Genetic diversity of zebrafish (*Danio rerio*, Hamilton, 1822) in Bangladesh using DNA barcoding and RAPD techniques

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

Submitted By

Examination Roll: Curzon-805MS Session: 2015-2016

Registration Number: 2011-712-772Registration Session: 2011-2012

Department of Fisheries

University of Dhaka, Dhaka-1000

Bangladesh

February 2017

Dedicated

To

My Beloved Parents

Declaration

I hereby declare that the dissertation entitled **"Genetic diversity of zebrafish (*Danio rerio*, Hamilton, 1822) in Bangladesh using DNA barcoding and RAPD techniques"** submitted to the Department of Fisheries, University of Dhaka for the degree of Master of Science (MS) is based on self-investigation, carried out under the supervision of **Dr. Md. GolamRabbane**and**Dr Mohammad ShamsurRahman**, Associate Professor, 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.

Name: Md. AbulKalam Azad

Examination Roll: Curzon-805 MS session: 2015-2016

Registration No: 2011-712-772Registration session: 2011-2012

Department of Fisheries, Faculty of Biological Science

University of Dhaka, Dhaka-1000, Bangladesh

CERTIFICATE

This is certify that the research study entitled ***"Identification of genetic diversity of zebrafish (Danio rerio, Hamilton, 1822) in Bangladesh using DNA barcoding and RAPD techniques"*** *was done by* ***Md. AbulKalam Azad****, Examination Roll:Curzon-805, Registration No: 2011-712-772,MS session: 2015-16, under our supervision.*

This is further to certify that it is an original work and suitable in partial fulfillment of the requirements for the degree of Master of Science(MS) in Fisheries from University of Dhaka, Dhaka-1000, Bangladesh.

We wish every success in his life.

Dr. Md. GolamRabbane

(Supervisor)
Associate Professor
Department of Fisheries
Faculty of Biological Sciences
University of Dhaka
Dhaka-1000, Bangladesh

Dr MohammadShamsurRahman

(Co-supervisor)
Associate Professor
Department of Fisheries
Faculty of Biological Sciences
University of Dhaka
Dhaka-1000, Bangladesh

ACKNOWLEDGEMENT

Thanks to **Almighty Allah** who creates such a beautiful earth, controls each and every thing. From the core of my heart, I would like to reveal my earnest awe, loyalty and reverence to him who enable me to complete the thesis work in spite of numerous distresses.

My sincere indebtedness, deep sense of gratitude and honor to my supervisor, **Dr. Md. GolamRabbane**, Associate Professor, Department of Fisheries, University of Dhaka for constant guidance, advice, constructive criticisms, encouragement and valuable discussions during the course of the study.

I am grateful to my co-supervisor **DrMohammad ShamsurRahman**, Associate professor, Department of Fisheries, University of Dhaka for his incessant supervision, guidance and constructive criticism throughout the work.

Special thanks to our beloved Chairperson, **Dr. KanizFatema**, Professor & Chairman, and **MdAlamgirKabir,** Assistant Professor, Department of Fisheries, University of Dhaka for providing necessary suggestions and giving valuable encouragement.

I also acknowledged to all of my honorable and respected teachers of the Department of Fisheries, University of Dhaka for their helpful suggestions, encouragement, academic collaboration and blessing to well wishes.

Personal thanks to all of my friends for their co-ordination especially **Utpal Chandra Ray**, thanks to my all well wishers, classmates and other friends to Department and Hall for their encouragement during the study.I am also grateful to Ministry of Science and Technology for funding to conduct my thesis work.

I am highly delighted to express my cordial gratitude and veneration to my parents and sisters for their affection, blessing and sacrifices. All of their helps, inspiration and encouragement have facilitated me to reach at this stage of education.

The Author **February, 2017**

Abstract

Zebrafish is one of the most important model fish species used in aquaculture research. The study of genetic diversity of wild zebrafish (*Danio rerio*) by RAPD markers and DNA barcoding are important tools which will help in developing commercial fish stock for the sustainable aquaculture management.

The present investigation was undertaken to study the morphometric variation and genetic diversity of the zebrafish collected from 4 locations in Bangladesh (Mymensingh, Faridpur, Khulna and Munhigonj) and 12 of the samples were analyzed by RAPD marker and DNA barcoding techniques. Three random decamer primers were used for RAPD marker and two were used for DNA barcoding to amplify DNA fragments.

112 bands were scored by the primers, 22 were polymorphic. The percent polymorphic loci and gene diversity values varied within a range of 10.60–33.33 and 0.0645–1.2685 respectively. Nei's genetic similarity between populations across all the primers ranged from 0.2812 to 1.0000. The dendrogram based on Nei's genetic distance showed 11 clusters; the population of Faridpur was found to have maximum genetic diversity with 33.33% polymorphic loci and the Khulna population had a minimum with 10.60% polymorphic loci. Genetic diversity also found among the populations of Mymensingh and Munshigonj with 15.15% and 19.69% polymorphic loci. The clusters also revealed strong correlation with the species.

For DNA barcoding, we amplified the cytochrome oxidase c subunit 1(COI) barcode sequence (680 bp long) for 4 specimens, and we compared them with reference sequences from different databases (GenBank and BOLD). Though the database is undergoing continual development, it was able to provide species matches of >99% sequence similarity for 4 samples tested. The overall sequence alignment similarity among our sampled specimens was 99.35%.

Although both of the techniques are costly, recently DNA barcoding and RAPD marker has achieved support as a rapid, cost-effective and broadly applicable molecular diagnostic technique for this purpose. It can be conclude that the amplified sample used for the experiment appeared significant genetic variation among them.

Table of contents

List of tables

Lists of figures

List of appendices

List of abbreviation

Abbreviation	Elaboration
%	Percentage
CoI	Cytochrome Oxides Sub-unit 1
Kb	Kilo Base
NaOH	Sodium Hydroxide
MgCl	Magnesium Chloride
DoF	Department of Fisheries
FAO	Food and Agricultural Organization
mg/L	Milligram per liter
GDP	Gross Domestic Production
ng/µl	Nano-gram per micro-litre
°C	Degree Celsius
Yr	Year
Hrs	Hours
Cm	Centimeter
RAPD	Random Amplified Polymorphic DNA
G	Gram
PCR	Polymerase Chain Reaction
Ml	Micro-litre
SDS	Sodium Dodecyl Sulfate
M	Molarity
DNA	Deoxyribo Nucleic Acid
BLAST	Basic Local Alignment Search Tool
NCBI	National Centre for Biotechnology Information
MEGAM	Molecular Evolutionary Genetics Analysis
bp	Base Pair
EDTA	Ethylenediaminetetra acetic acid
HCl	Hydrochloric Acid
NaCl	Shodium chloride

Chapter 1

Introduction

1.1 General Introduction

Bangladesh has enriched fresh water fisheries resources with its vast marine area. The fisheries sector plays a major role in national GDP, about 3.69% (DoF, 2016). The water area of inland fisheries includes rivers, beels, Kaptai Lake and flood plain areas which normally called capture based fisheries resources and the ponds, ditches, oxbow lakes, coastal shrimp farms are mainly called culture based fisheries resources. The country has the largest deltaic plain dominated by the major river systems like the Ganges, the Brahmaputra and the Meghna and is endowed with unique water resources comprising of both inland and marine waters. Along with potential water resources, the country is also rich in the diversity of various fish species. Bangladesh is ranked third in aquatic biodiversity in Asia after China and India (Hossain and Mazid, 2001). There are about 260 species of freshwater fish, zebrafish (*Danio rerio*) is one of them and 475 species of marine fish and 12 species of exotic fish in this country. There are also 56 species of shrimp, prawn, crab, oyster, snail, frog, turtle, crocodiles and various types of small and big aquatic animals and birds available in this country (DoF, 2016).

The aquaculture contributes about 2.01% of total export earning, next to the ready- made garments (DoF, 2015). Mainly polyculture system is performed in our country with Indian major carps like Rui (*Labeo rohita*, Hamilton), catla (*Catla catla*, Hamilton), mrigal (*Cirrhinus mrigala*, Hamilton) and kalibaus (*Labeo calbasu*, Hamilton). Due to their fast growth, relatively better taste and higher market price, they take a prime position in the aquaculture scenario of Bangladesh. In our daily feeding habit fish contributes about 60% of protein. According to DoF, currently a person consuming 53 g of fish per day, but the daily requirement is approximately 60 g of fish which is very much closer to the present uptake rate.

Zebrafish (*Danio rerio*) is not an important food fish .It is a model fish species like three spine stickle back, guppy fish, medaka etc. They are used in various types of scientific research and especially in aquaculture development at several place, it is referred as model organism. It has been reported that zebrafish can be reared in small tanks (Huang et. al., 2013).

1.1.1 Zebrafish

Dania rerio or zebrafish, is small fish that is making a big revolution in fisheries research. It's a fresh water living organism. It has four bluish and three silvery very narrow stripes in its body (Shafi, 1982). The anal fin of the fish also contains 3-4 bluish stripes. It lays hundreds of eggs that develop externally, allowing scientists to perform genetic manipulations and monitor early phenotypes in a complex organism. Since they are easy available and their rearing activities can be done within a limited space, zebrafish research is helping us on our way to understanding and treating various fish disease. That's why it is called a model organism worldwide (Lieschke and Currie 2007; Spence et al., 2008). The scientific classification of zebrafish is given below –

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Cypriniformes

Family: Cyprinidae

Genus: *Danio*

Species: *Danio rerio* (Hamilton, 1822)

Figure 1: *Danio rerio* (Hamilton, 1822)

A pair of zebrafish can produce up to 300 fertilized eggs in a week (Statura et al., 2013). This extraordinarily high fecundity (the ability to produce lots of offspring) allows scientists to have vast number of samples (Lieschke and Currie, 2007). It is also making a big splash in biomedical research. They share much of their genome with humans, so zebrafish research is also beneficial to discover and treat human disease. The zebrafish is very common to the slow stream, ponds, river and paddy fields in our country. In these waters, they rarely grow larger than 4 cm long. Zebrafish has a far shorter relationship with science than many other animals. They were first used in research in the late 1960s. By 1976, the USSR [the Soviet Union] had launched a zebrafish into orbit on the Salyut 5 space mission (Genes, Genomes and Health, l6-l9). In the wild, most zebrafish live up to one year old. In captivity, zebrafish have a mean lifespan of 42 months. The maximum age observed in captivity was 66 months. Captive zebrafish develops spinal curvature

after its second year, which is not observed in natural populations. The zebrafish can grow to 6.4 cm (2.5 in) in length, although it seldom grows larger than 4 cm (1.6 in) in captivity (Lieschke and Currie, 2007).

George Streisinger the father of zebrafish genetics, who worked at the University of Oregon began the fish frenzy. Streisinger a fish hobbyist was the first to recognize the potential for using zebrafish in the lab in the late 1960 (This resource was first published in 'Genes, Genomes and Health' in December 20l4). The first clone of zebrafish was created by Streisinger and his colleagues in 1981 and it was published in "Nature" one of the most prestigious Journal. Recent studies have begun to examine questions in ecology and evolution using natural populations of zebrafish, which occur in India, Nepal and Bangladesh. These include: behavioral genetics of shoaling, activity level, boldness and aggression (Allendorf FW and Hohenlohe PA 2010) reproductive behavior color and pattern variation as it relates to speciation, genetic effects of domestication (Robison and Rowland, 2005; Robison, 2007).

Variation in individual growth rates and the number of recessive lethal in wild-caught populations (McCune et al., 2002) are very high. Much more potential extensive knowledge of development and phenotypic expression with genes and gene network needs to develop. The zebrafish (*Dania rerio*) is a prominent model organism in developmental genetics, neurophysiology and biomedical research (Lieschke and Currie, 2007; Spence et al., 2008). Currently, over 400 laboratories worldwide conduct research with zebrafish from established lab strains largely because of its short generation interval, rapid development, high fecundity, transparent embryos and ease of genetic manipulation (Licschke and Currie, 2007.)

George Streisinger pioneered his research using zebrafish (*Danio rerio*), at the University of Oregon in 1972. The zebrafish has not only become a unique animal model in basic research, due to its fine embryonic and (molecular) genetics technique/tool but also developed globally. He believe that this direction of zebrafish research will lead to a better understanding of some nasty human diseases and their pathogenic mechanisms, and eventually help to achieve a better health of human beings (Huang et al.,2013).

Zebrafish is a teleost fish species included under the family cyprinidae and order Cypriniformes and constitutes its karyotype with 25 pairs of diploid (2n) chromosomes

which differ with regard to the number of submetacentric and acentric types (Manna and Khuda-Buksh, 1977; referred by Reddy, 1990). It has also been possible to produce gynogens and polyploids (triploid and tetraploid) through chromosome manipulation or chromosomal engineering, with varied degree of success (John et al., 1984; Reddy et al., 1990), and transgenic kalibaus by gene transfer or genetic engineering (FAO, 1999). However, with such response to diversified manipulation, zebrafish has ranked itself the most popular research aquaculture species all over the world.

1.1.2 Zebrafish as a model organism

Zebrafish can be used in research as an alternative to all other species. Dr Caroline Brennan, a professor of Queen Mary University of London, has performed his research using the NC3Rs to study the genetic mechanisms of zebrafish (Brennan, 2014). Dr Brennan has showed the top five reasons why zebrafish are model organism.

At the time of using animals in research, it is more important to minimize animal suffering by using the least perceptive organism possible to answer the question. Although there are scientific reasons why 'lower' organisms, such as zebrafish, can create a better model system. Some of these reasons are outlined below:

1.1.2.1 Genetic similarity to humans

Zebrafish are vertebrates and therefore share a high degree of sequence and functional homology with mammals including humans. Due to the conservation of cell biological and developmental processes across all vertebrates, studies in fish can give great insight into human disease processes. For example, to date all proteins studied have a similar function in fish and mammals.

1.1.2.2 Easier to house and care

Due to their small size and relatively simple nature in natural environment, zebrafish is easier to keep in what appear to be more natural conditions than it is possible to simulate for mammals. This minimizes housing stress and the impact such stress may have the outcome of experiments. Not only does this add to the refinement of animal usage, it also

minimizes the number of animals that need to be used because it reduces the between subject variation that can be caused by stress.

1.1.2.3 Impact of any genetic mutation or drug treatment is easy

Zebrafish embryos and larvae are completely transparent, meaning that it is possible to follow the impact of a genetic manipulation or pharmacological treatment using non-invasive imaginary techniques. Less intrusive techniques minimize animal suffering. The invasive procedures not only avoid the impact on welfare, but may also affect the experimental outcome, so the transparent nature of zebrafish larvae also mean that results are more accurate and easier to reproduce.

1.1.2.4 Lots of offspring

Ensuring a ready supply of animals for research is also easier with zebrafish. Zebrafish has larger number of offspring in each generation than rodents. Rodents have 5-10 offspring per pair, in comparison to the 200-300 obtained from fish. Zebrafish offspring also grow and develop very quickly.

1.1.2.5 Easier to introduce genetic changes

Zebrafish embryos are able to absorb chemicals that are added to their water, so it means that it is easy to introduce changes to their genes using nothing more than chemical mutagens. Zebrafish are able to withstand much higher levels of chemical mutagens than that of rodents so it is possible to induce a much higher density of mutations in their genome.

1.2 Genetic Variation

The differences in the hereditary constitutions of the individuals of a species can be revealed by genetic variation. It is important in maintaining the developmental stability and biological potential of fish stocks (Oster et al., 1988 and Tave, 1993). High genetic variability within populations and significant genetic differentiation between populations indicate rich genetic resources of a species. According to Allendorf (1983), the knowledge of the distribution of genetic variation within and between populations is

essential for an evolutionary interpretation of interactions and for the management of endangered or commercially important taxa. However, knowing the genetic variations of a population fishery managers have an opportunity to understand the evolutionary processes better which have led to genetic differences among populations. This may assist making decisions on whether fisheries require management on a multi-stock, rather than a single-stock basis (Ovenden, 1990).

Genetic variation is the basic resource for any successful fish-breeding program. Information on genetic variation within hatchery stocks indicates the level of success in their management and also the status of their brood stock. Genetic diversity between stocks is also critical when one considers hatcheries as Gene Bank for conserving genetic resources (Allendorf and Phelps, 1980; Cross and King, 1983 and Kincaid, 1983). Proper utilization of the gene pool of an organism requires utilization of biochemical genetic markers to monitor stock purity i.e., to quantify the genetic variability, to identify parents and progeny in single pairs or complex crosses (Moav et al., 1976) and to monitor introgression. This is particularly true for zebrafish, which gives rise to a large number of offspring and is thus subject to rapid breakdown of stock purity and loss of genetic integrity.

However, the Random Amplified Polymorphic DNA markers have added new dimension in the field of fisheries and aquaculture where many populations are recently separated or have gone through severe bottleneck events or inbreeding, and as a consequence exhibit low variation and traditional markers cannot be used. In brief, the knowledge of the genetic Variation of zebrafish could help in formulating more effective strategies for managing this aquaculture species and also in evaluating the potential genetic effects induced by hatchery operations for selective breeding.

1.2.1 Intra-specific genetic variation

Alam and Khan (2001) performed RAPD analysis using ten decamer random primers to assess the extent of genetic diversity within and between four populations of the Japanese loach, *Misgurnusanguilli caudatus*. They found three out of 10 primers producing consistent and reproducible pattern of RAPD products and reported insignificant difference in the degree of similarly and polymorphism among the population. Difference was observed in the primers in producing similarities in the

population. The size of minigel (7 X 10cm), decrease number of primers and fish samples limited the chance to obtain better discrimination of fish populations. However, this investigation uncovered a good number of polymorphism for population analysis and revealed that nuclear DNA variation in four populations of the Japanese loach is very high.

Bielawski and Pumo (1996) reported sufficient nuclear DNA variation in Atlantic Coast striped bass, *Morone saxatilis* (highly conserved species) in the frequencies of 32% of surveyed RAPD markers. Band sharing-based similarity indices were higher for intra-river system samples than for inter-river systems (in Delaware River 92.6% and Roanoke River 95.6%). They suggest that Atlantic Coast striped bass are genetically subdivided and gene flow is present among the sampled Atlantic Coast striped bass. Chong et al. (2000) carried out an investigation to study the genetic variation within and among five geographical populations of Malaysian river catfish, *Mystus nemurus*, revealed by RAPD analysis and amplified fragment length polymorphism (AFLP) techniques. A total of 158 and 42 polymorphic markers were detected by using 4 AFLP primer combinations and 9 RAPD primers. The results of both markers provide similar conclusions as far as the population clustering analysis is concerned. The Sarawak population was isolated from the rest of the population located in Peninsular Malaysia. High genetic variability was observed within different populations. Three subgroups each from Kedah, Perak, and Sarawak populations were detected by AFLP but not by RAPD. This indicates the more efficiency of AFLP for identifying genotypes within populations than RAPD.

Degani et al. (1997) compared the patterns of DNA fingerprint (DFP) and RAPD results in three strains of angle fish to analyze the variations among them. Band-sharing within the strains (0.60-0.80) was higher than between strains (0.43-0.48). They observed different patterns of DNA among three strains. Therefore, the calculated band-sharing of DFP and RAPD are useful parameters, not only to measure variation between strains, but also the genetic relationship between them.

Li et a1. (2003) employed random amplified polymorphic DNA (RAPD) to detect the genetic variation of two groups of primary parent shrimp, *Litopenaeus vannamei*, collected from Hawaii. They used 140 random primers and got 16 polymorphic primers where 78 polymorphic loci for the first group and 48 for the second. The proportion of polymorphic loci was 61.54% and the average genetic distance was 0.1960 ± 0.0392 and

0.0922 ± 0.0189 for the first and second group respectively. They found different bands in the two parents amplified with primers OPF-09, OPV-19 and OPZ-11 and some differences between two groups.

Mamuris et al. (1999) carried out an experiment on intra-specific genetic variation of Stripped red mullet (*Mullussur muletus*) in six locations of the Mediterranean Sea using 20 putative enzyme-coding loci (allozyme technique) and RAPD analysis on 154 markers, by 8 decamer random primers. They observed significant differences among samples using both methods and Nei´s genetic distance (D) among samples was low (mean D = 0.011 for allozyme; mean D = 0.018 for RAPD) with data from both approaches revealing the sample from France to be most distinct from the Greek samples. In this experiment, the RAPD method showed a more pronounced effect of isolation-by-distance in comparison with allozymes because a correlation between genetic affinities and geographical area was found with RAPDs.

Parenrengi et al. (2001) collected grouper, *Epinephelus merra* form Makassar Strait, South Sulawesi, Indonesia and investigated genetic polymorphisms by RAPD analysis. They showed that ten primers generated a total of 72 loci with 41 polymorphic loci (56.9%). The number of genotypes produced for each primer varied from three to six. The OPA15 produced the highest polymorphism (85.7%) while OPA18 produced the lowest polymorphism (33.3%). RAPD bands of *E. merra* ranged from five to eight and its size range between 200-3000%. They calculated average of genetic distance between individuals of 0.2737 ± 0.1051.

Tassanakajon et a1. (2007) investigated the genetic variation in three geographically different samples of wild black tiger prawn, *Penaeus monodon* in Thailand using six out of 200 ten-base primers and reported 48% and 45% polymorphic bands in Satun-Trang and Trat respectively, suggesting a high genetic variability of the two samples. On the other hand, only 25% polymorphic bands were found in the Angsila sample, indicating the lowest polymorphic level among the three samples examined. In this experiment, primer 428 detected a RAPD marker that was found only *P. monodon* originating from Satun-Trang, suggesting the potential use of this marker as a population-specific marker for this species.

Wasko et al. (2004) used random amplified polymorphic DNA fingerprinting to assess the genetic variability of a wild Stock from the Amazon River and of three captive stocks that correspond to consecutive generations from the fishery culture. They found considerably lower genetic variation in farmed stocks than the wild population and a significantly higher level of polymorphism in the third hatchery generation. They revealed that their results seemed to reflect a common breeding practice on several hatchery fish program that use a small number of parents as brood stocks, obtaining reproductive success with few non-identified mating couples.

Yang et al. (2004) applied random amplified polymorphic DNA (RAPD) technique to assess genetic variations in two wild populations of *Spams lotus* from Fujian and Zhujiang River. They reported that the intra-population similarities of populations were 0.8821 and 0.8785 respectively and the intra-population genetic distances were 0.1179 and 0.1215 respectively. The inter-population genetic distance was 0.1314 and these two populations assembled into two branches according to the methods of UPGMA and NJ on the basis of genetic distances indicating that there was obvious genetic differentiation between them.

Yoon and Park (2002) used RAPD analysis with 5 primers based on numerous polymorphic bands to investigate genetic similarity and diversity among and within 2 cultured and wild populations of Crucian carp, *Carassius carassius.* They found 273 out of 442 polymorphic bands to be specific to a wild population and the average number of polymorphic bands in each population was found to be different and was higher in the wild than in the cultured population. They further reported that the average number of polymorphic bands in the wild population was approximately 1.5 times as diverse as that in the cultured population and the average level of band sharing values was 0.40 ± 0.05 in the wild population as compared to 0.69 ± 0.08 in the cultured.

1.2.2 Inter-specific genetic variation

Ali et a1. (2004) used random amplified polymorphic DNA (RAPD) analysis to assay polymorphisms within and among four fish families in Egypt using 20 random primers. They observed different RAPD fragment patterns and the results demonstrated that there were great differences among the three families.

Chang et al. (2001) studied inter and intra-population genetic variation of *Misgurnusanguilli caudatus* and *Paramisgurnus dabganus* collected from middle reaches of Huanghe, Changjiang and Zhujiang rivers using RAPD markers. They showed that the average band sharing coefficient of conspecific populations and the corresponding genetic distance were 0.730-0.938 and 0.089-0.245 respectively. The average band sharing coefficient of inter-species populations was 0.392-0.505 and the corresponding genetic distance was 0.620 - 0.800. In conclusion, the genetic distance among individuals of Wuhan population was higher.

Dinesh et a1. (1993) performed RAPD fingerprinting to assess the genetic diversity and species differentiation in three species of tilapia with 10-mer random primer and reported high genetic similarity by pair-wise comparisons based on the method of Nei´s and Li (1979) within arch of the tilapia species. The average inter-specific genetic similarities among the three species were also determined and the estimates were lower than the inter-specific genetic similarity. DNA profiles generated in each species of tilapia were unique. Finally, they detected a total of 13 RAPD markers differentiating the three species of tilapia.

Huang and Cheng (2003) assessed RAPD markers for *Clarias fuscus*, *C. mossambicus* and *C. batrachus* to provide a reliable DNA fingerprint for the preservation of the pure strain *C. fuscus*. Genetic variation in wild caught *C. fuscus* was analyzed according to the fragment sharing method. They found different levels of polymorphisms both among the 3 *Clarias* species and within *C. fuscus* samples from different collection sites.

Jug et al. (2004) detected genetic variation by RAPD analysis of Marble trout, *Salmo marmoratus* from hybrids (marble trout x brown trout) of the Po river system and the Adriatic river system of western Balkan. Of 200 RAPD primers, they tested 14 species specifically showing a clear inter-specific polymorphism.

Tinni et al. (2007) carried out comparative karyotype analysis with different staining in two forms of *Anabas testudineus*. Bloch in which differential staining with Giemsa, CMA and DAPI was compared in two forms (non-spotted and spotted form) of the species. The somatic chromosome number of these forms was determined as 2n = 46. The chromosomal length of non-spotted and spotted form; 1.86-5.33µm and 1.60-5.32µm respectively, indicates the gradual decrease in chromosome length of both the

forms. Sedre and Almeida (2002) conducted a comparative study on six species of the Pimelodidae family collecting at four sites in the Tibagi river basin using RAPD. They found a defined separation among six species, with a genetic similarity of 0.084, obtained from dendrogram by analysis of the RAPD profiles.

Bardakci and Skibinski (1994) uncovered the systematic investigation at the level of species and subspecies of tilapia by assaying polymorphisms within and between populations using 13 random 10-mer primers. They identified different RAPD fragment patterns for different species, although not always for different subspecies.

Callejas and Ochando (2001) used RAPD to identify the eight *Barbus* species of the Iberian peninsula. Ten random primers were employed to generate RAPD markers. Four species-specific markers were found in *B. bocagei*, seven in *B. comizo*, five in B. *graellsii*, three in *B. guiraonis*, eight in *B. haasi*, 13 in *B. meridionalis*, four in *B. microcephalies* and four in *B. sclateri*. They proved that RAPD markers constitute useful tools for accurate taxonomic identification of Spanish barbels spp.

Wang et al. (2001) exploited many informative fragment of RAPD from *Aphyocypris chinensis* (Japan and China), *Hemigrammocypris sp*. (Japan), *Gobiocypris rarus* (China) by amplifying with 20 primers. The results indicated that the species *Hemigrammocypris sp*. was an independent one and the species *Aphyocypris* in China and Japan was the same species.

Dong et al. (2003) identified of clone of pengze crucian carp, *C. auratus* by using isozyme and RAPD genetic markers. The results of RAPD were the same with the results of isozymes. Zheng et al. (1999) identified the mud crab, Indian mrigal and Indian rohu using decamer random primers. They noted polymorphic fragments in all the 3 species with the size ranging from 200 to 2300bp. In this study, they established RAPD labeling procedure for identifying the 3 species using OPN6, OPM8 and OPM12 respectively.

Ahmed et al. (2004) applied RAPD analysis to four-tilapia species (three genera): red belly tilapia (Tilapia zillii), white tilapia (*Sarotherodon salilaeus*), blue tilapia (*Oreochromis aureus*) and Nile tilapia (*Oreochromis niloticus*). They used twenty random primers, fifteen (10-mer) and five (20-mer), to assay polymorphisms among three genera and between two species. They used observed different RAPD fragment patterns for different genera species. Results showed that there are great differences

among the three genera of tilapia fish. Also, the data demonstrated that RAPD markers are useful for the systematic investigation at the level of tilapia species.

Brahmane et al. (2006) used RAPD to delineate the hilsa populations of Ganga, Yamuna, Hooghly and Narmada Rivers at with six degenerate primers. All primers were highly polymorphic and generated high numbers of amplification products. Nei's genetic distances were calculated between locations. The overall average genetic distance among all the six locations was 0.295. The Fst value within the Ganga was 0.469 and within the Hooghly it was 0.546. The overall Fst value for the six populations analyzed was 0.590. The UPGMA dendrogram clustered the hilsa into two distinct clusters: Ganga and Yamuna populations and the Hooghly and Narmada populations.

Exadactylos et al. (2003) employed RAPD analysis to estimate genetic variation within and between 6 Northeast Atlantic populations of Dover sole *Solea solea*. They used a total of 16 fish randomly selected from each population and compared the results with allozyme variation within and between populations. Here, the results from the RAPD technique revealed higher levels of genetic variation than allozyme.

Kuusipalo (1994) carried out an investigation to assess the genetic structure of pelagic fish populations of Lake Tanganyika. Specimens of *Limnothrissa miodon* and *Stolothrissa tanganicae* collected from 5 different localities were tested on 4 and 3 random primers respectively. In case of *L. miodon,* they observed that the strains of Malagarasi and Moba share some characters that are missing elsewhere. Similarly, the strains from Chituta and Nsumbu express some characters not found among others. In case of *S. tanganicae*, on one character, the strains from Rusizi, Malagarasi and Moba differ from Chituta and Nsumbu. They also found that the strains from Chituta and Nsumbu resemble the strain from Rusizi. In this experiment, very small number of individual was analyzed. Finally, they recommended that definite conclusions could be made only after at least 30 individuals of each population have been analyzed.

Nazrul et al. (2005) assessed the genetic variation of Walking catfish, (*Clarias batrachus*) in beel (Kella beel, Mymensingh; Shobornokhali beel, Sherpur), haor (Hakaluki haor, Maulovibazar) and hatchery (Brahmaputra Fish Seed Complex, Mymensingh) using microsatellite DNA markers. The microsatellite loci were polymorphic and varied within and between the four stocks of *Clarias batrachus.*

Shubina et al. (2004) studied the intra-specific structure of major populations of the walleye Pollock, *Theragrachalco gramma* from the northwestern part of the Bering Sea using PCR-RAPD and microsatellite analysis. They revealed the existence of cluster with low level of bootstrap support, which generally corresponds to geographic localization of the shoals and me deviation from HWE and differentiation between the samples. The results indicated the high levels of gene flow with the inter-population variance of 0.02.

Zhang et al. (2001) conducted a study on Silver carp (*Hypophthalmichthys molitrix*) and gass carp (*Ctenopharyngodon idella*) from the Yangtze River using RAPD technique for analysis of four populations. They found no consistent population relationships of two species with each other and low genetic differentiation among populations. They concluded that that low genetic differentiation and population relationship might be due to sampling location and frequent gene flow among population.

1.2.3 Genetic variation in some major fish species

The molecular investigation of Indian fish species especially by RAPD markers is limited in zebrafish (*Danio rerio*). However, researchers in some of the Asian countries particularly in India have initiated genetic characterization of these species by applying both biochemical and molecular techniques. Most of these studies are an attempt based on isozyme pattern and some on mtDNA or DNA fingerprinting by using RAPD or RFLP techniques. Recently, a few numbers of molecular genetic studies of Indian major carps have executed by RAPD markers in Bangladesh.

Barman et al. (2003) applied the random amplified polymorphic DNA assay for studying genetic relationships and diversities in four species of Indian major carps using 34 random primers. They detected distinct and highly reproducible RAPD profiles with a great degree of genetic variability among the species.

Das et al. (2005) carried out an investigation involving the comparative analysis of RAPD profiles of six Labeo species Viz*., L. bata* (bata), *L. calbasu* (calbasu), *L. dyocheilus* (dyocheilus), *L. fimbriatus* (fimbriatus), *L. gonius* (gonius) and *L. rohita* (rohu) at the nuclear DNA variation level. They chose 15 decamer random primers from 40 and amplified a total of 449 DNA fragments ranging in size from 400 to 3000 bp. Their results revealed that the highest intra-speeies genetic similarity value was in

calbasu (0.93). The cluster analysis showed two main clusters, one with calbasu, rohu, flmbriatus and gonius and another with bata and dyocheilus. This study provides evidence that RAPD could be used for genetic differentiation of closely related species.

Islam and Alam (2004) assessed the genetic variation in rivers (the Halda, the Jamuna and the Padma) and hatchery population of Indian major carp, *Labeo rohita* using RAPD assay. They used 6 decamer random primers and found the polymorphic loci proportions of 0.33, 0.28. 0.28 and 0.26 and Nei's gene diversity values of 0.06, 0.07, 0.06 and 0.05 for the Halda, the Jamuna, the Padma and the hatchery populations, respectively and a low level of genetic differentiation between the population pairs. They also found a correlation between genetic affinities and geographical area based on Nei's genetic distances. The results concluded that the RAPD technique can be introduced as a tool in the population genetics of Indian major carps.

Islam et al. (2005) conducted a study to find out genetic variations of Indian major carp, *C. catla* in three river populations via; the Halda, the Jamuna and the Padma by RAPD markers. Using 4 decamer primers, they detected the existence of high level of genetic variation in Halda River population. They also found some degree of differentiation among three catla populations and suggested that the RAPD technique could be used to discriminate different river populations of catla.

Macdonald (1995) applied RAPD-PCR technique to analyze genetic variation within and between three aquaculture species of Indian major carp: *Labeo rohita*, *Cirrhinus mrigala* and *Catla catla* and four strains of *C. catla* using four random decamer primers. He observed low variation within species and strains. However, variation between species was found to be higher than between strains.

Simonsen et al. (2005) studied with the allozyme and mtDNA in wild-caught (the Halda, the Jamuna and the Padma) and hatchery-reared Indian major carps of Bangladesh. They observed 21 allozyme loci and their Bayesian model-based analysis revealed the four taxa, corresponding to the three known species along with a fourth unknown taxon present in two hatchery samples. Individual admixture coefficient showed that 24% of hatchery reared fishes were hybrids. Mitochondrial DNA analysis revealed that catla x rohu hybridization primarily involved with catla male and rohu females, whereas mrigal x rohu hybrid primarily resulted from rohu males and mrigal females. They showed so

close genetic relationship among the Halda, the Jamuna and the Padma populations of catla and rohu.

From the above discussion it is clear that RAPD constitutes very useful markers for the genetic diversity of the populations as well as for species identification purposes, which is essential for the development of efficient strategies for stock management as well as in conservation programs.

1.3 RAPD (Random Amplified Polymorphic DNA) marker

An organism's DNA contains the blueprint of its characteristics (Abed, 1995). Recently, the term DNA fingerprinting or profiling is used to describe the combined use of several single locus detection systems and is being used as versatile tools for investigating various aspects of organism genomes. These include characterization of genetic variability, genome fingerprinting, genome mapping, gene localization, analysis of genome evolution, population genetics, taxonomy, fish breeding etc. Genetic analysis using molecular marker technologies can provide a powerful approach to understanding the organization and distribution of genetic resources in natural and managed populations of fishes. At the species level, the identification of taxonomic units and the determination of the uniqueness of species are Essential information for conservation, systematic, ecological, and evolutionary studies (Schierwater et a1., 1994).

Random amplified polymorphic DNA (RAPD) markers, generated by the polymerase chain reaction (PCR), is widely used since late 80's to assess intra-specific genetic variation on a nuclear level (Welsh and McClelland, 1990 and Williams et al., 1990). Since the RAPD technique involves enzymatic amplification of target DNA by PCR using arbitrary primers, it is also called Arbitrarily Primed Polymerase Chain Reaction (AP-PCR) or DNA Amplification fingerprinting (DAF). This technique always allows the examination of genomic variation without prior knowledge of DNA sequences (Hadrys et al., 1992), and is especially useful for revealing variation in species with low genetic variability when other techniques such as isozyme analysis and mitochondrial DNA control region sequencing fail to reveal differences among individuals (Dawson et al., 2008).

In fishes, the RAPD method has been used in the identification of species and sub-species in tilapia (Bridakci and Skibinski, 1994); in pengze crucian carp, *Carassius auratus* (Ding et al., 2003) and the monitoring of genetic polymorphism in sea bass after acclimation to freshwater (Allegrucci et al., 1995) and in the evaluation of intra-specific genetic variation of red mullet (Mamuris et al*.,* 1998); Malaysian river cattish, *Mystus nemurus* (Chong et al., 2000); Japanese loach, *Misgurmsanguilli caudatus* (Alam and Khan, 2001); grouper, *Epinephelus merra* (Parenrengi et al., 2001); crucian carp, *Carassius carassius* (YoonandPark, 2002); Neotropical fish, *Bryconlundii* (Wasko and Galetti, 2002); wild black tiger prawn, *Penaeus monodon* (Tassanakajon et a1., 1997); and shrimp, *Litopenaeus vannamei* (Li et al*.,* 2003). RAPD also has been applied in inter-specific genetic variation by Chang et al., 2001; Chiari and Sodre, 2001; Sodre and Almeida, 2002; Huang and Chen, 2003; Ali et al., 2004; Jug et al., 2004, and used as an efficient method of DNA fingerprinting in many fish species (Dinesh et al., 1993; Degani et a1., 1997; Wasko et a1., 2004). In addition, a comparative study of RAPD and multi locus DNA fingerprinting on strains of *Oreochromis niloticus* revealed similar genetic relationships (Naish et a1., 1995). Therefore, it is useful tool to know the intra and inter-population variation of fish species.

1.4 DNA barcoding

DNA barcoding are already a powerful tool for the identification of species. It concludes that barcodes have broad applicability for authenticity testing and the phylogeographic patterning of genetic diversity. It can also inform aspects of traceability. DNA barcoding aims to provide an efficient method for species identifications using an array of species specific molecular tags derived from the 5' region of the mitochondrial cytochrome c oxidase I (COI) gene. The efficiency of the method hinges on the degree of sequence divergence among species and species-level identifications are relatively straight forward when the average genetic distance among individuals within a species does not exceed the average genetic distance between sister species. Fishes constitute a highly diverse group of vertebrate development. In this context, the identification of fish species is challenging and DNA barcoding provide new perspectives in ecology and systematics of fishes.

DNA bar-coding is designed to provide accurate and automated species identifications through the use of molecular species tags based on short, standardized gene regions.

While humanity is facing increasing evidence of the erosion of earth's biodiversity, this approach is proving its effectiveness in characterizing the complexity of the biodiversity realm at a pace unequalled by other characters. The primary goal of DNA bar-coding focus on the assembly of reference libraries of barcode sequences for known species in order to develop reliable, molecular tools for species identification in nature.

DNA barcoding relies on the sequencing and comparison of a standardized portion of the genome to aid in specimen identification and species discovery. The DNA bar-coding method now represents the largest effort to catalogue biodiversity using molecular approaches. Although initially regarded as controversial, numerous cases have been reported where the analysis of DNA sequence variation in the cytochrome c oxidase subunit 1 (C0I) region of mtDNA has proven highly effective for the delineation and identification of animal species in general and fish in particular.

DNA bar-coding, the analysis of sequence diversity in a standardized gene region, has gained considerable validation as a tool for species identification and discovery. Several studies have demonstrated its effectiveness for identifying both marine and freshwater fishes provoking an effort to build a barcode library for all fish species. Currently, records are available for 41771 fishes, representing 6566 fish species on the Barcode of Life Data System, BOLD. DNA bar-coding also provides an independent means of testing the validity of existing taxonomic systems, revealing cases of inappropriate synonymy or overlooked taxa. These results suggest that the species boundaries need to examine for the heavily exploited populations targeted by the aquarium trade, to properly inform conservation strategies and planning.

DNA sequencing is the process of determining the precise order of nucleotides within a DNA molecule. It includes any method or technology that is used to determine the order of the four bases—adenine, guanine, cytosine, and thymine—in a strand of DNA. The advent of rapid DNA sequencing methods has greatly accelerated biological and medical research and discovery. The rapid speed of sequencing attained with modern DNA sequencing technology has been instrumental in the sequencing of complete DNA sequences, or genomes of numerous types and species of life, including the human genome and other complete DNA sequences of many animal, plant, and microbial species.

The first DNA sequences were obtained in the early 1970s by academic researchers using laborious methods based on two-dimensional chromatography. Following the development of fluorescence-based sequencing methods with a DNA sequence, DNA sequencing has become easier and orders of magnitude faster. Several new methods for DNA sequencing were developed in the mid to late 1990s and were implemented in commercial DNA sequencers by the year 2000. Together these were called the "next-generation" sequencing methods.

On October 26, 1990, Roger et al., filed a patent describing stepwise ("base-by-base") sequencing with removable 3 blockers on DNA arrays (blots and single DNA molecules). In 1996, Pal Nyren and his student Mostafa Ronaghi at the Royal Institute of Technology in Stockholm published their method of pyro-sequencing. On April 1, 1997, Pascal Mayer and Laurent Farinelli submitted patents to the World Intellectual Property Organization describing DNA colony sequencing. The DNA sample preparation and random surface-PCR arraying methods described in this patent, coupled to Roger Tsien et al. "base-by-base" sequencing method, is now implemented in Illumina's Hi-Seq genome sequencers. Lynx Therapeutics published and marketed massively parallel signature sequencing (MPSS), in 2000. This method incorporated a parallelized, adapter/ligation-mediated, bead-based sequencing technology and served as the first commercially available "next-generation" sequencing methods; though no DNA sequencers were sold to independent laboratories.

1.5 Rationale of the study

The analysis of the genetic diversity of wild zebrafish by RAPD markers and DNA barcoding are appropriate for the study of genetic variation of zebrafish which will help in developing commercial fish stock for the sustainable aquaculture management. Their genetic information will also be helpful in taxonomical analysis and Species identification. In this way a better understanding of population structure of zebrafish will be obtained and this will be great important for sound management and conservation of this valuable species. Moreover, this method will be used as a tool to simplify the more traditional methods of selective breeding, by identifying what are known as markers. Thus, the present study is a fundamental stone for all sorts of future research in the field of population genetics of the species.

1.6 Research aims and objectives

1.6.1 Overall objective

The aim of the present study was to create base line information for genetic diagnostic of wild zebrafish (*Danio rerio*) in Bangladesh using random amplified polymorphic DNA (RAPD) and DNA barcoding technique, as it is very much important for the sustainable aquaculture development and other biomedical research.

1.6.2 Specific objectives

To attain this aim, the present study was carried out with the following specific objectives:

I. to identify the genomic DNA-random amplified polymorphic DNA (RAPD) variation in the wild zebrafish population;
II. to identify the DNA sequence variation of *D. rerio* in Bangladesh using DNA barcoding technique;
III. to utilize the observed variations;
IV. to study the population structure of this species in Bangladesh water body;
V. to know the genetic relatedness among the populations of *Danio rerio.*

Chapter 2

Materials and Methods

2.1 Sample collection

2.1.1 Fish sample sources

The samples were collected from different geographical region of the country: from four different districts like Moyuri canal of Khulna, Kumar river of Faridpur, Nariakhal of Munshigonj and from Brahmaputra river ofMymensingh district.

Table1. Samples of the *Danio rerio* used in the present study

Sampling No.	Sampling Districts	Sampling Upazila	Sampling Reservoirs	Date of Sampling
1.	Khulna	Khulna Sadar	Moyuri Khal	15 July, 2016
2.	Mymensingh	Mymensingh Sadar	Brahmaputra River	02 August, 2016
3.	Munshigonj	Naria	Nariakhal	20 August, 2016
4.	Faridpur	Vanga	Kumar River	12 September, 2016

2.1.2 Sampling procedure

With the help of local people and mechanized boat, involving artisanal fishers, the fish were caught with traditional nets. Immediately after catching the fishes, they were

transported to aquarium lively with the help of 5L plastic jar. For proper oxygenation the jar were aerated and the mouth was kept open during the transportation period. After that it was kept in separate aquarium and reared. Then it was used for the morphometric measurements of the fishes. At last it was sampled in small sampling bags and storedin-20ºC.

Figure 2: Map of Bangladesh showing the sampling zone of *Danio rerio* (Hamilton, 1822)

2.2 Morphometric measurements of fishes

Geographically, populations of *Danio rerio*were caught from 4 freshwater bodies of Bangladesh. About 50fish specimens were collected from all the locations with the help of local fishermen. For morphometric measurements and estimating genetic variations 12 fish sample were used. For the morphometric measurements, total 16 parameters that is, total length, standard length, body weight, body width, body length, body depth, head length, length of anal fin, length of pelvic fin, length of pectoral fin, length of dorsal fin and 5 fin rays numberwere taken. Fish specimens were morphologically identified with taxonomic keys (Shrivastava, 2000; Jayaram, 1999). The whole fish sample then preserved at -20°C for further use.

Figure 3: Morphometric measurements of fishes (*Danio rerio*). (A: Munshigonj; B: Mymensingh; C: Faridpur; D: Khulna species

2.3 RAPD markers Amplification by PCR

2.3.1 Principle of the amplification of RAPD

For performing amplification of RAPD, a single oligonucleotide of an arbitrary DNA sequence is mixed with genomic DNA in the presence of a thermo stable DNA polymerase and a suitable buffer, and then is subjected to temperature cycling conditions typical of the polymerase chain reaction (PCR). The products of the reaction depend on the sequence and length of the oligonucleotide, as well as the reaction

conditions. At an appropriate annealing temperature during the thermal cycle, the single primer binds to sites on opposite strands of the genomic DNA that are within an amplifiable distance of each other (e.g., within a few thousand nucleotides) and a discrete DNA segment is produced. The presence or absence of this Specific product, although amplified with an arbitrary primer, will be diagnostic for the oligonucleotide binding sites on the genomic DNA. In practice, the DNA amplification reaction is repeated on a set of DNA samples with several different primers, under conditions that result in several amplified bands from each primer. Polymorphic bands are noted and the polymorphisms can be mapped in a segregating population. Often a single primer can be used to identify several polymorphisms, each of which matches to a different locus.

2.3.2 Primer selection

Three decamer primers of random sequences were screened in this study. The details of the primers are given in Table2. Firstly, primers were screened on the sub sample of one fish from the each population. Finally, four primers out of twelve that exhibit the highest quality banding patterns and sufficient variability for population analysis were then retained for further analysis.

Table 2.Parameters of the random primers used for DNA screening in this study

Serial No.	Primers code	Nucleotide length	Sequences(5´ to 3´)
1.	OPA03	10- mer	AGTCAGCCAC
2.	OPA04	10 -mer	AATCGGGCTG
3.	OPF01	10-mer	ACGGATCCTG

2.3.3 Genomic DNA isolation

The key objective of the isolation process is to recover the maximum yield of high molecular weight DNA devoid of protein and other restriction enzymes (Sambrook et al. 1989). In this study genomic DNA was prepared using the body portion of the fish. For the isolation of total genomic DNA, a short procedure was applied according to a modified protocol than reported (Wu et. al., 1995). For the isolation procedure Automated DNA extractor were used (Model: Maxwell 16, Origin: Promega, USA).

Tissues (200-500 mg) were placed in a 1.5 ml micro centrifuge tube and homogenized by using Eppendorf micro-pestle. In the homogenized tissue, 0.5 ml of lysis buffer (4 mMNacl, 0.5 mM EDTA, 0.1% SDS and 0.02 NP 40) and 0.01% proteinase K were added, mixed gently and incubated at 55°C on dry bath for 45 - 60 min for complete lysis of cells. After incubation, chloroform (250 µl) and phenol (250 µl) were added, mixed gently and centrifuged at 30,000 rpm at room temperature (High speed brushless centrifuge, MPW-350R, Poland) for 5 min. The supernatant was then transferred to a new micro-centrifuge tube and half volume of 7.5 M ammonium acetate and 2 volume of 100 % chilled ethanol was added, mixed well and centrifuged at 10,000 rpm for 5 min at room temperature. 1 ml of 70 % ethanol was added to the tube for washing. Again, centrifuged sample for 10 min at 10,000 rpm at 4°C. The DNA pellet was then washed with 70% ethanol, dried and dissolved in a Tris EDTA buffer (10 mMTrisHCl, 1 mM EDTA, pH 7.6). UV-VIS spectrophotometer (Nano-Drop ND-1000, USA) was used to check quality as well as quantity ofisolated DNA. The concentration of extracted DNA was adjusted to 25 ng/µl for PCR amplification.

2.3.4 Confirmation of the presence of genomic DNA

DNA extraction following the above protocol often contains a large amount of RNA and pigments that can usually cause over estimation of DNA concentration by the NanoDrop Spectrophotometer, Model ND2000. For this reason, 1% agarose gels were used for assessing both the quality (presence of RNA or degradation of DNA or others) and quantity of the DNA samples.

2.3.5 Preparation of working solution of DNA samples

Before PCR amplification of DNA, the DNA concentrations were adjusted to 25ng/µl using the following formula:$S_1\ V_1 = S_2\ V_2$

Where,

S_1 = Initial strength (ng/µl)

V_1 = Initial volume of DNA solution (µl)

S_2 = Final strength (ng/µl)

V_2 = Final volume of DNA solution (µl)

2.3.6 Standardization of different PCR parameters

At the very beginning, experiments were carried out to test the effect of DNA, dNTPs, Mg++, and Tag polymerase concentrations and to determine the optimum annealing temperature. During the experiment, PCR buffer, dNTPs, and primer solutions were thawed from frozen stocks, mixed by overtaxing and placed on ice plate. DNA samples were also thawed out and mixed gently. The primers were pipetted first into PCR tubes compatible with the thermocycler used (0.2ml). For each DNA sample being tested, a pre-mix was then prepared including, in the following order, sterile distilled water, buffer, dNTPs and DNA. Polymerase was then added to the pre-mix. The pre-mix was vortexed briefly, then taken into the tubes already containing primers. The tubes were then sealed and placed in an oil-free thermoocycler (Appendorfmastercycler gradient) and the cycling was started immediately. After completion of cycling program, the reactions were run using 1% agarose gel electrophoresis and photographed for documentation.

Table 3: Thermal Profile for PCR with M7431 Master Mix

Number of cycle	Step name	Temperature	Time
1	Pre Heat	95°C	2 min
	Denaturation	95°C	30 sec
40 Cycle	Annealing	54°C	30 sec
	Extension	72°C	1 min
1	Final Extension	72°C	5 min
1	Hold	4°C	Over Night.

Equation for Annealing Temperature:

$$\text{Annealing Temp} = \frac{(Tm\,F + Tm\,R)}{2} - (1\ to\ 5)$$

The amplification conditions originally recommended by Williams et al. (1990) were applied with slight modifications regarding annealing temp, amount of genomic DNA and primer following Alam and Khan (2010) as recommended by the manufacturer. The modified reaction conditions and PCR profiles were followed to amplify DNA samples. The PCR conditions were optimized by manipulating different parameter

before performing final amplification reactions. Then PCR reactions were performed on each DNA sample in a l0µl reaction mix containing lµl of 10x Taq DNA Polymerase buffer (Japan), 0.25µl of 0.4µM primer, lµl of l0µM dNTPs (Japan), 0.2µl of 3u/µl of Taq DNA polymerase (Japan) and 2µl of fish genomic DNA (25ng/µl). The desired volume was restored by adding a suitable amount of sterile distilled water. The reaction mix was preheated at 95°C for 2min for denaturation followed by 40 cycles of 30sec at 95°C, annealing at 31°C for 30sec and elongation or extension at 72°C for 1min. A final step of 5min at 72°C was maintained to allow complete extension of all amplified fragments followed by holding at 4°C.

Then the RAPD reactions were taken out from the thermocycler and placed into refrigerator at 4°C. The PCR products were electrophoresed carefully on agarose gel as quickly as possible.

2.4. Gel Electrophoresis

Gel electrophoresis is a method for separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on their size and charge. In molecular biology to separate a mixed population of DNA and RNA fragments by length, to estimate the size of DNA and RNA fragments or to separate proteins gel electrophoresis is used.

Table 4: Reagents and instruments used for gel electrophoresis

Reagents	Cat	Origin
Agarose	V3125	Promega
100 bp DNA Ladder	G2101	Promega
1 kb DNA Ladder	G5711	Promega
Ethidium Bromide	H5041	Promega
TAE Buffer	V4251	Promega

2.4.1 DNA sample preparation for electrophoresis

All the samples were in the same concentration of buffer. For each sample, 6ul of 1x TAE buffer was placed on a piece of aluminum foil paper and 2ul loading dye (0.25% xylene anocyl, 0.25% bromophenol blue, 30% glycerol and lmM EDTA) was added to

it using 20µl adjustable micropipette. Loading dye was used for monitoring loading and the progress of the electrophoresis and to increase the density of the sample so that it stayed in the well. Finally 2µl extracted DNA was added to it and mixed well using same micropipette. Then well mixed samples were loaded carefully in the gel to approach them lo the bottom of the wells.

2.4.2 Preparation of Agarose gel

For preparing 1% gel, 1.5g agarose powder (AGAROSE superior grade, type II) was taken into a 500ml Erlenmeyer flask containing 150ml of electrophoresis buffer (1x TAE buffer) prepared by adding 30ml of 5x TAE buffer in 120ml of distilled water. The top of the flask was covered with aluminum foil paper to prevent excessive evaporation and heated in a microwave oven with occasional swirling for generating uniform suspension until no agarose particles was seen and the agarose solution became transparent. When the agarose cooled to about 50°C (flask was cool enough to comfortably hold with bare hand), 4µl ethidium bromide (l0µg/µl) DNA stain was added to make the DNA visible under ultraviolet light box (transilluminator) and mixed well by gentle shaking. Then the gel was poured on to the gel bed (15 x 15 x 2cm^3 in size) that was placed on a level bench and the appropriate comb was inserted and finally the bubbles were removed. When the gel became completely cooled and solidified, the comb was removed gently and kept in an airtight packed for future use.

Composition of 5x TAE buffer (1000 m1)

Trisbase: 54 g, Acetic acid: 27.5 g,EDTA: 4.65g (Brodyet al., 2004)

2.4.3 DNA sample documentation

For documentation, the gel was taken from the electrophoresis chamber and placed on the high performance ultraviolet transilluminator (UVP, Inc.) to observe the quality of the extracted genomic DNA. Then the gel was examined and finally photographed using Polaroid film (Type 667) with the help of a gel cum camera and ZUV transilluminator (UVP Biotech - It^{TM} System).

2.4.4 DNA concentration Quantification

One of the important variables for PCR amplification is the concentration of DNA. As different DNA extraction methods produce DNA of widely different purity, it may be necessary to optimize the amount of DNA used in the RAPD assay to achieve reproducibility and a strong signal. Below a certain critical concentration level of genomic DNA, RAPD amplification is no longer reproducible (Williams et al., 1993). Therefore, it is essential to stay above this critical concentration level. On the other hand, excess amount of DNA is likely to produce poor resolution or "smears" resulting in a lack of clearly defined bands in the gel. It is best to do a series of RAPD reactions using a couple of primers and a set of serial dilutions of each genomic DNA to identify empirically the useful range of DNA concentrations, for which reproducible RAPD patterns are obtained. During the quantification of DNA concentration, at first the spectrophotometer (SPECTRONIC® GENESYSTMS) UV-lamp was turned on and after it had warmed up, the wavelength was set at 260nm.

After washing the cuvette carefully with deionized water and wiping with tissue paper, it was filled with 2ml sterile distilled water and placed in Spectrophotometer and fixed as blank or zero. Then 2µl of extracted DNA sample was in that cuvette containing 2ml sterile distilled water and uniformly mixed with water and finally the absorbance reading was taken at 260nm and recorded. Cuvette was rinsed out with sterile water, wiped with tissue paper, and absorbance reading for each sample was recorded in the same way. The absorbance readings of extracted DNA samples of different populations are shown in Appendix II. Using the above absorbance readings, the original sample concentrations were determined according to the formula below:

$$\text{DNA conc. } (\mu g/\mu l) = \text{Absorbance} \times \frac{\text{Volume of Distilled water}(\mu l)}{\text{Amount of DNA}(\mu l)} \times \text{Conversion factor } (0.05)$$

2.4.5 Electrophoresis of amplified products

An aliquot of 10µl of amplified product from each sample was separated electrophoretically on 1% agarose gel containing ethidium bromide in 1x TAE buffer. DNA bands were observed under UV light on a trans-illuminator and photographed by a Gel Cam Polaroid camera and 2UV trans-illuminator (UV P Biotech It^{TM} System). Two DNA molecular weight markers (100bp DNA Ladder, Promega, U.S.A and 1kb

DNA Ruler, Promega, U.S.A) were electrophoresed alongside the RAPD reactions in separate wells of the gel.

2.4.6 RAPD data analysis

When electrophoresis was completed, the sizes of the amplification products were estimated by comparisons of distance traveled by each fragment with distance traveled by known size fragments of the DNA molecular weight markers (100 bp DNA ladder and 1kb DNA Ladder, both from U.S.A). All distinct bands or fragments (RAPD markers) were given identification numbers according to size and scored visually on the basis of their presence (1) or absence (0), separately for each fish for each primer. For more accuracy, band scoring was performed by two independent persons. Bands or RAPD markers not identified by all two persons or readers were considered as non-scorable. The scores obtained using all primers in the RAPD analysis were then pooled for constructing a single data matrix. This was used to estimate polymorphic loci (Nei´s,1972), gene diversity, population differentiation (G_{ST}), gene flow (N_m), genetic distance (D) and to construct a UPGMA (Un-weighted Pair Group Method of Arithmetic means) dendogram among populations with 1000 simulated samples using POPGENE (Version 1.31; Yeh et al., 1999) computer program.

This procedure avoids problems created by the occurrence of any cell with an expected frequency of less than 1.0 and/or by more than 20% of the cells having an expected frequency of less than 5.0 such as occurs with rare polymorphisms and small sample sizes. The sizes of the RAPD markers were estimated by using the software DNAftag (Version 3.03) (Nash, 1991). Gene frequency for RAPD loci was estimated based on the assumption of a two-allele system. Of the two alleles, only one is capable of amplification of a RAPD-band by primer annealing at an unknown genomic position (locus). The other is the 'null' allele incapable of amplification, mainly because of loss of the primer-annealing site by mutation. The two allele assumption is in most cases acceptable, because co-dominant loci showing band shifts are few (Eloet al., 1997; Welsh and McClelland, 1990). In this system only a null-homozygote is detectable as negative for the RAPD-band of interest. Under the assumption of Hardy-Weinberg equilibrium, the null allele frequency (q) may be $(N/n)^{1/2}$, where N and n are the number of band-negative individuals observed and the sample size, respectively. The

frequency of the other allele (p) is 1-q. The assumption of the two-allele system enables us to calculate the Nei's genetic distance (Nei, 1972) from the RAPD pattern.

The frequencies of polymorphic loci across primers in different populations were analyzed by two-way analysis of variance.The genetic-similarity (SI) values are defined as the fraction of shared bands between the RAPD profiles of any two individuals on the same gel SI values were calculated from RAPD markers of the same molecular weight on the data matrix according to the following formula;

$$\text{Similarity Index (SI)} = 2N_{AB} / (N_A + N_B)$$

Where, N_{AB}, N_A and N_B are the total number of RAPD band shared by individuals; A and B, the total number of band produced by individual A and the total number of band produced by individual B respectively (Lynch, 1990). SI values range from O to 1.

When SI = 1, the two DNA profiles are identical and when SI = 0, there are no common bands between the two profiles. Within population similarity [S_i] was calculated as the average of SI across all possible comparisons between individuals within a population. Between population similarity (S_{ij}), was calculated as the average similarity between randomly paired individuals from populations i and j (Lynch, 1991).

Gene flow (N_m) was estimated according to the following formula using the POPGENE Computer package (Yeh et al., 1999):

$$\text{Gene flow, } N_m = 0.5 (1 - G_{ST}) / G_{ST}$$

Where, G_{ST} is the proportion of total genetic diversity attributable to subpopulation. It is also known as co-efficient of gene differentiation.The G_{ST} values were calculated by using the following formula with the help of POPGENE (Version 1.31) (Yehet al., 1999) computer package:

$$G_{ST} = 1-Hs/H_T$$

Where, Hs are the average heterozygosity of the total population and H_T is the mean of Hardy-Weinberg expectation of heterozygosity obtained with populations average allele frequencies. Information index (equivalent to the Shannon-Weaver Index of Ecology) in terms of Shannon information index (I) was calculated by the following formula:

$$I = -\Sigma P_i \ln P_i$$

Where, ln = the natural logarithm, and

Pi = the frequency of the i^{th}allele.

Nei's (1972) original measures of genetic distance values were computed from frequencies of polymorphic markers using the POPGENE (Version 1.31) computer package (Yehetal., 1999). Genetic distance values (D) (Neiś, 1972) were calculated using the following formula:

$$D = - \ln J_{XY}/\sqrt{\Sigma} J_X J_Y$$

Where, $J_X = \Sigma X_i^2 / r$ in population X

$J_Y = \Sigma Y_i^2 / r$ in population Y

$J_{XY} = \Sigma X_i Y_i$, X_i and Y_i are the frequency of the i^{th} allele of a given locus in the four populations of fishes compared and

r = the number of loci frequency for all possible pairs of populations

To estimate genetic relationships between the studied populations using the UPGMA (Sneath and Sokal, 1973) the dendrogram was constructed using POPGENE (Version 1.31; Yeh et al., 1999) and Tree View (Version 1.6.6).

2.5 DNA barcoding of the sample

Among the 12 sample from 4 regions, 4 samples(Mymensingh1, Faridpur1, Khulna3 and Munshigonj1) were selected for the sequencing. Each of the samples was taken from individual stock. The main purpose of this sequencing was to bring molecular evidence of genetic diversity, parentage and evolution within *D. rerio* population, studying individuals from isolated aquatic habitat in Bangladesh.

2.5.1 Primer selection for DNA barcoding

Primers used for PCR barcoding is given in table 5.

Table 5: Random primers used in the screening

Primer code	Sequence (5´-3´)	G+C

		content (%)
Fish F1	TCAACCAACCACAAAGACATTGGCAC	46.154
Fish R1	TAGACTTCTGGGTGGCCAAAGAATCA	46.154

2.5.2GenomicDNA isolation

The DNA isolation process was explained in 2.3.3

2.5.3 Materials used for gel electrophoresis

The following equipment's and chemicals were used for gel electrophoresis:

a) A horizontal electrophoresis chamber and power supply

b) Gel casting tray and combs

c) Gel documentation system

d) Pipette and tips

e) DNA markers

i. Gene Ruler^{TM} 1Kb Plus DNA Ladder

ii. Gene Ruler^{TM} 1Kb DNA Ladder

f) Electrophoresis buffer (TAE pH 8.0)

g) Agarose

h) DNA stain (Ethidium bromide)

2.5.4 Agarose gel electrophoresis

i. The standard method used to separate, identify and purify DNA fragments through electrophoresis was followed according to the method described by Sharp et al. 1973.

ii. 1.0 g of agarose was heated to melt into 100 ml of TAE buffer, ethidium bromide was added (10 ug/ml) and poured into gel casting tray fixed with appropriate combs.

iii. After the gel was solidified it was placed into gel-running kit containing lxTAE buffer.

iv. Digested plant DNA solutions were loaded with 6x gel loading dye and electrophoresis was continued until DNA fragments were separated well.

2.5.5 Preparation of PCR reaction mixture

The following components were used to prepare PCR reaction mixture. The total volume of PCR reaction mixture was for 4 samples.

Table 6:Components of PCR reaction mixture (for 4 reactions)

Sl. No.	Reagents	Amount per sample	Total
1	Distilled water	18.7	74.8
2	Taq Buffer A 10X	2.5	10.0
3	Primer	1.0	4.0
4	dNTPs 10 mM	0.25	1.0
5	Taq DNA Polymerase	0.05	0.2
6	Template	2.5	10.0
Total		**25.0 µl**	**100 µl**

2.6PCR of the sample

PCR amplification was done in an oil-free thermal cycler. The optimum amplification cycle was as follows:

Table 7: PCR condition table for DNA bar-coding

Number of cycle	Step name	Temperature	Time
1	Pre Heat	94°C	4 min
32	Denaturation	94°C	45 s
	Annealing	54°C	1 min
	Extension	72°C	1 min

Final Extension	72°C	5 min

After completion of cycling program, the reactions were held at 4°C.

2.6.1Electrophoresis of the amplified products and documentation

The amplified products were separated electrophoretically on 1% agarose gel. The gel was prepared using 1.0 g agarose powder containing ethidium bromide and 100 ml 1XTAE buffer. Agarose gel electrophoresis was conducted in 1X TAE buffer at 90 Volts for 30 minutes. Molecular weight marker 1kb plus or1IKb DNA ladder was electrophoresed alongside the reactions. DNA bands were observed on UV-trans-illuminator and photographed by a gel documentation system.

2.6.2 PCR product purification

i. PCR product was transferred to 1.5ml Eppendrof.
ii. 5 times FADF buffer added and centrifuged at 11000 rpm for 1 min.
iii. Flow through discarded and 750 ml was buffer added and centrifuged at 11000 rpm and liquid discarded.
iv. Again centrifuged at 14000 rpm for 10 minutes.
v. Then 40µl of Elution buffer added to the membrane center of F SDF column and waited for 3 minutes for fully absorption.
vi. Centrifuged at full speed at 14000 rpm for 2 minutes to elote the DNA.
vii. Then the liquid further placed in column tube and centrifuged at 14000 rpm for 2 minutes.

2.6.3 Quantification of DNA concentration

1. Qubit™ working solution was prepared by diluting the Qubit™ reagent 1:200 in Qubit™ buffer and 200 µlof working solution prepared for each sample.

2. Assay tubes prepared according to the table below

	Standard assay tubes	**User sample assay tube**
Volume of working solution (from step 1)	190	180-190

Volume of standard solution	10	
Volume of user sample		1-20
Total volume	**200µl**	**200µl**

3. Vortexed all tubes for 2 to 3 seconds.

4. Incubated the tubes for 2 minutes at room temperature (15 minutes for theQubit™ protein assay).

5. Inserted the tubes in the Qubit® 2.0 Fluorimeter and readings were taken.

6. Fluorimeter, determine the stock concentration of the original sample

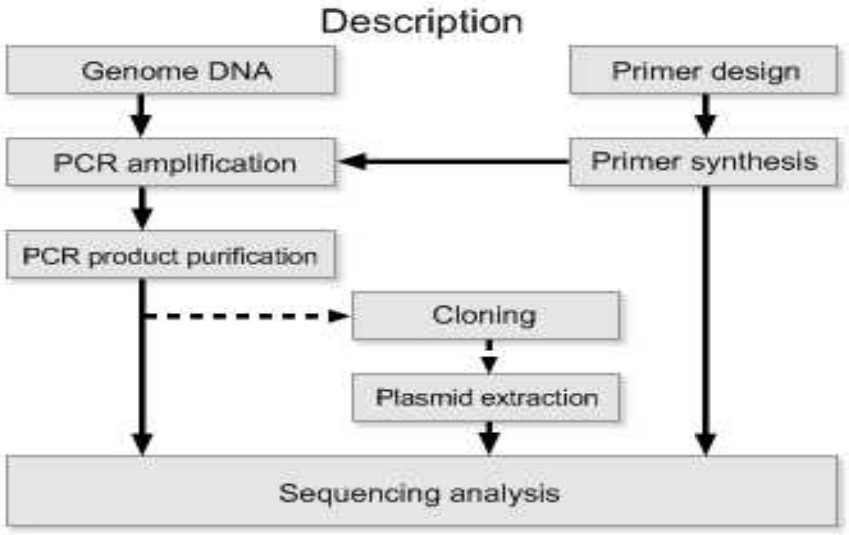

Figure 4: A view of genomic DNA sequencing

2.6.4 Sequencing reaction

Sequences of amplicons was performed using the kits: GenomeLab Method Development Kit and DTCS Quick Start Kit (Beckman Coulter). The program used had a primers alignment temperature of 50ºC and a total of 30 cycles of replication.

2.7 Sequences analysis

Alignment of all sequences for one gene from different individuals (individuals of the same population), was performed by ClustalW method using the MegAlign module of Laser gene v.7 software. Comparison of sequences, and drawing phylogenetic trees was done using Laser gene v.7 and MEGA 5.1 software. Phylogenetic trees were

constructed based on the similarity degree and through distance based method Neihgbor-Joining (NJ)**.**

2.7.1 Software used for Sequence analysis

2.7.1.1 NCBI Blast

BLAST is like doing an experiment to get good, meaningful results, everyone needs to optimize the experimental conditions. BLAST on Windows Azure is a cloud-based implementation of the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI). BLAST is a suite of programs that is designed to search all available sequence databases for similarities between a protein or DNA query and known sequences. BLAST allows quick matching of near and distant sequence relationships, providing scores that allow the user to distinguish real matches from background hits with a high degree of statistical accuracy. Scientists frequently use such searches to gain insight into the function and biological importance of gene products.

BLAST on Windows Azure extends the power of the BLAST suite of programs by allowing researchers to rent processing time on the Windows Azure cloud platform. The availability of these programs over the cloud allows laboratories, or even individuals, to have large-scale computational resources at their disposal at a very low cost per run. For researchers who don't have access to large computer resources, this greatly increases the options to analyze their data. They can now undertake more complex analyses or try different approaches that were simply not feasible before.

2.7.1.2 MEGA 6

The Molecular Evolutionary Genetics Analysis (MEGA) software is developed for comparative analysis of DNA and protein sequences that are aimed at inferring the molecular evolutionary pattern of genes, genomes and species over timeMEGA is currently distributed in two editions: a graphical user interface (GUI) edition with visual tools for exploration of data and analysis results and a compound line edition (MEGA-CC), which is optimized for iterative and integrated pipeline analyses (Kumar et al,. 2012).

2.7.1.3 CLUSTALW

ClustalW is a general purpose multiple alignment program for DNA or protein. The program performs simultaneous alignment of many nucleotide or amino acid sequences. It is typically run interactively, providing a menu an online help. CLUSTALW (Thomson et al., 1994) is the most widespread among many available algorithms. It can be access through several website or downloaded locally in personal computer. CLUSTALW must be searched in the NPS@ section.

Chapter 3

Results

3.1 Morphometric measurement data of zebrafish

In the present study 16 classical morphometric characteristics were studied in the 4 populations of *D. rerio*. The morphometric characteristics did not vary much among the populations. However, all values of morphometric characteristics of fishes of the four districts showed little variation from one stock to another stock. Themorphometric measurements data table are given in table 9 and 10.

3.2 RAPD profiling using primer

There are various types of universal RAPD primers. Among them three primers were used in this study. They are OPA-03, OPA-04 and OPF-01 which yielded comparatively maximum number of application products with high intensity and minimal smearing. Each of the primer produced separate pattern of amplified DNA (Figure 5-7).

Table 8. RAPD primers with corresponding bands scored for the studied*Danio rerio*populations.

Primer code	Sequence of the primers	Total number of bands scored
OPA-03	5′-AGTCAGCCAC-3′	50
OPA-04	5′-AATCGGGCTG-3′	32
OPF-01	5′-ACGGATCCTG-3′	30
Total bands		112

The three primers (OPA-O3, OPA-O4 and OPF-O1) yielded a total of 112 reproducible and consistentlyscorable RAPD bands of which 22 (19.64%) were considered as polymorphic.

Table 9: Range of variation, mean and standard deviation of morphometric measurements of *Danio rerio*

S/N	Measurements	Munshigonj (n=3)			Faridpur (n=3)			Mymeningh (n=3)			Khulna (n=3)		
		Min.	Max.	Mean±SD	Min.	Max.	Mean±SD	Min.	Max.	Mean±SD	Min.	Max.	Mean±SD
1.	Total length(cm)	4.5	5.1	4.7±0.071	3.3	3.4	3.33±0.012	3.9	4.2	4.03±0.014	3.6	4	3.77±0.021
2.	Standard length(cm)	3.8	4.6	4.2±0.710	2.9	3.1	3.0±0.000	3.4	3.7	3.56±0.007	3.1	3.4	3.23±0.021
3.	Body width(cm)	0.9	1.1	1±0.000	0.56	0.6	0.59±0.007	0.7	0.9	0.8±0.000	0.6	0.7	0.67±0.014
4.	Body depth(cm)	0.4	0.6	0.53±0.021	0.3	0.4	0.37±0.014	0.4	0.5	0.43±0.014	0.3	0.4	0.37±0.007
5.	Head length(cm)	0.5	0.7	0.60±0.000	0.4	0.5	0.43±0.014	0.5	0.6	0.53±0.014	0.3	0.4	0.33±0.015
6.	Body weight(gm)	0.87	1.13	1.03±0.710	0.31	0.41	0.37±0.007	0.46	0.59	0.54±0.011			±
7.	Length of dorsa fin(cm)	0.4	0.6	0.53±0.021	0.4	0.5	0.47±0.014	0.5	0.6	0.53±0.014	0.5	0.7	0.53±0.021
8.	Length of caudal fin(cm	0.4	0.7	0.53±0.015	0.5	0.6	0.53±0.014	0.5	0.7	0.6±0.000	0.5	0.7	0.57±0.021
9.	Length of pelvic fin(cm)	0.3	0.4	0.37±0.014	0.3	0.5	0.37±0.021	0.3	0.5	0.4±0.000	0.3	0.5	0.37±0.021
10.	Length of pectoral fin(cm)	0.5	0.6	0.53±0.014	0.4	0.5	0.43±0.012	0.4	0.5	0.43±0.015	0.4	0.6	0.5±0.000
11.	Length of anal fin(cm)	0.4	0.5	0.43±0.012	0.3	0.5	0.4±0.000	0.3	0.4	0.37±0.014	0.3	0.4	0.37±0.017

Table 10: Number variation, mean and standard deviation of fin rays of *D. rerio*

S/N	Name of Fins	Munshigonj (n=3)			Faridpur (n=3)			Mymensingh (n=3)			Khulna (n=3)		
		Min.	Max.	Mean±SD	Min.	Max.	Mean±SD	Min.	Max.	Mean±SD	Min.	Max.	Mean±SD
1.	Dorsal fin	6	9	8.0±0.354	10	12	11.0±0.000	7	11	9.0±0.000	8	9	8.67±0.120
2.	Caudal fin	19	23	21.0±0.000	25	27	25.67±0.236	22	25	23.33±0.120	17	22	19.33±0.120
3.	Pectoral fin	6	11	8.0±0.353	10	15	12.0±0.354	6	8	7.0±0.000	6	9	7.0±0.354
4.	Pelvic fin	5	8	7.0±0.060	9	9	9.0±0.000	6	8	6.67±0.233	8	11	9.0±0.354
5.	Anal fin	12	13	12.33±0.120	14	16	15.33±0.237	13	19	15.67±0.233	15	19	16.67±0.233

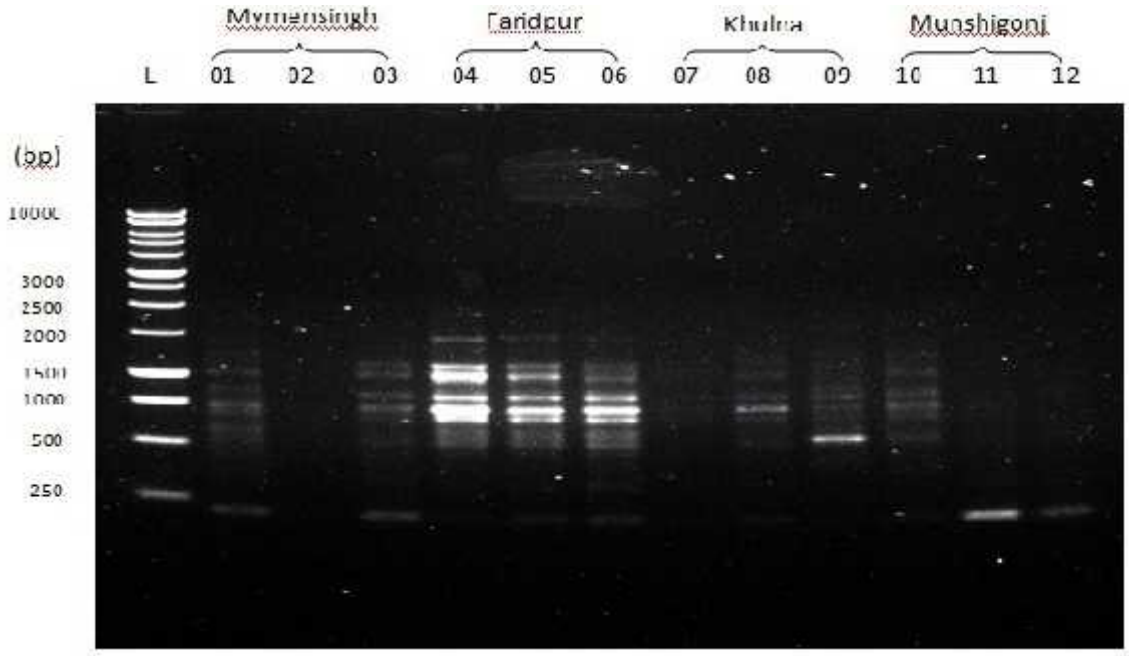

Figure 5: RAPD profile of Zebrafish (*Danio rerio*, Hamilton, 1822) at four different habitat using OPA03 primer.

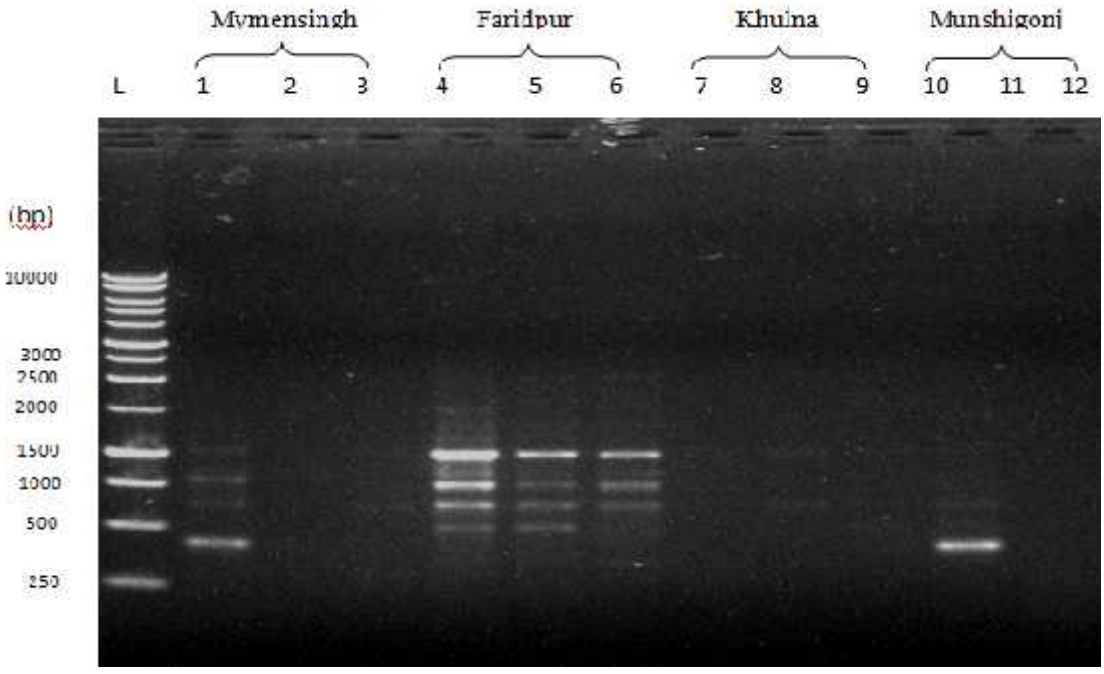

Figure 6: RAPD profile of Zebrafish (*Danio rerio*, Hamilton, 1822) at four different habitat using OPA04 primer.

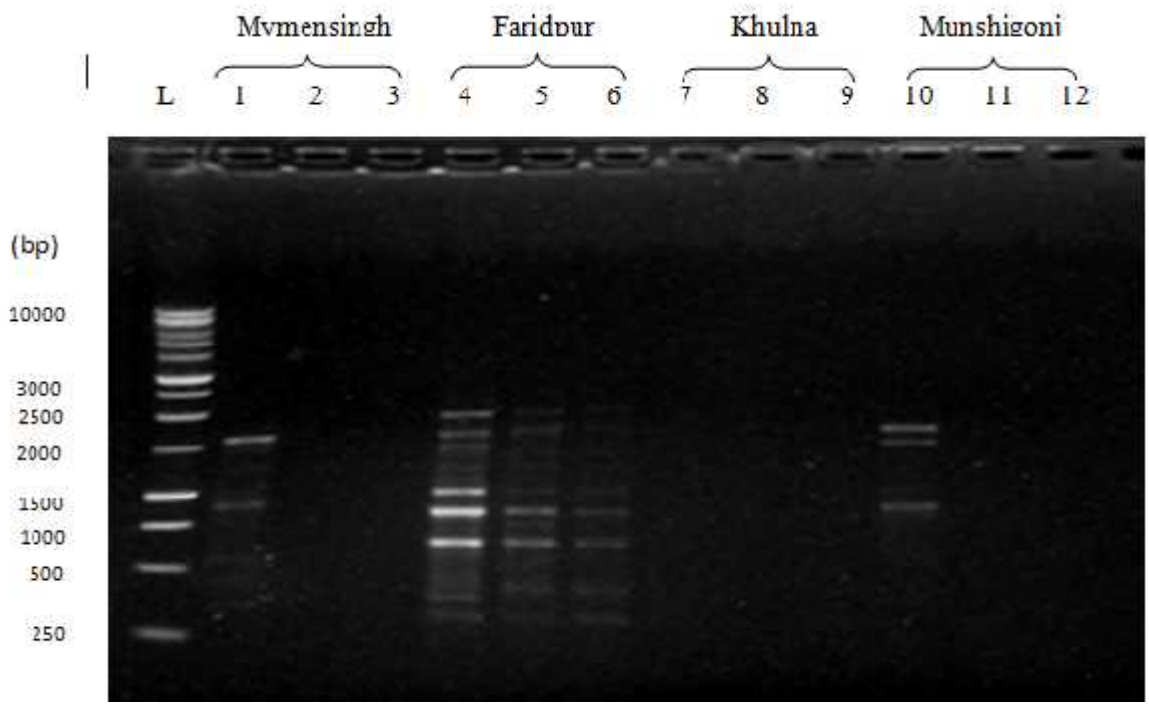

Figure 7: RAPD profile of zebrafish (*Danio rerio*, Hamilton, 1822) at four different habitat using OPF01 primer.

3.3 Polymorphic loci

The representative RAPD profiles of 12 individual zebrafish samples from all 4 populations generated by primer OPA03, OPA04 and OPF01 are depicted in figure 4, 5 and 6. It is clearly evident from these figures as well as data generated using other primers, that the RAPD profiles of Mymensingh, Faridpur, Khulna and Munshigonj were clearly distinct. The RAPD profiles of zebrafish samples obtained by 3 random primers are summarized in table 9. From all the 3 primers, 112 bands were scored, of which 22 bands (19.64%) were polymorphic (Table 12). Number of the scored fragments varied from 1 to 9 with a size range of 200–2000 bp (Table 12). Genetic distance within populations varied from 0.0645 to 1.2685 and genetic identity varied from 0.2812 to 1.0000. The population of Faridpur was found to have maximum genetic diversity with 33.33% polymorphic loci and the Khulna population had a minimum with 10.60% polymorphic loci. Genetic diversity also found among the populations of Mymensingh and Munshigonj with 15.15% and 19.69% polymorphic loci.

Table 11: No. of unique bands counted in three primers (OPA03, OPA04 and OPF01)

Primers code	Unique bands											
	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	S_{11}	S_{12}
OPA-03	1	-	1	-	-	-	-	-	-	1	-	-
OPA-04	-	-	-	2	-	-	-	-	-	-	-	-
OPF-01	3	-	-	-	1	-	-	-	-	-	-	-

Table 12: Pattern of polymorphism and uniqueness in 3 primers (primer wise) in 12 individuals of *D. rerio*

Sl. No.	Primers code	Size range (bp)	Total bands	Polymorphic bands	Polymorphism (%)	Avg. Polymorphism (%)
1	OPA03	250-1400	50	7 (250,300,400, 500,1000,1100, 1400)	14	19.64
2	OPA04	200-2000	32	7 (200,250, 300,500,600, 1000,2000)	21.88	
3	OPF01	200-2000	30	8 (200,350, 750,1000,1200, 1500,1700, 2000)	26.67	

3.4 Genetic identity and genetic distances

The genetic similarity among populations ranged from 0.2812 to 1.0000. Highest similarity was found between the population of Munshigonj3 & Mymensingh2; Khulna1 & Mymensingh2 and Munshigonj3 & Khulna1. On the other hand lowest similarityfoundbetween the population of Munshigonj3 & Faridpur1; Khulna1 & Faridpur1 and Faridpur1 & Mymensingh2. Again the genetic distance varies from 0.0000 to 1.2686. The highest genetic distance found between the population of Mymensingh2 and Faridpur1 but the lowest distance were found between Mymensingh2 & Munshigonj2; Mymensingh2 & Khulna1 and Faridpur1 & Mymensingh2. The genetic identity and genetic distance information are given in Table 13.

Table 13: Genetic identity and genetic distance between four populations of *Danio rerio*(Nei´s original measure of genetic identity (above diagonals) and genetic distance (below diagonals)).

PoP ID	Mymensingh 1	Mymensingh 2	Mymensingh 3	Faridpur 1	Faridpur 2	Faridpur 3	Khulna 1	Khulna 2	Khulna 3	Munshigonj 1	Munshigonj 2	Munshigonj 3
Mymensingh1	****	0.5625	0.6875	0.4062	0.4688	0.5625	0.5625	0.7188	0.6562	0.5312	0.5938	0.5625
Mymensingh2	0.5754	****	0.7500	0.2812	0.3438	0.4375	1.0000	0.7812	0.7812	0.6562	0.9062	1.0000
Mymensingh3	0.3747	0.2877	****	0.4688	0.5312	0.6250	0.7500	0.9062	0.9062	0.7188	0.7188	0.7500
Faridpur1	0.9008	1.2685	0.7577	****	0.8125	0.7812	0.2812	0.5000	0.5000	0.5625	0.3125	0.2812
Faridpur2	0.7577	1.0678	0.6325	0.2076	****	0.9062	0.3438	0.5000	0.5000	0.5000	0.3750	0.3438
Faridpur3	0.5754	0.8267	0.4700	0.2469	0.0984	****	0.4375	0.5938	0.5938	0.5938	0.4062	0.4375
Khulna1	0.5754	0.0000	0.2877	1.2685	1.0678	0.8267	****	0.7812	0.7812	0.6562	0.9062	1.0000
Khulna2	0.3302	0.2469	0.0984	0.6931	0.6931	0.5213	0.2469	****	0.9375	0.8125	0.7500	0.7812
Khulna3	0.4212	0.2469	0.0984	0.6931	0.6931	0.5213	0.2469	0.0645	****	0.7500	0.7500	0.7812
Munshigonj1	0.6325	0.4212	0.3302	0.5754	0.6931	0.5213	0.4212	0.2076	0.2877	****	0.5625	0.6562
Munshigonj2	0.5213	0.0984	0.3302	1.1632	0.9808	0.9008	0.0984	0.2877	0.2877	0.5754	****	0.9062
Munshigonj3	0.5754	0.0000	0.2877	1.2685	1.0678	0.8267	0.0000	0.2469	0.2469	0.4212	0.0984	****

3.5 UPGMA dendrogram

The UPGMA dendrogram was prepared based on genetic distance indicating the segregation of the *Danioreri*opopulations collected from the 4 sites of Bangladesh. The unweighted pair group method of arithmetic means (UPGMA) dendrogram based on Nei's (1972) original measures of genetic distance (D) was constructed (Figure 8). The unweighted dendrogram divided all the 12individuals in 11 clusters but the samples were collected from 4 locations. The intra-specificgenetic similarity was more amongfishes of Mymensingh than the other locations.

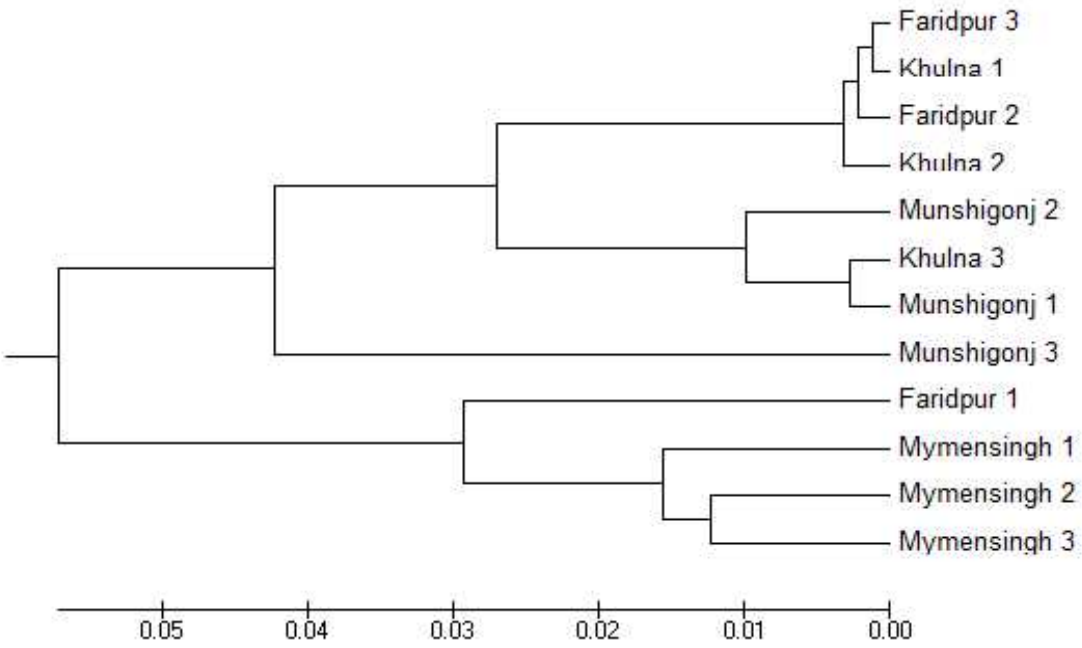

Figure 8: UPGMA dendrogram based on Nei´s (1972) original measures of genetic distance, summarizing the data on differentiation between *D. rerio*populations according to RAPD analysis (Using PopeGene software).

The intra-species genetic distance was higher among the populations of Faridpur. It is evident from the dendrogram that 4 populations of Faridpur and Khulna have more similarity in their genetic distances. Another population of Faridpur showed similarity with the populations of Mymensingh and one population from Khulna showed similarity with the populations of Munshigonj.

On the geographical point of View, as the populations located near to each other or the 4 individual districts are not so far, they showed more similarity among them. The kumar

river of Faridpur has internal junction between the Brahmaputra river of MymensinghandMoyuri canal of Khulna. Perhaps, they have internal junction among their habitats, so they showed genetic similarity among the populations. The UPGMA dendrogram based on Nei´s (1972) genetic distance, also Showed that the individuals from the same stock has genetic distance between their populations.

3.6 DNA sequencing results

3.6.1 DNA sequence interpretation

DNA sequence results obtained from the fish samples collected from 4 districts of Bangladesh. Out of 12, 4 were successfully amplified with the FishF1 FishR1 primer. The resulting PCR products were sequenced to produce full length DNA barcodes averaging 680 bp (base pairs) in length, with no detectable insertions, deletions or stop codons.All the 4 samples were amplified with the primers and they produced significant bands. Then it was processed and sent to Malaysia for sequencing.

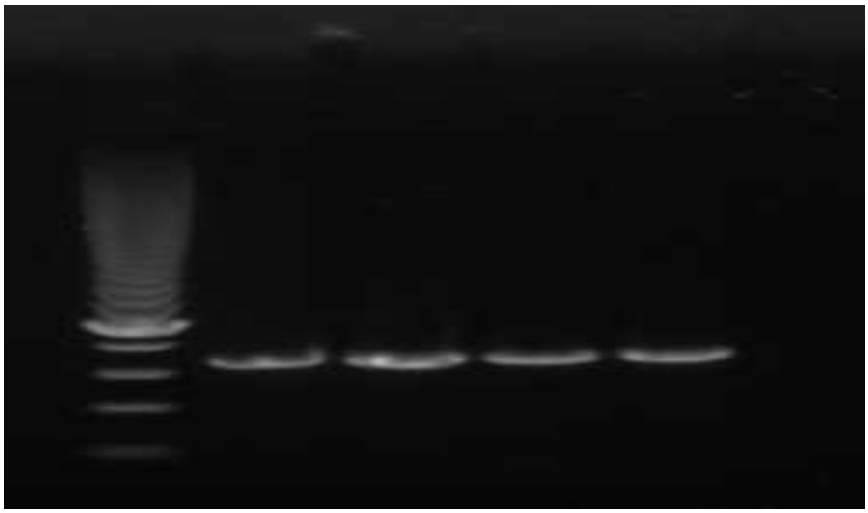

Figure 9: PCR amplification by COI gene by using FishF1 and FishR1 primer (1, 2, 3 and 4 indicating the sample from Mymensingh1, Faridpur1, Khulna3 and Munshigonj1)

Full length DNA barcodes (680 bp) were recovered using the Fish primers Fish F1 and Fish R1. Out of 12 samples, 4fish DNA was amplified using the primer set and used for bi-directional sequencing (both forward and reverse). Peak intensities and sequencing

qualities of the generated barcodes were compared to the sequences downloaded from NCBI GeneBank and its database.

Table 14: List of all identification results using the GeneBank as search engine

Sample Name	Fish Name	Type	Gene Bank References	Gene Bank Accession No.
Mymensingh 1	Zebrafish (*Danio rerio*)	Whole Fish	*Danio rerio*	KT624626.1
			D. rerio	AY996924.1
			D. rerio	JF915578.1
			D. catenatus	KT199751.1
Faridpur1	Zebrafish (*D. rerio*)	Whole Fish	*D. rerio*	HQ141077.1
			D. rerio	JF459455.1
			M. erythromicron	LC190394.1
Khulna 3	Zebrafish (*D. rerio*)	Whole Fish	*D. rerio*	KM207081.1
			D. albolineatus	HM224143.1
			D. kyathit	JF915573.1
Munshigonj 1	Zebrafish (*D. rerio*)	Whole Fish	*D. rerio*	JF915578.1
			D. annulosus	KT199744.1
			D. rerio	KT624627.1

3.6.2 Sequence analysis

From the sequenced data (nucleotides), a well sequenced portion was taken using the Finch TV software and blast using NCBI Blast. All the samples showed 100% similarity with *Danio rerio*species. After that the highest similarity species and other 2/3 species sequence was downloaded. This process was done for each of the 4 samples. The aligned species information is given in table 12-13.

Table 15: Identification of collected samples using the Gene Bank

SL.	Sample ID	Description	Max. score	Total score	Query cover	E value	Identification	Accession No.
1.	Mymensingh1	*D. rerio*	1158	1158	100%	0.0	100%	KT624626.1
2.	Faridpur4	*D. rerio*	1158	1158	100%	0.0	100%	HQ141077.1
3.	Khulna9	*D. rerio*	1158	1158	100%	0.0	100%	KM207081.1

4.	Munshigonj10	*D. rerio*	1158	1158	100%	0.0	100%	JF915578.1

Table 16: Identification of downloaded samples using Gene Bank

Sl. No.	Species Name	Accession No.
1.	*Danio rerio*	AY996924.1
2.	*D. rerio*	JF915578.1
3.	*D. catenatus*	KT199751.1
4.	*D. rerio*	JF459455.1
5.	*M. erythromicron*	LC190394.1
6.	*D. albolineatus*	HM224143.1
7.	*D. kyathit*	JF915573.1
8.	*D. annulosus*	KT199744.1
9.	*D. rerio*	KT624627.1

From the above data the phylogenetic tree was contructed using MEGA6 software.

3.6.3Phylogenetic tree

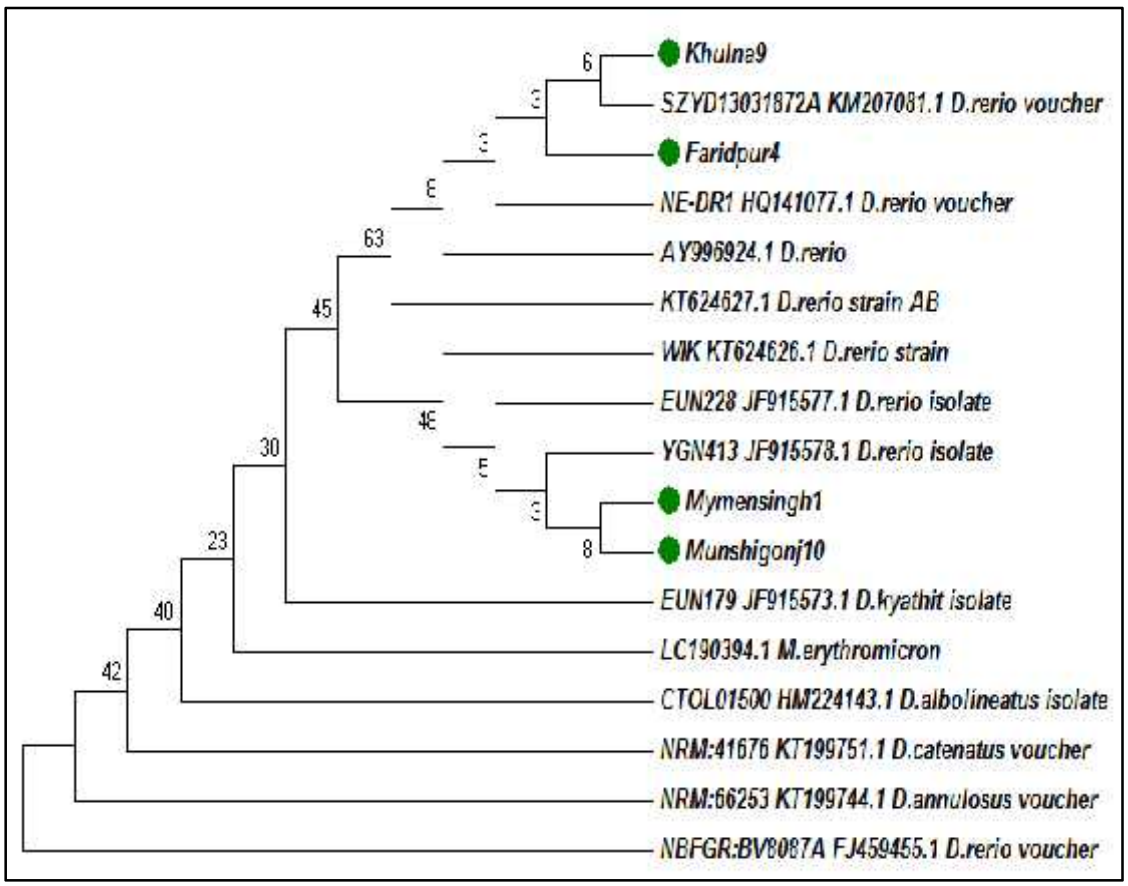

Figure 10: The neighbor joining Phylogenetic tree based on partial COI gene sequences using MEGA6 software. The evolutionary distances were compared by Maximum Composite Likelihood method. Numbers in tree are bootstrap value.

3.6.4 DNA sequence alignment

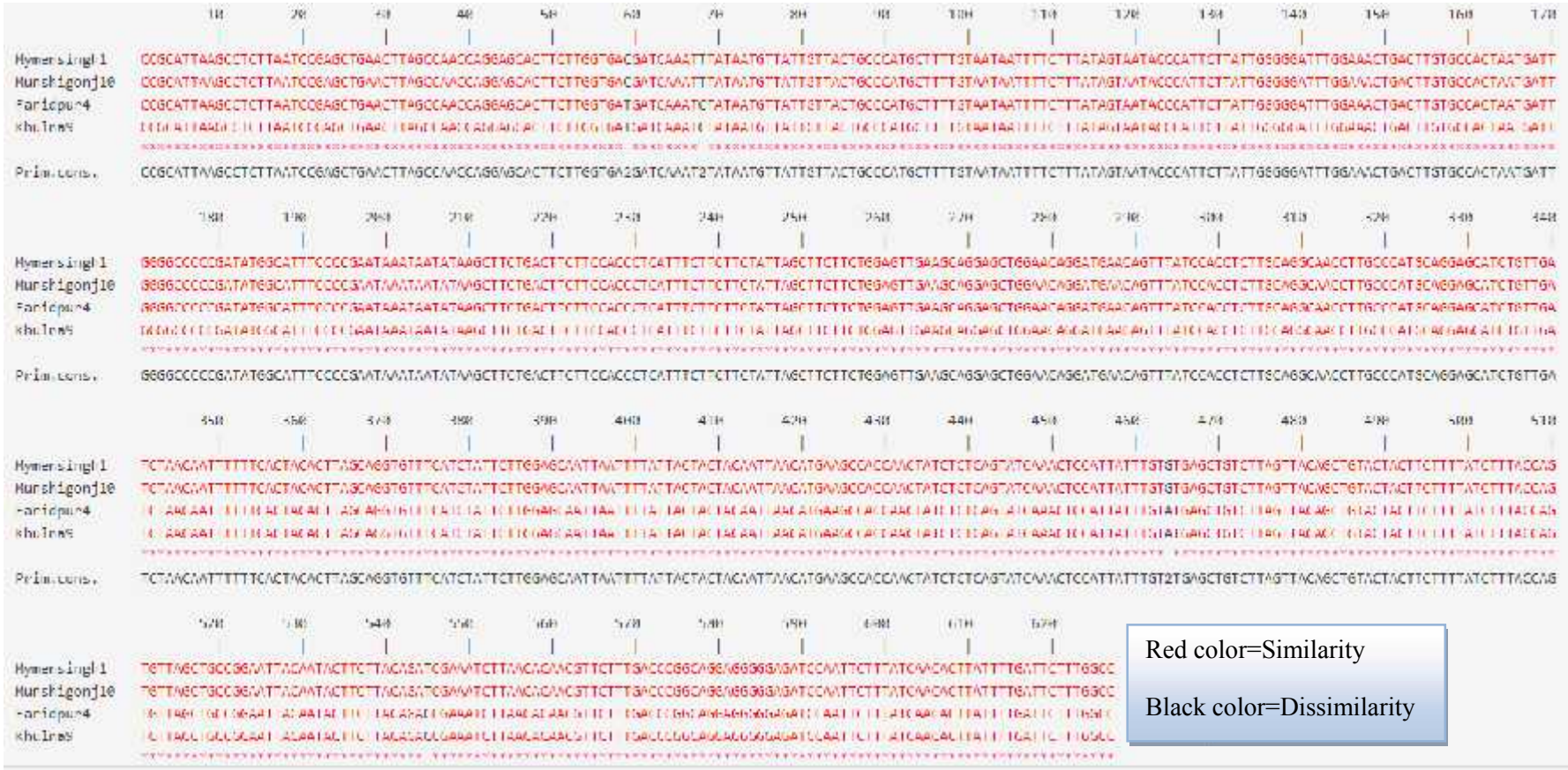

Figure 11: Multiple sequence alignment of COI gene fragment 4 substitute fish spp. Some representative polymorphic sites are indicated by nucleotide position.

Alignment data:Alignment length: 620 bp; Identity (*): 616 bp (99.35%); Different: 4 bp (0.65%)

Table 17: Polymorphic sites observed among four sequences of samples (BOLD database)

Position (bp)	Mymensingh1	Faridpur1	Khulna 3	Munshigonj1
59	C	T	T	C
68	T	C	C	T
464	G	A	A	G
545	T	C	C	T

At last it can be concluded that the fish sample has huge similarity with each other because 4 out of 620 nucleotide bases of the sequences were found polymorphic. So the *D. rerio* fish species found in Bangladesh are very similar to each other.

From the neighbor joining Phylogenetic tree given in 3.6.3 it can be concluded that the sequences exceeded 620 nucleotides in length showed similarity with each other. The populations of Mymensingh has significant similarity with the populations of Munshigonj and populations of Faridpur has similarity with populations of Khulna.

Chapter 4

Discussion

The present study shows genetic variation within twelve zebrafish (*Danio rerio*) species collected from four different locations of Bangladesh that is one of the most important aquaculture research model fish species all over the world. But a little information is available about its genetic structure found in Bangladesh. Das et al. (2005) carried out an investigation involving the comparative analysis of RAPD profiles of six *Labeo*species viz., *L. calbasu* (kalibaus),*L. bata* (bata), *L. dyocheilus* (dyocheilus), *L. fimbriatus* (fimbriatus), *L. gonius* (gonius) and *L. rohita* (rohu) at the nuclear DNA variation level. They chose 15 decamer random primers from 40 and amplified a total of 449 DNA fragments ranging in size from 400 to 3000 bp. Their results revealed that the highest intra-species genetic similarity value was in calbasu (0.93). In the present study, the RAPD and DNA barcoding technique has been found to be suitable in discriminating the four population of zebrafish obtained from different habitats.

RAPD markers DNA barcoding have been found to have a wide range of applications in gene mapping, population genetics, molecular evolutionary genetics and plant and animal breeding (Bardakci and Skibinski, 1994; Ertas and Seker, 2005). The RAPD and DNA barcoding technique consists of amplification by PCR of random segments of genomic DNA using a single-short primer of arbitrary sequence. There is no requirement of prior knowledge of the sequence of DNA. Its cost effectiveness provides an advantage in population genetic studies. RAPD technique has been applied to the study of phylogenetic relationship in tilapia and cichlid species (Bardakci and Skibinski, 1994). The presence of variability among populations as well as individuals within a population is essential for their ability to survive and successfully respond to environmental changes (Ryman et al., 1995). Intra-population genetic variation in tilapia was studied using different RAPD primers (Bardakci and Skibinski, 1994). This technique is more sensitive than the mt-DNA analysis, which failed to reveal variations within the tilapia populations (Capili, 1990; Seyoum and Kornfield, 1992). Genetic variation was studied between 4 different populations of Hilsa Shad from Ganga, Yamuna, Hoogly and Narmada rivers of India using DNA barcoding technique (Brahmaneet al., 2006). Thus, RAPD and DNA barcoding has been used in population studies in fisheries and can be used efficiently for variation analysis of populations with differential degrees of geographic isolation.

In this study, morphometric variations were observed through the normal visual inspection. In the present study, the RAPD technique has been found to be suitable in discriminating the twelvezebrafish populations obtained from different habitats of Bangladesh. All the primers tested in this study were not so consistently reproducible during DNA amplification, which is one of the very common features in RAPD methods. The results of the present study agree with those of Sahooet a1.(2013), Faddaghet al. (2012) and Ramellaet al. (2006). Technical problems from amplification of the RAPD and DNA sequencing techniques in the field of population genetic research have also been reported by many authors (Schierwater and Ender, 1993; Lynch and Milligan, 1994; and Naishet al., 1995).

Thethree primers produced considerable reproducible and polymorphic amplification with DNA of *Danio rerio* populations. In this study, no specific markers were found to discriminate zebrafish populations but the results analyzed from the data, scored from existing polymorphic bands (15), revealed some degree of divergence among the populations. The substantial difference in the number of polymorphic bands suggests that thelevel of genetic variation among the four zebrafish populations may be sufficient for developing intra-specific populations.

Three random primers were finally used, 19.64% of polymorphic loci were detected during this study indicating the effectiveness of RAPD technique to study polymorphism. The percentage polymorphic loci in the studied four different populations of *Danio rerio*, Which was highly deviated from the result drawn by Das et al. (2005), where they obtained 75% of polymorphic loci after screening four RAPD primers in three wild populations of *Labeocalbasu*. In contrast, Barman et al. (2003) detected on an average 45% polymorphic loci in four Indian major carps (rohu, *Labeorohita*; catla, *C. catla*; kalbasu, *Labeocalbasu* and mrigal, *Cirrhinusmrigala*). In another studies, Islam and Alam (2004) investigated in four different populations of Indian major carp, *Labeorohita* and found 46.59% of polymorphic loci by five RAPD primers. Among all the populations, Mymensingh2 and Faridpur1 population showed maximum intra-population genetic diversity, i.e., 1.2685 with 33.33% loci being polymorphic. Although the present data are unable to explain this high degree of variation, it was noticed that there was sufficient variations and similarities among the populations.Highest similarity found between the populations of Munshigonj3 & Mymensingh2; Khulna1 & Mymensingh2 and Munshigonj3 & Khulna1. On the other hand lowest similarity found

between the population of Munshigonj3 & Faridpur1; Khulna1 & Faridpur1 and Faridpur1 & Mymensingh2. Again the genetic distance varies from 0.0000 to 1.2686. The highest genetic distance found between the population of Mymensingh2 and Faridpur1 but the lowest distance were found between Mymensingh2 & Munshigonj2; Mymensingh2 & Khulna1 and Faridpur1 & Mymensingh2.

RAPD analysis revealed varying degree of polymorphism from 3 primers and showed that nuclear DNA variation in the populations is very high. UPGMA dendrogram based on Nei's (1972) genetic distance for zebrafishsp. of 12 populations showed variation among them.This may be attributed to the maintenance of a limited number of individuals sampled from the wild and their repeated propagation over a long period. Inbreeding may be another reason for reduced genetic variation in the population, as was also reported by Ekanth and Doyle (1990) on the basis of effective population size. All the populations showed almost similar level of intra-population similarity indices in the present study implies that individual within each population are genetically close to each other.

The similar result was also established in different Indian major carps and other fin fishes like *L. calbasu* (93%) (Das et al., 2005); *C. catla* (88.53%) (Islam et al., 2005); *L. rohita* (94.88%) (Islam and Khan, 2004; Barman et al., 2003); Atlantic coast striped bass (*Moronesaxatilis*) in Delaware River 92.6% and Roanoke River 95.6% (Bielawski and Pumo, 1997); Xinggus red carp (72.2%), Russian mirror carp (78.7%); German mirror carp (82.7%) (Dong et al., 1999) and *Sparus latus* (88.21% and 87.85%) from Fujian and Zhujiang river respectively (Yang et al., 2004). This implies that individuals within each population are genetically more similar to each other, as was expected, than to individuals from all other populations (Macdonald, 2005). Similar results were provided by Das et al., (2005) in *Labeo* species, Islam et al. (2005) in *C. catla*, Islam and Alan (2004) in *L. rohita*, and also by Alam and Khan (2001) in case of Japanese loach, *Misgurnusanguillicaudatus*. The pair-wise inter-population polymorphism indices was 19.64% compared to the whole populations. This is the cause that, they are from separate stock or from separate habitat.The percentage of polymorphic loci was found to be higher in the population of Faridpur is indicative of relatively high level of genetic variation, as well as lower level of percentage of polymorphic loci was in Munshigonj population. Like percentage of polymorphic loci, othermeasurements such as gene diversity and Shannon's information index were also higher in the Faridpur (Kumar

river) population than other individuals of studied populations but comparatively lower in the Munshigonj (Nariakhal) population. The higher value of the percentage of polymorphic loci and gene diversity in the individuals of the Kumarriver are usually expected, because it is well known to all that the small fish species are more available in the Kumar river. Therefore, in Bangladesh, genetically diversified more *D. rerio* individuals can be found in the Kumarriver.

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 sequences in forensics has already been investigated for reproducibility, heteroplasmy, mixed DNA samples, chemical treatments, environmental conditions and other factors showing consistent results in which a great range of reference data exist (Dawson et al. 2007).

In this study, we identified zebrafish samples through the comparisons of COI mtDNA sequences using the BLAST engine to search Gene Bank. The resulted sequences matched 100% with *D. rerio* by NCBI Blast searching. Mymensingh1 matched with accession no. KT624626.1, Faridpur1 matched with accession no. HQ141077.1, Khulna3 matched with accession no.KM207081.1 and the Munshigonj1 sample matched with accession no.JF915578.1. All the four samples are *D. rerio* species. In the multiple sequence alignment, the four fish samples showed 99.35% similarity with each other in their nucleotide sequences. So it can be easily concluded that zebrafish spp. found in Bangladesh are very similar.

RAPD markers and DNA barcoding technique have been proved as effective tools to monitor the genetic variation in different populations of organisms. Using only three primers and twelve live samples for populations, the present study revealed a remarkable level of intraand inter-population genetic variation of *Danio rerio* and the DNA barcode results showed massive similarity among the populations. Although no specific markers were detected to discriminate studied *Danio rerio* populations but the fact that 19.64% of the bands were found to be polymorphic and indicating that the RAPD marker system may be more useful to generate molecular markers for genetic characterization in this species.

Chapter 5

Conclusion and Recommendations

5.1 Conclusion

The level of genetic variation provides the raw material for the selective improvement of a stock for sustainable aquaculture production. The present study was carried out to assess the genetic variation in four wild populations zebrafish (*Danio rerio*, Hamilton, 1822) using randomly amplified polymorphic DNA (RAPD) and DNA barcoding techniques. Three different primers were used for the RAPD technique and two other primers were used for the DNA barcoding technique. Significant genetic diversity was observed among 12 individuals from 4 habitats. The RAPD primerswere found as polymorphic and were selected. RAPD markers were amplified by PCR technique. Genetic distance within populations varied from 0.0645 to 1.2685 and genetic identity varied from 0.2812 to 1.0000. The population of Faridpur was found to have maximum genetic diversity with 33.33% polymorphic loci and the Khulna population had a minimum with 10.60% polymorphic loci. Genetic diversity also found among the populations of Mymensingh and Munshigonj with 15.15% and 19.69% polymorphic loci.

For DNA barcoding, we amplified the cytochrome oxidase c subunit 1(COI) barcode sequence (680 bp long) for 4 specimens, and we compared them with reference sequences from different databases (GenBank and BOLD). Though the database is undergoing continual development, it was able to provide species matches of >99% sequence similarity for 4 samples tested. The overall sequence alignment similarity among our sampled specimens was 99.35%.

The DNA barcoding technology and reference library is an improvement over the existing DNA-based techniques in detecting*D. rerio* species variance.DNA barcoding offers a new level of precision in the application of species names, which is increasingly expanding in international level. The ease of generating DNA barcodes and a focus on high quality data records instill increasing confidence in the technique.

From the study, it is concluded that RAPD markers and DNA barcoding can be useful and efficient tools in the investigation of intraand inter-population genetic variation of *Danio rerio*. Thus this study holds enormous prospect to the development and application of molecular markers for zebrafish. For the management of endangered

inland wild populations, which are commercially harvested, a clear picture of the genetic structure of the population is necessary for the future conservation and management (Allendorf et al., 1986). The results of the present study will be a pre-requisite to know the genetic variation, population structure, stock purity etc. of the studied species before undertaking any breeding program and also will be used as a base for further study.

However, there were a few limitations in the study. Due to of highly expensive reagents, it was not possible to amplify whole DNA from huge number of individuals per population. Only four samples were analyzed using three primers, which reduced the chance to know the genetic variation more conclusively. In future, additional populations, Sampling sites and individuals with more number of primers need to be included in the analysisto make conclusive remarks about the genetic structure of different populations of this species.

5.2 Recommendations

From this study it can be recommended that

1. Study was conducted only in very short period and for that reason few number of replica were used so further study is needed.

2. As DNA barcoding technology is very important tool in detecting species variance so it is very essential to apply it to identify populations.

3. Cost should be minimized for species identification.

4. Government should monitor regularly fish biodiversity.

References

Abed, Y., 1995.Variation of RAPD fingerprinting patterns using different DNA extraction methods with Gram-positive bacteria. World J. Microbial Biotechnol. 11, 238-247.

Ahmed, M.M.M., Ali, B.A., El-Zaeem, S.Y., 2004. Application of RAPD markers in fish: Part I -some genera (Tilapia*, Sarotherodon* and *Oreochromis*) and species (*Oreochromis aureus* and *Oreochromis niloticus*) of Tilapia. International Journal of Biotechnology (IJBT). 6, 210-218.

Alam, M.A., Alam, M.S., 2003. Comparative studies on developmental instability, growth and genetic variability of selected hatchery and one natural population of rohu (*Labeo rohita*). Bangladesh Agric. Univ. Prog. 13, 131-132.

Alam, M.A., Akanda, M.S.H., Khan, M.M.R., Alarm, M.S., 2002. Comparison of genetic variability between a hatchery and a river population of rohu (*Labeo rohita*) by allozyrne electrophoresis. Pak J. Biol. Sci. 5(9), 959-961.

Alam, M.S., Islam, M.S., 2005. Population genetic structure of *Catla catla* (Hamilton) revealed by microsatellite DNA markers. Aquaculture. 246, 151-160.

Alam, M.S., Khan, M.M.R., 2010. Intra-specific genetic variation in the Japanese Loach (*Misgurnus anguillucaudatus*) revealed by random amplified polymorphic DNA (RAPD) analysis. Pak J. Biol. Sci. 4 (7), 877-880.

Alam, M.S., Popplewell, A., Maclean, N., 1996. Germ line transmission and expression of a lack containing transgene in tilapia (*Oreochromis niloticus*). Transgenic Res. 5, 87-95.

Ali, B.A., Ahmed, M.M.M., El-Zaeem, S.Y., 2004. Technical note: application of RAPD markers in fish: Part II - Among and within family; Chichlidae (fresh water), Mugilidae (catadromous), Sparidae and Serrandae (marine). Int. J. Biotech. 6(4), 393-401.

Ali, M.Y., 1997. Induced breeding of major carps in ponds by pituitary hormone injection. Agric. Inform. Serv., Dhaka pp 3.

Allegrucci, G., Caccone, A., Cataudella, S., Powel, J.R., 1995. Acclimation of the European sea bass to freshwater: monitoring genetic changes by RAPD polymerase chain reaction to detect DNA polymorphisms. Mar. Biol. 121, 591-599 .

Allendorf, F.W., 1983. Isolation of gene flow and genetic differentiation among populations. In: Genetics and Conservation: A Reference for Managing Wild Animal and Plant Populations, eds. C. Schonewald-Cox, S.M. Chambers, B. McBryde and W.L. Thomas. Benjamin/Cummings Pubs. Co. Menlo Park, Cal. pp. 51-65.

Allendorf, F.W., Phelps, S.R., 1980. Loss of genetic variation in a hatchery stock of trout. Trans. Am. Fish. Soc. 109, 530-537.

Azadi, M.A., 2002. Management of spawn fishery of major carps (*Calla catla, Labeo rohita and Cirrhinus mrigala*) in the Halda River, Bangladesh. SUPER project, DFID, BUGC.

Azadi, M.A., Kibria, M.M., Jahangir, S., Akhteruzzaman, M., 2003. Management of spawn fishery of major carps (*Catla catla, labeo rohila* and *Cirrhimus mrigala*) in the Halda River, Bangladesh.

Banik, R.C., Humayun, K., 2012. Fishery resources statistics. Fish Week 98 Compendium. Department of Fisheries, Ministry of Fisheries and Livestock, Dhaka. pp. 100-102.

Bardakci, F., Skibinski, D.O.F., 1994. The application of the RAPD technique in tilapia fish: species and subspecies identification. Heredity. 73, 117-123.

Barman, H.K., Barat, A., Yadav, B.M., Banerjee, S., Meher, P.K., Reddy, P.V.G.K., Jana, R.K., 2003. Genetic variation between four species of Indian major carps as revealed by random amplified polymorphic DNA assay. Aquaculture. 217, 115-123.

Basavaraju, Y., Devaraj, K.V., Ayar, S.P., 1995. Comparative growth of reciprocal carp hybrids between *Catla catla* and *Labeo fimbriatus*. Aquaculture. 129, 187-191.

Bentzen P.E., Taylor, B., Wright, J.M., 1993. Minisatellite sequences in the Salmonidae and related family. Journal of Fish boil. 43, 313-316.

Bhowmick, R.M., Jana, R.K., Gupta, S.D., Kowtal, G.V., Rout, M., 1981. Studies on some aspects of biology and morphometry of the intergeneric hybrid, *Catla catla*, Hamilton; *Labeo rohita*. Hamilton. Produced by hypophysation. Aquaculture. 23, 367-371.

Bielawski, J.P., Pumo, D.E., 1996. Randomly amplified polymorphic DNA (RAPD) analysis of Atlantic Coast striped bass. Heredity. 78, 32-40.

Bowditch, B.M., Albright, D.G., Williams, J.G.K., Braun, M.J., 1994. Use of randomly amplified polymorphic DNA markers in comparative genome studies. Methods Enzymol. 224, 294-309.

Brahmane, M.P., Das, M.K., Sinha, M.R., Sugunan, V.V., Mukherjee, A., Singh, S.N., Prakash, N., Maurye, P., Hajra, A., 2006. Use of RAPD fingerprinting for delineating populations of hilsa shad, *Tenualosa ilisha* (Hamilton, 1822). Genet. Mol. Res. 5 (4), 643-652.

Brody, J.R., Kern, S.E., 2004. History and principlesof conductive media for standard DNA electrophoresis. Anal Biochem. 333(1), 1-13.

Callejas, C., Ochando, M.D., 2001. Molecular identification (RAPD) of the eight species of the genus *Barbus* (Cyprinidae) in the Iberian Peninsula. J. Fish Biol, 59(6), 1589-1599.

Capili, B.W., 1990. Systematic biodiversity in the freshwater fishes of Iran. Italian J. Zool. 65, 101-108.

Chacko, P.I., 1948. A note on carp spawning in the Godavari and Krishna (Madras). Proc. Indian Sci. Congr, 33(3), 128-135.

Chang, Z., Zhou, R., Yu, Q., 2001. Genetic variation of two loach species revealed by RAPD analysis. Acta Zool. Sin. 47(1), 89-93.

Chiari, L., Sodre, L.M.K., 2001. Study of eight species of the Anostomidae family (Pisces, Characiformes) by RAPD analysis. Acta. Scientiarum. 23(2), 445-451.

Chong, L.K., Tan, S.G., Yusoff, K., Siraj, S.S., 2000. Identification and characterization of Malaysian river catfish, *Mystus nemurus* (C&V): RAPD and AFLP analysis. Biochem.Genet. 38(3), 63-76.

Clark, A.G., Lanigan, C.M.S., 1993. Prospects for estimating nucleotide divergence with RAPDs. Mol Bio Evol. 10, 1096-4111

Cross, T.E., King, J., 1983. Genetic effects of hatchery rearing in Atlantic salmon. Aquaculture. 33, 830-840.

Das, P., Barat, A., 1990. Fish habitat degradation necessitating conservation. Environmental Series. 4, 85-89.

Das, P., Prasad, H., Meher, P., Barat, A., Jana, R.K., 2005. Evaluation of genetic relationship among six *Labeo* species using randomly amplified polymorphic DNA (RAPD). Aquacult. Res. 36, 564.

Dawson, I.K., Chalmers, K.J., Waugh, R., Powell, W., 1993. Detection and analysis of genetic variation in *Hordeum spontaneum* populations from Israel using RAPD markers. Mol. Ecol. 2, 151-159.

Degani, G., Pitcovski, J., Dobski, T., Plotsky, Y., 1997. DNA fingerprint bands to analysis of variations in angel fish (*Pterophyllum scalare*) (Cichlidae) strains. J. Aquacult. Trop. 12(1), 43-51.

Dinesh, K. R., Lim, T. M., Chua, K. L., Chan, W. K., Phang, V.P.E., 1993. RAPD analysis: An efficient method of DNA fingerprinting in fishes. Z001. Sci. 10, 849-854.

Ding, S., Su, Y., Wang, J., Quan, C., 2003. Genetic diversity of nature and reared group *of Nibeamiich thioides* by RAPD analysis. Mar. Sci. 27(8), 63-66.

DoF., 2015. Fish fortnight 2015 compendium.Department of Fisheries, Ministry of Fisheries and Livestock, Government of the Peoples Republic of Bangladesh, Dhaka.

DoF., 2016. Fisheries Statistical Yearbook of Bangladesh 2015-2016. Department of Fisheries, Ministry of Fisheries and Livestock, Government of the Peoples Republic of Bangladesh, Dhaka

Dong, S., Wang, Q., Qian, Z., 2003. The identification of clone in pengze crucian carp, *C. auratus* pengzesis using isozyme and RAPD markers. J. Iimei. Univ. Nat. Sci. 3(3), 208-212.

Ekanth, A.E., Doyle, R.W., 1990. Effective population size and rate of inbreeding in aquaculture of Indian major carps. Aquaculture. 85, 293-305.

Elo, K., Ivanoff, S., Vuorinen, J.A., Piironen, J., 1997. Inheritance of RAPD markers and detection of inter-specific hybridization with brown trout and Atlantic salmon. Aquaculture. 152, 55-65.

Ertas., H.B., Seker,S ., 2005. Isolation of *Listeria monocytogenes* from Fish Intestines and RAPD Analysis. Turk J. Anim. Sci. 29, 1007-1011.

Exadactylos, A., Geffen, A.J., Panagiotaki, P., Thorpe, J.P., 2003. Population structure of Dover sole Sdeasolea: RAPD and allozyme data indicate divergence in European stocks. Mar. Ecol. Frog. Ser. 246, 253-264.

Faddagh, M.S., Hussain, N.A., Al-Badran, A.I., 2012.Advances in Life Sciences. 2(2), 9-16.

FAO., 2012. Genetic resources of Indian major carps. No. 387. Rome, Italy.

FAO., 2014. World aquaculture production of fish, crustaceans, mollusks, etc by principal species in 2002.Yearbook of fishery statistics. Rome, Italy.

Goldschmilt, C.R., 1953. Catla fishing in powai Lake, Greater Bombay. J. Bombay Nat. Hirt. Soc. 55(3), 473-480.

Gupta, S.D., Rath, S.C., Dasgupta, S.,Tripathi, S.D., 1995. A first report on quadruple spawning of *Catla catla* (Ham).Vet. arhiv. 65(5), 143-148.

Hadrys, H., Balick, M., Schierwater, B., 1992. Application of random amplified polymorphic DNA (RAPD) in molecular ecology. Mol. Ecol. 1, 55-63.

Homel, J., 1924. Administrative report for the year 1922-1972. Madras Fish. Bull. 18, 1-58.

Huang, p., Dong, W., Li, H.H., Liu, D., 2013. A brief history of zebrafish research. 35(4), 410-420.

Huang, C., Cheng, J., 2003. Evaluation of genetic variability in Taiwan endemic catfish *Clarias fuscus* species using RAPD markers. J. Fish. Soci. Taiwan. 30(3), 253-262.

Hussain, M.G., Mazid, M.A., 2001. Genetic improvement and conservation of carp species in Bangladesh.BFRI, Mymensingh, Bangladesh and ICLARM, Penang, Malaysia.

Islam. M.S., Ahmed, A.S.I., Azam, M.S., Alam, M.S., 2005. Genetic analysis of three river populations of *Catla catla* (Hamilton) using Randomly Amplified Polymorphic DNA markers. Asian Aust. J. Anim. Sci. 18(4), 453-457

Islam, M.S., Alam, M.A., 2004. Randomly amplified polymorphic DNA analysis of four different populations of the Indian major carp, *Labeo rohita* (Hamilton). 1. Appl. Ichthyol. 20, 407-4l2.

Jana, R.K., 1993. Studies on some aspects of biology and cytogenetics of rohu-catla and catla-rohu hybrids. Thesis submitted for the degree of Doctor of philosophy (Science) of the University of Calcutta.

Jayaram, K.C., 1999. The Freshwater Fishes of the Indian Region. Narendra Publishing House, Delhi, p. 551.

Jhingran, V.G., 1968. Synopsis of Biological data on catla, *Catla catla* (Ham. 1822). FAO Fisheries Synopsis No. 32 Rev. I.

Jhingran, V.G., Khan, H.A., 1979. Synopsis of Biological data on the mrigal, *Cirrhinus mrigala* (Hamilton 1822). FA0 Fisheries Synopsis No. 120. p 78.

John, G., Reddy, P.V.G.K., Gupta, S.D., 1984. Artificial gynogenesis in two Indian major carps, *Labeo rohita* (Ham) and *Catla catla* (Ham). Aquaculture. 42, 161-168.

Jug, T., Dove, P., Pohar, J., Snoj, A., 2004. RAPD analysis as a tool for discriminating marble trout from hybrids (marble trout x brown trout) in the zones of hybridization. J. Anim. Breed. Genet. 121(3), 156-162.

Khan, H.A., Jhingran, V.G., 1975.Synopsis of biological data on rohu *Labeo rohita* (Ham, 1822). FAO fisheries synopsis, No. 1. p100.

Khan, M.M.R., Alam, M.S., Bhuiyan, M.M.H., 2005. Allozyme variation of hatchery and river populations of rohu (*Labeo rohita*, Hamilton) in Bangladesh. Aqualt.Res. (in press).

Kincaid, E.L., 1983. Inbreeding in fish populations used for aquaculture. Aquaculture. 33, 215- 227.

Kumar, S., Tamura, K., Nei, M., 1994. MEGA: molecular evolutionary genetics analysis software for micro-computers. Comput. Appl. Biosci. 10, 189-191.

Kuusipalo, L., 1994. Assessing genetic structure of pelagic fish population of Lake, Tanganyika Bujumbura Burundi. FAO. pp.13-17.

Lieschke, L., Currie, J., 2007. Zebrafish (*Dania rerio*) vary by strain and sex in their behavioral and transcriptional responses to selenium supplementation. Comparative Biochemistry and Physiology A-Molecular and Integrative Physiology. l57, 310-3l8.

Li, F., Liu, C., Lin, J., 2003. Genetic diversity analysis of two groups of primary parents of *Litopenaeus vannamei*. J. Zbanjiang Ocean Univ. 23(4), 1-5.

Lynch, M., 1990. The similarity index and DNA fingerprinting. Mol. Biol. Evol. 7, 478-484.

Lynch, M., 1991. Analysis of population genetic structure by DNA fingerprinting. In: DNA fingerprinting Approaches and Applications, eds. T. Burke, G. Dolf, A.J. Jeffreysand R Wolf. Basel, Switzerland, pp. 113-126.

Lynch, M., Milligan, B.S., 1994. Analysis of population genetic structure with RAPD markers. Mol. Ecol. 3, 91-99.

Macdonald, C., 1995. The use of random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) to analyze genetic variation within and between species and strains of Indian major carp. University of Wales, p- 29.

Mamuris, Z., Apostolidis, A.P., Theodorou, A.I., Triantaphyllidis, C., 1998. Application of random amplified polymorphic DNA (RAPD) markers to evaluate intraspecitic genetic variation in red mullet (*Mullus barbatus*). Mar. Biol. 132, 171-178.

Mamuris, Z., Stamatis, C., Triantaphyllidis, C., 1999. Intraspecific genetic variation of striped red mullet (*Mullussur muletus*) in the Meditenanean Sea assessed by allozyme and random amplified polymorphic DNA (RAPD) analysis. Heredity. 83, 30-38.

McCune, S.K.J., Leamon, J., Skibinski, D.O.F., Mair, G.C., 2001. Microsatellite markers form the Indian major carp species, *Catla catla.* Mol. Ecol. Notes I. 115-116.

Moav, R., Brody, T., Wohlfanh, G., Hulata, G., 1976. Applications of electrophoretic genetic markers to fish breeding. 1. Advantages and methods. Aquaculture. 9, 217-228.

Mostafa, M.G., Istiaq, A.S., Rabbane, M.G., Islam, M.N ., 2009. Genetic diversity of wild and farmed Kaliboush (*Labeo calbasu*, Hamilton, 1822) by RAPD analysis of the genomic DNA.

Naish, K.A., Warren, M., Bardakei, F., Skibinski, D.O.F, Carvalho, G.R., Mair, G.C., 1995. Multilocus DNA fingerprinting and RAPD reveal similar genetic relationships between strains of *Oreochromis niloticus* (Pisces: Cichlidae). Mol. Ecol. 4, 271-274.

Naish, K.A., Skibinski, D.O.F., 1998. Tetra nucleotide microsatellite loci for Indian major carp. J. Fish Biol, 53, 886-889.

Nash, J.H.E., 1991. DNA frag, Verstion 3.03. Institute of Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada.

Nataranjan, A.V., Jhingran, A.G., 1963. On the biology of *Catla catla* (Ham) from the river Yamuna. Proc. Inst. Sci. India (B). 29(3), 326-355.

Nazrul, M,, Alam, S.M., Hussain, M.G., 2005. Study of genetic variation in wild and hatchery stocks of walking catfish, (*Clarias batrachus*) using microsatellite DNA markers. A thesis for MS degree in Biotechnology. Dept. of Biotechnology. BAU. Mymensingh.

Nei, M., 1972. Genetic distance between populations. Am. Nat. 106, 283-292.

Nei, M., 1973. Analysis of gene diversity in subdivided populations. Proc. Natl. Acad Sci. 70, 3321-3323.

Oster, G.F., Shubin, N., Maury, J.D., Alberch, P., 1988. Evolution and morphogenetic rule: the shape of the vertebrate limb in ontogeny and phylogeny. Evolution. 42, 862-884.

Ovenden, J.R., 1990. Mitochondrial DNA and marine stock assessment: a review. Aust. J. Mar. Freshwater Res. 41, 865-853.

Page, R.D.M., 1996. TREEVIEW: an application to display phylogenic trees on personal computers. Comput. Appl. Biosci. 12, 357-358

Parenrengi, A., Shamsudin, L., Ismail, P., Amin, N.M., 2001. A study on DNA the polymorphic markers in the grouper *Epinephelus merra.* Asia Pacific J. Mol. Biol. Biotechnol. 9(1), 52-59.

Ponniah, A.G., 1997. Conservation of carp genetic resources of India In: Proc. Collaborative Research and Training on Genetic Improvement of Carp species in Asia eds. M.V. Gupta, Dey M. M., Dunham, R. and Bimbao G. Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar, mass. P-96.

Powell, W., 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsattelite) markers for germplasm analysis. Molecualr Breeding. 2, 225-238.

Qui, I.J., Li, Y.P., 1999. Random amplified polymorphic DNA analysis of eel genome. Cell Res. 9(3), 217-23.

Ramella, M.S., Kroth, M.A., Meurer, S., Filho, E.Z., 2006. Genetic Variability in Four Fish Species (*Pimelodus maculatus*, *Prochildus lineatus*, *Salminus brasiliensis* and *Steindachner idionscripta*) from Uruguay River Basin. BABT.49(3), 589-598.

Reddy, P.V.G.K., 1990.Genetic resources of Indian major carps. FAO fisheries technical Papers. T-387.pp. 76

Reddy, P.V.G.K., Kowtal, G.V.,Tantia, M.S., 1990. Preliminary observations on induced polyploidy in Indian major carps, *Labeo rohita* (Hamilton, 1822) and *Catla catla* (Ham). Aquaculture. 87, 279-287.

Reiter, R.S., 1992. Global and local genome mapping in shad by using recombinant inbred lines and RAPDs. Proc Natl Acad Sci. USA 89, 1477-1495.

Robinson, G., Roland, M.S., (2007). Species identification and authentication of tissues of animal origin using mitochondrial and nuclear markers. Meat Science 76, 666-674.

Ryman. N., Utter, F., Laikre, L., 1995. Protection of intra-specific biodiversity of exploited Fishes. Rev. Fish. Biol. Fish. 5, 417-446.

Sahoo, P.K., Mohanty, J., Garnayak, S.K., Mohanty, B.R., Kar, B., 2013. Genetic diversity and species identification of Argulus parasites collected from major aquaculture regions of India using RAPD-PCR. J. Of Aq. 44, 220-230.

Schierwater, B., Ender, A., 1993. Different thermostable DNA polyerases may amplify different RAPD products. Nucleic Acids Res. 21, 4647-4648.

Schierwater, B., Ender, A., Schwent, K., Spaak, P., Streit, B., 1994. Molecular ecology evolution: Approaches and Applications. Birkh user Verleg. Basel, Boston, pp. 495-500.

Seyoum, S., Kornfield, I., 1992. Identification of the sub-species of *Oreochromis niloticus* (Pisces: Cichlidae) using restriction endonuclease analysis of mitochondrial DNA. Aquaculture, 102, 29-42.

Shah, M.S., Biswas, B.K., 2004. Growth and reproduction of wild and hatchery produced strains of Indian major carps. Bangladesh J. Fish. 27, 15-16.

Shamsudin, L., Shima, T.I.S., Norzehan, Z., 2001. The diversity and genetic population study on the mudskipper (*Periophthalmus schlosseri*) in the sea surrounding Peninsular Malaysia. 6th Asian Fisheries Forum Book of Abstracts, Philippines Asian Fisheries Society, pp. 225.

Shafi, M., Quddus, M.M.A., 1982. Bangladesher Matsho Shampad. Bangla Academy, Dhaka. First Edition.

Shrivastava, G., 2000. Fishes of UP and Bihar, Seventh Edition, Vishwavidalaya Prakashan, Chowk Varanasi-221001, India.

Shubina, E.A, Melnikova, M.N., Glubokov, A.I., Mednikov, B.M., 2004. Analysis of the genetic structure of northwestern Bering Sea walleye pollock, Theragrachalcogramma. Environ Biol Fish. 69(14), 177-185.

Simonsen, V., Hansen, M.M., Mensberg, K.L.D., Sarder, M.R.I., Alam, M.S., 2005. Widespread hybridization among species of Indian major carps in hatcheries, but not in the Wild. J. Fish Biol. 67, 794-808.

Sneath, P.H.A., Sokal, R.R., 1973. Numerical taxonomy. W. H. Freeman and Co, San Francisco, pp. 21-28.

Sodre, L.M.K., Almeida, F.S., 2002. Comparative study by RAPD analysis of six species of the Pimelodidae family (Osteichthyes, Siluriformes) from the Tibagi River, state of Parana, Brazil. Acta Sci. 24(2), 513-517.

Statura, D.L., Svoboda, O., Campbell, C.A., 2013. The zebrafish granulocyte colony stimulating factors: two paralogous cytokines and their role in hematopoietic development and maintenance. Asia Pacific J. Mol. Biol. Biotechnol. 9(1), 52-59.

Sultmann, H., Mayer, W.E., Figueroa, F., Tichy, H., Klein, J., 1995. Phylogenetic analysis of cichlid fishes vising nuclear DNA markers. Mol. Biol. Evol. 12, 1033-1047.

Tassanakajon, A., Pongsomboon, S., Rimphanitchayakit, V., Jarayabhand, P., Boonsaeng, V., 1997. Random amplified polymorphic DNA (RAPD) markers for determination of genetic variation in wild populations of the black tiger prawn (*Penaeus monodori)* in Thailand. Mol. Mar. Biol. Biotechnol. 6 (20), 110-115.

Tave, D., 1993. Genetics for fish hatchery managers (second edition). AVI, Westport, Connecticut. pp. 415.

Tinni, S.R., Jessy, N.S., Hasan, M.M., Mustafa, M.G., Alam, S.S., 2007. Comparative karyotype analysis with differential staining in two forms of *Anabas testudineus* Bloch. The Japan Mendel Society. Cytologia. 72(1), 71-75.

Wadeh, H., Alsarakibi, M., Li, G.Q., 2010. Analysis of genetic variability within Argulusjaponicus from representatives of Africa, Middle East, and Asia revealed by sequences of three mitochondrial DNA genes. Parasitology Research. 107, 547–553.

Walples, R.S., 1987. A multispecies approach to the analysis of gene flow in marine shore fishes. Evolution, 41, 385-400.

Wang, W., He, S., Nakajima, T., 2001. The RAPD analysis for cyprinid fish (*Aphyocypris and Hemigrammo cypris*).Acta Hydrobiol, Sin. 25(4), 358-361.

Ward, R.D., Woodwork, M., Skibinski, D.O.F., 1994. A comparison of genetic diversity levels in marine, freshwater, and anadromous fishes. J. Fish. Biol. 44, 2l3-232.

Wasko, A.P., Galetti, R.M., 2002. RAPD analysis in the Neotropical fish *Bryconlundii*: genetic diversity and its implications for the conservation of the species. Hydrobiologia. 74(3), 131-137.

Wasko, A.P., Martins, C., Oliveira, C., Senhorini, J.A., Foresti, F., 2004. Genetic monitoring of the Amazonian fish matrincha (*Brycon cephalus*) using RAPD markers: insights into supportive breeding and conservation programmes. J. Appli. Ichthyol. 20(1), 48-52.

Welsh, J., McClelland, M., 1990. Fingerprinting genomes using PCR with arbitary primers. Nucleic Acids Res. 18, 7213-7218.

Williams, J.G.K., Hanafey, M.K., Rafalski, J. A., Tingey, S.V., 1993. Genetic analysis using random amplified polymorphic DNA markers. Methods Enzymol. 218, 704-740.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V., 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18, 6531-6535.

Yang, H., Jiang, S., Su, T., Lv, J., 2004. Genetic diversity of two wild populations of *Sparus latus* by RAPD. J. Trop. Oceanogr. 23(2), 55-61.

Yoon, J.M., Park, H.Y., 2002. Genetic similarity and variation in the cultured and wild *Carassius carassius* estimated with random amplified polymorphic DNA. Asian Aust. J. Anim. Sci. 15(4), 470-476.

Zhang, S., Deng, H., Wang, D., Yu, L., 2001. Population structure and genetic diversity of silver carp and grass cap from populations of Yangtze River system revealed by RAPD. Acta Hydrobiol. Sin. 25(4), 324-330.

Zheng, G.M., Zhu X.P., Zhang, Y., Luo, J.R., Xia, S.L., 1999. Identitication of Indian mrigal and Indian rohu using RAPD. J. Huazhong. Agric. Univ. 18(4), 371-374.

APPENDICES

Appendix 1. Reagents used for RAPD and DNA barcoding technique

1. Extraction buffer: TEN buffer + 1% SDS
2. TEN buffer (TrisHCl, EDTA (Ethylenediaminetetra acetic acid), NaCl, pH = 8.0 (adjusted with HCl))
3. 1% SDS (Sodium Dodecyl Sulfate):
   20 g SDS (20%) were dissolved in 2000m1 of distilled water
4. Proteinase K:
   It was dissolved in sterile distilled water at a concentration of 20 mg/ml.
5. Phenol: Chloroform: Isoamyl alcohol::25: 24: 1(equilibrated to pH near 8.0 with TEN buffer.
6. Isopropanol
7. Ethanol:
   100% ethanol (for 100ml): 100 ml absolute ethanol
   95% ethanol (for 100ml): 95 ml absolute ethanol + 5 ml distilled water
   70% ethanol (for 100ml): 70ml absolute ethanol + 30ml distilled water
8. Sodium acetate (3M) (for 250ml):
   102g of sodium acetate (1M) were dissolved in 250ml of distilled water pH = 5.2
9. 5 x TAE buffer (pH 8.3)
10. 6 x Loading Dye
11. Ethidium Bromide Solution

Appendix 2. Solution Preperation

1M Stock Solution of Tris-HCl pH 8.0 (100 ml)

12.14 g of Trizma base (MW=121.14) was dissolved in 75 ml of distilled water. The pH of this solution was adjusted to 8.0 by adding about 5 ml of concentrated HCl. The volume of the solution was adjusted to a total of 100 ml with de-ionized distilled water. Then it was sterilized by autoclaving and stored at 4°C.

0.5 M Stock Solution of EDTA pH 8.0 (100 ml)

18.61 g of EDTA (EDTA.2H_20, MW = 372.24) was added to 75 ml of distilled water and stirred vigorously with a magnetic stirrer. Approximately 2 g of NaOH pellets was added to adjust the final pH to 8.0. The final volume of the solution was adjusted to 100 ml by adding sterile de-ionized distilled water. The solution was sterilized by autoclaving and stored at 4°C.

5 M Stock Solution of NaCl (100 ml)

29.22 g of sodium chloride (NaCl, MW = 58.44) was dissolved in 75 ml of distilled water. The total volume of the solution was adjusted to 100 ml with distilled water. The solution was heated in an oven for 15 seconds and stirred vigorously on a magnetic stirrer to dissolve NaCl. It was then sterilized by autoclaving and stored at 4°C. NaCl was added in small amount at once to be dissolved in solution.

70% Ethanol (100 ml)

30 ml double distilled water was added in 70 ml absolute ethanol to prepare 100 ml 70% ethanol.

Stock Solution of TE (Tris-HCl EDTA) Buffer pH 8.0 (100 ml)

1 ml of l M Tris-HCI was added with 0.2 ml (200 µl) of 0.5 M EDTA. The final volume was adjusted to 100 ml with sterile de-ionized distilled water. The solution was sterilized by autoclaving and stored at 4°C.

3 M Sodium acetate pH 5.2 (100 ml)

40.824 g of sodium acetate with 70 ml of dH_2O and adjusted the final Volume to 100 ml with dH_2O and pH was adjusted to 5.2. Then it was sterilized by autoclaving.

5 x TAE Buffer (pH 8.3) (1 liter)

242 g Trizma base (MW=121.14) was dissolved into 900 ml of sterile de-ioni/ed distilled water. Then 57 ml glacial acetic acid was added to the solution. Finally, 100 ml 0.5 LDTA (pH 8.0) was added in it. They were mixed well. The pH of the Solution was adjusted by mixing concentrated HCl at pH 8.3. The final volume of the solution was adjusted to I000 ml.

6x Loading Dye

T his is required to load samples in gel electrophoresis for further visualization.

Ethidium Bromide Solution

For 1 ml solution, Ethidium Bromide 10 mg was added to 1 ml of sterile de-ionized distilled water. It was then mixed by hand shaking. The solution was then transferred to a dark bottle and stored at room temperature. Stock solution of 10 mg/ml can also be purchased directly from companies.

Appendix 3. All sequences of collected sample, suspected species sequences and real species sequence

>Mymensingh1

CAGGAGCACTTCTTGGTGACGATCAAATTTATAATGTTATTGTTACTGCCCATGCTTTTGTAATAATTTTCTTTA
TAGTAATACCCATTCTTATTGGGGGATTTGGAAACTGACTTGTGCCACTAATGATTGGGGCCCCCGATATGGCA
TTTCCCCGAATAAATAATATAAGCTTCTGACTTCTTCCACCCTCATTTCTTCTTCTATTAGCTTCTTCTGGAGTTG
AAGCAGGAGCTGGAACAGGATGAACAGTTTATCCACCTCTTGCAGGCAACCTTGCCCATGCAGGAGCATCTGTT
GATCTAACAATTTTTTCACTACACTTAGCAGGTGTTTCATCTATTCTTGGAGCAATTAATTTTATTACTACTACAA
TTAACATGAAGCCACCAACTATCTCTCAGTATCAAACTCCATTATTTGTGTGAGCTGTCTTAGTTACAGCTGTAC
TACTTCTTTTATCTTTACCAGTGTTAGCTGCCGGAATTACAATACTTCTTACAGATCGAAATCTTAACACAACGT
TCTTTGACCCGGCAGGAGGGGGAGATCCAATTCTTTATCAACACTTATT

>Faridpur4

CAGGAGCACTTCTTGGTGATGATCAAATCTATAATGTTATTGTTACTGCCCATGCTTTTGTAATAATTTTCTTTA
TAGTAATACCCATTCTTATTGGGGGATTTGGAAACTGACTTGTGCCACTAATGATTGGGGCCCCCGATATGGCA
TTTCCCCGAATAAATAATATAAGCTTCTGACTTCTTCCACCCTCATTTCTTCTTCTATTAGCTTCTTCTGGAGTTG
AAGCAGGAGCTGGAACAGGATGAACAGTTTATCCACCTCTTGCAGGCAACCTTGCCCATGCAGGAGCATCTGTT
GATCTAACAATTTTTTCACTACACTTAGCAGGTGTTTCATCTATTCTTGGAGCAATTAATTTTATTACTACTACAA
TTAACATGAAGCCACCAACTATCTCTCAGTATCAAACTCCATTATTTGTATGAGCTGTCTTAGTTACAGCTGTAC
TACTTCTTTTATCTTTACCAGTGTTAGCTGCCGGAATTACAATACTTCTTACAGACCGAAATCTTAACACAACGT
TCTTTGACCCGGCAGGAGGGGGAGATCCAATTCTTTATCAACACTTATT

>Khulna9

CAGGAGCACTTCTTGGTGATGATCAAATCTATAATGTTATTGTTACTGCCCATGCTTTTGTAATAATTTTCTTTA
TAGTAATACCCATTCTTATTGGGGGATTTGGAAACTGACTTGTGCCACTAATGATTGGGGCCCCCGATATGGCA
TTTCCCCGAATAAATAATATAAGCTTCTGACTTCTTCCACCCTCATTTCTTCTTCTATTAGCTTCTTCTGGAGTTG
AAGCAGGAGCTGGAACAGGATGAACAGTTTATCCACCTCTTGCAGGCAACCTTGCCCATGCAGGAGCATCTGTT
GATCTAACAATTTTTTCACTACACTTAGCAGGTGTTTCATCTATTCTTGGAGCAATTAATTTTATTACTACTACAA
TTAACATGAAGCCACCAACTATCTCTCAGTATCAAACTCCATTATTTGTATGAGCTGTCTTAGTTACAGCTGTAC
TACTTCTTTTATCTTTACCAGTGTTAGCTGCCGGAATTACAATACTTCTTACAGACCGAAATCTTAACACAACGT
TCTTTGACCCGGCAGGAGGGGGAGATCCAATTCTTTATCAACACTTATT

>Munshigonj10

CAGGAGCACTTCTTGGTGACGATCAAATTTATAATGTTATTGTTACTGCCCATGCTTTTGTAATAATTTTCTTTA
TAGTAATACCCATTCTTATTGGGGGATTTGGAAACTGACTTGTGCCACTAATGATTGGGGCCCCCGATATGGCA
TTTCCCCGAATAAATAATATAAGCTTCTGACTTCTTCCACCCTCATTTCTTCTTCTATTAGCTTCTTCTGGAGTTG
AAGCAGGAGCTGGAACAGGATGAACAGTTTATCCACCTCTTGCAGGCAACCTTGCCCATGCAGGAGCATCTGTT
GATCTAACAATTTTTTCACTACACTTAGCAGGTGTTTCATCTATTCTTGGAGCAATTAATTTTATTACTACTACAA
TTAACATGAAGCCACCAACTATCTCTCAGTATCAAACTCCATTATTTGTGTGAGCTGTCTTAGTTACAGCTGTAC
TACTTCTTTTATCTTTACCAGTGTTAGCTGCCGGAATTACAATACTTCTTACAGATCGAAATCTTAACACAACGT
TCTTTGACCCGGCAGGAGGGGGAGATCCAATTCTTTATCAACACTTATT

>KT624627.1_D.rerio strain AB

CAGGAGCACTTCTTGGTGATGATCAAATCTATAATGTTATTGTTACTGCCCATGCTTTTGTAATAATTTTCTTTA
TAGTAATACCCATTCTTATTGGGGGATTTGGAAACTGACTTGTGCCACTAATGATTGGGGCCCCCGATATGGCA
TTTCCCCGAATAAATAATATAAGCTTCTGACTTCTTCCACCCTCATTTCTTCTTCTATTAGCTTCTTCTGGAGTTG
AAGCAGGAGCTGGAACAGGATGAACAGTTTATCCACCTCTTGCAGGCAACCTTGCCCATGCAGGAGCATCTGTT
GATCTAACAATTTTTTCACTACACTTAGCAGGTGTTTCATCTATTCTTGGAGCAATTAATTTTATTACTACTACAA
TTAACATGAAGCCACCAACTATCTCTCAGTATCAAACTCCATTATTTGTATGAGCTGTCTTAGTTACAGCTGTAC
TACTTCTTTTATCTTTACCAGTGTTAGCTGCCGGAATTACAATACTTCTTACAGACCGAAATCTTAACACAACGT
TCTTTGACCCGGCAGGAGGGGGAGATCCAATTCTTTATCAACACTTATT

>WIK_KT624626.1_D.rerio strain

CAGGAGCACTTCTTGGTGACGATCAAATTTATAATGTTATTGTTACTGCCCATGCTTTTGTAATAATTTTCTTTA
TAGTAATACCCATTCTTATTGGGGGATTTGGAAACTGACTTGTGCCACTAATGATTGGGGCCCCCGATATGGCA
TTTCCCCGAATAAATAATATAAGCTTCTGACTTCTTCCACCCTCATTTCTTCTTCTATTAGCTTCTTCTGGAGTTG
AAGCAGGAGCTGGAACAGGATGAACAGTTTATCCACCTCTTGCAGGCAACCTTGCCCATGCAGGAGCATCTGTT
GATCTAACAATTTTTTCACTACACTTAGCAGGTGTTTCATCTATTCTTGGAGCAATTAATTTTATTACTACTACAA
TTAACATGAAGCCACCAACTATCTCTCAGTATCAAACTCCATTATTTGTGTGAGCTGTCTTAGTTACAGCTGTAC
TACTTCTTTTATCTTTACCAGTGTTAGCTGCCGGAATTACAATACTTCTTACAGATCGAAATCTTAACACAACGT
TCTTTGACCCGGCAGGAGGGGGAGATCCAATTCTTTATCAACACTTATT

>AY996924.1_D.rerio

CAGGAGCACTTCTTGGTGATGATCAAATCTATAATGTTATTGTTACTGCCCATGCTTTTGTAATAATTTTCTTTA
TAGTAATACCCATTCTTATTGGGGGATTTGGAAACTGACTTGTGCCACTAATGATTGGGGCCCCCGATATGGCA
TTTCCCCGAATAAATAATATAAGCTTCTGACTTCTTCCACCCTCATTTCTTCTTCTATTAGCTTCTTCTGGAGTTG
AAGCAGGAGCTGGAACAGGATGAACAGTTTATCCACCTCTTGCAGGCAACCTTGCCCATGCAGGAGCATCTGTT
GATCTAACAATTTTTTCACTACACTTAGCAGGTGTTTCATCTATTCTTGGAGCAATTAATTTTATTACTACTACAA
TTAACATGAAGCCACCAACTATCTCTCAGTATCAAACTCCATTATTTGTATGAGCTGTCTTAGTTACAGCTGTAC
TACTTCTTTTATCTTTACCAGTGTTAGCTGCCGGAATTACAATACTTCTTACAGACCGAAATCTTAACACAACGT
TCTTTGACCCGGCAGGAGGGGGAGATCCAATTCTTTATCAACACTTATT

>EUN228_JF915577.1_D.rerio isolate

CAGGAGCACTTCTTGGTGACGATCAAATTTATAATGTTATTGTTACTGCCCATGCTTTTGTAATAATTTTCTTTA
TAGTAATACCCATTCTTATTGGGGGATTTGGAAACTGACTTGTGCCACTAATGATTGGGGCCCCCGATATGGCA
TTTCCCCGAATAAATAATATAAGCTTCTGACTTCTTCCACCCTCATTTCTTCTTCTATTAGCTTCTTCTGGAGTTG
AAGCAGGAGCTGGAACAGGATGAACAGTTTATCCACCTCTTGCAGGCAACCTTGCCCATGCAGGAGCATCTGTT

GATCTAACAATTTTTTCACTACACTTAGCAGGTGTTTCATCTATTCTTGGAGCAATTAATTTTATTACTACTACAA
TTAACATGAAGCCACCAACTATCTCTCAGTATCAAACTCCATTATTTGTGTGAGCTGTTTAGTTACAGCTGTACT
ACTTCTTTTATCTTTACCAGTGTTAGCTGCCGGAATTACAATACTTCTTACAGATCGAAATCTTAACACAACGTT
CTTTGACCCGGCAGGAGGGGGAGATCCAATTCTTTATCAACACTTATT

>YGN413_JF915578.1_D.rerio isolate

CAGGAGCACTTCTTGGTGACGATCAAATTTATAATGTTATTGTTACTGCCCATGCTTTTGTAATAATTTTCTTTA
TAGTAATACCCATTCTTATTGGGGGATTTGGAAACTGACTTGTGCCACTAATGATTGGGGCCCCCGATATGGCA
TTTCCCCGAATAAATAATATAAGCTTCTGACTTCTTCCACCCTCATTTCTTCTTCTATTAGCTTCTTCTGGAGTTG
AAGCAGGAGCTGGAACAGGATGAACAGTTTATCCACCTCTTGCAGGCAACCTTGCCCATGCAGGAGCATCTGTT
GATCTAACAATTTTTTCACTACACTTAGCAGGTGTTTCATCTATTCTTGGAGCAATTAATTTTATTACTACTACAA
TTAACATGAAGCCACCAACTATCTCTCAGTATCAAACTCCATTATTTGTGTGAGCTGTCTTAGTTACAGCTGTAC
TACTTCTTTTATCTTTACCAGTGTTAGCTGCCGNNANTACAATACTTCTTACAGATCGAAATCTTAACACAACGT
TCTTTGACCCGGCAGGAGGGGGAGATCCAATTCTTTATCAACACTTATT

>NRM:41676_KT199751.1_D.catenatusCAGGAGCACTTCTTGGTGATGATCAAATTTATAATGTTA

TTGTTACTGCCCATGCCTTTGTAATAATTTTCTTTATAGTGATACCAATCCTTATCGGGGGATTTGGAAATTGAC
TTGTACCACTAATAATTGGAGCCCCCGACATAGCATTCCCACGAATAAATAACATAAGCTTTTGACTTCTCCCCC
CGTCATTCCTTCTCCTATTAGCCTCTTCAGGGGTTGAAGCAGGAGCCGGAACAGGATGGACAGTCTACCCACCT
CTTGCGGGTAATATTGCTCATGCAGGAGCATCTGTTGACCTGACAATTTTCTCGCTACATTTGGCAGGTGTTTCA
TCAATCTTAGGGGCAATTAATTTTATTACTACTACAATTAATATAAAACCACCAACTATTTCTCAATATCAAACCC
CTCTATTTGTATGAGCTGTTCTAGTAACGGCCGTACTACTTCTTTTATCATTACCAGTATTAGCTGCTGGAATTA
CAATACTTCTAACAGATCGAAACCTCAACACAACATTCTTTGACCCGGCTGGAGGGGGAGATCCCATTCTTTAC
CAACACTTATT

>NBFGR:BV8087A_FJ459455.1_D.rerio voucher

CCTGTATCTAGTATTTGGTGCTTGAGCCGGAATAGTAGGGACCGCATTAAGCCTCTTAATCCGAGCTGAACTTA
GCCAACCAGGAGCACTTCTTGGTGATGATCAAATTTATAATGTTATTGTTACTGCCCATGCTTTTGTAATAATTT
TCTTTATAGTAATACCCATTCTTATTGGGGGATTTGGAAACTGACTTGTGCCACTAATGATTGGGGCCCCCGATA
TGGCATTTCCCCGAATAAATAATATAAGCTTCTGACTTCTTCCACCCTCATTTCTTCTTCTATTAGCTTCTTCTGG
AGTTGAAGCAGGAGCTGGAACAGGATGAACAGTTTATCCACCTCTTGCAGGCAACCTTGCCCATGCAGGAGCAT
CTGTTGATCTAACAATTTTTTCACTACACTTAGCAGGTGTTTCATCTATTCTTGGAGCAATTAATTTTATTACTAC
TACAATTAACATGAAGCCACCAACTATCTCTCAGTATCAAACTCCATTATTTGTGTGAGCTGTCTTAGTTACAGC
TGTACTACTTCTTTTATCTTTACCAGTATTAGCTGCCGGAATTACAATACTTCTTACAGACCGAAATCTTAACAC
AACGTTCTTTGACCCGGCAGGAGGGGGAGATCCAATTCTTTATCAACACTTATT

>LC190394.1_M.erythromicron

CAGGAGCACTTCTTGGCGATGATCAAATTTATAATGTCATTGTTACCGCCCACGCTTTCGTTATAATCTTCTTTA
TAGTAATACCCATTCTTATTGGAGGATTTGGAAACTGACTAGTGCCCTTAATAATTGGGGCCCCGGACATAGCA
TTCCCACGAATAAACAACATAAGCTTCTGACTTCTTCCTCCCTCGTTTCTCCTTTTATTGGCTTCTTCTGGTGTTG
AAGCAGGAGCTGGAACAGGGTGAACAGTATATCCACCTCTTGCAGGCAACCTCGCCCACGCAGGAGCATCCGT
TGACTTAACTATTTTCTCACTACATTTAGCAGGTGTTTCATCAATTTTAGGGGCAATCAATTTTATTACCACTACA
ATTAATATAAAACCACCAACCATCTCCCAATACCAAACCCCACTATTTGTATGAGCTGTTCTAGTAACAGCTGTG
CTGCTTCTTTTATCTTTACCAGTATTAGCTGCTGGAATTACAATACTTCTCACGGACCGAAACCTTAACACAACA
TTCTTTGACCCGGCAGGAGGGGGAGACCCAATTCTTTACCAACACTTATT

>CTOL01500_HM224143.1_D.albolineatus isolate

CAGGGGCCCTCCTTGGAGACGACCAAATTTATAATGTAATTGTTACTGCCCACGCTTTTGTAATAATTTTCTTTA
TAGTAATGCCTATTCTTATTGGGGGTTTTGGAAACTGATTAGTTCCGTTAATAATTGGAGCCCCTGACATAGCAT
TCCCACGAATAAATAATATAAGTTTTTGACTTCTCCCCCCATCTTTTCTTCTTTTATTAGCTTCCTCTGGGGTTGA
AGCAGGAGCTGGGACAGGATGAACAGTATACCCACCTCTTGCAGGCAACCTCGCCCATGCAGGAGCATCTGTT
GATTTAACAATTTTTTCACTACACTTAGCAGGTGTCTCATCAATTCTTGGGGCAATTAATTTTATTAYGACTACG
ATTAACATAAAACCACCAACTATTTCACAGTATCAAACCCCTTTGTTTGTATGGGCTGTTCTAGTTACAGCCGTA
CTTCTTCTCTTATCTTTACCTGTTTTAGCTGCTGGAATTACAATGCTTCTTACAGATCGAAACCTTAATACGACG
TTTTTTGACCCGGCAGGAGGAGGAGATCCAATTCTCTACCAACACTTGTT

>NE-DR1_HQ141077.1_D.rerio voucher

CAGGAGCACTTCTTGGTGATGATCAAATCTATAATGTTATTGTTACTGCCCATGCTTTTGTAATAATTTTCTTTA
TAGTAATACCCATTCTTATTGGGGGATTTGGAAACTGACTTGTGCCACTAATGATTGGGGCCCCCGATATGGCA
TTTCCCCGAATAAATAATATAAGCTTCTGACTTCTTCCACCCTCATTTCTTCTTCTATTAGCTTCTTCTGGAGTTG
AAGCAGGAGCTGGAACAGGATGAACAGTTTATCCACCTCTTGCAGGCAACCTTGCCCATGCAGGAGCATCTGTT
GATCTAACAATTTTTTCACTACACTTAGCAGGTGTTTCATCTATTCTTGGAGCAATTAATTTTATTACTACTACAA
TTAACATGAAGCCACCAACTATCTCTCAGTATCAAACTCCATTATTTGTATGAGCTGTCTTAGTTACAGCTGTAC
TACTTCTTTTATCTTTACCAGTGTTAGCTGCCGGAATTACAATACTTCTTACAGACCGAAATCTTAACACAACGT
TCTTTGACCCGGCAGGAGGGGGAGATCCAATTCTTTATCAACACTTATT

>EUN179_JF915573.1_D.kyathit isolate

CAGGAGCACTTCTTGGCGATGATCAAATTTATAATGTTATTGTTACTGCCCATGCCTTTGTAATAATTTTCTTTA
TAGTAATACCTATTCTTATTGGGGGTTTTGGAAACTGACTCGTGCCACTAATGATTGGAGCCCCAGACATGGCA
TTCCCACGAATAAATAATATAAGCTTTTGACTTCTGCCACCTTCATTCCTTCTCCTATTGGCCTCTTCTGGGGTT
GAAGCGGGGGCTGGAACAGGATGAACAGTTTACCCACCTCTTGCAGGCAACCTCGCCCATGCAGGAGCATCCG
TTGATTTAACGATTTTTTCCCTGCACTTAGCAGGTGTCTCATCAATTCTTGGAGCAATTAACTTTATTACTACCA
CAATTAACATGAAACCGCCAACTATCTCTCAGTATCAAACTCCACTATTCGTTTGAGCTGTTTTAGTGACTGCCG
TACTTCTTCTTCTATCTTTACCTGTCTTAGCTGCCGGAATTACAATACTTCTTACAGATCGTAATCTTAACACAAC
ATTCTTTGACCCGGCAGGAGGGGGAGACCCAATCCTTTATCAACACTTGTT

>SZYD13031872A_KM207081.1_D.rerio voucher

CAGGAGCACTTCTTGGTGATGATCAAATCTATAATGTTATTGTTACTGCCCATGCTTTTGTAATAATTTTCTTTA
TAGTAATACCCATTCTTATTGGGGGATTTGGAAACTGACTTGTGCCACTAATGATTGGGGCCCCCGATATGGCA
TTTCCCCGAATAAATAATATAAGCTTCTGACTTCTTCCACCCTCATTTCTTCTTCTATTAGCTTCTTCTGGAGTTG
AAGCAGGAGCTGGAACAGGATGAACAGTTTATCCACCTCTTGCAGGCAACCTTGCCCATGCAGGAGCATCTGTT
GATCTAACAATTTTTTCACTACACTTAGCAGGTGTTTCATCTATTCTTGGAGCAATTAATTTTATTACTACTACAA
TTAACATGAAGCCACCAACTATCTCTCAGTATCAAACTCCATTATTTGTATGAGCTGTCTTAGTTACAGCTGTAC
TACTTCTTTTATCTTTACCAGTGTTAGCTGCCGGAATTACAATACTTCTTACAGACCGAAATCTTAACACAACGT
TCTTTGACCCGGCAGGAGGGGGAGATCCAATTCTTTATCAACACTTATT

>NRM:66253_KT199744.1_D.annulosus voucher

CAGGAGCACTTCTTGGTGATGATCAAATTTATAATGTTATTGTTACTGCCCATGCCTTTGTAATAATTTTCTTTA
TAGTGATACCGATTCTTATCGGGGGATTTGGAAATTGACTTGTACCATTAATAATTGGAGCTCCTGACATAGCAT
TCCCACGAATAAATAACATAAGCTTTTGACTTCTCCCTCCCTCATTCCTCCTCCTGTTAGCCTCTTCAGGGGTTG
AAGCGGGAGCCGGAACAGGATGAACAGTCTACCCACCTCTTGCGGGTAATATTGCTCATGCAGGAGCATCTGTT
GATTTAACAATTTTCTCGCTACATTTGGCAGGTGTTTCATCAATCTTAGGAGCAATTAATTTTATTACTACTACA
ATTAATATAAAACCACCAACTATTTCTCAATATCAAACCCCTTTATTCGTATGAGCTGTTCTAGTAACAGCCGTA
CTACTTCTTTTATCACTACCAGTATTAGCTGCTGGGATTACAATACTTCTAACAGATCGAAACCTCAACACAACA
TTCTTTGACCCGGCTGGAGGAGGAGATCCCATTCTTTACCAACACCTATT