both the intra-specific and intra-generic levels of sequence variability and divergence in some cases when compared to full length barcodes (Hajibabaei et al.,2006). Full-length 650bp COI barcodes can exhibit up to 98% species resolution, with smaller regions 100bp and 250 bp producing correspondingly lower rates of identification success (Meusnier et al.,2008) but when employed in ecological or environmental contexts where the number of species per genus is often low, they can producerates of identification that are very high. In silico studies have been utilized to corroborate the empirical tests of the rates of identification success for DNA barcodes, but also point to the need to carefully design experiments in environmental contexts where primer bias may affect the results (Min and Hickey,2007; Ficetola et al.,2010).

Although some potential limitations of DNA barcoding have previously been recognized, the method has more recently been validated for use in forensic and regulatory fields (Dawnay et al.,2007). Momentum for the initiative has further been aided by, inter alia, the establishment of the Consortium for the Barcode of Life (COBL) - an international initiative devoted to developing DNA barcoding as a global standard for the identification of biological species. Barcode of Life Database (BOLD) was created and is

maintained by University of Guelph in Ontario. It offers researchers a way to collect, manage, and analyze DNA barcode data (http://www.barcodeoflife.org/). The Fish Barcode of Life Initiative (FISH-BOL, http://www.fishbol.org/) is one of such campaign aiming to assemble a COI reference library for all fishes.

1.1.4 RAPD (Randomly Amplified Polymorphic DNA)

The random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) technique allows detection of polymorphisms by randomly amplifying independent multiple regions of the genome through PCR using single arbitrary primers (Williams et al., 1990; Welsh and McClelland, 1990).Molecular markers derived from polymerase chain reaction (PCR) amplification of genomic DNA are an important part of the toolkit of evolutionary geneticists. RAPD (randomly amplified polymorphic DNA) markers that were shown togenetically link to a trait of interest could be usedfor individual and pedigree identification, pathogenic diagnostics, and trait improvement in geneticsand breeding programmes(Yoon and Kim, 2001; Holsinger et al., 2002).

Random oligonucleotide primers produce RAPDs that have been used extensively as molecular markers (Koh et al., 1999; Shikano and Taniguchi, 2002). RAPDs also have the advantage that no prior knowledge of the genome is necessary for successful application (Welsh and McClelland, 1990; Williams et al., 1990; Fischer et al., 2000; Klinbunga et al., 2000).Information on the genetic structure of fish species is useful for optimizing identification ofstocks, stock enhancement, breeding programs, management for sustainable yield and preservation of genetic diversity (Dinesh et al., 1993; Garcia and Benzie, 1995;Tassanakajon et al., 1997, 1998). RAPD markers have been used for phylogenetic studies for species and subspecies identification of fish (Bardakci and Skibinski, 1994; Borowsky et al., 1995; Sultmann et al., 1995; Partis and Wells, 1996).DNA polymorphisms have beenextensively employed as a means of assessing genetic diversity in aquatic organisms. RAPDfingerprinting offers a rapid and efficient method for generating a new series of DNA markers in fishes (Foo et al., 1995).The RAPD technique has an advantage over other systems of genetic documentation because it usesuniversal sets of primers, and no preliminary work such as probe isolation, filter preparation, or nucleotide sequencing is necessary. These primers adhere to a specific nucleotide segment of the genomic DNA. The DNA is cut into many segments of a specific length which can be measured using gel electrophoresis. For a mutation to

change the RAPD pattern, it must occur in the priming region or must change the length of the DNA between priming regions. In this way the RAPD analysis can provide a simple and reliable method for measuring genomic variation. Because the RAPD approach is a relatively straight-forward technique to apply, and the number of loci that can be examined is unlimited, RAPD analysis is viewed as having a number of advantages over RFLP's and other techniques (Lynch and Milligan, 1994).

In many instance, only a small number ofprimers are necessary to identify polymorphism within species. The ease of the RAPD technique could lead to the automation of genetic mapping and to the extension of genetic analysis to cover organisms which lack an ample number of phenotypic markers to completely describe their genome (Williams et al., 1990). For any population a selective process can produce change only if there is variation to select among. No amount of reproduction can affect a population's genetic composition if all`individuals are identical. From an evolutionary standpoint the progressive accumulation of genetic variation is thought to have given rise, beginning with common ancestors, to the diversity of life. The process of continued evolution is critically dependent on renewed variation. Thus, genetic variation can be thought of as the ‘‘fuel’’ for evolution. Genetic differences within populations were easily detectable using RAPD analyses with single-primer DNA amplifications (Mulcahy et al., 1993, 1995; Vicario et al., 1995). RAPD methods showed a more pronounced effect of isolation-by-distance in comparison with allozymes(Mamuris et al., 1999). Additional findings supported the use of RAPD analysis as an effective tool in species identification and cross-contamination test among different cell lines (Guo et al., 2001). The RAPD-PCR method can be applied to detect genetic diversity and similarity in numerous organisms using various primers (Welsh et al., 1991; Levin et al., 1993; Cagigas et al., 1999; Bernardi and Talley, 2000). For all of these reasons,the RAPD assay has been used to construct phylogenetic trees for resolving taxonomic problems in many organisms (Chalmers et al., 1992; Bardakci and Skibinski, 1994; Greef and Triest, 1999). It has been concluded that although AFLP analysis is superior in terms of efficiency, RAPDs may still be used as reliable markers in small lowtechlaboratories (Kjolner et al., 2004).

Some limitations restrict practical application of RAPD analysis (e.g. dominance, reproducibility, homology inferences and artifact fragments). Dominance is a major limitation of the RAPD approach. RAPD markers are thought to be dominant, with polymorphisms detected as either band presence or absence. Dominant markers arenot as

efficient as co-dominant markers for population genetics studies (Lewis and Snow, 1992; Lynch and Milligan, 1994). Lynch and Milligan(1994) estimated that 2–10 times more individuals need to be sampled per locus for dominant markers compared to co-dominant markers. Krauss and Peakall(1998) suggested that this disadvantage may be overcome because of the large number of available polymorphisms, typically over 100 polymorphismsper gel lane are possible. Zhou et al. (2001) reported that gynogenetic silver crucian carp are very sensitive to reaction conditions, but this problem can be overcome using cloning and sequencing techniques. Concerns about reproducibility of RAPDs have limited their wider use in environmental biology. Several studies have reported poor reproducibility for RAPD markers (Weeden et al., 1992; Penner et al., 1993; Skroch and Nienhuis, 1995).

In the case of investigation of polymorphism inclosely related stocks, strains, or groups the highest possible complexity of the patterns obtainable by RAPD-PCR is required to capture limited polymorphism. Most parameters (reaction components concentration, additives, different polymerases, and thermal profiles) affecting RAPD-PCR should be examined, in an effort to increase pattern complexity (Diakou and Dovas, 2001). Fraga et al. (2002) analyzed the effect of changing concentrations of the primer, template DNA and TaqDNA polymerase with the goal of determining their optimum concentration for the standardization of the RAPD technique for genetic studies of *Trichomonasvaginalis*. To ensure that amplified DNA bands derive from genomic DNA, and not primer artifacts, negative control should be run for each primer/breed combination (Ali, 2003). No amplification was detected in control reactions and all amplification products were found to be reproducible when reactions were repeated using the same reaction conditions. Despite all of these limitations (dominance and low reproducibility due to low stringent PCR), RAPD analysis has been used effectively for initial assessment of genetic variation among fish species (Dinesh et al., 1993; Johnson et al., 1994; Foo et al., 1995; Bielawski and Pumo, 1997; Caccone et al., 1997; Cunningham and Mo, 1997; Barman et al., 2002).

1.2 Rationale

"Jatka" are caught in large quantity by artisanal fishers using small mesh nets or current jal. The fish are sold on local markets as Chapila. It is very similar to Indian rivers shad. Though ilish and chapila are different species, but it is very difficult to distinguish them. Unfortunately the consumers are unable to detect jatka and chapila by their morphological features.Morphologically they are very similar but they must have some genetic difference.

Though many works have been done in the world and Bangladesh to identify the genetic diversity of many fish species but in Bangladesh insufficient work has been done to detect the genetic diversity of ilish and chapila species by RAPD and DNA barcoding yet.

1.3 Objectives

The overall objectives of the study were morphological identification and genetic characterization of chapila and different species of hilsa.

The specific objectives were:

1. Morphological identification of chapila and hilsa.
2. Molecular identification of hilsa and chapila by DNA barcoding.
3. To find out the genetic diversity of chapila and hilsa by RAPD fingerprinting.

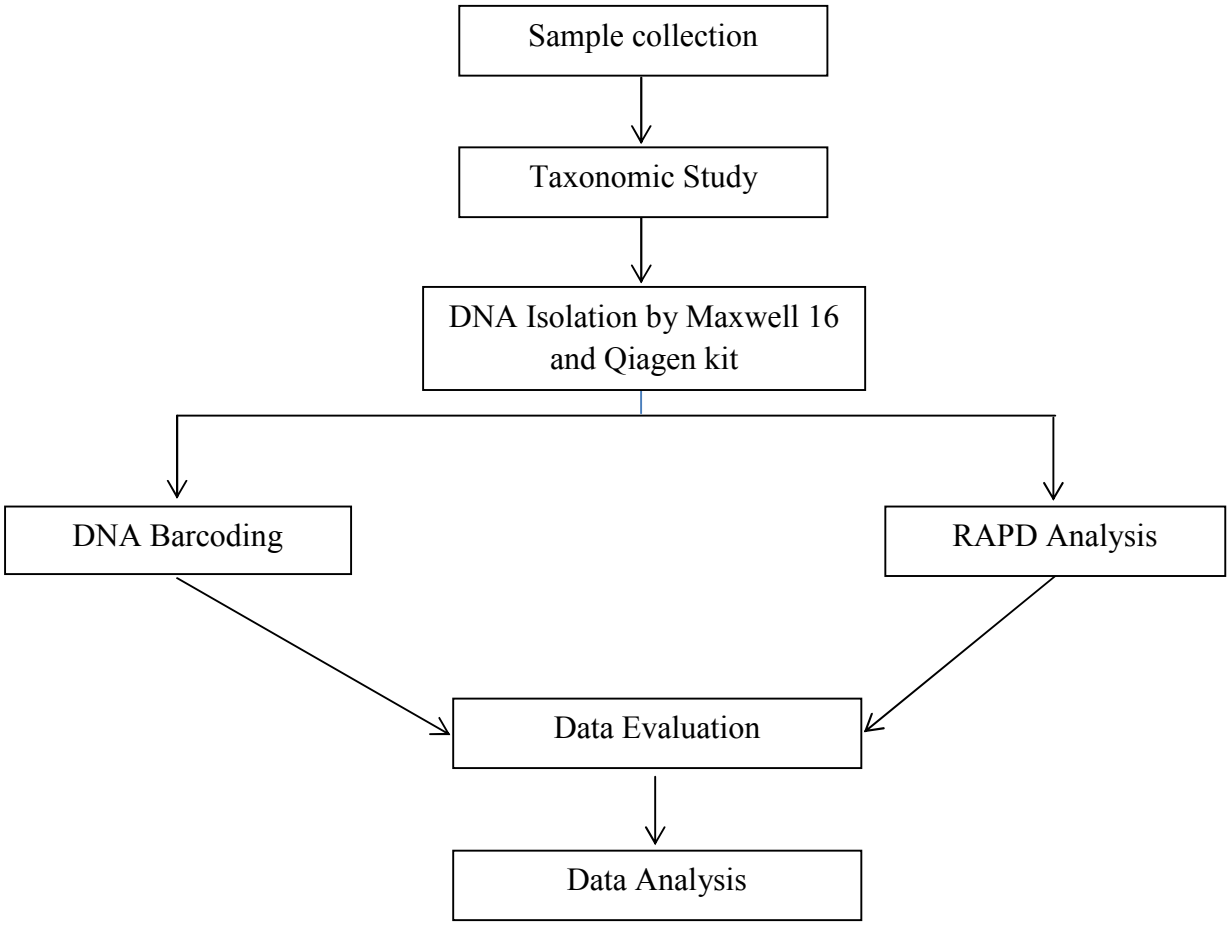
Chapter 2

Materials and Methods

2.1 Collection of sample

Chapila (*Gudusia chapra*), Padma ilish (*Tenualosa ilisha*), Chandanailish (*Tenualosa toli*), Chokkailish (*Ilisha megaloptera*) samples were collected from different markets of Dhaka city such as ShwoariGhat, Nayabazar, Jatrabari, Karwan Bazar, Ananda Bazar, New Market. During the transportation fish sample were taken in ice box and another necessary measures were taken.

Flowchart 1: Overview of the current study



2.2 List of studied fish sample

Sl.	English Name	Collection Place	Origin
01	Chapila	Jatrabari	Chittagong
02	Chapila	Karwan Bazar	Chittagong
03	Chapila	Karwan Bazar	Chittagong
04	Hilsa	Showarighat	Bhola
05	Hilsa	Karwan Bazar	Chittagong
06	Hilsa	Karwan Bazar	Barisal
07	Toli shad	Nayabazar	Chittagong
08	Bigeye ilisha	Nayabazar	Chandpur
09	Bigeye ilisha	Karwan Bazar	Oman

2.3 Sample preservation

Fresh fish sample were collected carefully and preserved at -20^0C in lab freezer at Department of Fisheries, University of Dhaka.

2.4 Taxonomic study

Fishes were examined while still fresh. Total length (TL), standard length (SL), head length (HL), eye diameter (ED), pre orbital length (ProL), post orbital length (PosL), upper jaw length (UJL), lower jaw length (LJL), pectoral fin length (PFL), pelvic fin length (PVFL), body depth (BD), caudal peduncle depth (CPD) were measured in cm using measuring tape. The taxonomic study was done according toShafi and Quddus(1982); Rahman(2005) and Talwar and Jhingran(1991).(Appendix 1).

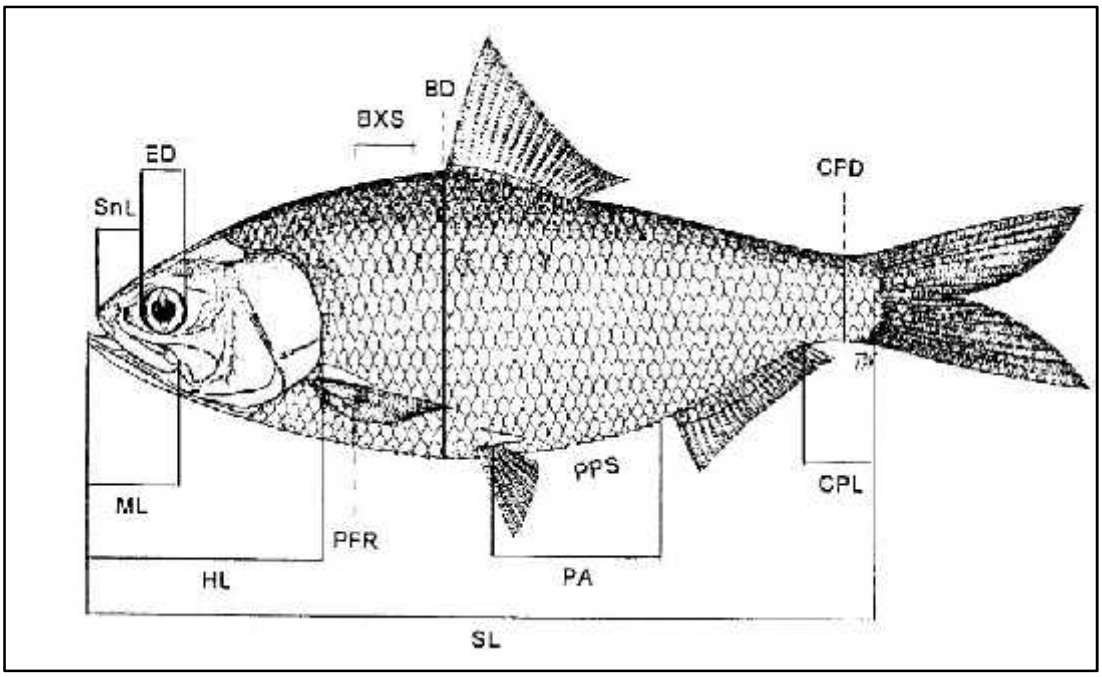


Fig. 2.1. Morphological characters measured. SL: standard length; HL: head length; SnL: snout to start of eye orbit; ED: eye diameter (horizontal); IOX: width of head across eyes; ML: snout-jaw length; BXS: body width at anterior dorsal fin; BD: body depth anterior to the dorsal-pelvic fin; PA: inner pelvic fin–anus distance; CPD: caudal peduncle height; CPL: caudal peduncle length post-anal fin to tail length; PFR: pectoral fin rays; PPS: post-pelvic scutes.

2.5 DNA isolation

2.5.1 DNA isolation by Maxwell R16

First the samples were cut by scissor. Then the tissue sample were collected and taken into the DNA kits with plungers. After that, 300µl of elution buffer was taken into the elution tube. DNA kits were placed into the Automatic nucleic acid purification system (Maxwell® 16 MDx Research Instrument, Promega, USA) for 35 minutes for washing.

Fig. 2.2.Photograph of Maxwell® 16 DNA extraction kit

2.5.2 DNA isolation by QIAGENDNeasy® Blood & Tissue Kit

Equipment and reagents required:

- DNeasy Blood & Tissue Kit (cat. no. 69504 or 69506)
- Pipets and pipet tips
- Vortexer
- Micro centrifuge tubes (1.5 ml)
- Micro centrifuge with rotor for 1.5 ml and 2 ml tubes
- Thermo mixer, shaking water bath, or rocking platform for heating at 56°C
- Ethanol (96–100%)
- Mortar and pestle
- Liquid nitrogen

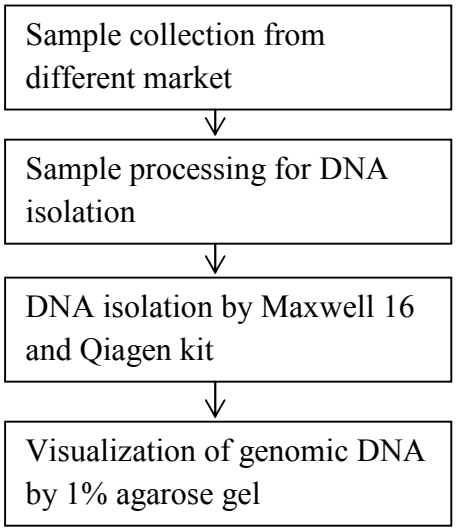
Procedure — using a mortar and pestle

1. 50 mg tissue sample was grinded in liquid nitrogen using a mortar and pestle and then the powder was placed in a 1.5 ml micro centrifuge tube. To prevent cross-contamination, the mortar and pestle was cleaned thoroughly.
2. 180µl Buffer ATL was added in the micro centrifuge tube.
3. 20µl proteinase K was added. Then mixed thoroughly by vortexing, and incubate at 56°C until the samples were completely lysed. Vortex was done occasionally during incubation to disperse the sample, or placed in a thermo mixer, shaking water bath, or on a rocking platform. Lysis time varies depending on the type of sample processed. Lysis was usually completed in 1–3 h. If it is more convenient, samples can be lysed overnight.

4. Vortexing was done for 15 s. 200µl Buffer AL was added to the sample, and mixed thoroughly by vortexing. Then 200µl ethanol (96–100%) was added, and mixed again thoroughly by vortexing.
5. The mixture from step 4 (including any precipitate) was placed into the DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at ≥6000 x g (8000 rpm) for 1 min. After centrifuge, the flow-through and collection tube was discarded.
6. The DNeasy Mini spin column was placed in a new 2 ml collection tube (provided), 500 µl Buffer AW1 was added, and centrifuged for 1 min at ≥6000 x g (8000 rpm). Then the flow-through and collection tube was discarded.
7. Again the DNeasy Mini spin column was placed in a new 2 ml collection tube (provided), 500 µl Buffer AW2 was added, and centrifuged for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Then the flow-through and collection tube was discarded.
8. The DNeasy Mini spin column was placed in a clean 1.5 ml or 2 ml micro centrifuge tube, and 200 µl Buffer AE was pipetted directly onto the DNeasy membrane. Then incubated at room temperature for 1 min, and centrifuged for 1 min at ≥6000 x g (8000 rpm) to elute.

Fig. 2.3.Photograph of QIAGENDNeasy® Blood & Tissue Kit for DNA extraction

Flowchart 2: Overview of DNA isolation



2.6 DNA quantification

DNA quantification was done by Nano Drop spectrophotometer (Nano Drop 2000 UV-Vis spectrophotometer, Thermo Fisher Scientific Inc., USA) at 260/280nm to determine the purity of DNA of fish samples was around 1.8. Ratio less than 1.8 indicate that the preparation was contaminated, either with protein, RNA or with phenol (Appendix 2).

Fig. 2.4.Photograph of Nano Drop 2000 UV-Vis spectrophotometer for DNA quantification

2.7 DNA Barcoding

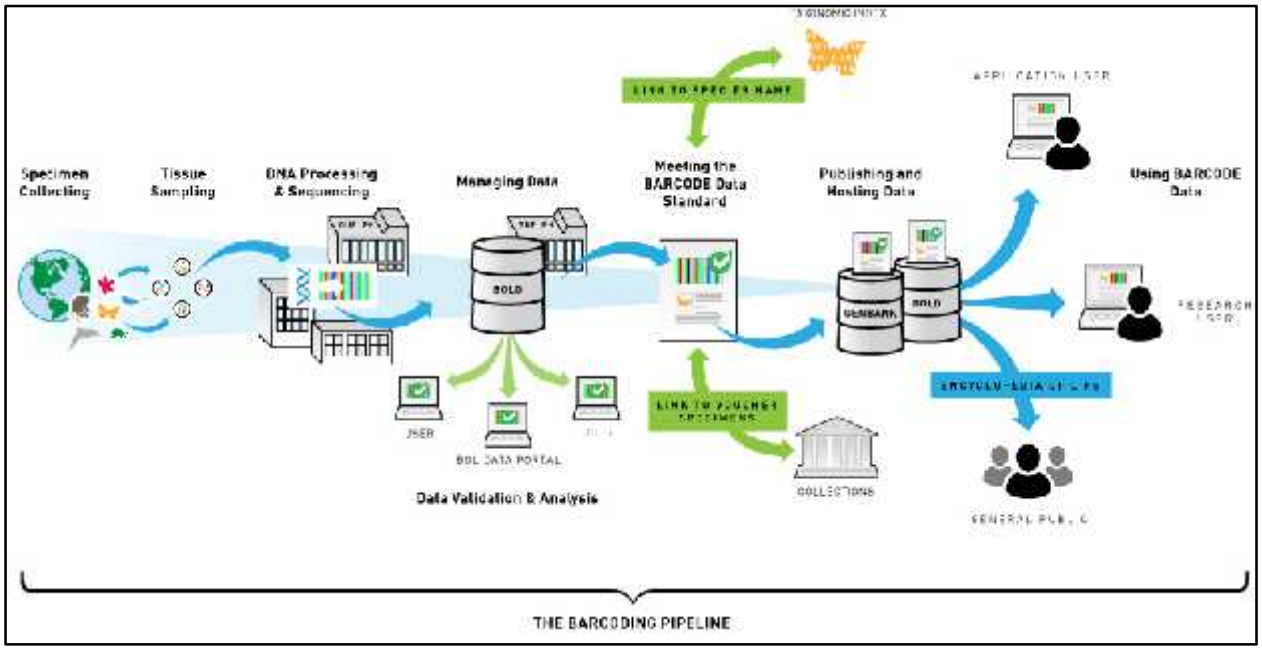


Fig. 2.5.Photograph of DNA Barcoding Pipeline (Source: http://www.barcodeoflife.org/content/about/what-dna-barcoding)

2.7.1 List of Primers

Sl. No.	Primer codes	Type	Sequences (5´-3´)
01	F1	**Forward**	TCAACCAACCACAAAGACATTGGCAC
02	R1	**Reverse**	TAGACTTCTGGGTGGCCAAAGAATCA

2.7.2 PCR reaction mixture

PCR reaction mixture was prepared by mixing the specific volume of the components in an appropriate sized tube provided in the following table.

- 12.5µl of Hot Start Green Master Mix containing- dNTPs, Buffer, $MgCl_2$, Taq polymerase (Cat: M7432, Promega, USA). Template DNA was prepared and aliquoted into PCR tubes.
- Specific template was added into a labeled PCR tube. PCR tube containing reaction mixture and template DNA was capped properly followed by vortex and centrifugation briefly to mix the mixture well.

- The PCR tubes were then placed in a thermal cycler and the cycling was started immediately. PCR amplification was done in an oil-free thermal cycler (Applied Biosystems® 2720 Thermal Cycler).

Component	Volume (µl)	Total volume (µL)
GoTaq® G2 Hot Start Green Master Mix, 2X	12.5	12.5×8=100
Forward Primer	1	1×8=8
Reverse Primer	1	1×8=8
DNA template	1	1×8=8
Nuclease-Free Water to	9.5	9.5×8=76
Total reaction volume	25	200

2.7.3 PCR reaction condition

The reaction mixtures containing PCR tubes were pre heated for 4 minutes at 94^0C in the thermal cycle to ensure the denaturation of all DNA templates. The PCR reaction was then continued for 32 cycles according to the following table.

PCR Condition		
Stage	**Temperature**	**Time**
Initial Heating	94^0C	2 min
Denaturation	94^0C	30 sec
Annealing	54^0C	30 min
Extension	72^0C	1 min
Final Extension	72^0C	5 min

2.7.4 Gel electrophoresis

The successful amplification of the desired genes was visualized by resolving the PCR products in 1% agarose gel (w/v) depending on the size of amplicon.

- The gel was prepared using 0.32g 1% Agarose powder (V3125, Promega, USA) and 40ml 1X TAE buffer (V4251, Promega, USA).

- The mixture was heated in a hot plate and magnetic stirrer (VS- 130HS, Vision scientific Inc., LTD, Korea) for about 3 minutes to dissolve the agarose.
- Boiled mixture was allowed to cool about 45^0 C and 2µL of Ethidium Bromide (H5041, Promega, USA) was added.
- Then the gel was poured onto gel casing preset with well former (comb) and allowed to set on a flat surface for about 15 minutes.
- After solidification of the gel, the comb was removed and buffer (1X TAE) was poured onto tank to submerge to solidified gel.
- The samples were prepared by mixing 5µL PCR product with 1µL loading dye and each 6µL prepared PCR product was loaded into the wells formed in the gel.
- The electrophoresis was conducted in 1X TAE buffer at 100 Volts for 40 minutes.
- 1kb DNA ladder was also electrophoresed along the side of the amplified sample DNA.
- DNA bands were observed and photographed by Alphalmager MINI Gel-documentation system (ProteinSimple, USA).

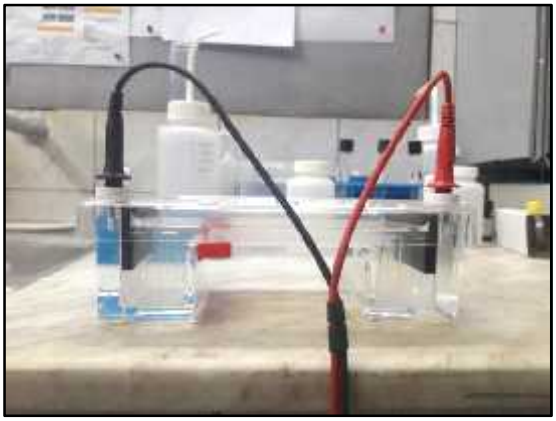


Fig. 2.6.Photograph of Gel electrophoresis of PCR product at 120V for 60min

2.7.5 PCR product purification

PCR products were resolved by agarose gel electrophoresis to confirm successful amplification of desired sequence. The PCR product of specific genes were purified with the Wizard PCR SV Gel and PCR Clean-Up System Kit (Promega, USA) according to the manufacturer's instruction. The steps of purification are given below.

a) Processing of PCR products

An equal volume of Membrane Binding Solution was added to the PCR amplification. Binding of DNA A SV Minicolumn was inserted into collection tube. The prepared PCR product was transferred to the Minicolumn assembly and incubated at room temperature for 1 minute. The preparation was centrifuged at 16000X g for 1 minute using the centrifuge (Sigma, USA). The flow through was discarded and the Minicolumn was reinserted into collection tube.

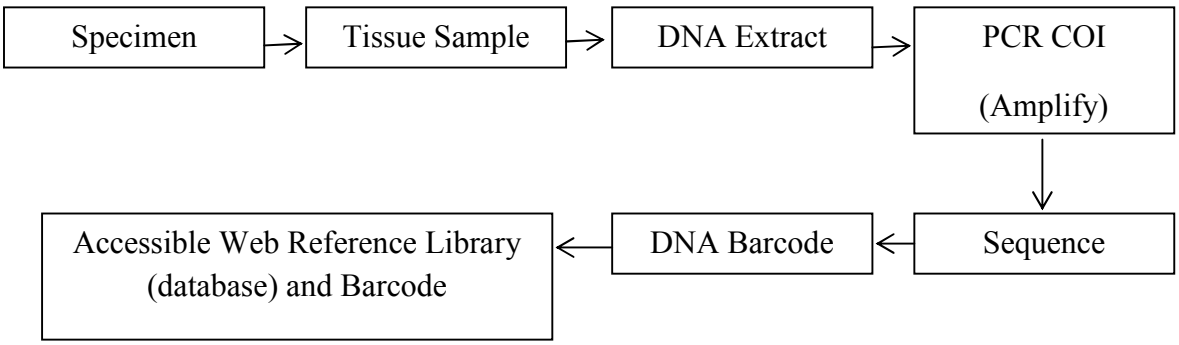
b) Washing

700µl Membrane Wash Solution was added into the Minicolumn and centrifuge at 16000Xg for 1 minute. The flow through was discarded and the Mini column was reinserted into Collection Tube. The previous step was repeated with 500µl Membrane Wash Solution and centrifuged at 16000 X g for 5minutes. The Collection Tube was emptied and the column assembly was re-centrifuged for 1 minute with the micro-centrifuge lid open to allow evaporation of any residual ethanol.

c) Elution

The Minicolumn was carefully transferred to a clean 1.5 mL micro centrifuge tube. 50 µL Nuclease-Free Water was added to the Minicolumn and incubated at room temperature for 1minute followed by centrifugation at 16000 X g for 1 minute. The Minicolumn was discarded and DNA was stored at 4°C or -20°C.

Flowchart 3: Overview of DNA barcoding process



2.7.6 Automated Sequencing and Bioinformatics analysis

The sequences were analyzed by different bioinformatics tools. These tools are given below-

Finch TV version 1.4

Geospiza'sFinchTV is the popular way to view DNA sequence. It leads the way with raw data views, BLAST searching and the ability to reverse complement sequences and traces.

Nucleotide BLAST

In bioinformatics, BLAST for Basic Local Alignment Search Tool is an algorithm for comparing primary biological sequence information, such as the amino-acid sequences of proteins or the nucleotides of DNA sequences.

CLUSTALW

CLUSTALW is a tool to align three or more sequences together in a computationally efficient manner. Aligning multiple sequences highlights areas of similarity which may be associated with specific features that have been more highly conserved than other regions. These regions in turn can help classify sequences or to inform experiment design. Multiple sequence alignment is also an important step for phylogenetic analysis, which aims to model the substitutions that have occured over evolution and derive the evolutionary relationships between sequences.

MEGA 7

MEGA （Molecular Evolutionary Genetics Analysis）is an integrated tool for automatic and manual sequence alignment, inferring phylogenetic trees, mining web-based databases, estimating rates of molecular evolution, and testing evolutionary hypotheses.

2.8 Randomly Amplified Polymorphic DNA (RAPD)

Amplification of the isolated DNA sample was performed by Polymerase Chain Reaction (PCR) for further analysis.

2.8.1 List of primers for RAPD

Sl. No.	Primer codes	Sequences (5´-3´)
01	OPA-03	AGTCAGCCAC
02	OPA-04	AATCGGGCTG
03	OPA-09	GGGTAACGCC
04	OPAL-04	ACAACGGTCC
05	OPAW-09	ACTGGGTCGG
06	OPAK-04	AGGGTCGGTC
07	OPA-02	TGCCGAGCTG
08	OPG-04	AGCGTGTCTG
09	OPF-01	ACGGATCCTG
10	OPG-05	CTGAGACGGA

2.8.2 PCR reaction mixture

PCR reaction mixture was prepared by mixing the specific volume of the components in an appropriate sized tube provided in the following table.

- 7.5µl of Hot Start Green Master Mix containing- dNTPs, Buffer, $MgCl_2$, Taq polymerase (Cat: M7432, Promega, USA). Template DNA was prepared and aliquoted into PCR tubes.
- Specific template was added into a labeled PCR tube. PCR tube containing reaction mixture and template DNA was capped properly followed by vortex and centrifugation briefly to mix the mixture well.
- The PCR tube were then placed in a thermal cycler and the cycling was started immediately. PCR amplification was done in an oil-free thermal cycler (Applied Biosystems® 2720 Thermal Cycler).

Component	Volume (µl)	Total volume (µL)
GoTaq® G2 Hot Start Green Master Mix, 2X	7.5	7.5×40 = 300
Upstream primer, 10µM	1.5	1.5×40 = 60
DNA template	2	2×40 = 80
Nuclease-Free Water to	14	14×40 = 560
Total reaction volume	25	1000

2.8.3 PCR reaction condition

The reaction mixtures containing PCR tubes were pre heated for 5 minutes at 95^0C in the thermal cycle to ensure the denaturation of all DNA templates. The PCR reaction was then continued for 40 cycles according to the following table.

PCR Condition		
Stage	**Temperature**	**Time**
Initial Heating	95^0C	5 min
Denaturation	95^0C	30 sec
Annealing	34^0C	30 sec
Extension	72^0C	1min
Final Extension	72^0C	5min

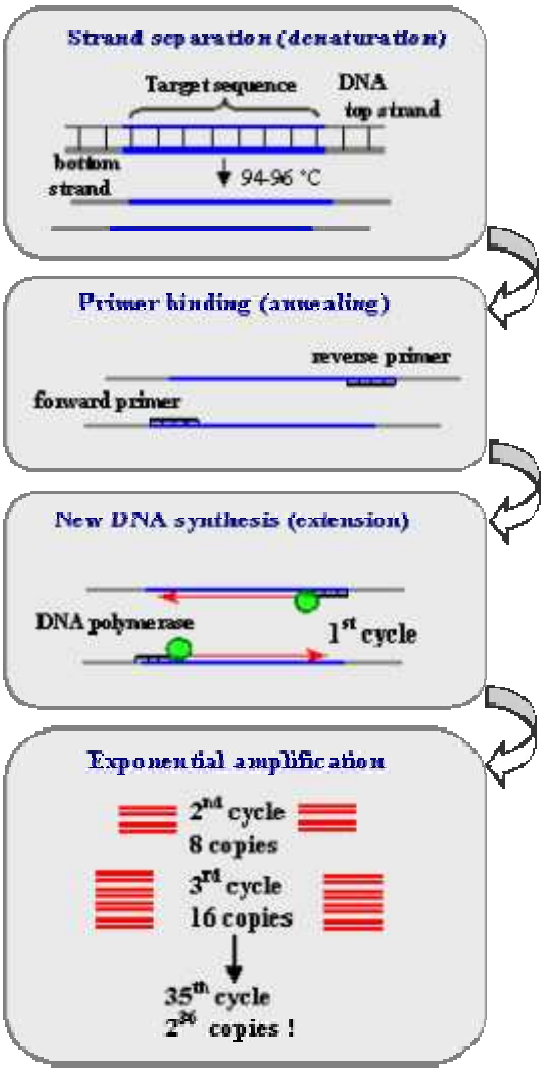


Fig. 2.7.Photograph of PCR amplification steps (Source: https://www.ncbi.nlm.nih.gov/probe/docs/techpcr/)

2.8.4 Gel electrophoresis and documentation

Gel electrophoresis was done according to section **2.7.4**

2.8.5 Data analysis

POPGENE 32

Popgene - Population Genetic Analysis is a software application whose purpose is to aid people in analyzing genetic variations within the population, using co-dominant or dominant markers.

MEGA 7as described**2.7.6**

Chapter 3

Results

3.1 Taxonomic identification

Total length (TL), standard length (SL), head length (HL), eye diameter (ED), pre orbital length (ProL), post orbital length (PosL), upper jaw length (UJL), lower jaw length (LJL), pectoral fin length (PFL), pelvic fin length (PVFL), body depth (BD), caudal peduncle depth (CPD) of the fish samples were measured in cm and given below in the table 3.1.

Table 3.1Morphological data of collected samples in centimeter (cm.)

Sl.	English Name	Collection Place	Origin	T.L	F.L	S.L	P.D.L	B.H	P.L	H.L	P.O.L	I.O.L
1	Chapila	Jatrabari	Chittagong	15.9	14.5	14	6	3.5	1.3	2.5	0.6	0.9
2	Chapila	Karwan Bazar	Chittagong	15.5	13.6	13	6.1	3.4	1.2	2.4	0.5	0.9
3	Chapila	Karwan Bazar	Chittagong	15.7	13.5	12.5	5	3.5	1.4	2.6	0.5	1
4	Hilsa	Showarighat	Bhola	27	22.6	21.5	10.9	7.4	1.9	5.5	1.8	1.3
5	Hilsa	Karwan Bazar	Chittagong	11	9.6	9	3.2	3.5	1.4	2.2	0.4	0.7
6	Hilsa	Karwan Bazar	Barisal	28	22.3	21.3	9.7	7	2	3.9	1.5	0.8
7	Toli shad	Nayabazar	Chittagong	23.8	19.9	19	8.7	7	2	4.2	1.8	1.1
8	Bigeye ilisha	Nayabazar	Chandpur	23.2	20.8	19.5	9.5	4.6	1.6	4.2	2	0.9
9	Bigeye ilisha	Karwan Bazar	Oman	22.9	20.6	19.3	9.3	4.4	1.5	4	1.9	0.8

Sl.	English Name	E.D	L.U.J	L.L.J	G.W	L.D.F/F.B	L.P.F/F.B	L.A.F/F.B	L.C.F	L.U.L	L.L.L	Species Name
1	Chapila	0.5	0.5	0.6	0.9	2.2	0.2	2.2	3.5	0.5	0.5	*Gudusia chapra*
2	Chapila	0.5	0.4	0.5	0.8	2.2	0.2	2.1	3.4	0.5	0.5	*Gudusia chapra*
3	Chapila	0.6	0.5	0.6	0.8	2.3	0.2	2.3	3.5	0.6	0.6	*Gudusia chapra*
4	Hilsa	0.6	1.6	1.4	0.8	3.8	0.6	3.6	5.5	1.8	2	*Tenualosa ilisha*
5	Hilsa	0.3	0.3	0.2	0.7	2	0.2	1.8	2.9	0.4	0.3	*Tenualosa ilisha*
6	Hilsa	0.5	2.4	2.2	1.6	2.5	0.4	2.4	5.7	1.9	1.8	*Tenualosa ilisha*
7	Toli shad	0.6	2	1.8	1.1	2.8	0.3	2.9	5.3	1.6	1.8	*Tenualosa toli*
8	Bigeye ilisha	0.5	2.1	1.6	1.1	2.9	0.7	2.4	4.3	1.2	1.2	*Ilisha megaloptera*
9	Bigeye ilisha	0.4	1.9	1.2	0.9	2.8	0.6	2.2	4.2	1	1	*Ilisha megaloptera*

[N.B: T.L = Total length F.L= Fork length S.L= Standard length

P.D.L= Pre dorsal length B.H= Body height P.L= Peduncle length

H.L= Head length P.O.L= Pre orbital length I.O.L= Inter orbital length

E.D= Eye diameter L.U.J= Length of the upper jaws G.W= Gap Wide

L.L.J= Length of the lower jaws L.D.F/F.B= Length of dorsal fin/ fin base

L.P.F/F.B= Length of pelvic fin/ fin base L.A.F/F.B= Length of anal fin/ fin base

L.C.F= Length of the caudal fin L.U.L= Length of the upper lobe L.L.L= Length of the lower lobe.]

3.1.1 Taxonomic study of Chapila

According to Rahman(2005); Talwar and Jhingran(1991) and the measured morphometric and meristic data showed that the sample 1, 2 and 3 were *Gudusia chapra*. The taxonomic classification of chapila is given below.

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Clupeiformes

Family: Clupeidae

Genus: *Gudusia*

Species: *Gudusia chapra* (Hamilton, 1822)

Fig. 3.1.Photograph showing the specimen sold as chapila (*Gudusia chapra)*

3.1.2 Taxonomic study of Hilsa

According to Shafi and Quddus(1982); Rahman(2005) and the measured morphometric and meristic data showed that the sample 4, 5 and 6 were *Tenualosa ilisha.* The taxonomic classification of hilsa is given below.

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Clupeiformes

Family: Clupeidae

Subfamily: Alosınae

Genus: *Tenualosa*

Species: *Tenualosa ilisha* (Hamilton, 1822)

Fig. 3.2. Photograph showing the specimen sold as Padma ilish(*Tenualosa ilisha)*

3.1.3 Taxonomic study of Toli shad

According to Shafi and Quddus(1982); Rahman(2005) and the measured morphometric and meristic data showed that the sample 7 was *Tenualosa toli.* The taxonomic classification of toli shad is given below.

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Clupeiformes

Family: Clupeidae

Genus: *Tenualosa*

Species: *Tenualosa toli* (Valenciennes, 1847)

Fig. 3.3. Photograph showing the specimen sold as chandanailish(*Tenualosa toli)*

3.1.4 Taxonomic study of Big eye ilish

According to Rahman(2005); Talwar and Jhingran(1991) and the measured morphometric and meristic data showed that the sample 8 and 9 were *Ilisha megaloptera.* The taxonomic classification of big eye ilish is given below.

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Clupeiformes

Family: Pristigasteridae

Genus: *Ilisha*

Species: *Ilisha megaloptera* (Swainson, 1839)

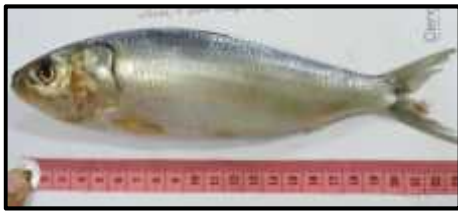


Fig. 3.4. Photograph showing the specimen sold as Bangla ilish(*Ilisha megaloptera)*

3.2 DNA Barcoding

3.2.1 PCR amplification

The extracted DNA from four fish samples were amplified by PCRamplification for Cytochrome Oxidase subunit 1 (COI) gene using COI specific primer F1 and R1. All the samples showed bright band.

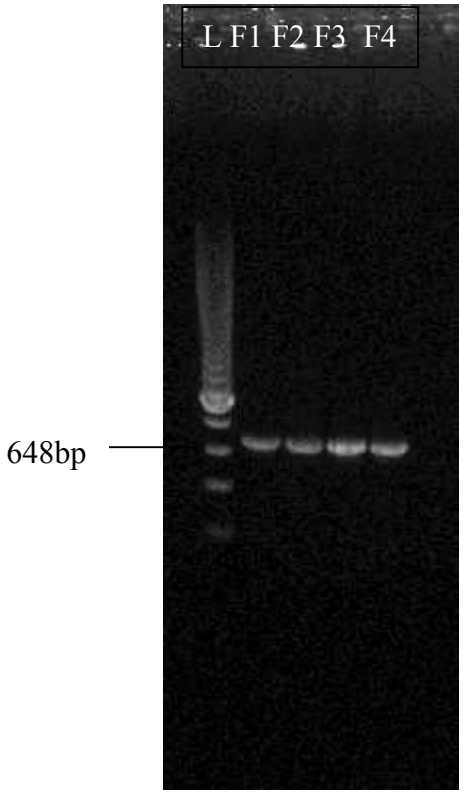


Fig. 3.5. PCR amplification of four fish samples by COI gene using FishF1 and FishR1 primers. Lane F1 denote chapila (*Gudusia chapra),* F2 denotes hilsa (*Tenualosa ilisha),* F3 denotes toli shad (*Tenualosa toli),* F4 denotes big eye ilish (*Ilisha megaloptera)* and L denotes 1kb ladder

3.2.2 DNA Sequence interpretation

DNA sequencing results obtained from the fish samples collected from the local fish market. The resulting PCR products were sequenced to produce full length DNA barcodes averaging 648bp in length, with no detectable insertions, deletions or stop codon. Among the four samples, F1 sample did not produce quality sequence. This failure could most likely be attributed to DNA degradation or the poor concentration of DNA. There may be some concern as to whether the DNA was degraded and unrecoverable due to long term preservation.From the chromatogram, the sequence data

were transferred to FASTA format and blast within nucleotide database to identify species using NCBI blast. Peak intensities and sequencing qualities of the generated barcodes were compared to the sequences downloaded from NCBI Gene Bank.

Table 3.2Identification of collected samples using the Gene Bank database

Sl.	Sold as	Description	Max score	Total score	Query cover	E value	Identity	Accession
F2	*Hilsa*	*Sardinella longiceps* cytochrome oxidase subunit I (COI) gene, partial cds; mitochondria	1050	1050	100%	0.0	99%	KR905704.1
F3	Chandanailish	*Hilsa kelee* voucher ADC54.5-4 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	1079	1079	100%	0.0	99%	JF493644.1
F4	Bangla ilish	*Sardinella longiceps* isolate 4 cytochrome c oxidase subunite 1 gene, partial cds; mitochondrial	1050	1050	100%	0.0	99%	KM016230.1

Specimen F2 sold as hilsa (*Tenualosa ilisha)* and specimen F4 sold as banglailish (*Ilisha megaloptera)* but identified in GeneBank as Indian oil sardine (*Sardinella longiceps)* which accession numbers are KR905704.1 and KM016230.1. Both the collected species were 99% similar with *Sardinella longiceps.Sardinella longiceps*found in Indian Ocean especially in the northern and southern parts. Highly school forming fish and feeds on phytoplankton and small crustaceans.

Sample F3 sold as chandanailish (*Tenualosa toli)* but identified in GeneBankaskelee shad(*Hilsa kelee)* which accession numbers is JF493644.1. Sample F3 was 99% similar with *Hilsa kelee.* Kelee shad is native to the coasts and estuaries of the Indian Ocean and the western Pacific, generally in tropical waters. It feeds on diatoms and dinoflagellates, and any other small plankton.

3.2.3 DNA Sequence alignment

Alignment among three species F2, F3 and F4

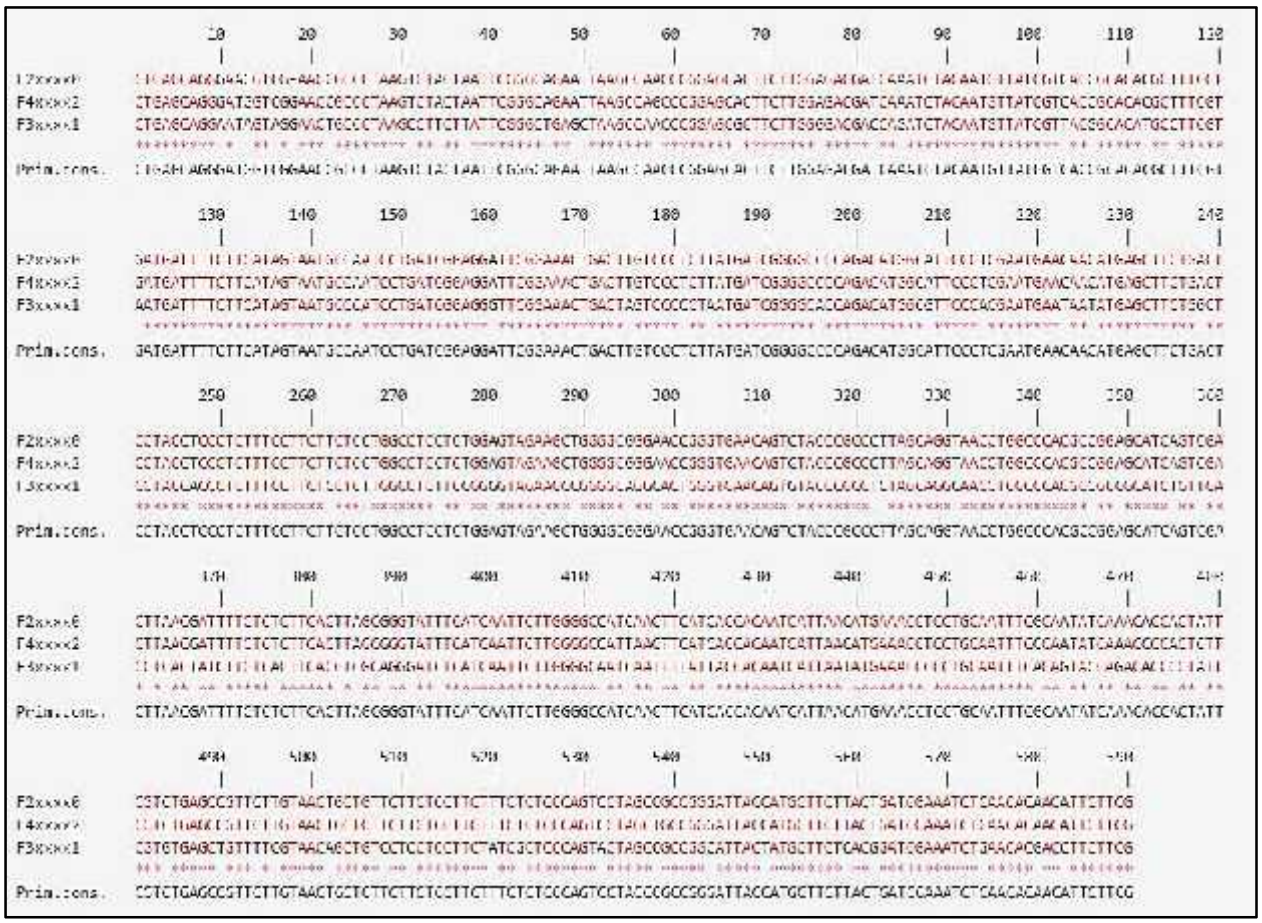


Fig. 3.6. Multiple sequence alignment of COI gene fragment of fish sample F2, F3 and F4

Comparison of COI gene sequence of sample F2 (*Sardinella longiceps*), F3 (*Hilsa kelee*) and F4 (*Sardinella longiceps*). Alignment lengths were 590bp and the differences among them were 15.76%.

Alignment data :
Alignment length : 590
Identity (*) : 497 is 84.24 %
Strongly similar (:) : 0 is 0.00 %
Weakly similar (.) : 0 is 0.00 %

Different : 93 is 15.76 %
Sequence 0001 : F2xxxx0 (589 residues).
Sequence 0002 : F4xxxx2 (590 residues).
Sequence 0003 : F3xxxx1 (590 residues).

Alignment between fish sample F2 and F4

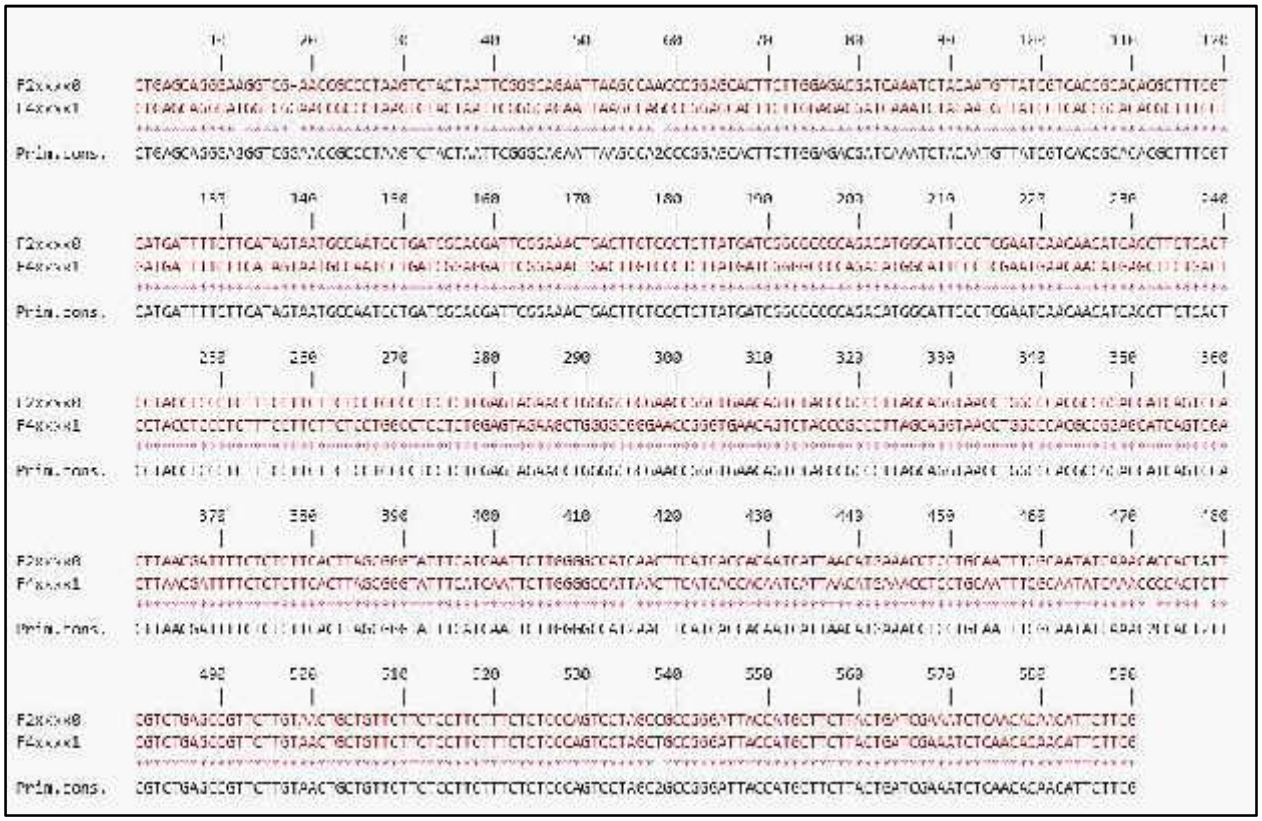


Fig. 3.7. Pairwise sequence alignment of COI gene fragment of fish sample F2 and F4

The collected fish sample F2 and F4 were *Sardinella longiceps*. There alignment length was 590bp and they had the identity of 98.81%.

Alignment data :
Alignment length : 590
Identity (*) : 583 is 98.81 %
Strongly similar (:) : 0 is 0.00 %
Weakly similar (.) : 0 is 0.00 %
Different : 7 is 1.19 %
Sequence 0001 : F2xxxx0 (589 residues).
Sequence 0002 : F4xxxx1 (590 residues).

Alignment between sample F3 and *Tenualosa ilisha*

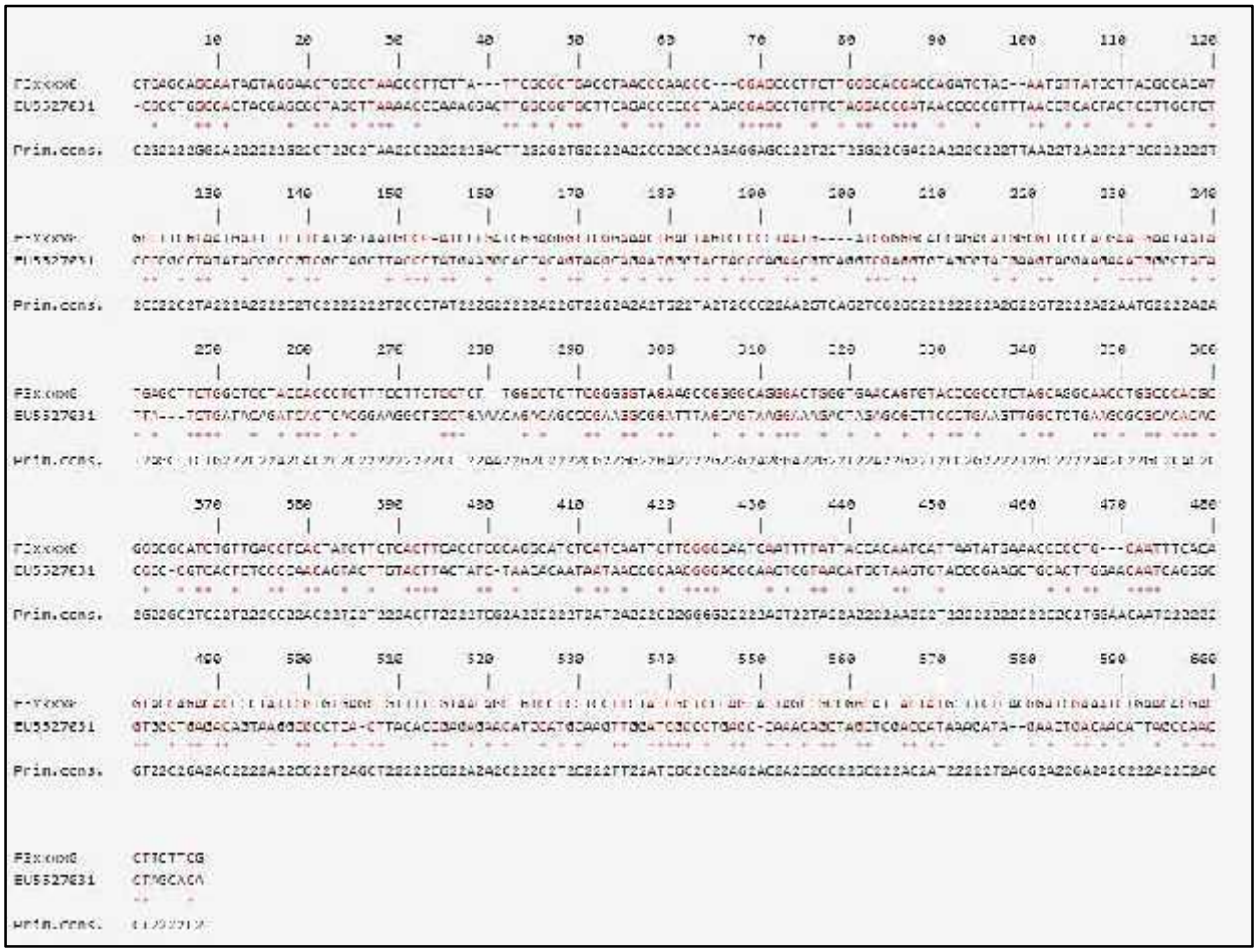


Fig. 3.8. Pairwise sequence alignment of COI gene fragment of sample F3 and *Tenualosa ilisha*

The collected fish sample F3 (*Hilsa kelee)* was aligned with *Tenualosa ilisha.* There alignment length was 608bp. They were very much different from each other with difference of 60.03%.

Alignment data :
Alignment length : 608
Identity (*) : 243 is 39.97 %
Strongly similar (:) : 0 is 0.00 %
Weakly similar (.) : 0 is 0.00 %
Different : 365 is 60.03 %
Sequence 0001 : F3xxxx0 (590 residues).
Sequence 0002 : EU5527031 (598 residues).

3.2.4 Phylogenetic tree analysis

Phylogeny Test

Test of Phylogeny: Bootstrap method

No. of Bootstrap Replications: 1000

Substitutions Type: Nucleotide

Model/Method: Maximum Likelihood method

Rates among sites: Uniform rates

ML Heuristic Method: Nearest-neighbor-Interchange (NNI)

No. of sites: 328

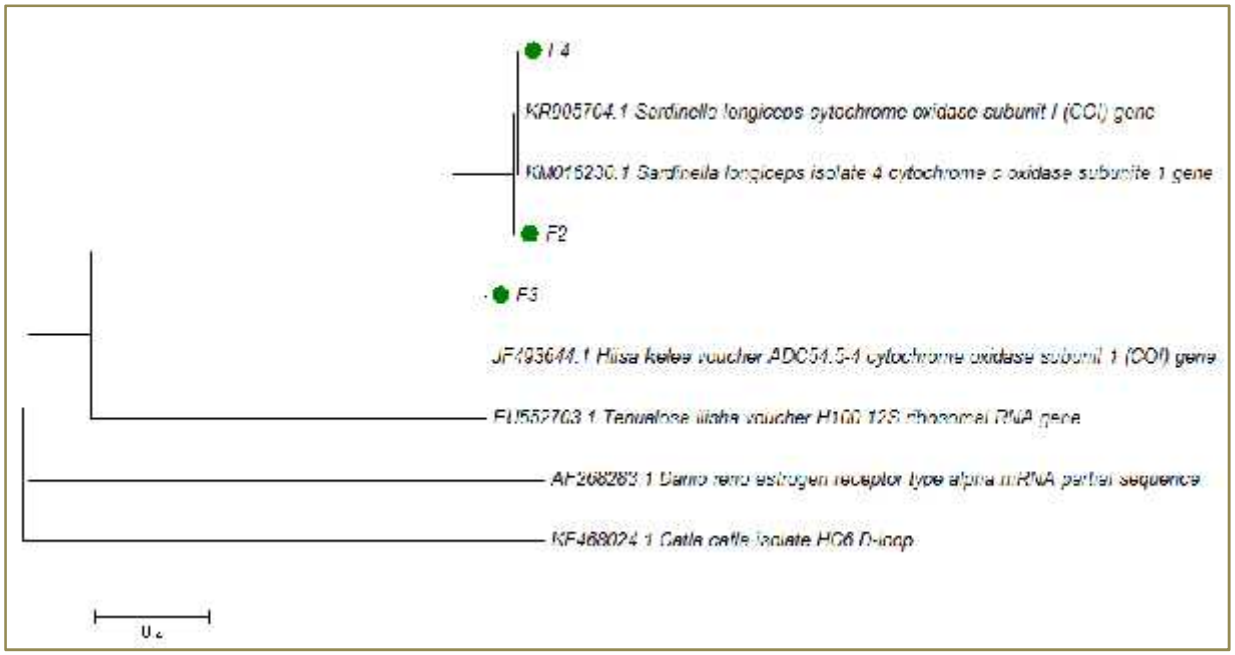


Fig. 3.9. Molecular Phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-3371.2930) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 9 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 565 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

The maximum likelihood phylogenetic tree shows that sample F2 and F4 were clustered together with Indian oil shad *Sardinella longiceps* and sample F3 clustered with kelee shad (*Hilsa kelee*). For constructing the phylogenetic tree *Tenualosa ilisha, Daniorerio*and *Catlacatla*were taken as outgroup (Fig 3.9).

3.3 RAPD Analysis

The selected ten primers were OPA-03, OPA-04, OPA-09, OPAL-04, OPAW-09, OPAK-04, OPA-02, OPG-04, OPF-01 and OPG-05. They produced clear and reproducible bands. These primers were selected for further RAPD analysis of chapila, Indian oil shad and kelee shad(Table 3.3). These ten primers produced a total of 134 bands of which 23 were polymorphic with 18.48% polymorphisms among four species of chapila, Indian oil shad and kelee shad. The size of the amplified DNA fragments was ranging from 200 to 1480bp. In addition to polymorphic bands, 83 unique bands were observed. The number, size, population and respective primer for each unique band were shown in Table 3.3 and Figure 3.10 (a-f), 3.11 (g-j).The unique bands were stable and specific for the respective population and thus could be used as a tool for characterization of a specific population. These results also indicate some degrees of genetic diversityamong four species of chapila, Indian oil shad and kelee shad.

Table 3.3 Compilation of RAPD analysis among four species of chapila and ilish.

Sl. no.	Primer Codes	Size ranges (bp)	Total bands	Polymorphic bands	Unique Band				Polymorphism (%)	Average Polymorphism (%)
					F1	F2	F3	F4		
01	OPA-03	270-1100	19	2 (600, 1050)	4 (310, 500, 720, 850)	4 (350, 530, 700, 1100)	3 (420, 710, 940)	4 (270, 390, 800, 900)	10.53	18.48
02	OPA-04	280-1480	17	3 (600, 700, 800)	2 (280, 500)	2 (350, 590)	4 (450, 720, 980, 1480)	1 (400)	17.65	
03	OPA-09	300-1300	17	2 (300, 880)	2 (390, 700)	3 (800, 960, 1250)	3 (500, 1000, 1300)	5 (420, 580, 690, 750, 900)	11.76	
04	OPAL-04	390-1350	10	1 (390)	2(480, 590)	2 (430, 580)	2 (600, 700)	1 (500)	10	
05	OPAW-09	500-1100	7	3 (500, 510, 600)	-	1 (1100)	-	-	42.86	
06	OPAK-04	480-1100	11	2 (480, 800)	-	1 (650)	4 (520, 700, 860, 1100)	-	18.18	
07	OPA-02	200-1400	14	4 (200, 300, 320, 1400)	-	1 (480)	3 (890, 1100, 1200)	2 (400, 810)	28.57	
08	OPG-04	300-1300	11	2 (600, 1150)	1 (900)	2 (500, 700)	3 (550, 850, 1300)	1 (300)	18.18	
09	OPF-01	250-1200	12	1 (900)	2 (300, 750)	2 (250, 600)	5 (450, 640, 700, 1000, 1200)	1 (400)	8.33	
10	OPG-05	310-1400	16	3 (310, 800, 1100)	1 (1200)	2 (500, 700)	3 (600, 900, 1400)	4 (410, 520, 720, 1300)	18.75	
Total		200-1480	134	23	83					18.48

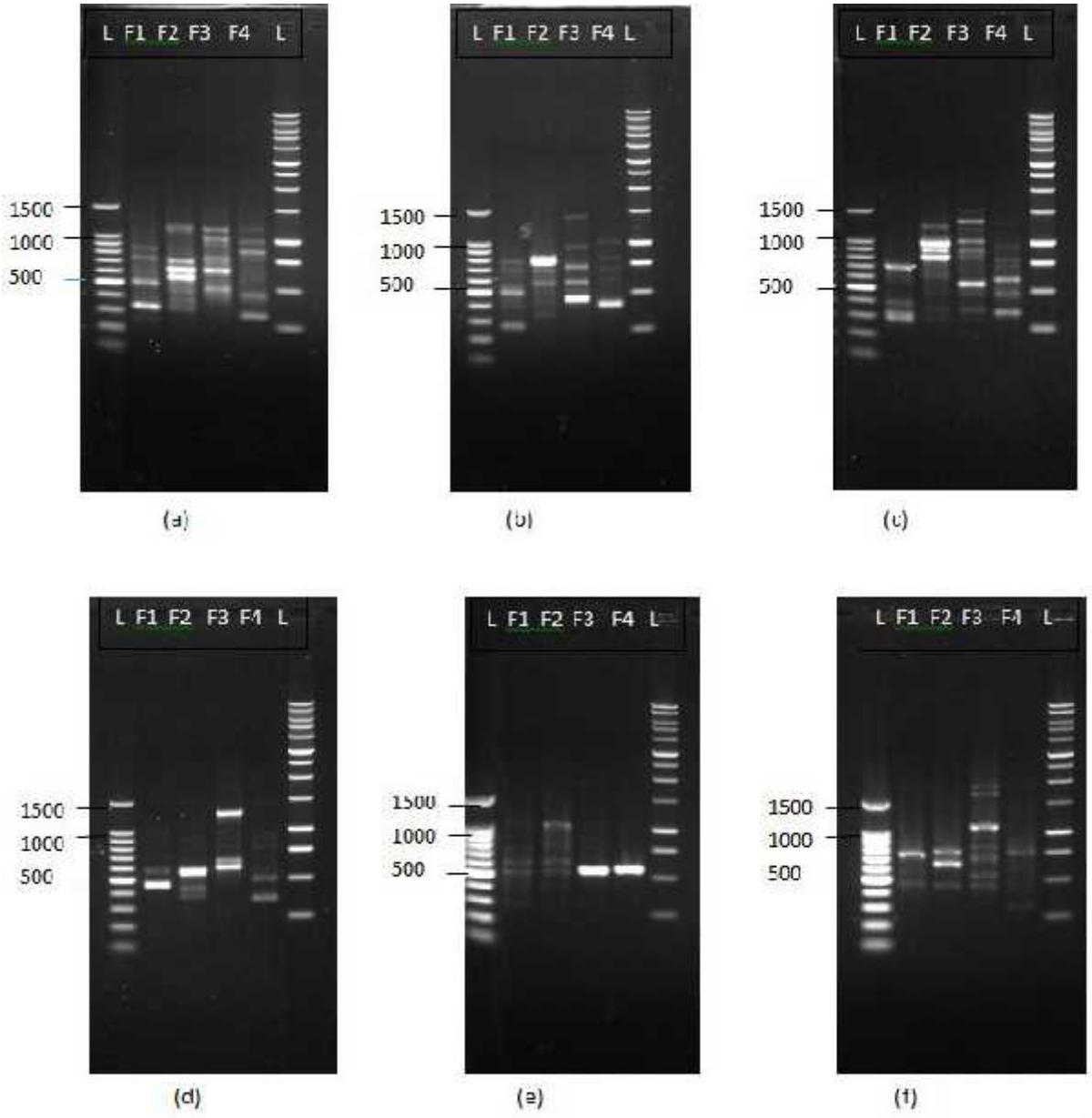


Fig. 3.10. (a-f). RAPD analysis with ten primers of four different fish species. a. Primer OPA-03, b. Primer OPA-04, c. Primer OPA-09, d. Primer OPAL-04, e. Primer OPAW-09, f. Primer OPAK-04, L= 1 kb DNA ladder, F1= chapila (*Gudusia chapra*), F2 and F4= Indian oil shad (*Sardinella longiceps)* and F3= kelee shad (*Hilsa kelee*).

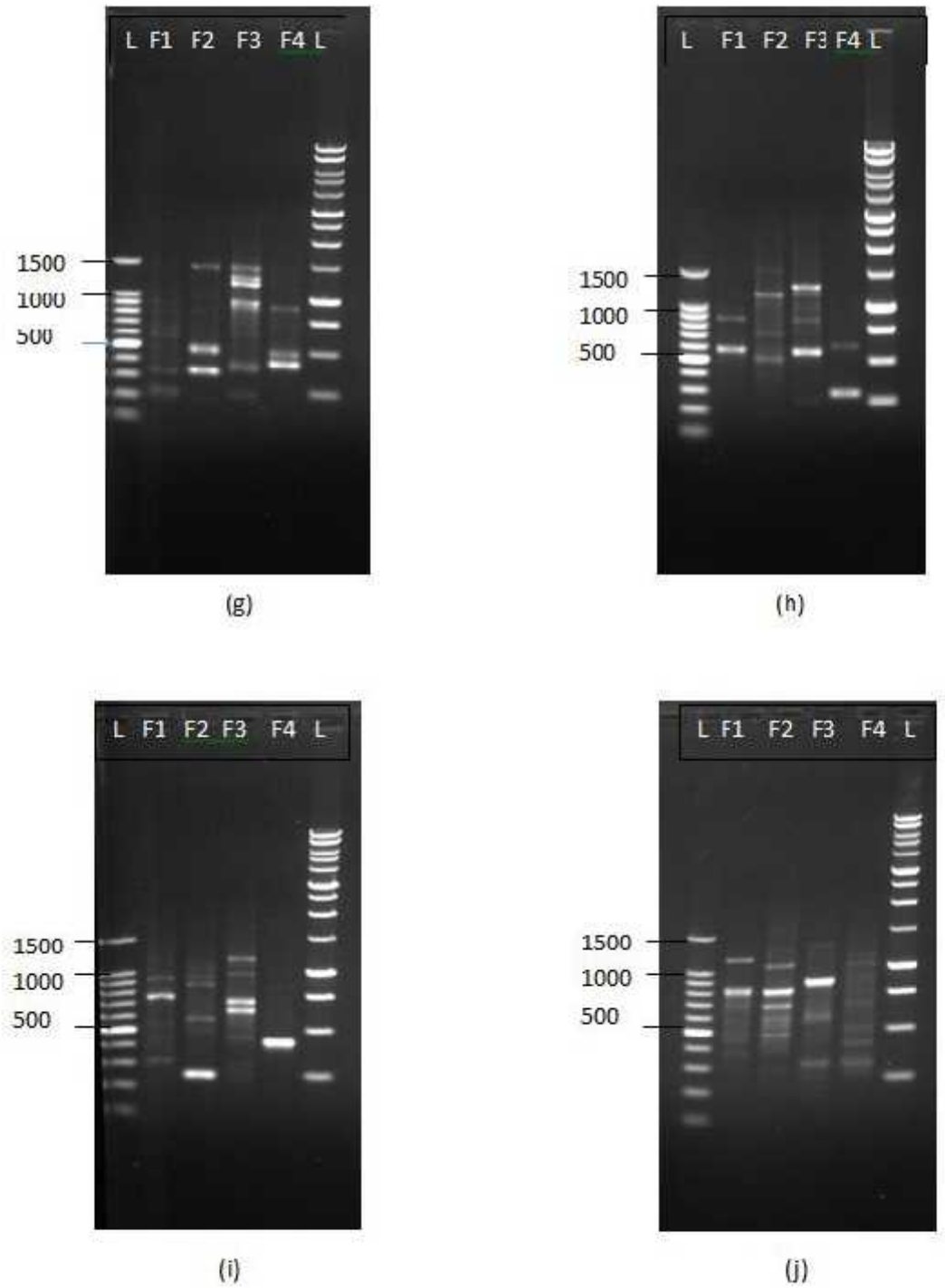


Fig. 3.11. (g-j). RAPD analysis with ten primers of four different fish species. g. OPA-02, h. OPG-04, i. OPF-01, j. OPG-05. L= 1 kb DNA ladder, F1= chapila (*Gudusia chapra*), F2 and F4= Indian oil shad (*Sardinella longiceps)* and F3= kelee shad (*Hilsa kelee*).

3.3.1 Genetic distance

The values of pair-wise genetic distances were analyzed by using computer software "POPGENE32" (version 1.31) using total RAPD fragments. The genetic distances ranged between 0.5077 and 0.9933 (Table 3.4). The highest genetic distance (0.9933) was found between *Gudusia chapra* and *Sardinella longiceps*. While the lowest genetic distance (0.5077) was found between *Sardinella longiceps* and *Hilsa kelee.*

Table 3.4 Genetic distance and genetic identities among four species

Sample Name	*Gudusia chapra*	*Sardinella longiceps*	*Hilsa kelee*	*Sardinella longiceps*
Gudusia chapra	****	0.3704	0.3981	0.3981
Sardinella longiceps	0.9933	****	0.6019	0.4907
Hilsa kelee	0.9209	0.5077	****	0.5741
Sardinella longiceps	0.9209	0.7118	0.5550	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal) calculated by POPGENE 32

3.3.2 Genetic identity

The values of pair-wise genetic identity were analyzed by using computer software "POPGENE32" (version 1.31) using total RAPD fragments. The genetic identity ranged between 0.6019 and 0.3704 (Table 3.4). The highest genetic identity (0.6019) was found between *Hilsa kelee* and *Sardinella longiceps*. While the lowest genetic identity (0.3704) was found between *Sardinella longiceps* and *Gudusia chapra.*

3.3.3 Cluster analysis (Tree diagram)

Cluster analysis on the basis of DNA fingerprinting by RAPD was carried out by POPGENE 32 (version 1.31) among four different species of chapila and hilsa. Dendrogram based on Nei's (1972) genetic distance was separated in three major clusters *viz.* C1, C2 and C3. Clusters C1 and C2 comprised *Sardinella longiceps and Hilsa kelee.* On the other hand, *Gudusia chapra* created the new distant cluster C3 (Fig 3.12).

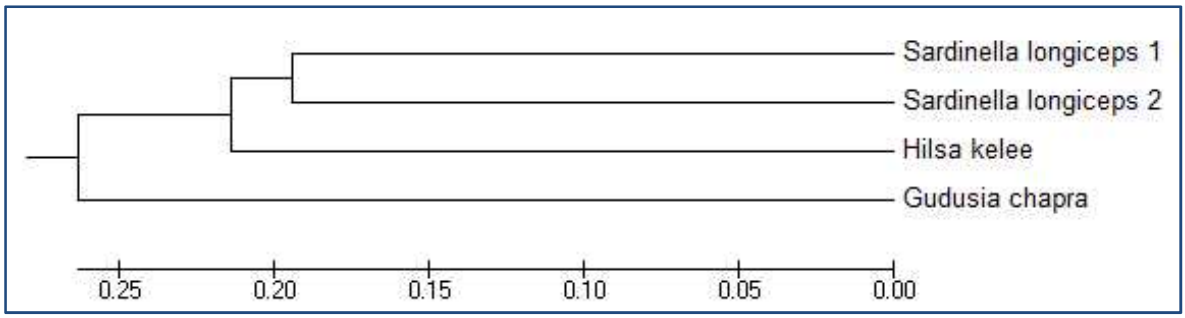


Fig. 3.12.UPGMA dendrogram based on RAPD analysis of four species of fish sample.Dendrogram was conducted in MEGA7 (Kumar et al., 2016).

Chapter 4
Discussion

4.1 Morphometric Study

Morphological descriptors are important for taxonomic classification of organism and estimation of species diversity (Dean et al., 2004). One of the major key in fish biology are morphometric characters as they are important for systematics, estimation of growth variability (Kovac and Copp, 1999) and population level studies (Verep et al., 2006). Variations in morphology are less obvious at the intra-specific level, whereas phenotypic variation is less obvious under genetic control and more subject to environmental influences (Clayton, 1981). Risch(1986) used both morphometric and meristic characters to cluster species and subspecies of the genus *Chrysichthy*into thee valid species: *C. auratus, C. maurus*and *C. nigrodigitatus*.

The analysis of morphometric characters obtained in this study shows that there are four morphologically distinct populations of *Gudusia chapra, Tenualosa ilisha, Tenualosa toli and Ilisha megaloptera.* Morphological characters were used in this work to make essential information about the differences among the four species. This information was used in the further molecular studies.

4.2 DNA Barcoding

DNA barcoding is effective in identifying species and provided a straightforward identification system when a perfect match existed between the morphology based taxonomy and genetic divergence. This study demonstrated the ability of DNA barcoding to calibrate the current taxonomic resolution and to shed new light on the fish diversity. The application of COI sequence in forensic has already been investigated for reproducibility, mixed DNA samples, chemical treatment, environmental conditions and other factors showing consistent results in which a great range of reference data exist (Dawny et al., 2007).

Despite the innovative applications of DNA barcoding, it has beencontroversial in some scientific circles (Ebach&Holdredge, 2005; Will andRubinoff, 2004). Interestingly recent resultsillustrated some straightforward benefits from the use of a standardized species-specific molecular tags derived from COI gene for species-level identifications (Hebert

et al., 2003; Lakra et al., 2011; Ward et al., 2005). DNA barcoding aims to provide an efficient method for species-level identifications using an arrayof species-specific molecular tags derived from COI gene (Pradhan et al., 2015). Kochzius et al. (2010) evaluated the applicability of the three mitochondrial genes for the identification of 50 European marine fish species by combining techniques of DNA barcoding and microarrays.DNA barcoding appears to hold greatpotential for fish species authentication monitoring system by both regulatory bodies and industry, the utilization of which could enhance transparency and fair trade on the domestic fisheries market in South Africa (Cawthorn et al., 2012). DNA barcoding was used to detect improper labeling and supersession of crab food served by restaurants in India (Vartak et al., 2015).As an emerging toolfor species identification, DNA barcoding can reliably assign unknown specimens to known species, also flagging potential cryptic species and genetically distant populations (Radulovici et al., 2010). There was a 27-fold more pronounced K2P distance differenceamong congeneric species of Canadian freshwater fish species than among conspecific individuals (Hubert et al., 2008).

In this study specimen F2 sold as hilsa (*Tenualosa ilisha)* and specimen F4 sold as banglailish (*Ilisha megaloptera)* but identified in GeneBank as both of them are Indian oil sardine (*Sardinella longiceps)* which accession numbers are KR905704.1 and KM016230.1. Sample F3 sold as chandanailish (*Tenualosa toli)* but identified in GeneBankaskelee shad(*Hilsa kelee)* which accession numbers is JF493644.1. This result is very much different from the morphometric and meristic study of the same fish sample. That means the morphometric and meristic study cannot identify any species accurately. But DNA barcoding can produce accurate data in species identification. Phenotypic characters can be changed due to environmental factors. That's why DNA barcoding is more important for species identification.

4.3 RAPD Analysis

The four populations of chapila, Indian oil shad and kelee shad produced different banding pattern with ten primer combinations. The average polymorphism was about 18.48%, revealing a low range of polymorphisms among these four populations.

Polymorphisms based on RAPD analysis were reported in other fishes earlier. Mostafa et al. (2009) reported 57.69% polymorphisms among two riverine and one hatchery stock of *Labeocalbasu*collected from Padma, Jamuna and hatchery. Barman et al.

(2003)indicated 45% polymorphisms in four different populations of *Labeorohita*, *Catlacatla*, *Labeocalbasu*and *Cirrhinusmrigala*. Islam and Alam(2005) found 46.5% of polymorphisms in four different populations of *L. rohita*. Akter et al. (2010) reported a high level of polymorphisms (89.58 %) in four barb individuals.

Besides polymorphic bands, 83 unique bands were found.The term unique band means that a band found with a specific primer in a species is absent in other individuals with the same primer. Unique bands are very stable and specific to each species. These unique bands could be used as a marker for respective species.

The dendrogram shows that the genetic distance (0.9933) between Indian oil shad (*Sardinella longiceps)* and chapila (*Gudusia chapra)* was highest, that means Indian oil shad and chapila are geographically distant from each other. The lowest genetic distance (0.5077) was found between *Sardinella longiceps* and *Hilsa kelee.* That means Indian oil shad and kelee shad has a close geographical relationship. Geographical distance is an important factor influencing thegenetic relatedness of populations (Wright, 1943). Bhat et al. (2014) reported that genetic diversity is primarily dependent on geographical isolation and there is a significant correlation between genetic diversity and geographical distance.

Genetic distances based on RAPD analysis were reported in other fishes earlier. Callejas et al. (1998) tried to identify Spanish barbel species using the RAPD technique. Cluster analysis of the genetic similarity values obtained from RAPD data indicated that the species *B. bocagei*and *B. graellsii*are more related to each other than to *B. sclateri*. According to Liu et al. (1998) overall polymorphism was low among strains of catfish and no difference in RAPD profiles between channel catfish and blue catfish F1 hybrids. RAPD fingerprintingwere used for discriminating among three populations of Hilsa shad (*Tenualosa ilisha*). There were high degree of polymorphism both within and between populations and individuals sampled in the same locations were clustered together(Dahle et al., 1997). Elo et al. (1997) applied RAPD marker to detect interspecific hybridization between brown trout and Atlantic salmon. DNA markers separate nonanadromous salmon & brown trout.Brahmane et al. (2006) used RAPD fingerprinting for delineating populations of hilsa shad *Tenualosa ilisha* (Hamilton, 1822) from six different locations. They found overall average genetic distance of 6 locations was 0.295 and overall Fst value was 0.590. Kabir et al. (2012)found Thai spotted-released form is quite different

from the spotted and wild formamong three forms of *Anabas testudineus*Bloch wild (native, non-spotted), Thai (introduced from Thailand, spotted) and Thai (a spotted-released form from local hatcheries).

One major drawback of the RAPD polymorphisms is unclear inheritance patterns. It is not possible to determine if an individual is a homozygote or a heterozygote, and two fragments occupying the same position on a gel might not be identical. The RAPD technique might not be ideal for genetic studies, but the approach seems useful for identification and phylogenetic studies. A major advantage with RAPD is that the entire procedure is very fast compared with other DNA-based methods (e.g., Southern blotting, sequencing). RAPD analysis can show high levels of polymorphisms in species with low electrophoretical (allozyme) variation.

The use of other genetic methods, such as minior microsatellite studies, should be undertaken in order to gain a more precise knowledge about the population structure. These methods are however, more laborious, expensive, and require more technical skill. The RAPD method is best suited for identification of species, and for differentiating among conspecific populations, particularly in cases where the morphological characters do not permit an unambiguous or a rapid identification of species (Dahle et al., 1997).

Chapter 5
Conclusion and Recommendations

5.1 Conclusion

Morphometric study and DNA barcoding were used for species identification. Results have shown that there were some difficulties in species identification by morphometric study. Species identification based on the DNA sequence of a fragment of the cytochrome c oxidase subunit I gene in the mitochondrial genome, DNA barcoding, is widely applied to assist in sustainable exploitation of fish resources. It provides accurate data in species identification. The morphometric study shows that there were four morphologically distinct species of *Gudusia chapra, Tenualosa ilisha, Tenualosa toli and Ilisha megaloptera.* But DNA barcoding shows that there were two different species of Indian oil sardine (*Sardinella longiceps)* and kelee shad(*Hilsa kelee)*.

RAPD (Randomly Amplified Polymorphic DNA) analyses were used to find out the genetic distance by ten arbitrary oligonucleotide RAPD primers. Results have shown that the values of pair-wise genetic distances ranged between 0.5077 and 0.9933with some degrees of genetic variation among the populations.Cluster analysis showed that cluster C1 and C2 comprised *Sardinella longiceps and Hilsa kelee.* On the other hand, *Gudusia chapra* created the new distant cluster C3. The results prove the existence of genetic variability within and between species. RAPD method is the mostly used method for identifying genetic variability within and between species.

5.2 Recommendations

Based on the current study, several recommendations could be mentioned.

- The study was conducted only in very short period and for that reason few number of species were used, so further study is needed.
- Robust study is needed to find out the genetic diversity among all species of hilsa found in Bangladesh.
- Meta barcoding approach can be used to identify the all species under order Clupeidae found in Bangladesh.

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Appendices

A.1 Identification of A Bony Fish

Date:…….. Serial number:……..

Place of collection:…… Habitat:…….

Local name:…….

A. EXTERNAL DESCRIPTIVE CHARACTERISTICS

1. COLOUR

a) BODY:
Light/Dark/Dull/Burnished/Coppery/Steel/Stone/Blackish/Leaden/Greenish/Whitish/Yellowish/Silvery/Brownish/Olivaceous/Golden/Grayish/Pinkish/Bluish/Buff/Scarlet/Reddish/Violet/With brassy reflection/With purplish reflection/With metallic reflection/Others..

b) FINS:
Blackish/Greenish/Whitish/Yellowish/Silvery/Brownish/Reddish/Light/Dark/Others……

c) DECORATIONS:…….
   i. Spots:……
   ii. Blotches:……...
   iii. Stripes:……..
   iv. Bands:……...
   v. Ocelli:……

2. SHAPE

a) MOUTH:
Crescentic/Rounded/Pointed/Blunt/Triangular/Protractile/Wide/Small/Moderate…

b) SNOUT:
Pointed/Blunt/Triangular/Compressed/Depressed/Obtuse/Prominent/Conical/Tubular/Short/Thick/Hooked/Rounded/Trilobed/Spatulate/Directed upward/Directed downward……..

c) HEAD:
Anteriorly/Laterally/Compressed/Depressed/Shield/Spatulate/Conical/Median groove on head

d) TRUNK:
Somewhat/Strongly/Rather/Very/Moderately/Fairly/Laterally/Dorso-Ventrally/Dorsally/Ventrally/Compressed/Depressed/Elongated/Slender/Rounded/Deep/Fusiform/Oblong/Oval/Short/Extremely long/Asymmetrical/Cylindrical/Flattened/Sub-Cylindrical/Tapering posteriorly

3. HEAD SIZE

Large/Moderate/Small

4. MOUTH POSITION

Terminal/Superior/Inferior/Sub-terminal/Ventral/Oblique

5. JAWS

Equal/ Unequal/ Beak-like. If unequal,

Upper/ Lower: Longer and shorter than upper/Lower one with/without teeth

6. BARBELS

Present/ Absent. If present number in pairs…….

a) SIZE: Elongated/ Moderate/ Short/ Reduced……..
b) POSITION: Rostral/Mandibular/Maxillary/Nasal
Remark. …….

7. EYES

a) POSITION: Laterally/ Dorsally/ Dorso-laterally/ Termino-dorsal.
b) SIZE: Large/ Moderate/ Small/ Very Small
c) CONDITION: Free orbital margin/ sessile/ Covered with adipose/ Not covered with adipose

8. NOSTRILS

Present/Absent. If present, one pair/two pairs

POSITION: Termino-dorsal/ Termino-lateral/ Termino-ventral/ Dorso-lateral

9. OPERCULAM

Spiny/ Non-spiny/ Scaly/ Non-scaly/ Large/ Moderate/ Small

10. LATERAL LINES

Present/Absent. If present,

a) Single/ Double
b) Complete/ Incomplete/ Interrupted/ Irregular
c) Curved: Upward/ Downward/ Straight/ Waved
d) Smooth/ Spine/ Others. ……

11. SCUTES

Present/ Absent. If present,

a) Number of scutes on lateral line…….

b) Number of scutes on ventral margin…….
Before…….and behind……..of the pelvic fins.

12. SCALES

Present/ Absent. If present

a) TYPE: Cycloid/Ctenoid
b) SHAPE: Round/ Oval/ Rectangular
c) SIZE: Large/ Moderate/ Small/ Minute

13. DORSAL FINS

a) Single/ Double/ Free/ Confluent
b) Spiny/ Non-spiny/ Rayed/ Soft/ Hard
c) Adipose fins: Present/ Absent
d) Origin and modification: Nearer to head/ Caudal/ Central
    i. First dorsal…….
    ii. Second dorsal…….

14. PECTORAL FINS

Present/ Absent. If present

One part/ Two parts/ Upper/ Lower Rays Filamentous/ Spinous

Origin: Nearer to head/ Caudal/ Central

15. PELVIC FINS

Present/ Absent. If present

Free/ A single filiform ray

Origin: Nearer to head/ Caudal/ Central

16. ANAL FINS

Present/ Absent. If present,

Origin: Nearer to head/ Caudal/ Central

17. CAUDAL FINS

Present/ Absent. If present,

Free/ Confluent/ Filamentous.

Type: Homocercal/ Heterocercal

Shape: Forked/ Rounded/ Oval/ Lanciate/ Pointed/ Emergent/ Trunked/ Lunet

Size: Small/ Large/ Moderate/ Elongated

B. MORPHOMETRIC CHARACTERISTICS

1. BODY

i. Total length (T.L)……cm.
ii. Forked length (F.L)…….cm.
iii. Standard length (S.L)……..cm.
iv. Pre-dorsal length (P.D.L)……cm.
v. Body height (B.H)……...cm.
vi. Peduncle length (P.L)……...cm.

2. HEAD

i. Head length (H.L)……cm.
ii. Pre orbital length (P.O.L)/ (Snout length)……cm.
iii. Inter orbital length (I.O.L)…….cm.
iv. Eye diameter (E.D)……cm.
v. Jaws.
   a) Length of the upper jaws (L.U.J)……..cm.
   b) Length of the lower jaws (L.L.J)……..cm.
   c) Gape wide…….cm.

3. FINS

i. Length of the dorsal fin / fin base……cm.
ii. Length of the pelvic fin / fin base……cm.
iii. Length of the anal fin / fin base……cm.
iv. Length of the caudal fin……..cm.
   a) Length of the upper lobe (L.U.L)……..cm.
   b) Length of the lower lobe (L.L.L)……..cm.

4. RELATIONSHIP (IN PROPORTION)

i. T.L. / S.L.=……cm/……..cm=……i.e., Standard length is……of the total length.
ii. T.L. / F.L. =……cm/…….cm=……i.e., Forked length is…….of the total length.
iii. T.L. / H.L. =……cm/…….cm=……i.e., Head length is…….of the total length.
iv. T.L. / E.D. =……cm/…….cm=……i.e., Eye diameter is…….of the total length.
v. T.L. / P.O.L. =……cm/…….cm=……i.e., Snout length is…….of the total length.

C. MERISTIC STUDY FOR THE ESTABLISHMENT OF A TAXONOMIC FORMULA

1. **BRANCHIOSTEGAL RAYS (B.)** Number…….

2. FINS

i. Number of dorsal fin rays (D)……
   (D_1)……...
   (D_2)……
ii. Number of pectoral fin rays (P)……..
iii. Number of pelvic fin rays (V/P_2)…….
iv. Number of anal fin rays (A)……..
v. Number of caudal fin rays (C)……..

3. SCALES

i. Number of pre-dorsal scales……

ii. Number of lateral line scales (L.l)……...
iii. Number of transverse rays of scales (L.r)……....
iv. Number of longitudinal rays of scales (L.t.r)……...

4. TAXONOMIC FORMULA

B.…… **D_1**..... **D_2**……..

P. ……... **V.** ……. **A.**…….. **C.** …….

L.l. ……. **L.r.** ……... **L.t.r.** ……..

D.FIGURE WITH MEASUREMENT

E. IDENTIFYING CHARACTERISTICS

Class: 1.

2.

Order: 1.

2.

Family: 1.

2.

Genus: 1.

2.

Species: 1.

2.

3.

4.

F. CONCLUSION: FROM THE ABOVE CHARACTERISTICS, IT MAY CONCLUDE THAT THE SUPPLIED FISH SPECIMEN IS…….

AND CLASSIFIED AS FOLLOWS

G. CLASSIFICATION

Phylum	**:**	**Chordata**
Division	**:**	**Craniata**
Sub-phylum	**:**	**Vertebrata**

Super-class	**:**	**Pisces**
Class	**:**	**Osteichthyes**
Sub-class	**:**	**Actinopterygii**
Order	**:**	
Family	**:**	
Genus	**:**	
Species	**:**	

H. REFERENCE BOOKS

A.2 DNA Extraction Report

Sl.	Sample ID	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Factor
1	F1	7/29/2016 1:26:06 PM	9.5	ng/µl	0.19	0.126	1.51	0.34	50
2	F2	7/29/2016 1:27:42 PM	15.4	ng/µl	0.307	0.17	1.81	0.26	50
3	F3	7/29/2016 1:28:59 PM	12.7	ng/µl	0.253	0.136	1.86	0.45	50
4	F4	7/29/2016 1:30:34 PM	6	ng/µl	0.121	0.063	1.92	0.15	50
5	F4	7/29/2016 1:30:50 PM	6.1	ng/µl	0.122	0.065	1.88	0.14	50

A.3 All Sequence of Collected Sample, Suspected Species Sequence and Outgroup Sequence

>F2

CTGAGCAGGGAAGGTCGAACCGCCCTAAGTCTACTAATTCGGGCAGAATTAAGCCAACCCGGAGCACTTCTTGGAGAC
GATCAAATCTACAATGTTATCGTCACCGCACACGCTTTCGTGATGATTTTCTTCATAGTAATGCCAATCCTGATCGGAG
GATTCGGAAACTGACTTGTCCCTCTTATGATCGGGGCCCCAGACATGGCATTCCCTCGAATGAACAACATGAGCTTCTG
ACTCCTACCTCCCTCTTTCCTTCTTCTCCTGGCCTCCTCTGGAGTAGAAGCTGGGGCGGGAACCGGGTGAACAGTCTAC
CCGCCCTTAGCAGGTAACCTGGCCCACGCCGGAGCATCAGTCGACTTAACGATTTTCTCTCTTCACTTAGCGGGTATTT
CATCAATTCTTGGGGCCATCAACTTCATCACCACAATCATTAACATGAAACCTCCTGCAATTTCGCAATATCAAACACC
ACTATTCGTCTGAGCCGTTCTTGTAACTGCTGTTCTTCTCCTTCTTTCTCTCCCAGTCCTAGCCGCCGGGATTACCATGCT
TCTTACTGATCGAAATCTCAACACAACATTCTTCG

>F3

CTGAGCAGGAATAGTAGGAACTGCCCTAAGCCTTCTTATTCGGGCTGAGCTAAGCCAACCCGGAGCGCTTCTTGGGGA
CGACCAGATCTACAATGTTATCGTTACGGCACATGCCTTCGTAATGATTTTCTTCATAGTAATGCCCATCCTGATCGGA
GGGTTCGGAAACTGACTAGTCCCCCTAATGATCGGGGCACCAGACATGGCGTTCCCACGAATGAATAATATGAGCTTC
TGGCTCCTACCACCCTCTTTCCTTCTCCTCTTGGCCTCTTCGGGGGTAGAAGCCGGGGCAGGGACTGGGTGAACAGTGT
ACCCGCCTCTAGCAGGCAACCTGGCCCACGCGGGGGCATCTGTTGACCTCACTATCTTCTCACTTCACCTCGCAGGGAT
CTCATCAATTCTTGGGGCAATCAATTTTATTACCACAATCATTAATATGAAACCCCCTGCAATTTCACAGTACCAGACA
CCCCTATTCGTGTGAGCTGTTTTCGTAACAGCTGTCCTCCTCCTTCTATCGCTCCCAGTACTAGCCGCCGGCATTACTAT
GCTTCTCACGGATCGAAATCTGAACACGACCTTCTTCG

>F4

CTGAGCAGGGATGGTCGGAACCGCCCTAAGTCTACTAATTCGGGCAGAATTAAGCCAGCCCGGAGCACTTCTTGGAGA
CGATCAAATCTACAATGTTATCGTCACCGCACACGCTTTCGTGATGATTTTCTTCATAGTAATGCCAATCCTGATCGGA
GGATTCGGAAACTGACTTGTCCCTCTTATGATCGGGGCCCCAGACATGGCATTCCCTCGAATGAACAACATGAGCTTCT
GACTCCTACCTCCCTCTTTCCTTCTTCTCCTGGCCTCCTCTGGAGTAGAAGCTGGGGCGGGAACCGGGTGAACAGTCTA
CCCGCCCTTAGCAGGTAACCTGGCCCACGCCGGAGCATCAGTCGACTTAACGATTTTCTCTCTTCACTTAGCGGGTATT
TCATCAATTCTTGGGGCCATTAACTTCATCACCACAATCATTAACATGAAACCTCCTGCAATTTCGCAATATCAAACCC
CACTCTTCGTCTGAGCCGTTCTTGTAACTGCTGTTCTTCTCCTTCTTTCTCTCCCAGTCCTAGCTGCCGGGATTACCATGC
TTCTTACTGATCGAAATCTCAACACAACATTCTTCG

A.4 Loci Data for RAPD Analysis

OPA-03

Chapila (F1)	Padma Ilish (F2)	Chandanailish (F3)	Chokkailish (F4)
0	1	0	0
1	0	0	1
1	0	0	0
0	0	0	1
0	0	1	0
0	0	0	1
0	0	1	0
1	0	0	0
0	1	0	0
1	1	0	0
0	1	0	0
0	0	1	0
1	0	0	0
0	0	0	1
0	1	0	0
0	0	1	0
0	0	0	1

OPA-04

F1	F2	F3	F4
1	0	0	0
1	0	0	0
0	1	1	1
1	0	0	0
0	1	1	1
1	0	0	1
0	1	0	0
0	0	1	0
1	0	0	0
0	0	0	1
0	1	0	0
0	0	1	0

OPA-09

F1	F2	F3	F4
1	0	0	0
0	1	0	0
1	0	0	0
0	1	0	0
0	0	0	1
1	1	0	0
0	1	0	0
0	0	0	1
0	0	1	0
0	0	0	1
0	0	0	1
1	0	0	0
0	0	0	1
0	0	1	0
0	0	1	1

OPAL-04

F1	F2	F3	F4
1	0	0	0
1	0	0	0
1	0	0	0
0	0	1	0
0	1	0	0
1	0	0	0
0	0	0	1
0	0	1	0
0	1	0	0
0	1	0	1

OPAW-09

F1	F2	F3	F4
0	1	0	0
0	1	1	0
1	0	0	1
0	1	1	0

OPAK-04

F1	F2	F3	F4
1	0	0	0
1	0	0	0
0	1	1	1
1	0	0	0
0	1	0	0
1	0	0	0
1	1	1	0

OPA-02

F1	F2	F3	F4
1	1	0	0
1	0	0	0
1	0	0	0
1	0	0	0
0	0	0	1
0	1	0	0
0	0	0	1
1	0	0	1
0	1	1	0
1	0	1	0

OPG-04

F1	F2	F3	F4
1	0	0	0
1	1	0	0
0	0	1	0
1	0	0	0
0	1	0	0
0	0	1	1
1	0	0	0
0	1	0	0
0	0	0	1

OPF-01

F1	F2	F3	F4
1	0	0	0
1	0	0	0
0	1	1	0
0	0	1	0
1	0	0	0
1	0	0	0
0	1	0	0
1	0	0	0
0	0	0	1
0	0	1	0
0	1	0	0

OPG-05

F1	F2	F3	F4
1	0	0	0
0	0	0	1
0	0	1	0
0	1	0	1
1	0	0	0
0	1	1	0
0	0	0	1
0	1	0	0
1	0	0	0
0	0	0	1
0	1	0	0
0	0	0	1
1	0	0	0