PHYLOGENETIC RELATIONSHIP OF *Amblypharyngodon mola* (DANIONINAE) AND *Labeo bata* (CYPRININAE) BY CYTOCRHOME b GENE SEQUENCE ANALYSIS

A Thesis
By

MD. ARAFATH HOSSAIN
Examination Roll No: 1605197
Session: 2016-2017
Semester: January-June, 2017

MASTER OF SCIENCE
IN
FISHERIES BIOLOGY AND GENETICS

Department of Fisheries Biology and Genetics

Hajee Mohammad Danesh Science and Technology University

Dinajpur

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Dinajpur

June, 2017

Dedicated
To
My Beloved
Parents

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The Author

June, 2017

ABSTRACT

Amblypharyngodon mola, locally known as "Mola" and *Labeo bata*, as "Bata" are fish of small indigenous species (SIS), belonging to subfamily Danioninae and Cyprininae, family Cyprinidae. Now a days both fishes are being considered as high aquaculture potential in Bangladesh. Hence, attempt was taken to reveal the evolutionary relationship of both species by using partial sequences of mitochondrial gene *Cytochrome b* (Cyt b). Sequences were amplified through PCR and submitted to the NCBI Genbank database after determined the final consensus size through multiple sequence alignment. The nucleotide sequences of related fishes were also retrieved from the NCBI genebank databases. The analyses were performed using computer software packages MEGA (Version 6.01). The length of consensus Cyt b were 357, 364 and 356 bp in *A. mola*, *L. bata* and *A. testudineus* respectively. The Transition/Transversion bias (*R*) was 0.91, where the rate of transitional substitution from A to G, T to C, C to T, G to A were 5.49, 24.93, 29.19 and 7.82 respectively. The rate of transversional substitution from A to T, A to C, T to A, T to G, C to A, C to G, G to T, G to C were 4.37, 4.37, 4.77,4.77, 3.07 and 3.07 respectively. The highest sequence divergence was found in between *A. mola* and a marine species *Opsarius keta* (10.003), and the lowest in between *L. bata* and a freshwater fishes of Australia *Melanotaenia duboulayi* (3.516). In Maximum likelihood method, studied taxa were grouped into three clades; clade 1consisted of *Danio rerio*, *M. splendida*, *O. keta*, *Barilius bendelisis* and *L. carbasu*; clade 2 formed by including *O. bakeri*, *C. mrigala* and *L. gonius*; clade 3 with *M.dubolulayi*, where *A. mola* did not form sister with any taxa. In Neighbour joining tree, three clades were also formed with similar cluster formation. Three clades were also observed in Maximum parsimony (MP) method. Interestingly in MP method *A. mola* formed clade 1 with *B. bendelisis* and *L. bata*. Same as previous two method *D. rerio* and *O. keta* formed sister group here. The time tree based on maximum likelihood method showed that there were the least times of divergence in between *C. mrigala* and *L. gonius*. There was the highest times of divergence (4.89 MYA) in between *D. rerio* and outgroup taxa *A. testiduneus*. The study concluded that *A. mola* and *L. bata* showed considerable variation in Cyt b gene sequences. A detailed phylogenetic and evolutionary analyses of entire fishes of the famiy Cyprinidae including more genes such as Cytochrome C Oxidase subunit-I, and nuclear genes are highly required to conserve the gene pools of these fishes.

Keywords: *Amblypharyngodon mola* , *Labeo bata*, Cytochrome b, Phylogenetic tree

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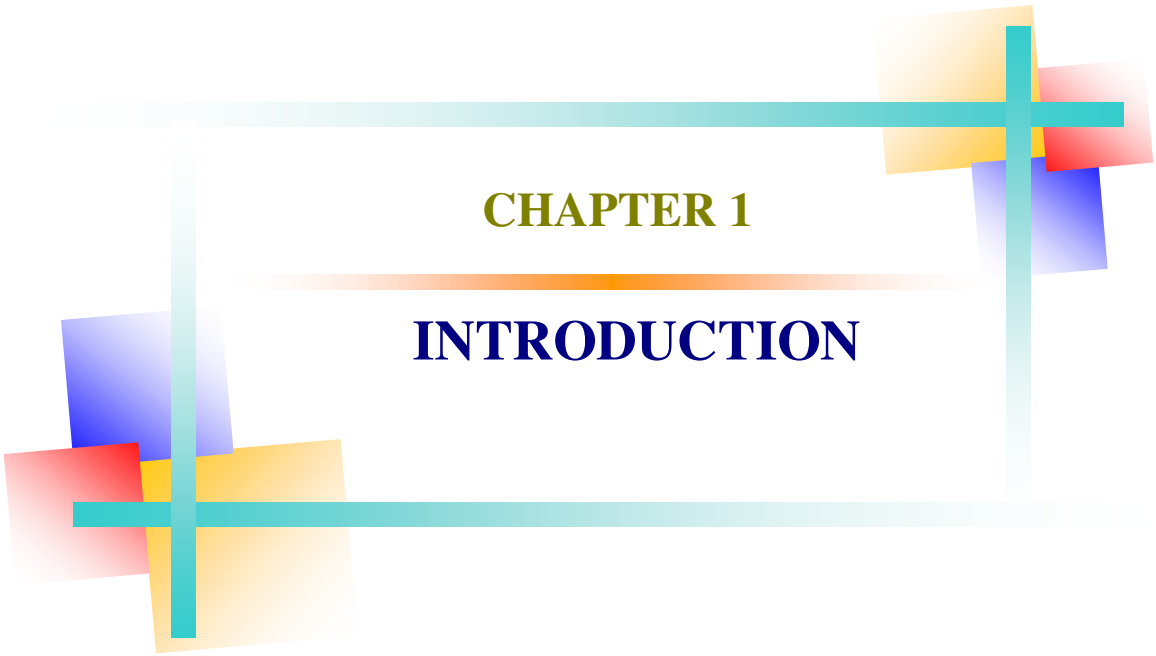
LIST OF ABBREVIATIONS

A	:	Adenine
BLAST	:	Basic Local Alignment Search Tool
bp	:	Base pair
C	:	Cytosine
Cyt b	:	*Cytochrome b*
DAMBE	:	Data Analysis in Molecular Biology and Evolution
DNA	:	Deoxyribonucleic acid
DoF	:	Department of Fisheries
FAO	:	Food and Agriculture Organization
FASTA	:	Fast Adaptive Shrinkage Thresholding Algorithm
FRSS	:	Fisheries Resources Survey System
G	:	Guanine
GDP	:	Gross Domestic Product
HSTU	:	Hajee Mohammad Danesh Science and Technology University
IUCN	:	International Union for Conservation of Nature
K2P	:	Kimura-2-Parameter
kbp	:	Kilo base pairs
MCL	:	Maximum Composite Likelihood
MEGA	:	Molecular Evolutionary Genetic Analysis
ML	:	Maximum Likelihood
min	:	Minute
mM	:	milli Mole
ME	:	Minimum Evolution
MT	:	Metric ton
MP	:	Maximum Parsimony
mt genome	:	mitochondrial genome
mtDNA	:	mitochondrial Deoxyribonucleic Acid
MYA	:	Million Years Ago
NCBI	:	National Centre for Biotechnology Information
NJ	:	Neighbor Joining algorithm
PCA	:	Principal Component Analysis
PCR	:	Polymerase Chain Reaction

LIST OF ABBREVIATIONS (Contd.)

Pi	:	Parsimony Informative
sec	:	Seconds
SNP	:	Single Nucleotide Polymorphism
T	:	Thymine



CHAPTER 1

INTRODUCTION

Chapter 1

INTRODUCTION

1.1 General information

In this world, 100 million people entirely depend on fish and fisheries for food and nutrition, income and livelihoods. Due to enormous growth of aquaculture, the world per capita fish supply increased up to 20kg (FAO, 2016). Within the production cyprinidae family plays significant role due to worldwide diversification and culture performances. Cyprinids are highly important as food fish; they are fished and farmed across Eurasia. Without the fast-flowing rivers majority in the land lock countries, cyprinidae is main species used for consumption due to covering the largest part of the fish biodiversity (Eschmeyer and Fong, 2015) and also used for drying and salting. Besides this to use as food fishes, in many areas including Bangladesh, cyprinidae fishes used as recreational fishing for centuries deliberately stocked in ponds and lakes (Kottelat, 2013). Several species of cyprinids have been introduced outside of their native habitat to provide food, sport or biological control for some pest species (Steven *et al.* 2005).Other than food fishes, there are significant number of popular aquarium cyprinids that include danionins, rasborines, and true barbs. Larger species are bred by the thousands in outdoor ponds, particularly in Southeast Asia and trade in these aquarium fishes is of considerable commercial importance. The small rasborines and danionines are perhaps only rivalled by characids and poecilid livebearers in their popularity for community aquaria (Riehl and Baensch, 1996).One particular species of these small and undemanding danionines is the zebra fish (*Danio rerio*). It has become the standard model species for studying developmental genetics of vertebrates, in particular fish (Helfman *et al.* 1997). Bangladesh is one of the richest countries of the world in respect of her vast and diverse inland water bodies in the form of rivers, flood plain, canals beels, haors, ponds, ox-bow lakes and coastal area. Fish and fisheries have been the integral part of the people of Bangladesh and play major role in employment generation providing nutritional security foreign exchange earnings and other aspect of the economy (DOF, 2016).

1.2 Cyprinid fishes in Bangladesh

Cyprinids fishes are native in North America, Eurasia and Africa and may have originated in Asia in the Eocene (Nelson, 2006). About 80% of the world fish covered by cyprnid including carp and minnows and it has over 2400 species in 220 genera (Nelson, 2006). In Bangladesh, cyprinidae is outstanding freshwater fishes which are the most numerous as regards genera and species and most abundant as regards individuals. There are records of at least of 57 species of cyprinids under 23 genera in our country (Rahman, 2005). The carp species are widely distributed in river, canal, lake, beel or haor and pond in Bangladesh. Fish secure the demand of 60% animal protein in our country (FRSS, 2016).

In total pond production of Bangladesh, carp species are contributing about 85.29% (FRSS, 2016). Among recorded 67 species, there are at least 13 endemic and 8 introduced species of interest to aquaculture in Bangladesh (Hussain and Mazid, 2001). Due to higher growth rate and disease resistance it grabs as most important freshwater aquaculture species in our country. The most important indigenous species of carp that enormously contributed to our aquaculture sector is Rohu, Mrigal, Katla contributed about 20.54%, the minor carp such as gonia, bataetc contributed about 2.26% and exotic carps such as Silver carp, Bighead carp, Common carp etc. contributed 11% of the country's total production (DOF, 2016).

1.3 General information about selected two fishes

1.3.1 *Amblypharyngodon mola*

Figure 1. ***Amblypharyngodon mola***

Amblypharyngodon mola is a small indigenous fish species (SIS) belonging to the family cyprinidae and considered as native primary freshwater fish species. The species locally known as Mola, Moa, Moshi and commonly known as Molacarplet and Pale carplet (English); Molacarplet (Fishbase, 2014). The Maximum length reported 8

cm (Bhuiyan, 1964), 20 cm (Talwar and Jhingran, 1991), 9.2 cm (Hussain, 1999) and 9 cm (Rahman, 2005). Highest length measured 6.5 cm in 2007 in the Chalan beel of Bangladesh. The species considered as highly nutritious. This fish is very important due to its self-recruiting ability in the farmer's ponds.

Systematic position

Phylum: Chordata
Class: Actinopterygii (Ray-finned fishes)
Order: Cypriniformes (Carps)
Family: Cyprinidae (Carps and minnows)
Sub-family: Danionie
Genus: *Amblypharyngodon*
Species: *A. mola*

Synonyms

Amblypharyngodon pellucidus (Mc Clelland, 1839), *Leuciscu spellucidus* (Mc Clelland, 1839), *Cyprinus mola* (Hamilton, 1822), *Leuciscus mola* (Hamilton, 1822), *Mola buchanani* (Blyth, 1860), *Amblypharyngodon saranensis* (Chaudhuri, 1912).

1.3.2 *Labeo bata*

Figure 2. *Labeo bata*

Labeo bata is locally known as Bata. Minor carp *Labeo* is a freshwater subtropical species which is commonly known as 'Bangon Bata'. This fish is commercially important and target species for commercial small and large-scale farming in Bangladesh, India and Pakistan. *L. bata* is in great demand in the market because of its high nutritional value and good taste (Bhuiyan, 1964). This fish contains about 15.42% of protein and 3.73% of lipid (Ahmed *et al.* 2012).

Taxonomic position

Phylum: Chordata
Class: Osteichthyes
Order: Cypriniformes
Family: Cyprinidae
Sub family: Cyprininae
Genus: *Labeo*
Species: *L. bata*

Synonyms

Cyprinus bata: Hamilton-Buchanan, 1822; *Labeo bata*: (Day, 1878, and Day,1889).

1.3.3 Significance of two selected fish

In Bangladesh, to promote the further aquaculture production several exotic fishes are introduced in the country. Day by day the introduced exotic fishes are becoming popular due to their higher growth and survival. On the other hand the interests of aquaculture of indigenous fishes are declining. In such situation, *Labeo bata* is still favoured by the farmers of Bangladesh in carp polyculture system because of its delicious food value. At the same time, now a days *A. mola* is in trial for aquaculture in the farmers pond. The very unique feature is that this fish is self-recruiting, hence once a farmers introduced in his/her ponds it continue its recruitment. Culture of two selected species needs special attention because these fish provide us plenty of vitamin, iron, calcium, and minerals (Kohinoor *et al.* 2005).

1.4 Phylogenetic analysis

Without phylogenetic trees we can't make much sense of the pattern of life. The phylogenetic tree of multi-cellular organisms reveals that the fishes are known from 450 million years ago. Phylogenetic analysis may be considered to be a highly reliable and important bioinformatics tool as it lies in its simple manifestation and easy handling of data. The simple tree representation of the evolution makes the phylogenetic analysis easier to comprehend and represent as well. The varied applications of phylogenetics in different fields of biology make this analysis an absolute necessity.

Cytochrome b is a gene found in the mitochondria of eukaryotic cells. It functions as part of the electron transport chain and is the main subunit of transmembrane cytochrome b c1 and b6f complexes (Esposti, 1993). Due to having the sequence variability the mitrochondrial DNA *Cytochrome b* is widely used for resolution of the evolutionary or phylogenetics relationships among the organisms (Alam, 2015). Mitochondrial gene cytochrome b gene has been more used for resolution of the systematic diversity in animals because it contains both slowly and rapidly evolving codon positions and have been used to assess phylogenetic relationships involving ancient divergences (Mindell and Honeycutt, 1990; Zardoya and Meyer, 2010; Abouheif *et al.* 1998; Zardoya *et al.* 1999), the majority of research on animals has used single mitochondrial DNA genes to assess population or low-level taxonomic relationships (Rocha-Olivares *et al.* 1999; Lovejoy and de Araujo, 2000; Tsigenopoulos and Berrebi, 2000).

Cytochrome b (cyt b) has been considered one of the most useful genes for phylogenetic work and is probably the best-known mitochondrial gene with respect to structure and function of its protein product (Esposti *et al.,* 1993). Early attempts at using phylogenomic data to extrapolate evolutionary relationships of fishes were on the basis of few available complete genomic sequences or of cDNA and ESTs for the relatively few taxa representing fish model systems (Chen W-J, 2010). More recently, it has become possible to construct multilocus data sets for large numbers of taxa with the increasing availability of "universal" PCR primers and the development of standard laboratory protocols for analyzing existing and novel nuclear gene markers useful for phylogenetic inference in ray-finned fishes (Li C, 2007; Chen *et al.* 2008).

1.5 Rationale of the study

Phylogenetic relationships among fishes are gradually being resolved with new sources of genetic data. Increasing resolution for the tree of life of fishes is emerging from recent studies using a modest number (10 to 20) of molecular markers. With the passing of the time fish populations are altered and may split into separate branches, hybridize together or terminate by extinction, as a result the evolution occurred. Thus, a phylogenetic context can provide the most meaningful insights into biology. It is obvious that phylogenetic studies are used in the systematics, because phylogeny is representing morphological, biochemical characteristics, gene sequence and the genealogical relationships of living organisms. The molecular studies describe the phylogenetic

systematics. Phylogenetic studies have been conducted for several fish families (cite with reference). Previously Alam (2015) have studied the phylogenetic relationship of cyprinid fishes of Bangladesh through morphological data and *Cytochrome b* sequence data from NCBI gene bank database. This is the first attempt to study the phylogenetic relationship of any cyprinid fishes by direct sequencing of Cyt b sequences. *L. bata* is widely cultured in the aquaculture pond of Bangladesh and *A. mola* also started to culture. To establish the secure insight about the stock of *L. bata* and *A. mola* it is high time to know the phylogenetic relationship of these fishes.

1.6 Objectives

The aim of this study is to establish the systematic position of selected fishes through the phylogenetic and evolutionary analysis of partial sequences of mitochondrial gene *Cytochrome b.* To achieve the aim following objectives were set:

- To reconstruct the phylogenetic relationships of *L. bata* (cyprininae) and *A. mola* (Danionane) of Bangladesh.
- To evaluate the evolutionary relationship between *L. bata* and *A. mola* and other fishes.

CHAPTER 2

REVIEW OF LITERATURES

Chapter 2

REVIEW OF LITERATURE

Phylogenetic studies using morphometric traits, is a traditional approach to understand the evolution in different groups of organisms. With the advancement of time, mostly after the discovery of polymerase chain reaction the nucleotide, RNA and amino-acid sequence data are mostly used in reconstruction of phylogenetic tree. In this chapter the current status of the phylogenetic studies in fishes and other related organism were reviewed. Briefly it was found that the phylogenetic studies in fishes had been conducted in many parts of the worlds including Asian countries. Unfortunately, still no studies except work of previous study of the same Department were available during the study.

2.1 Fish Phylogeny based on morphological traits

Gill (1872, 1893) divided the carps into five families: Catostomidae, Cyprinidae, Cobitidae, Homalopteridae, and Knerridae (Order: Gonorhynchiformes) and subsequently recognized the algae eaters (Gyrinocheilidae) as an independent family (Gill 1905). Subsequent workers used the familial classification of Gill, minus Knerridae but variously elevated or subsumed lineages, within these families to subfamilial or tribal rankings.

The cladistic analysis of cyprinidae family based on meristic counts was first reported by Howes (1987). Based on presence or absence of the anterior maxillary barbel with an accompanying maxillary foramen divided the cyprinidae family of fishes into two lineages that was cyprinins and leuciscins.

In 1970, authentic review on the cypriniform classification was conducted by Hessel. Generally, the first attempt was carried to classify regional faunas and resulted group were minnows, loaches, suckers into cyprinidae family.

An unresolved relationship was established on Cypriniformes relationships by Wu *et al.* (1981) using the Characiformesas outgroup. A dichotomous relationships were depicted in their phylogeny, examination of the distribution of synapomorphies indicates that there were no synapomorphies supporting the Catostomidae plus Gyrinocheilidae clade; two synapomorphies did support.

Cavender and Coburn (1992) provided the published phylogenetic analysis of the Cyprinidae. They also recognized two major lineages within the family; Cyprininae, containing three groups (barbins, cyprinins, and labeonins) and Leuciscinae, containing eight groups (tincins, rasborins, gobionin, acheilognathins, xenocyprins, cultrins, leuciscins, and phoxinins).

2.2 Use of cytochrome b in phylogenetic study

Due to several reasons the mitochondrial gene cytochrome b were widely used to establish the evolutionary relationship in animal mentioned by Irwin *et al.* (1991). Although the non-synonymous substitutions slowly occurred but the rate of evolution in silent positions is relatively fast.

Esposti *et al.* (1993) proved that cyt b has been widely used to determine the deep phylogenetic relationship due to it structure and function of its protein product. They established this was best known mitochondrial gene for determined the evolutionary relationships.

The simultaneous diversification of South American echimyid rodents (Hystricognathi) based on complete cytochrome b sequences was observed by Lara *et al.* (1996). Complete nucleotide sequence of the mitochondrial cytochrome b gene was examined for 32 individuals representing 12 supra specific taxa of South American rodents of the family Echimyidae (Hystricognathi) and the genus Hoplomys is closely related to Proechimys (sensu stricto), a finding supported by other molecular data in the study.

Identification of Five Primary Lineages of Extant Cetaceans by using cytochrome b gene was carried out by Arnason and Gullberg (1998). The study revealed that the level of divergence among the five cetacean lineages and that seen between cetaceans and artiodactyls, suggests that cetaceans and artiodactyls had a common ancestor before 60 million years ago (MYA) and it is a useful gene for identification.

A comparative summary of genetic distances in the vertebrates from the mitochondrial cytochrome b gene was revealed by Johns and Avise (1998). In the study 2,000 cyt b gene sequences from GenBank were calculate and compare levels of genetic distance between sister species, congeneric species, and confamilial genera within and across the major vertebrate taxonomic classes. Moreover, the study revealed that mitochondrial

cytochrome b (cyt b) is among the most extensively sequenced genes to date across the vertebrates.

The utility of cyt-b as a molecular marker for inferring phylogenetic relationship at various levels within the fish family Cichlidae by including 78 taxa, representing all the major groups in the family Cichlidae (72 taxa) and other families from the suborders Labroidei and Percoidei were investigated. Gene trees obtained from cyt-b are compared to a published total evidence tree derived from previous studies. Minimum evolution trees based on cyt-b data resulted in topologies congruent with all previous analyses. Parsimony analyses downweighting transitions relative to transversions (ts1:tv4) or excluding transitions at third codon positions resulted in more robust bootstrap support for recognized clades than unweighted parsimony. Despite some limitations of cyt-b as a phylogenetic marker, this gene either alone or in combination with other data sets yields a tree that is in agreement with the well-established phylogeny of cichlid fish (Farias *et al.* 2001)

In a study of mt DNA by Kocher (2011), it was found that the bird and fish sequences evolve with the same strong bias toward transitions that holds for mammals. However, because the light strand of birds is deficient in thymine, thymine to cytosine transitions are less common than in other taxa. Amino acid replacement in a segment of the cytochrome b gene is faster in mammals and birds than in fishes and the pattern of replacements fits the structural hypothesis for cytochrome b. The unexpectedly wide taxonomic utility of these primers offers opportunities for phylogenetic and population research.

2.3 Phylogenetic studies in fishes by using mitochondrial gene cytochrome b (Cyt b)

Phylogenetic relations among percid fishes as inferred from mitochondrial cytochrome b DNA sequence data Song *et al.* (1997). Maximum parsimony and minimum evolution analyses both recovered single shortest trees and the results of these analyses were generally congruent with one another. All analyses consistently recovered three monophyletic groups in Percidae: Etheostomatinae (Ammocrypta, Crystallaria, Etheostoma and Percina), Percinae (Perca and Gymnocephalus) and Luciopercinae (Stizostedion, Zingel and Romanichthys). A revised classification of Percidae and discuss the phylogenetic evidence for the independent evolution of small benthic species within Etheostomatinae and Luciopercinae.

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Phylogenetic analysis of mitochondrial cytochrome ***b*** genes was carried out. The phylogenetic trees obtained show that most species examined have diverged from each other almost simultaneously or during an extremely short period of time. The study concluded that a comprehensive estimation of the evolutionary scenario of all gobioid fishes using only morphological information is difficult and there were a wide ecological diversification, specialization and degeneration of morphological characters among these species (Akihito *et al.* 2000).

Gilles *et al.* (2001) established a phylogentic relationship in European carp species by using mitochondrial DNA sequences. To ensure the authentic work they used outgroup from the families Catostomidae, Cobitidae, Balitoridae and Gyrinocheilidae, from the order Cypriniformes but they have chosen also taxa from other closely related fish orders (Siluriformes and Characiformes). The results of their 7 study show that Cypriniformes form a monophyletic lineage with a clear split into two main lineages: one including all cyprinid fishes and the other the members of the families Botiidae, Cobitidae, Balitoridae and Gyrinocheilidae. The position of Catostomidae is neither shown nor discussed in the article. However, the study was focused on the internal relationships within Cyprinidae.

Dowling *et al.* (2002) used to examine the phylogenetic relationship based on the sequences of cytochrome b (cyt b) on the endemic cypriniade in Great Basin and Lower Colorado River in southwestern North America. A paraphyletic relationship was established by this author that were distinctly appeared from the morphological data.

Middle Eastern area as an important interchange area or a center of speciation for the freshwater fauna. By using cyt b sequence Durand *et al.,* (2002) observed the phylogenetic relationships of cyprinid species from the Middle East and neighboring

biogeographically areas. The study has been conducted by neighbor joining (NJ) method and suggested that the Cyprininae subfamily showed three highly divergent lineages.

Phylogenetic analysis of the Sparidae (Perciformes: Percoidei) from Cytochrome b sequences was studied by Orrel (2002). Mutational analysis revealed that third codon position transitions were saturated and therefore, of questionable use in phylogenetic analysis. The previously proposed composition of genera within the six sparid subfamilies (Boopsinae, Denticinae, Diplodinae, Pagellinae, Pagrinae, and Sparinae) were not monophyletic in all analyses.

The DNA sequence diversity of *Sardina pilchardus* (Walbaum *et al.* 1792) and some closely related species of Clupeomorpha was investigated using the mitochondrial DNA gene encoding cytochrome b. The study found that sequence divergence between species and genera was evenly distributed in the cytochrome b gene but rather high compared to reports for other fish species. Phylogenetic analyses on complete cytochrome b were used to study the relationships among the considered species. *S. pilchardus* was easily differentiated, showing a genetic distance of 0.25 with respect to Clupeidae species and 0.26 with respect to the other species (Jerome *et al.,* 2003).

Thai *et al.* (2007) resolved the contradictory phylogenetic relationship in Vietnam over 220 recognized species that were previously based on traditional morphological data. They conducted their study based on the cytochrome b gene sequences from 25 species of cyprinids collected from Vietnam were obtained and combined with sequences of cyprinids available in GenBank, in order to investigate the taxonomic validity of subfamilies within Cyprinidae and their phylogenetic relationships. The molecular data supported traditional division of the Cyprinidae into two major lineages: Cyprinines and Leuciscines. The position of the Danioninae in Cyprinidae as the sister lineage to this grouping was not supported. Many of the subfamily boundaries were questioned and doubt was raised on some of the generic level classifications. The validity of species designation in *Cyprinus*, *Tor* and *Cyclocheilichthys* was also questioned.

Farah *et al.* (2011) observed genetic variation of four Mahseer, *Tor spp.* population in Pahang National Park using cytochrome b mtDNA gene based on phylogenetic analysis. In the study, Neighbor-joining as well as maximum-parsimony methods were used to analyze the phylogenetic relationships between Mahseer, *Tor spp.* and the continuation

of study based on the findings should be taken in order to start proper management as well as conservation of this valuable fish.

Phylogenetic relationships of *Epinephelus fuscoguttatus* and *Epinephelus hexagonatus* inferred from mitochondrial cytochrome b gene sequences was observed by using bioinformatic tools. In the study, phylogenetic trees were constructed using bioinformatic tools with ME method, NJ method and MP method with bootstrapping using arithmetic average method. The study revealed that there are no differences or only small differences were detected between these two taxa. The study concluded that *Epinephelus fuscoguttatus* clustered with *Epinephelus hexagonatus,* based on the phylogenetic trees *Epinephelus hexagonatus* and *Epinephelus fuscoguttatus* consistently clustered together with following the suggestion referring on their morphological data, that they may be closely related or possibly placed within the same species (Baharum and Nurdalila 2011).

Goswami *et al.* (2012) conducted a study on twelve species of *Puntius* (*P. chola*, *P. sophore*, *P. filamentosus*, *P. fasciatus*, *P. vittatus*, *P. chelynoides*, *P. gonionotus*, *P. denisonii*, *P. ticto*, *P. gelius*, *P. conchonius* and *P. sarana*) were investigated using 60 partial sequences of the mitochondrial cytochrome *b* (*Cyt b*) gene to estimate genetic divergence and to establish the phylogenetic relationship. The average intraspecies diversity was estimated as 0.002, whereas the average interspecies diversity was estimated as 0.177. The sequence analysis of the *Cyt b* gene revealed four distinct groups, which are genetically distinct species and exhibited identical phylogenetic relationship. The present study validated the utility of the *Cyt b* gene in genetic diversity and phylogenetic studies.

By analyzed the different data such as nuclear dataset, mitochondrial dataset, combined dataset and the dataset for each nuclear gene performed by maximum likelihood method, the genera *Labeo, Garra, Bangana, Cirrhinus* and *Crosso cheilus* were monophyletic. This study was performed by Yang *et al.* (2012).

A total of 60 samples from twelve species of the genus *Puntius* were collected from eight sampling sites of eight Indian Rivers and were investigated using 60 partial sequences of the mitochondrial cytochrome *b* (*Cyt b*, 1096 bp) gene to estimate genetic divergence and to establish the phylogenetic relationship. The average intraspecies diversity was estimated as 0.002, whereas the average interspecies diversity was estimated as 0.177.

The sequence analysis of the *Cyt b* gene revealed four distinct groups, which are genetically distinct species and exhibited identical phylogenetic relationship. The present study validated the utility of the *Cyt b* gene in genetic diversity and phylogenetic studies (Pallavi et al. 2012).

Yacoub *et al.* (2013) used a cytochrome *b* gene as barcoding tool in distinguishing among chicken strains. They performed polymerase chain reaction (PCR) amplification using universal primer to amplify around 415 bp fragment of cytochrome *b* gene of mtDNA. The study confirmed that short fragment of cytochrome *b* gene of mtDNA as a universal DNA barcode region. They applied this fragment in different animal to ensure this gene much more accurate and efficient tool to discriminate interspecies than intra species.

Awad *et al.* (2014) studied molecular phylogeny of some avian species using cytochrome b gene sequence analysis. Sequences alignment and phylogenetic analyses were performed by CLC main workbench program. The obtained five sequences were deposited in GenBank and compared with those previously registered in GenBank in the study. The similarity percentage was 88.60% between *Gallus gallus* and *Coturnix japonica* and 80.46% between *Gallus gallus* and *Columba livia* and the percentage of identity between the studied species and GenBank species ranged from 77.20% (*Columba oenas* and *Anasplatyr hynchos*) to 100% (*Gallus gallus* and *Gallus sonneratii*, *Coturnix coturnix* and *Coturnix japonica*, *Meleagris gallopavo* and *Columba livia*). Amplification of the partial sequence of mitochondrial cytochrome b gene proved to be practical for identification of an avian species unambiguously.

Wang *et al.* (2014) carried out on a research on *Garra* genus in China to clarify the confusion of phylogenetic position of this species. In this study, 128 samples from the Jinshajiang, Red, Nanpanjiang, Lancangjiang, Nujiang Rivers as well as Hainan Island were measured while 1 mitochondrial gene and 1 nuclear intron of 24 samples were sequenced to explore the phylogenetic relationship of these five species. The results showed that *G. hainanensis*, *G. yiliangensis*, *G. alticorpora* and *G. imberba* were the same species with *G. imberba* being the valid species name, while *G. nujiangensis*is a valid species in and of itself.

Awad *et al.* (2015) conducted a research to ensure the feasibility of using molecular markers. They proved the DNA sequencing have been taken as good markers to classify

the taxonomy and phylogenetic relationships among species. Among mitochondrial genes, *cytochrome b* (mt *Cytb*) gene has been proved as an efficient tool with high power of discrimination for species identification and characterization in both taxonomy and forensic science.

The 396 base pairs of mitochondrial gene of cytochrome b gene were amplified and sequenced from 110 Hampla fishes, a group of freshwater cyprinid inhabit in Southeast Asia. The sequenced nucleotide sequences were subjected to phylogenetic analyses by using the neighbor-joining, maximum parsimony and maximum likelihood methods. All three methods revealed the reciprocally monophyletic relationship of *Hampalam acrolepidota* to the other *Hampala* forms, thus strongly supporting its status as a distinct species. Overall, the partial sequence of the mitochondrial cytochrome b gene was useful for resolving the phylogenetic relationships among Hampala fishes in Malaysia (Jeffrine and Yuzine, 2006).

The molecular phylogenetic relationships for all but one of the described subspecies/species of *Percocypris* were investigated based on three mitochondrial genes (16S; COI; Cyt *b*) and one nuclear marker (Rag2). The results of Maximum Likelihood and Bayesian Inference analyses showed that *Percocypris* is a strongly supported monophyletic group and that it is the sister group of *Schizothorax*. Combined with analyses of morphological characters, the results suggest that *Percocypris* needs to be reclassified (Wang *et al.* 2013).

CHAPTER 3

MATERIALS AND METHODS

Chapter 3

MATERIALS AND METHODS

3.1 Research Institutes

The experiments were conducted in "Fisheries Biology and Genetics Laboratory" Faculty of Fisheries, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur and at Animal Biotechnology Laboratory, National Institute of Biotechnology (NIB), Savar, Dhaka.

3.2 Fish collection

The live fish were collected from Atrai river of Dinajpur districts in Bangladesh.

3.3 Used instruments and chemicals

The following instruments were used in the study-

- Scissors
- Scalpel
- Forceps
- Autoclave machine
- Micro bottom
- Deep freeze
- Eppendorf tube
- Vortex mixer
- PCR tube
- PCR machine
- Biological safety cabinet
- Sequencer machine
- Gel doc
- Paraflim

The flowing chemicals were needed in the study-

- 100% Ethanol
- Distilled water
- Lifton buffer
- Proteinase k
- Phenol
- Chloroform
- 100% Ice cool Alcohole
- Amonium acetate
- Primer
- Template DNA
- Master mix

3.4 Methodologies

3.4.1 Sample collection and preservation

For each species 5 individual were selected and the collected tissue samples were preserved in 95% ethanol. At first fish hold and cut tissue with scalpel. The sample was taken in micro bottom and then the bottom filled with ethanol. Then the bottom was labeled by using marker for clear identification. All instruments were sterilized and one instrument was use for one sample collection. After taking the sample paraflim was used to close the mouth of all bottoms.

3.4.2 DNA extraction

A standard proteinase K, phenol-chloroform method was used for genomic DNA extraction from muscle tissue (Wu *et. al.* 1995). Approximately, 100 mg of muscle were taken 1.5 ml-Eppendorf tube and vortexing each tube 1min for keeping the sample in the bottom of the tube. Then, 350 µl Lifton buffer (100 mM EDTA, 25mM Tris HCL, 0.1% SDS) and 20 µl proteinase K were added, mixed gently and incubated at 55°C on dry bath for 120 min for complete lysis of cells. After incubation, chloroform (250 µl) and phenol (250 µl) were added, mixed gently and centrifuged at 10,000 rpm at 4°C temperature for 10 min. The supernatant was transferred to a new Eppendorf tube, were added 900-1000 µl ice cool alcohol and observed cotton like white color DNA structure. After freezing, all tube was freezed at kept at -20°C for 1 hr, subsequently centrifuged at 14,000 rpm at 4 °C for 10 min. The surface liquid was then kept out from tube and observed DNA pellet.

3.4.3 Purification of extract DNA product

For purification of DNA pellet, 70% alcohol (800-900µl) and 7.5m ammonium acetate (20-25µl) were added, freezed at -20°C for 15 min and centrifuged at 14,000 rpm at 4°C temperature for 10 min. After that, the liquid of all Eppendorf tube was kept out and purified DNA pellet were observed inside. All tube was dried carefully, added 150 µl TE buffer (10mM Tris CL and 1mM EDTA). After ensuring complete solubility of DNA, the purity factor (A260/A280 nm) was measured by UV spectrophotometer (Cole Parmer Ins. Company, US) and its integrity was checked by loading 10 µl DNA preparation (2 µl extracted DNA, 2 µl dye and 6 µl sterile water) on 0.1 percent agarose gel and stained

with ethidium bromide. The extracted DNA samples were then stored at -20°C till their further use. These DNAs were used as templates DNA in PCR reaction.

3.4.4 Amplification of cytochrome b gene

3.4.4.1 Primer selection

The following universal primer were used in the study.

Table 1. Selected primer used for amplification of Cyt b gene

Gene	Primers	Primer Sequence (5'-3')	Reference
Cyt b	Forward (F)	AAAAAGCTTCCATCCAACATCTC	Kocher *et al.* (2009)
	Reverse (R)	AAACTGCAGCCCCTCAGAATGAT	

3.4.4.2 Preparation of PCR reaction mixture

The following components were used to prepare PCR reaction mixture.

Table 2. Components of PCR reaction mixture

Sl. No.	Reagents	Amount per sample
1	Master mix (Tag DNA Polymerase, dNTPs, $MgCl_{2)}$	12.5 µl
2	Primer (Forward + Reverse)	2 µl
3	Template	1 µl
4	Distilled water	9.5 µl

During the experiment, exact amount of master mix were thawed from frozen stocks, mixed by overtaxing and placed on ice plate. The primers, distilled water were mixed with master mix. Then all of them were mixed gently. Then exact amount of mixture (24 µl) were taken each PCR tube. After that 1 µl of template DNA were added in each tube and vortexed briefly. All work were done in safety cabinet to avoid all types of contamination.

3.4.4.3 PCR reaction

After mixing all component in PCR tube, all tubes were put on PCR machine (Fig.5) very carefully. The number of cycle, temperature, time were set up and then run the PCR machine for amplification.

Table 3. PCR condition for amplification

Number of cycle	Step name	Temperature	Time
1	Pre heat	104°C	2 min
	Denaturation	94°C	4 min
35	Denaturation	94°C	1 min
	Annealing	48°C	1 min
	Extention	72°C	45 Sec
1	Final Extention	72°C	5 min

After completion of cycling program, the reactions were held at 4°C and then kept out

3.4.5 Electrophoresis of the amplified products and documentation

1.0 g agarose gel (1%) was prepared using agarose powder containing ethidium bromide and 100 ml 1XTAE buffer. Electrophoresis was conducted in 1X TAE buffer at 90 Volts for 30 minutes. 1kb DNA ladder was used to electrophore alongside the reactions. DNA bands were observed on UV-transilluminator and photographed by a gel documentation system.

3.4.6 Purification of PCR product

All PCR products were transferred to the SV Minicolumn assembly and incubate for 1 minute at room temperature. SV Minicolumn assembly was centrifuged in a micro centrifuge at 16,000 x g (14,000rpm) for 1 minute. SV Minicolumn was removed from the Spin Column assembly and discard the liquid in the collection tube. Then SV Minicolumn was returned to the collection tube. The column were washed by adding 700 µL of membrane wash solution, previously diluted with 95% ethanol, to the SV Mini column and centrifuge the SV Minicolumn assembly for 1 minute at 16,000 x g (14,000rpm). Washing was repeated with 500 µL of membrane wash solution and centrifuge the SV Minicolumn assembly for 5 minutes at 16,000 x g. SV Minicolumn assembly was then removed from the centrifuge, being careful on to wet the bottom of the column with the flow through. The collection tube was made empty and the column assembly was re-centrifuged for 1 minute with the micro centrifuge lid open (or off) to allow evaporation of any residual ethanol. SV Minicolumn was carefully transferred to a clean 1.5ml micro centrifuge tube and apply 50 µL of nuclease-free water directly to the center of the column without touching the membrane with the pipette tip, then incubate at room temperature for 1 minute. Then centrifuged for 1 minute at 16,000xg

(14,000rpm). SV Minicolumn was discarded the micro centrifuge tube containing and eluted DNA was stored at 4°C or -20°C.

3.4.7 Sequencing

The purified products were labeled using the Big Dye terminator 3.1 cycle sequencing kit (Sanger sequencer) in a total reaction mixture of 10 µl containing 4.94 µl of distilled sterile water, 1.94 µl of 5x Big Dye Buffer (400 mM Tris–HCl pH 9.0 and 10 mM $MgCl_2$), 2 µl of 10 p mol of M13F or M13R, 0.12 µl of Big Dye terminator and 1 µl ExoSAP products. Sequence-

3.4.8 Sequence analysis

Cyt b (352-368 bp) were collected and 10 partial sequences were used in the present study. The raw DNA sequences were aligned, truncated and edited manually using DnaStar sequence alignment editor version 7.0.5.2 software (Hall, 1999). Multiple alignments were performed with CLUSTAL W (Thompson *et al.,* 1994) alignment editor (Bootstraps 2000) and checked manually for obvious misalignments as implemented in BioEdit. Polypeptides were checked for erroneous stop codons. The sequences were submitted to GenBank using a programme "Bankit" (www.ncbi.nlm.nih.gov/bankit/index.html) for public access. The bankit submission id and sequences that were used from NCBI (http:// www.ncbi.nlm.nih.gov/) GenBank for analysis such as similar fish sequnce and outgroup given in Table-4.

Table 4. Details of BankIt submission and NCBI sequences used for study

Species	Accession Number
Labeo rohita	MG552727
Amblypharyngodon mola	MG552728
Danio rerio	AJ388456.1
Oncorhynchus keta	AJ314561.1
Melanotaenia splendida	HM007049.1
Labeo calbasu	GQ461851.1
Barilius bendelensis	HM636843.1
Labeo bata	JX0742260.1
Melanotaenia duboulayi	XX77507.1
Opsarius bakeri	KJ442609.1
Cirrhinus mrigala	JX986932.1
Labeo gonius	KP205283

3.4.9 Data analysis

Phylogenetic analyses (Exploratory data analysis of sequences) were performed using MEGA Version 6.01 (Tamura *et al.,* 2012). Pairwise sequence divergences within families were generated using Kimura's two-parameter method (Kimura, 1980). A cluster analysis within and between families was done with the Neighbour-Joining algorithm (NJ) as implemented in MEGA 6.01. Gaps were considered as missing data on the phylogenetic reconstructions. 2000 bootstraps were performed to assign confidence levels to the nodes in the branches of trees (Felsenstein, 1985). The simple NJ algorithm was considered at this juncture to be an appropriate starting point for the analyses, given that specimen identification was based entirely on sequence similarity, rather than on strictly phylogenetic relationships, and the speed of analysis that is necessary for biosecurity diagnostic purposes (Felsenstein, 1985).

CHAPTER 4

RESULTS

4.1 Nucleotide frequency

A total of 3 partial *Cytochrome b* gene sequences (both forward and reverse) from three species were sequenced. The final consensus size of the sequence was determined from the forward and reverse sequences of each species after multiple sequence alignment using BioEdit software packages. In the consensus cytochrome gene sequence, the amplified fragment length was 357 bp in *A. mola*, 364 bp in *L. bata* and 356 bp in *A. testudineus*.

The nucleotide frequencies of the *A. mola* and *L. bata* along with the other species of the study were shown in Table-5. The percent of Adenine (A) and Thiamin (T) were found more or less similar throughout the sequences, but the percent of Cytosine (C) and Guanine (G) were found different in the sequence of *A. mola* and *L. bata*. The percent of cytosine was 15.5 and 15.3 in *A. mola* and *L. bata* respectively but in other studied species the cytosine percent was higher that ranged from 22.1 to 29.6%. In case of guanine, the percent was 26.5 and 28.5 in *A. mola* and *L. bata* respectively but in other studied species the guanine percent was lower that ranged from 12.8 to 22.2 (Table-5).

Table 5. Nucleotide frequencies (%) of the Cyt b sequences in the study

Species Name	T(U)	C	A	G	Total
Amblypharyngodon mola (Sequenced)	30.1	15.5	27.9	26.5	355.0
Labeo bata (sequenced)	29.1	15.3	27.7	28.0	354.0
Danio rerio	34.8	22.1	26.7	16.4	330.0
Opsarius bakeri	29.5	29.2	28.5	12.8	421.0
Barilius bendelisis	29.9	27.1	20.8	22.2	288.0
Melanotaenia splendida	28.5	28.5	25.1	17.9	351.0
Melanotaenia duboulayi	29.3	29.3	24.5	16.8	542.0
Oncorhynchus keta	28.3	29.4	26.5	15.9	446.0
Labeo calbasu	29.7	28.1	28.7	13.5	303.0
Cirrhinus mrigala	29.0	28.3	28.3	14.3	307.0
Labeo gonius	28.7	29.6	27.0	14.7	307.0
A.testudineus (Sequenced outgroup)	25.4	16.3	29.3	29.0	355.0
Avg.	29.3	25.1	26.7	18.9	363.3

4.2 Transition/Transversion bias

Substitution pattern and rates were estimated under the Kimura (1980) 2-parameter model. For estimating ML values, a tree topology was automatically computed. The maximum Log likelihood for this computation was 4318.696. All positions containing gaps and missing data were eliminated. The estimated Transition/Transversion bias (*R*) was 0.91. The substitution matrix for the bases in the cytochrome b sequences on studied fish species was presented in Table-6. Each entry is the probability of substitution (r) from one base (row) to another base (column). Rates of different transitional substitutions are shown in **bold** and those of transversionsal substitutions are shown *in italics*. For simplicity, sum of r values is made equal to 100. The nucleotide frequencies were A = 26.87%, T/U = 29.27%, C = 25.00% and G = 18.86%. The rate of transitional stubstitution from A to G, T to C, C to T, G to A were 5.49, 24.93, 29.19 and 7.82 respectively (Table-6). On the other hand the rate of transversional substitution from A to T, A to C, T to A, T to G, C to A, C to G, G to T, G to C were 4.37, 4.37, 4.77, 4.77, 3.07 and 3.07 respectively (Table-6).

Table 6. Maximum likelihood estimate of substitution matrix

	A	**T/U**	**C**	**G**
A	-	*4.77*	*4.07*	**5.49**
T/U	*4.37*	-	**24.93**	*3.07*
C	*4.37*	**29.19**	-	*3.07*
G	**7.82**	*4.77*	*4.07*	-

4.3 Genetic distance

The genetic distance of *A. mola* and *L. bata* along with 9 other studied species were estimated in terms evolutionary divergence between sequences. The number of base substitutions per site from between sequences is shown and the standard error estimate(s) are shown above the diagonal Table-7. The highest sequence divergence of *A. mola* cytochrome b sequences was found from *O. keta* (10.003) that is a marine species and the lowest sequence divergence of *A. mola* was occurred from *C. mrigala* (5.430). The sequence divergence between pairs of *L. bata* and other species is shown in shaded areas of the Table-7. The highest sequence divergence of *L. bata* cytochrome b sequence was occurred from the *Danio rerio* (9.858) and the lowest sequence divergence of *L. bata* was occurred from *Melanotaenia duboulayi* (3.516) which is also a freshwater fishes of Australia.

Table 7. Estimates of evolutionary divergence between sequences

Sl No	Species List	1	2	3	4	5	6	7	8	9	10	11
1	*Amblypharyngodon mola* (Sequenced)		2.822	2.804	3.041	3.127	2.704	3.161	3.633	3.611	2.464	2.482
2	*Danio rerio*	6.611		3.071	3.115	3.630	1.828	3.223	1.584	2.584	2.354	2.373
3	*Opsarius bakeri*	6.509	6.578		2.795	3.364	2.723	3.447	3.132	3.165	2.263	2.453
4	*Barilius bendelisis*	6.853	7.567	7.169		2.365	1.942	2.357	2.973	3.252	3.016	3.110
5	*Labeo bata* (sequenced)	6.833	9.858	7.841	5.415		3.649	1.597	2.275	3.403	3.010	2.927
6	*Melanotaenia splendida*	6.102	3.637	6.442	4.595	9.660		2.500	1.694	1.923	2.886	3.076
7	*Melanotaenia duboulayi*	7.392	7.220	7.452	5.367	3.516	5.226		2.100	3.554	1.882	1.800
8	*Oncorhynchus keta*	10.003	3.657	7.092	7.018	5.539	3.742	4.828		2.410	3.175	3.254
9	*Labeo calbasu*	9.155	5.982	7.154	6.808	9.281	4.620	9.669	5.668		3.020	2.860
10	*Cirrhinus mrigala*	5.430	5.453	4.374	6.904	7.604	6.537	4.574	7.299	7.414		0.023
11	*Labeo gonius*	5.995	5.383	5.234	6.892	7.132	7.115	4.046	7.546	6.682	0.134	

4.4 Phylogenetic tree

To depict the molecular taxonomy and evolution of the studied fish species, Phylogenetic trees were constructed using three methods that included Maximum likelihood (ML), Neighbor Joining (NJ) and Maximum Parsimony (MP) method.

4.4.1 Tree based on Maximum Likelihood Methods

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches (Figure-3). The phylogenetic tree in ML method divided 12 studied taxa in three clades. Clade1 consisted of *Danio rerio*, *M. splendida*, *O. keta*, *B. bendelisis* and *L. carbasu*. *A. mola* did not form sister with any studied taxa. Clade 2 form by including *O. bakeri*, *C. mrigala* and *L. gonius*, *L. bata* form clade 3 with *M. dubolulayi*, where *A. mola* did not form sister with any taxa.

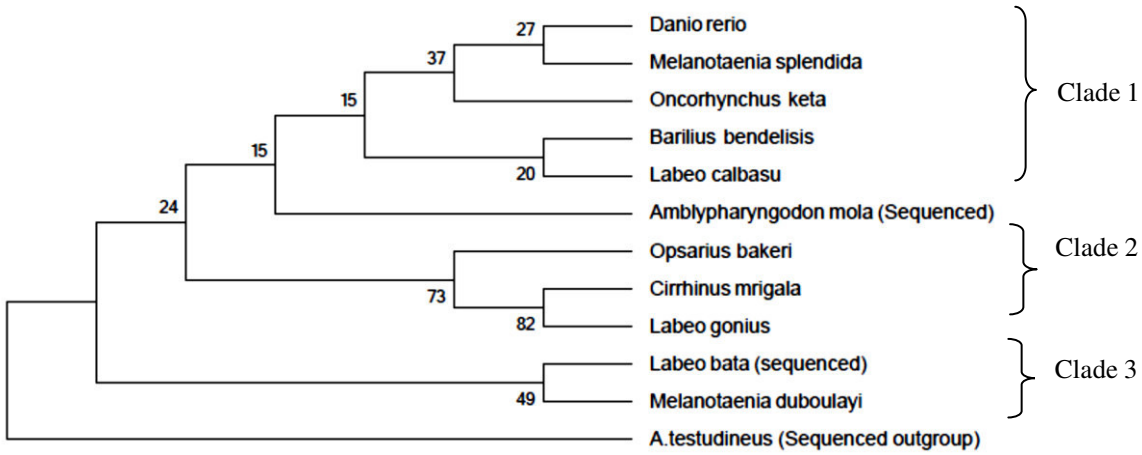

Figure 3. The bootstrap consensus phylogenetic tree through ML method

4.4.2 Tree based on Neighbour Joining Methods

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Figure-4). In this tree three clades were formed by the studied species. In NJ tree *A. mola* formed clade 1 with *C. mrigala*, *L. gonius*, *O. bakeri*.

L. calbasu, *M. splendid*, *D. rerio* and *O. keta* form clade 2. *L. bata* formed clade 3 with *B. bendelisis* and *M. duboulayi*.

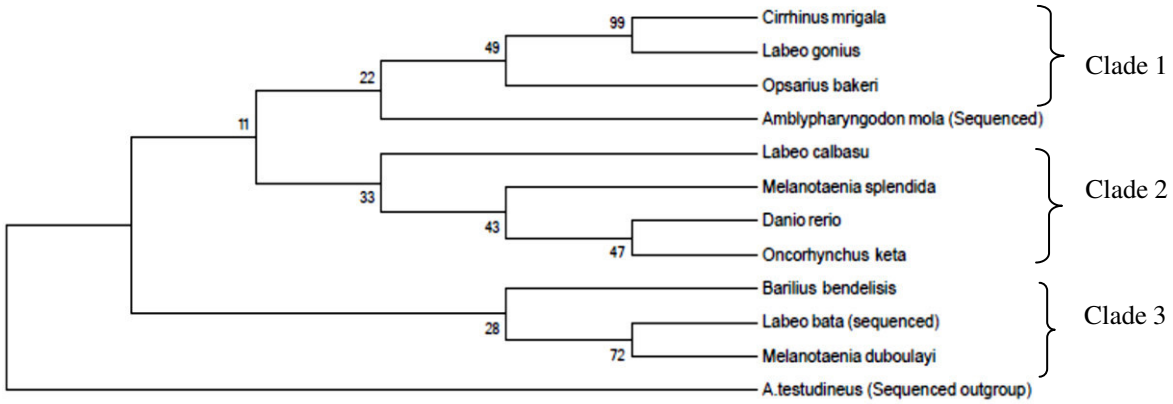

Figure 4. The bootstrap consensus phylogenetic tree through NJ method

4.4.3 Tree based on Maximum Parsimony Methods

The evolutionary history was inferred using the Maximum Parsimony method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Figure-5). Formation of three clades was also observed in this method also. Interestingly here *A. mola* form clade 1 with *B. bendelisis* and *L. bata*. Same as previous two method *D. rerio* and *O. keta* form sister group here also.

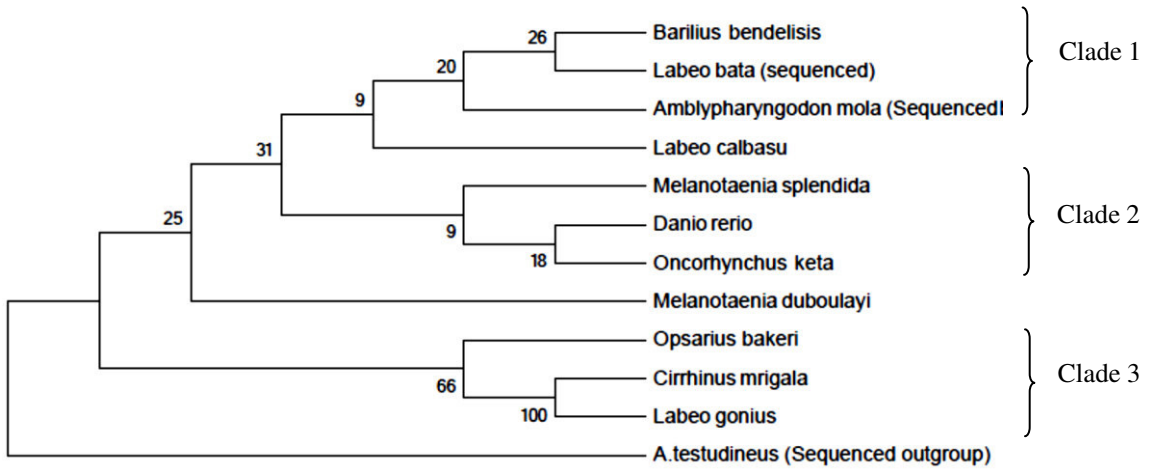

Figure 5. Time tree using maximum parsimony method

4.5 Time tree based on maximum likelihood method

The time tree shown was generated using the RelTime method. Divergence times for all branching points in the topology were calculated using the Maximum Likelihood method based on the Tamura-Nei model. The estimated log likelihood value of the topology shown in 4286.0642. The tree was drawn to scale, with branch lengths measured in the relative number of substitutions per site (Figure-6). The time tree based on maximum likelihood method showed that there were the least times of divergence in between *C. mrigala* and *L. gonius*. There was the highest times of divergence (4.89 MYA) in between *D. rerio* and outgroup taxa *A. testudineus*.

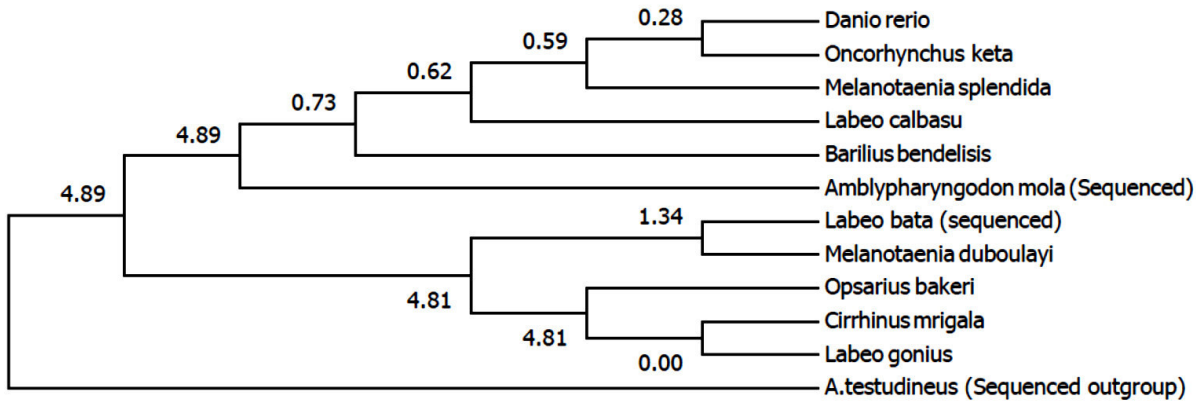

Figure 6. Time tree using maximum likelihood method

The divergance time between *Amblypharyngodon mola* and *Labeo bata* was 4.89 MYA.

CHAPTER 5

DISCUSSION

Chapter 5

DISCUSSION

5.1 Nucleotide frequency

In the consensus cytochrome gene sequence, the amplified fragment length was 357 bp in *A. mola*, 364 bp in *L. bata* and 356 bp in *A. testudineus*. The percent of Adenine (A) and Thiamine (T), Cytosine (C) and Guanine (G) were 26.7, 29.3, 25.4 and 18.9 respectively. Adenine and Thiamine were found more or less similar throughout the sequences, but the percent of and Guanine (G) were found different in the sequence of *A. mola* and *L. bata* (Table-5). The nucleotide frequency has been frequently reported in cyt b-based studies on a variety of fishes, including cyprinids and found the nucleotide composition was T 24.77%; C 27.77%; A 31.36%, G 16.09% similar to this study (Ketmaier *et al.* 2004; Li *et al.* (2015). The mean base composition of cytochrome b gene sequences was similar to that previously reported for cyprinid fishes (Briolay *et al.,* 1998; Durand *et al.,* 2002), with low G content (15.2%) and almost equal A, T and C content (28.1%, 28.9%, 27.7%, respectively). The content of A+T (56%) was higher than that of C+G (44%), which felt within the range of the GC content typical for vertebrates (Nei & Kumar, 2000) and similar of Yang *et al.* (2006).

5.2 Transition/Transversion bias

The estimated Transition/Transversion bias (*R*) was 0.91. The transition/transversion ratio 1.7 was reported by Yuzine *et al.* (2012) and 2.00 was reported by Zhu *et al.* (1994), which were slightly higher from the present study. The rate of transitional substitution from A to G, T to C, C to T, G to A were 5.49, 24.93, 29.19 and 7.82 respectively (Table-6). On the other hand the rate of transversional substitution from A to T, A to C, T to A, T to G, C to A, C to G, G to T, G to C were 4.37, 4.37, 4.77,4.77, 3.07, and 3.07 respectively (Table-6). Kocher e *et al.* (2007); Izeni *et al.* (2001); Zardoya *et al.* (1999) reported the similar result where they stated with increased divergence time the rate of transition is decreased and transversion is increased.

5.3 Genetic distance

The genetic distance of *A. mola* and *L. bata* along with 9 other studied species were estimated in terms evolutionary divergence between sequences with Kimura two-

parameter model (Kimura, 1980). The highest sequence divergence of *A. mola* cytochrome b seqequences was found from *O. keta* (10.003) that is a marine species and the lowest sequence divergence of *A. mola* was occurred from *C. mrigala* (5.430). The highest sequence divergence of *L. bata* cytochrome b sequence was occurred from the *Danio rerio* (9.858) and the lowest sequence divergence of *L. bata* was occurred from *Melanotaenia duboulayi* (3.516) which is also a freshwater fishes of Australia (Table 7). Briolay *et al.* (1998) found the highest genetic distance 1.3 in a study of fish phylogeny. Yuzine *et al.* (2012) found the lowest distance value of 6.2 and the highest genetic distance value of 18.8 among genera of cyprinidae fish family. Yang *et al.* (2006) showed that the pair-wise genetic divergence ranges from 0.4 to 23.8% observed. Similar statement of pairwise sequence divergence between taxa that ranged from 0.3 to 26% was described by Zardoya *et al.* (1999).

5.4. Phylogenetic tree and time tree

To find the reliable phylogenetic relationship among the studied taxa, three methods viz., Maximum likelihood (ML), Neighbor Joining (NJ), and Maximum Parsimony (MP) method were used to construct the phylogenetic tree. For the consensus tree, 1000 replicates were used in bootstrap reliability test for all three methods. In all three methods more or less similar clustering of the studied taxa were observed. In ML method studied taxa were grouped into three clades. Clade 1 consisted of *Danio rerio*, *M. splendida*, *O. keta*, *B. bendelisis* and *L. carbasu*. *A. mola* did not form sister with any studied taxa. Clade 2 form by including *O. bakeri*, *C. mrigala* and *L. gonius*, *L. bata* form clade 3 with *M.dubolulayi*, where A. mola did not form sister with any taxa (Figure 3). In NJ tree, three clades also were formed; *A. mola* formed clade 1 with *C. mrigala*, *L. gonius*, *O. bakeri*. *L. calbasu*, *M. splendid*, *D. rerio* and *O. keta* form clade 2. *L. bata* formed clade 3 with *B. bendelisis* and *M. duboulayi* (Figure 4). Formation of three clades was also observed in this method. Interestingly here *A. mola* form clade 1 with *B. bendelisis* and *L. bata*. Same as previous two method *D. rerio* and *O. keta* form sister group here (Figure 5). These results are consistent with other recent findings from molecular phylogenetic analyses by Mayden *et al.* (2007) and Koacher *et al.* (20087). The placement and relationships of species in the various subfamilies of Cyprinidae is not surprising (Conway *et al.* 2008). The time tree based on maximum likelihood method showed that there were the least times of divergence in between *C. mrigala* and *L. gonius*. There as the highest times of divergence (4.89 MY) in between *D. rerio* and

outgroup taxa *A. testiduneus*. Wang *et al.* (2013) mentioned the ancestor of Cyprininae originated about 32.3 MYA. This occurs due to the selection of outgroup. In this study close divergence time observed due to all living species and selection of close taxa from subfamily in cyprinidae family. The estimated divergence time from the fossil showed very high time than live species (Chen and Liu 2007; Cavender 1991; Chen 1987).

CHAPTER 6

SUMMARY AND CONCLUSION

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In Bangladesh, there are records of at least of 57 species of cyprinids under 23 genera**.** *Amblypharyngodon mola*, locally known as "Mola" and *Labeo bata*, as "Bata" are fish of small indigenous species (SIS), belonging to Danioninae and Cyprininae subfamily of Cyprinidae fish family. With the increased introduction of exotic fish species, interest of aquaculture of indigenous fishes is declining. In such situation, *Labeo bata* is still favoured by the farmers of Bangladesh in carp polyculture system because of its delicious food value. At the same time, now a days *A. mola* is in trial for aquaculture in the farmers pond. The very unique feature is that this fish is self-recruiting, hence once a farmer introduced in his/her ponds it continues its recruitment. With the passing of the time fish populations are altered and may split into separate branches, hybridize together or terminate by extinction, as a result the evolution occurred. Thus, a phylogenetic context can provide the most meaningful insights into biology. Phylogenetic studies have been conducted for several fish families**,** but still no molecular phylogeny of the freshwater fish species of Bangladesh was available. The aim of this study is to establish the systematic position of selected fishes through the phylogenetic and evolutionary analysis of partial sequences of mitochondrial gene *Cytochrome b*. The fish tissue samples were collected for the selected species and subjected to PCR amplification.. The amplified PCR products were purified with kit box and then forwarded for the sequencing. Cyt b (352-368 bp) were collected and 3 partial sequences were used in the present study. The raw DNA sequences were aligned, truncated and edited manually using DnaStar sequence alignment editor version 7.0.5.2 software (Hall, 1999). Multiple alignments were performed with CLUSTAL W (Thompson *et al.* 1994) alignment editor (Bootstraps 2000) and checked manually for obvious misalignments as implemented in BioEdit. Polypeptides were checked for erroneous stop codons. The sequences were submitted to GenBank using a programme "Bankit" (www.ncbi.nlm.nih.gov/ Bankit/index.html) for public access.

In the consensus cytochrome gene sequence, the amplified fragment length was 357 bp in *A. mola*, 364 bp in *L. bata* and 356 bp in *A. testudineus*. The percent of Adenine (A), Thiamine (T), Cytosine (C) and Guanine (G) were 26.7, 29.3, 25.4 and 18.9 respectively. Adenine and Thiamine were found more or less similar throughout the sequences, but the

percent of and Guanine b (G) were found different in the sequence of *A. mola* and *L. bata* (Table-5). The estimated Transition/Transversion bias (*R*) was 0.91. The rate of transitional substitution from A to G, T to C, C to T, G to A were 5.49, 24.93, 29.19 and 7.82 respectively (Table-6). On the other hand the rate of transversional substitution from A to T, A to C, T to A, T to G, C to A, C to G, G to T, G to C were 4.37, 4.37, 4.77,4.77, 3.07, and 3.07 respectively (Table 6). The genetic distance of *A. mola* and *L. bata* along with 9 other studied species were estimated in terms evolutionary divergence between sequences with Kimura two-parameter model (Kimura, 1980). The highest sequence divergence of *A. mola* cytochrome b seqequences was found from *O. keta* (10.003) that is a marine species and the lowest sequence divergence of *A. mola* was occurred from *C. mrigala* (5.430). The highest sequence divergence of *L. bata* Cyt b sequence was occurred from the *Danio rerio* (9.858) and the lowest sequence divergence of *L. bata* was occurred from *Melanotaenia duboulayi* (3.516) which is also a freshwater fishes of Australia (Table 7).

To find the reliable phylogenetic relationship among the studied taxa, three methods viz., Maximum likelihood (ML), Neighbor Joining (NJ), and Maximum Parsimony (MP) method were used to construct the phylogenetic tree. For the consensus tree, 1000 replicates were used in bootstrap reliability test for all three methods. In all three methods more or less similar clustering of the studied taxa were observed. In ML method studied taxa were grouped into three clades. Clade 1consisted of *Danio rerio*, *M. splendida*, *O. keta*, *B. bendelisis* and *L. carbasu*. *A. mola* did not form sister with any studied taxa. Clade 2 formed by including *O. bakeri*, *C. mrigala* and *L. gonius*. , *L. bata* form clade 3 with *M.dubolulayi*, where A. mola did not form sister with any taxa (Figure 3). In NJ tree, three clades also were formed; *A. mola* formed clade 1 with *C. mrigala*, *L. gonius*, *O. bakeri*. *L. calbasu*, *M. splendid*, *D. rerio* and *O. keta* form clade 2. *L. bata* formed clade 3 with *B. bendelisis* and *M. duboulayi* (Figure-4). Formation of three clades was also observed in this method. Interestingly here *A. mola* form clade 1 with *B. bendelisis* and *L. bata*. Same as previous two method *D. rerio* and *O. keta* form sister group here (Figure 5). The time tree based on maximum likelihood method showed that there were the least times of divergence in between *C. mrigala* and *L. gonius*. There as the highest times of divergence (4.89 MYA) in between *D. rerio* and outgroup taxa *A. testiduneus*.

The study concluded the *A. mola* and *L. bata* showed considerable variation in this Cyt b gene sequences. To protect the gene pool proper stock manage is highly required. The study suggested for a detail phylogenetic and evolutionary analyses of cyprinid family of fishes by including all the species of cyprinidae family. Along with the *Cytochrome b* gene, more genes such as Cytochrome C Oxidase subunit-I, nuclear genes also needed to added for a clear phylogeny and evolutionary relationship of the studied fishes.

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