Identification of oxidative stress responsive microRNA (miRNA) in economically important fishes of Bangladesh

A thesis submitted to the Department of Fisheries, University of Dhaka

in partial fulfillment of the requirements for the degree of

Master of Science (MS) in Fisheries

Submitted by

Examination Roll: 802 MS session: 2015- 16

Registration Number: 2010- 712- 845 Registration Session: 2010-11

Department of Fisheries

University of Dhaka, Dhaka- 1000

Bangladesh

February 2017

Declaration

I hereby declare that the dissertation entitled "**Identification of oxidative stress responsive microRNA (miRNA) in economically important fishes of Bangladesh**" 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. Mosammat Salma Akter, Assistant 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.

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Certification

We certify that the research work embodied in this thesis entitled "**Identification of oxidative stress responsive microRNA (miRNA) in economically important fishes of Bangladesh**" submitted by Jakir Hossain, roll number: 802, session: 2015-16, registration number: 2010- 712- 845 has been carried out under my supervision.

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

We wish every success in his life.

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Acknowledgements

On the accomplishment of the present study, all praises belong to the most Gracious, Merciful **ALMIGHTY ALLAH**, for successful completion of this work.

Words are inadequate in the available lexicon to avouch the excellent guidance given by my supervisor **Dr. Mosammat Salma Akter**, Assistant Professor, Department of Fisheries, University of Dhaka. Her words of encouragement, scholastic guidance, and excellent counsel help me to complete the research and thesis paper as accurately as possible.

I convey my heartfelt appreciation to **Dr. Haseena Khan**, Professor and **Ms. Ahlan Sabah Ferdous**, Lecturer, Molecular Biology Lab, Department of Biochemistry and Molecular Biology, University of Dhaka for their precious advice and technical support.

I would like to express my all sense of gratitude to **Mohammad Shahneawz Khan**, Assistant Professor, University of Dhaka for his ceaseless endurance and suggestions and for careful reading and reviewing the write-up the thesis.

My deepest appreciation to my co-supervisor **Dr. Md. Golam Rabbane**, Associate Professor,and **Goutam Kumar Kundu**, Assistant Professor, Department of Fisheries, University of Dhaka for their generous assistances and kind cooperation during the study period.

I would like to express my profound gratitude to **Dr Md. Ghulam Mustafa,** Professor, **Dr Mahmud Hasan**, Professor, and**Dr Mohammad Shamsur Rahman**, Associate Professor, Department of Fisheries, University of Dhaka for their kind advice during thesis work.

I convey my sincere gratitude to **Dr. Kaniz Fatema**, Professor & Chairperson, Department of Fisheries, University of Dhaka for her kind cooperation to continue my research.

I would like to acknowledge Ministry of Science and Technology for providing me the National Science and Technology (NST) fellowship.

Author

February 2017

Abstract

Distinct stress conditions, such as oxidative stress can alter microRNAs (miRNAs) expression that is known to regulate cellular processes. MicroRNAs (miRNAs) are a family of single-stranded RNA molecules about 22 nucleotides in length, which can regulate protein-coding gene expression in various organisms by post-transcriptional repression of mRNA. As there is no miRNAs study in fish species, this study was performed to identify the specific stress-responsive miRNAs in economically important fish of Bangladesh. Firstly, nucleotide databases such as ESTs (Expressed Sequence Tags) and GSSs (Genome Survey Sequences) of Rohu (*Labeo rohita*), Catla (*Catla catla),* Silver carp (*Hypophthalmichthys molitrix*), Climbing perch (*Anabas testudineus),* Hilsa (*Tenualosa ilisha*), and Gangetic mystus(*Mystus cavasius)* were mined against known fish miRNAs database to identify evolutionarily conserved miRNAs in these fish species. Secondly, a database on stress responsive miRNA in fish was built and then the study confirmed the presence of miR-21 (oxidative stress responsive miRNA) in these fish including Zebrafish (*Danio rerio*).The target genes of all studied miRNAs were further predicted using *in silico* approach and the possible regulating mechanisms of miRNA were discussed. This study deemed to employ experimental and computational methodsfor the first time to identify miRNAs in fish of Bangladesh. Future perspectives of this study are to focus on function, stress dose-responses and temporal expression of the identified miRNAs and to facilitate their use as initial markers for stress responses.

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Symbols and Abbreviations

Bp	**Base pair**
Cm	Centimeter
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra acetic acid
eg	For example (exempligratia)
et al	And others (et alliori)
EtBr	Ethidium Bromide
HMM	Hidden Markov Model
miRNAs	microRNAs
mL	Milliliter
mM	Millimolar
mRNA	Messenger RNA
NaCl	Sodium Chloride
NCBI	National Centre for Biotechnology Information
ng	Nanogram
PCR	Polymerase chain reaction
Pre-miRNAs	Precursor-microRNAs
Pri-miRNAs	Primary microRNAs
RNA	Ribonucleic acid
rpm	Rotations per minute
TAE	Tris-acetate EDTA
Taq	*Thermus aquaticus*
UTRs	Untranslated regions
w/v	Weight per volume
Mg	Microgram
µL	Microliter

Chapter 1

Introduction

1.1 Background

Aquatic ecosystems are enriched with a great diversity of organisms ranging from prokaryotes to higher vertebrates including fish and other aquatic organisms and these ecosystems also act as a basin for a boundless variety of anthropogenic contaminants that cause aquatic pollution (Ekambaram et al., 2014). Anthropogenic contaminants and pollutants impose stress to fish and other aquatic organisms that result in a serious problem in fish production (Devasagayam et al., 2004). Exposure to contaminants (i.e. industrial effluents, agricultural run-off, pesticides etc.) in aquatic ecosystems can intensify the cellular level of reactive oxygen species (ROS: peroxides, superoxide, hydroxyl radical, and singlet oxygen) which can generate oxidative stressin living cells (Devasagayam et al., 2004). This oxidative stress can induce oxidative damage (i.e. protein and lipid damage) to fish (Kong and Lin, 2010a). Recent studies indicate that ROS can interfere translational process through the oxidative modification of RNA, either microRNAs (miRNAs) or mRNA (messenger RNA), hence producing less protein and/or defective protein leading to injurious cellular function (Kong and Lin, 2010a).

MicroRNAs (miRNAs) are the class of highly conserved small non-coding RNA molecules with typically 22 nucleotides that regulate gene & protein expression by fine tuning mRNA translation (He and Hannon, 2004; Yang et al., 2011). miRNAs are involved in multiple intracellular processes by degrading targeted mRNAs or blocking mRNA translation in various organisms and control numerous biological processes, i.e. evolving timing, cell disparity, cell propagation, immune response and infection (Baltimore et al., 2008; Bicker and Schratt, 2008; Chen et al., 2005; Lodish et al., 2008; Saj and Lai, 2011). Emerging data suggest that distinct stress condition such as hypoxia, oxidative stress can alter the biogenesis and expression of miRNAs, the expression of mRNA targets and the activities of miRNA-protein complexes (Leung and Sharp, 2010a; Lin et al., 2009; Magenta et al., 2011).

1.1.1 Stress and response in fish: An overview of baseline information

1.1.1.1Stress and its causes in fish

Stress is any state that causes physical or biological distress that results in the release of stress-related hormones or results in specific physiological responses (Pasnik et al., 2010). Stress in fish consequences from biotic and /or abiotic challenges that act in modifying or changing the fish's natural or homeostatic state (Porchas, 2014;

Schneiderman et al., 2005). Stress exists in the lives of all living things and is the force that brings about physical change and adjustment. Stress can be physical, biological, or environmental; and it can either be short and abrupt, or prolonged and chronic. Mild, short-term stress has little health effects, but long-term stress or severe short-term stress contribute to many disorders and deaths of fish.

'Stressors' the causes of stress in fish

A stressor is a chemical or biological mediator, environmental disorder, external inducement or an event that causes stress to an organism. Such as environmental stressors (hypo or hyperthermic temperatures, Salinity, Pesticides, overcrowding etc.). Fish are exposed to biological and chemical stressors in the wild and captivity. Environmental pollutants, pesticides, agricultural run-off, disease, and various aspects of aquaculture are some examples of those stressors. Although pollutants are the common stressors, fish also can become physiologically stressed from psychological stressors such as exposure to predators and gathering (**Figure 1.1**). Moreoverother factors such as extreme temperature, pH, alkalinity, total hardness, ammonia, gas content, salinity and oxygen level etc. also contribute acute or chronic stress to fish depending on their intensity (Barton, 2002; Pasnik et al., 2010; Schneiderman et al., 2005).

Common fish stressors include alterations in the fish's immediate environment such as:

- Chemical changes - contaminants, pesticides, low oxygen, and acidification
- Physical changes - handling, capture, confinement and transport
- Perceived changes - startling or predators

The above stressors are characterized as either acute or chronic depending on their extent and incidence. At acute stress, fish experiences the high intensity of stress for a short time such as during excess handling or at a high level of pollutants. Chronic fish stressors are defined as a constant or habitual exposure that causes a persistent physiological response. Both types of fish stressors have severe bad effects on the fish growth and health. In most circumstances, stress can result in a change of habitat or a disruption in routine and behavior of fish.

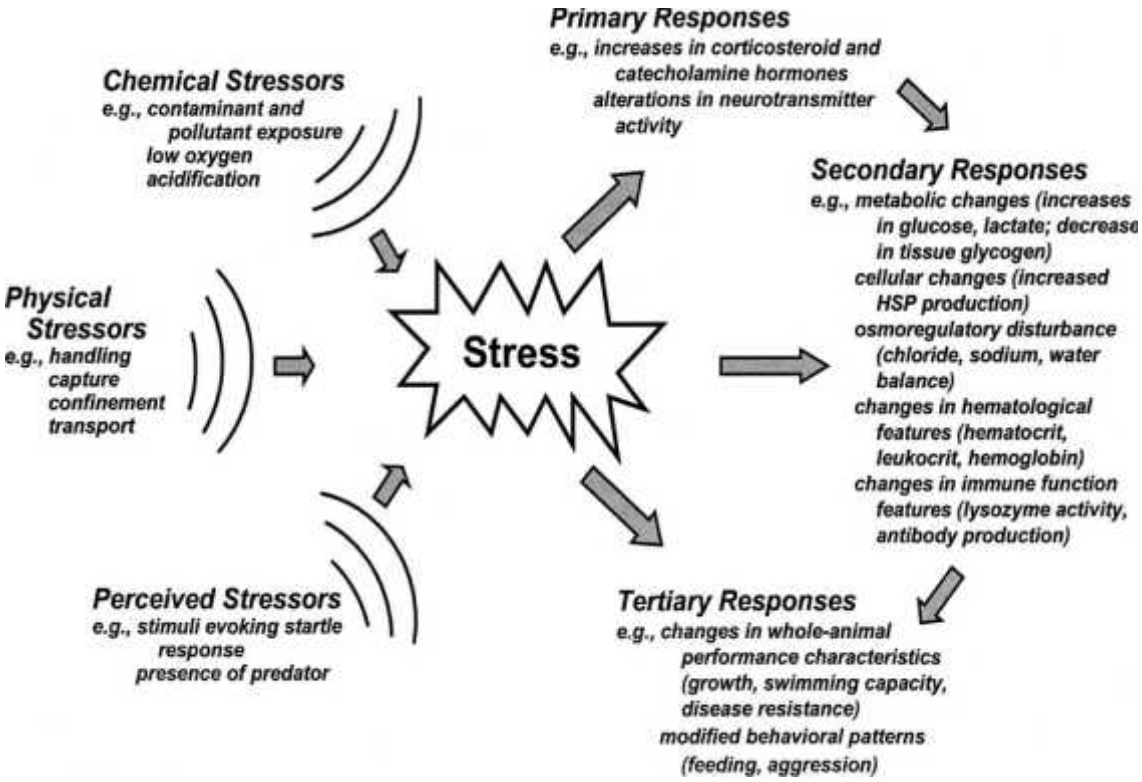

Figure 1. 1 Physical, chemical, and other supposed stressors act on fish to induce physiological and related effects, which are classified as primary, secondary and tertiary or whole-animal responses. In some cases, the primary and secondary responses, in turn, may actively affect secondary and tertiary responses, respectively, as shown by the arrows (Barton, 2002)

1.1.1.2 Physiological response to stress in fish

Stress responses in fish are chemical signaling systems linked with possibly adverse physiological consequences. The particular glucocorticoid, cortisol, is a prime hormone released in response to stressful stimuli in fish species (Anderson, 2016). Ray-finned fishes (teleosts) demonstrate cortisol-driven stress responses when exposed to certain stimuli (Anderson, 2016).Fishes show a widespread variation in their physiological responses to stress, which is undoubtedly apparent in the plasma corticosteroid changes, chiefly cortisol in actinopterygian fishes that occur following a stressful event. Generally, stress in fish is a non-specific response categorized as three different responses (Anderson, 2016; Barton, 2002; Iwama et al., 1999):

Primary response: The primary response is considered by the stimulation and the secretion of the hormones, corticosteroids (cortisol) and catecholamines, into the blood.

Secondary response: The release of hormones activates the secondary response which includes the relief of glucose into the blood for energy invention, followed by proliferations in heart rate, gill blood flow and metabolic rate which causes modifications in blood lactate and hematocrit.

Tertiary response: Changes in blood physiology eventually cascade into an entire body change or the tertiary stress responses. Changes related to the tertiary stress response contain decreased growth rate, reduced disease resistance, changed behavior and decreased survivability.

1.1.2Oxidative stress in fish

Aquatic organisms face a variety of stresses during their continuous dealings with the environment. Environmentally-induced stresses frequently stimulate the endogenous production of reactive oxygen species (ROS), most of which are produced as side products of tissue respiration that causes oxidative stress to fish (Stoliar and Lushchak, 2012a).

A disruption in the balance between the prooxidants and antioxidants leading to injurious biochemical and physiological effects is known as oxidative stress. This is a detrimental situation in which increase in free radical production, and/or drops in antioxidant levels can lead to potential damage of lipids, proteins, and DNA (Ekambaram et al., 2014).

1.1.2.1 Causes of oxidative stress in fish

Oxidative stress arises when the production of reactive oxygen species (ROS) beats the endogenous protection afforded by antioxidant enzymes like superoxide dismutase, a redox-sensitive thiol compound, catalase, glutathione S-transferase and compacted glutathione (Ekambaram, Narayanan, & Jayachandran, 2014).Reactive oxygen species (ROS) are biochemically reactive chemical species having oxygen. Examples include singlet oxygen, peroxides, hydroxyl radical, and superoxide (Hayyan et al., 2016). Reactive oxygen species are amplified by substances for instance pesticides, transitional metal ions and petroleum pollutants (Lushchak, 2011; Slaninová, Smutna, Modra, & Svobodova, 2009).

Aquatic ecosystems are under the pressure of multifaceted mixtures of contaminants released into the environment due to various human activities. They may originate from diverse sources such as chemical and drug manufacture, oil refineries, domestic sewage,

mining, polymer and petrochemical-based industries, glass blowing, battery manufacture and many others that cause oxidative stress in fish. Thus, aquatic environment is a sink for many environmental contaminants which can be absorbed by fish and cause oxidative stress in fish.

1.1.2.2 Effects of oxidative stress in fish

In fish, oxidative stress has been recognized in both field and laboratory exposure studies. This can be expressed in the form of upregulation of antioxidant enzymes as well as rises in oxidative damage, including DNA damage, protein carbonyls etc. Exposures to these different environmental toxicants can often result in cancer, not only in humans but in fish as well (Kelly et al., 1998)

Chemically, oxidative stress is related to thehigher production of oxidizing species or a substantial decrease in the efficiency of antioxidant defenses, such as glutathione (Schafer and Buettner, 2001). The effects of oxidative stress depend upon the size of these changes, with a cell being able to overwhelm small agitations and recover its original state. However, more severe oxidative stress can cause cell death, and even reasonable oxidation can activate apoptosis, while more extreme stresses may cause necrosis (Lennon et al., 1991). Under the severe levels of oxidative stress that cause the damage causes ATP depletion, preventing orderly apoptotic death and causing the cell to simply fall apart (Lee and Shacter, 1999; Lelli et al., 1998).

1.1.3 Oxidative stress and miRNA biology in fish

Environmental pollution is a major concern due to disorganized and increased industrialization and urban development. Oxidative stress in fish is a general result of the environmental pollution.

Mammalian studies have revealed that prenatal exposure to certain environmental stresses (e.g., hypoxia) and chemical contaminants (e.g., endocrine disrupting chemicals) can lead to modifications in phenotypes (e.g., delayed sexual and physical development, infertility, increased mortality and behavioral alterations) in successive peer group, despite the offspring have never been straightly exposed to the stress and contaminants before (Anway et al., 2005; Dunaeva et al., 2008). Such transgenerational effects may not be triggered by changes in DNA sequence but by alterations in the epigenome. For example, changes may happen in DNA methylation, covalent alteration of histones, or the activation or silencing of genes by miRNAs (Skinner et al., 2011). miRNAs have

been shown to modulate cellular differentiation and development (Baltimore et al., 2008; Bartel, 2004; Ketting, 2010; Schier et al., 2006).

Overproduction of free radicals initiating oxidative stress can impair cellular components resulting in progressive physiological dysfunction (Lin et al., 2009; Mendell, 2017; Pan et al., 2013). Oxidative damage to RNA got slight attention until the past decade. Recent studies specify that RNA, such as messenger RNA and ribosomal RNA, is very susceptible to oxidative damage (Kong and Lin, 2010a). Oxidative regulations to microRNAs (miRNA) are shown to result in disturbance of the translational process and impairment of protein synthesis, which can cause cell deterioration or even cell death (Gupta, 2014; Kong and Lin, 2010b; Simone et al., 2009).

1.1.4 MicroRNA

MicroRNA (miRNA), a class of highly conserved small non-coding RNA molecules with typically 22 nucleotides, is found in a wide variety of organisms. miRNAs act as transcriptional and post-transcriptional negative controller of gene expression through base-pairing with complementary sequences of mRNA molecules, leading to gene silencing through suppression of translation or mRNA degradation (Bicker and Schratt, 2008; He and Hannon, 2004). miRNAs are known to regulate cellular processes such as differentiation, cell cycle, immune functions, apoptosis, cancer cell formation and their expression can be changed by different stress conditions (Baltimore et al., 2008; Garzon et al., 2009; Pan et al., 2013; Tang et al., 2013).miRNA expression can be transformed by distinctive stress conditions such as hypoxia or oxidative stress (Lin et al., 2009; Magenta et al., 2011).

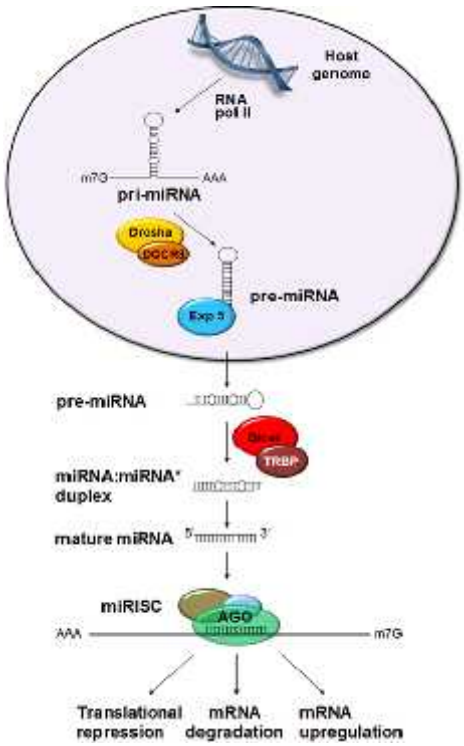

Figure 1. 2 Canonical biogenesis of miRNA

primary miRNAsliced by Drosha in conjunction with Pasha (= DGCR8 in vertebrates) and transported into the cytoplasm by Exportin-5 (Exp 5) where it is again treated by Dicer 1 in associationwith the *loquacious* protein (= TAR RNA binding protein in mammals, TRBP) to generate a ~22 nt miRNA:miRNA* duplex.RNA-induced silencing complex (RISC) having the argonaute (Ago) protein complex act together with the target sequences leading to suppression of translation, mRNA degradation, or upregulation of transcript levels(Asgari, 2011).

Biogenesis of miRNA:

1. miRNA synthesis starts in the nucleus, where miRNAs are generated from intergenic regions, from introns of protein-coding genes or from exons of non-coding genes(Saj and Lai, 2011).

2. miRNA genes are expressed generally by RNA polymerase II in the nucleus making the primary miRNA (pri-miRNA) (**Figure 1.2**). The size of the pri-miRNA varied from a few hundred base pairs to thousands.

3. The pri-miRNA may comprise one or several stems–loop structures that are sliced from the stem by the nuclear RNase III-type enzyme Drosha in conjunction with its cofactor Pasha (corresponding to the DGCR8 protein in vertebrates). The Drosha–Pasha/DGCR8 protein complex was denoted to as the microprocessor complex (Gregory et al., 2006).

4. The cleavage results in the generation of the hairpin precursor miRNA (pre-miRNA) which is nearly 70 nt (Winter et al., 2009). In certain cases, miRNAs may be made from introns, without the

participation of Drosha by splicing mechanisms (Ruby et al., 2007)

Although the most known function of miRNA is to diminish mRNA translation, animal miRNAs are engaged also in translational promotion and suppression of other noncoding RNAs. Post-transcriptional suppression by miRNAs can be attained through blockage of translation initiation, inhibition of translation elongation, premature termination of translation, or deadenylation (Ketting, 2010).

1.1.4.1Techniques for identification and confirmation of miRNAs

Most of the known miRNAs are very much evolutionarily preserved from species to species, ranging from insects to humans (Daido et al., 2014; Maher et al., 2006; Niwa and Slack, 2007). Conservation among species became one of the most important belongings of miRNAs. So, this feature would enable us to achieve the computational search for miRNAs based on the highly conserved sequence in the mature miRNAs and long stem-loop structures in miRNA precursors (Mishra and Lobiyal, 2011). There are some momentous advantages of detecting miRNAs by acomputationalmethod because it is accurate, fast, and inexpensive compared to the experimental method. For this reason, computational approaches offer an ideal technique for recognizing miRNAs in various organisms by using genome survey sequence (GSS) and expressed sequence tags (EST) databases, especially in species which genome sequences are not known. Using this method, a large number of miRNAs have been successfully identified in several plants and animal species (Akter et al., 2014; M. Y. K. Barozai, 2012; Luo et al., 2009; Yousef et al., 2009).

1.1.4.2*In Silico* analysis of miRNAs

MicroRNAs (miRNAs) are hairpin-derived RNAs that suppress the expression of target genes, typically by binding to the 3'-untranslated region (UTR) of mRNA (Chen and Rajewsky, 2007). More than 60% protein-coding genes are computationally predicted to be targets based on base-pairing between the 3'-UTR and the 5'-seed region of the miRNA (Friedman et al., 2009). MicroRNAs can show a vital role in gene modulation via posttranscriptional gene regulation during acclimation and adaptation, in the case of adverse environments or climate change for example. These are beneficial and considerable contributors to regulatory networks of expansion and adaptive plasticity in fishes (Rasal et al., 2016).

With the improvement and convenience of experimental technologies and computational methods, the field of miRNA biology has advanced over the last decade. In recent years,

a cumulative number of miRNAs have been recognized and deposited in the major miRNA databases. Although hundreds of miRNA have been identified, only a very small number of fish miRNAs have been discovered and functionally identified. Several approaches are used to identify new miRNAs in fish. Direct cloning is a typical technique that has facilitated the identification of miRNAs in zebrafish (Kloosterman et al., 2006) and rainbow trout (Ramachandra et al., 2008). However, it is difficult to clone low-abundance miRNAs because all miRNAs have similar secondary hairpin structures and many of these structures are evolutionarily conserved (Ambros et al., 2003).

Currently, *in silico* methods, high-throughput sequencing and computational methods have been commonly used to detect new miRNAs in fish. These sequencing technologies comprise genome-wide small RNA studies, thus, providing a global view of small RNA in different species. Using the small RNA deep sequencing protocol with *in silico* analysis, known or new miRNAs were identified in zebrafish (Soares et al., 2009), medaka (Li et al., 2010), Atlantic halibut (Bizuayehu et al., 2013), common carp (Zhu et al., 2012), and catfish (Xu et al., 2013). However, computational strategies based on expressed sequence tags (EST) and genome survey sequences (GSS), has been fruitful for identifying new miRNAs (mature and precursor) in fish (M. Barozai, 2012; Hairul-islam et al., 2014).

This will be helpful in the downstream analysis to recognize and enumerate miRNAs using computational tools and to expose their role in gene regulation using sequencing technology (Burnside et al., 2008; Kaeuferle et al., 2014). The data produced through this process have been used for gene innovation, variant documentation, marker detection and miRNA identification (Rasal et al., 2016).

1.1.4.3Computational tools/algorithms used for miRNA investigation

Advanced computational approaches are used for identifying miRNAs and their targets which perform an important role in gene regulation. In recent years, a large number of additional putative-miRNAs in various organisms including fishes have been identified. Many algorithms such as miRDeep, TargetScan, DIANAMicroT, RNAhybrid, MIReNA, miRExplorer, miRanalyzer, and miR Tools are being developed for miRNA and their target identification. The identification of miRNA targets is mainly established by Watson–Crick complementarity in the seed region between the miRNA and the target region of the gene. Those algorithms are based on the construction of a hairpin loop

secondary structure with lowest folding free energy and on the existence of mature miRNAs in the stem of the secondary structure and evolutionary conservation (Lim et al., 2003). Databases, such as miRBase(http://microrna.sanger.ac.uk/) containing miRNA sequence data, annotation and predicted miRNA gene targets have been used for miRNA identification. The selection of mature miRNAs was founded on various criteria such as mature miRNA having 0–4 mismatches in the sequence, stem-loop hairpin precursor structure or lower minimal free energy (MFE) (Huang et al., 2015). Further, the secondary configurations of all the selected miRNAs sequences need to generate using RNA Fold webserver (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). It provides results for the minimum free energy of miRNA, free energy of the thermodynamic ensemble, the frequency of the MFE structure and ensemble diversity. Thus, most of the algorithms are established on base pairing patterns and evolutionary conservation of the secondary structure of target transcript and nucleotide arrangement of target sequences (Grimson, 2010). The structural analysis of anticipated miRNAs could be carried out by means of web servers as well as offline or standalone tools.

Several computational tools are being utilized for the miRNA discovery and their target site identification. The computational methods are beneficial for accurate illustration of miRNA and to provide evidence for further studies. Below, a brief about the tools are described that have been used for miRNA identification and their target prediction based on different algorithms.

A. MicroRNA discovery tools

Numerous computational approaches have been implemented for miRNA gene prediction using methods based on sequence conservation and ⁄ or structural similarity. Some of these tools are listed in **Table 1.1**.

Table 1.1Computational tools for miRNA prediction

Algorithm	Web link	References
proMiR II	http://cbit.snu.ac.kr/~ProMiR2/	(C Xue et al., 2005)
mir-abela	http://www.mirz.unibas.ch/cgi/pred_miRNA_genes.cgi	(Sewer et al., 2005)
triplet-SVM	http://bioinfo.au.tsinghua.edu.cn/mirnasvm/	(Chenghai Xue et al., 2005)
Vmir	http://www.hpi-hamburg.de/fileadmin/downloads/VMir.zip	(Grundhoff et al., 2006)
RNA micro	http://www.bioinf.uni-leipzig.de/~jana/software/index.html	(Hertel and Stadler, 2006)
miRDeep	https://omictools.com/mirdeep-tool	(An et al., 2013)
RNAhybrid	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid	(Rehmsmeier et al., 2004)
Mfold	(http://www.bioinfo.rpi.edu/applications/mfold/)	(Zuker, 2003)

MirDeep

MirDeep was the first computational tool to be established for miRNA investigation using miRbase data (An et al., 2013). This software was developed to extract putative precursor structures and expect secondary structures using RNAfold after genome alignment of the sequences repossessed by NGS. It scores compatibility of the position and frequency of sequenced RNA with the secondary structures of miRNA precursor. Further, it identifies novel, conserved and non-conserved miRNAs with a high confidence score and based on their alignment using stem-loop sequences. The sequence

having highest countenance is recognized as a mature miRNA sequence. This software is standalone and can be run on a local machine using any operating system. Preinstallation of any other programs to support this software is not required.

MirEval

MirEval is used to expect miRNA precursor. These precursor sequences are used for BLASTx (http://www.ncbi.nlm.nih.gov) analysis for removing the protein-coding sequences and retain only non-protein encoding sequences.

RNAhybrid

The RNAhybrid is an addition of classical RNA secondary structure prediction software tools such as RNAfold and Mfold. The secondary structures of putative pre-miRNAs can be identified using RNAfold in the Vienna-RNA package.

miRanalyzer

The miRanalyzer is a machine learning approach for novel miRNA discovery based on the random forest method. It can be applied to miRNA discovery from different model organisms, including fish to build the final prediction.

B. Target identification tools

Although recent findings (X. H. Li et al., 2015; Schaeffer and Wold, 2010) suggest that miRNAs may affect gene expression by binding to either 5'- or 3'-UTRs of mRNA, most studies have found that miRNA mark their target mRNAs for degradation or suppress their translation by binding to the 3'-UTR and most target programs search there. These studies have suggested that the miRNA seed segment, which includes six to eight nucleotides at the 5' end of the mature miRNA sequence, is very important in the selection of the target site (**Figure 1.3**). Thus, most of the computational tools developed to identify mRNA target sequences depend heavily on complementarity between the miRNA seed sequence and the target sequence. Diana-microT (Kiriakidou et al., 2004) was one of the first computational tools for target prediction that identified specific interaction rules based on bioinformatics and experimental approaches.

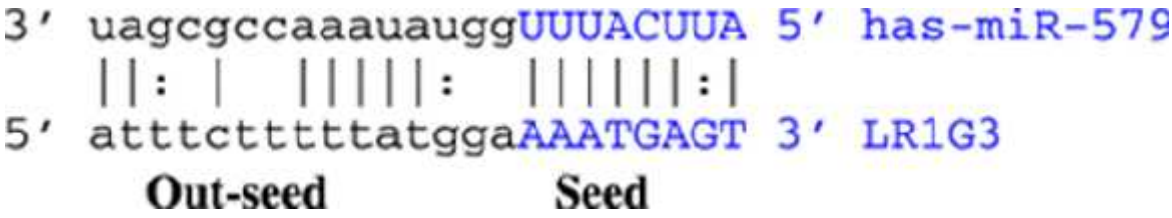

Figure 1. 3 The duplex for miRNA hsa-miR-579 and its target LRIG3 (Leucine-Rich Repeats and Immunoglobulin-like Domains 3) is partitioned into two parts, the seed part, and the out-seed part. The seed part is indicated by capital letters

Several additional tools (**Table 1.2**) for the prediction of miRNA targets have been subsequently developed. These tools mainly use sequence complementarities, thermodynamic stability calculations and evolutionary conservation among species to determine the likelihood of formation of a productive miRNA–mRNA duplex.

Table 1. 2 MicroRNA target prediction tools

Algorithm	Web link	References
TargetScanS	http://genes.mit.edu/targetscan	(Lewis et al., 2003)
miRanda	http://www.microma.org	(McWilliam et al., 2013)
PicTar	http://pictar.bio.nyu.ed	(Krek et al., 2005)
Target Boost	https://demo1.interagon.com/demo	(SaeTrom et al., 2005)
Rna22	http://cbcsrv.watson.ibm.com/rna22_targets.html	(Williamson et al., 2013)
Diana-microT	http://www.diana.pcbi.upenn.edu/cgi-bin/micro_t.cgi	(Kiriakidou et al., 2004)

TargetScan

TargetScan is an algorithm developed to recognize the targets of vertebrate miRNAs such as fish, human, mouse etc. TargetScan calculates the miRNAs targets by searching for the presence of conserved 8 and 7-mer sites that match the seed region of each

miRNA. The program participates thermodynamics- based modeling of miRNA–mRNA interactions and comparative sequence analysis to predict miRNA targets conserved across multiple genomes of species. The software is dependable because of a low rate of false positives. However, the software may have limited applicability as it is based on a prediction of miRNAs with replacement less than one between the species.

miRanda

This program predicts miRNA targets based on three properties such as sequence complementarity, conservation of target sites and free energies of RNA–RNA duplexes in related gene sequences. The disadvantages contain the occurrence of false negatives and unreliability of Smith–Waterman algorithm as it works better for comparison of sequences which are evolutionarily correlated.

DIANA-microT

DIANA-microT uses a window of 38 nucleotides that progressively go through a 3' UTR of the target. Here, mRNA UTR structure is integrated to predict microRNA targets, while MicroRNA targets are conserved across species. The DIANA-microT provides wide-ranging online connectivity by means of web-service to the biological resources. This server is mainly connected to a different online server, such as UniProt, iHOP, KEGG, and miRBase, respectively. On the other hand, DIANA-microT can sort out pre-miRNAs from pseudo hairpins.

PicTar

PicTar checks the alignments of 3' UTRs for those displaying seed site matches to miRNAs, filters the retained alignments based on their thermodynamic stability and estimates a Hidden Markov Model (HMM) maximum likelihood score (PicTar score) for each predicted target. It has advantages in that translation repression increases exponentially with increased miRNA binding sites in 3' UTR.

C. Databases for microRNA and targets

There is a variety of very useful databases that provide a significant amount of information on miRNA and Target predictions (**Table 1.3**). The most extensive database for both miRNA and target sequences is miRBase (Kozomara and Griffiths-Jones, 2011).

The miRBase database provides a searchable online repository for published microRNA sequences and associated annotation. miRBase also provides a gene naming and nomenclature function in the miRBase Registry. Release 21 of the database contains 28645 entries representing hairpin precursor miRNAs, expressing 35828 mature miRNA products, in 223 species including primates, rodents, birds, fish, worms, flies, plants, and viruses. The data are freely available to all through the web interface at http://www.mirbase.org/ and in flat file form from ftp://mirbase.org/pub/mirbase/. miRBase contains miRNA mature sequences, hairpin sequences of precursors and associated annotation.

Table 1. 3 Databases for microRNA and targets

Database	Web link
MiRBase	http://microrna.sanger.ac.uk/
Argonaute	http://www.ma.uni-heidelberg.de/apps/zmf/argonaute/
miRecords	http://mirecords.umn.edu/miRecords/

Several approaches are used for justification and confirmation of computationally identified miRNAs, such as profiling the miRNAs using microarray platforms or real-time PCR. The expression pattern of miRNA by RT-PCR is one of the ideal methods for confirmation and expression behavior of miRNAs. Identification of precursor miRNAs from genomic DNA by PCR is the reliable and inexpensive approach. Enzymatic alteration of miRNAs, such as RNA manipulation and 30 nucleotide trimmings has been used previously. Microarray techniques, quantitative real-time PCR, and RNA-seq are all widely used for explanation of miRNAs. Northern blotting, PCR, and 50 rapid amplification of cDNA ends (50RACE) are also used for miRNA confirmation. Recent progressions in gene editing technology such as TALEN and Cas9 systems in harmonization with homologous recombination facilitated transgene addition at precise locations within the genome has made it possible to identify the possible functions of the gene of interest. It was revealed that miRNA vector is operational in triggering enhanced

Green Fluorescent Protein (eGFP) knockdown in a transient in vivo eGFP assay via gene knockdown in zebrafish (Leong et al., 2012).

1.1.4.4miRNAs in fish

According to miRBase version 21(the microRNA database), identified precursors and mature miRNAs in fish is listed in **Table 1.4**(Kozomara and Griffiths-Jones, 2014),

Table 1. 4 Total miRNAs recorded in fishes (miRBase version 21)

Name of fish	miR name	No. of precursor miRNAs	No. of mature miRNAs
Cyprinus carpio	>ccr	134	146
Danio rerio	>dre	346	350
Fugu rubripes	>fru	131	108
Hippoglossus hippoglossus	>hhi	40	37
Ictalurus punctatus	>ipu	281	205
Oryzias latipes	>ola	168	146
Paralichthys olivaceus	>pol	20	38
Salmo salar	>ssa	371	498
Tetraodon nigroviridis	>tni	132	109

1.1.4.5 Roles of miRNAs in fish biology

miRNAs involved in developmental time and morphogenesis

miRNA profile of the zebrafish (*Danio rerio)* resolved their role in the developmental, cellular processes and their role varied cell type-specific manner (Chen et al., 2005). In zebrafish,total 154 distinct miRNAs expressed from 343 miRNA genes were identified (Chen et al., 2005). miRNAs have modulatory roles in evolving time at rainbow trout (Ramachandra et al., 2008). miRNAs expression was shown to regulate embryogenesis in fish (Flynt et al., 2009; A. J. Giraldez et al., 2006; Kapsimali et al., 2007).

miRNAs in environmental stress

Various sorts of environmental stresses, such as drought, salinity, osmotic stress, oxidative stress, hypoxic stress, high temperature, low temperature, UV light, heavy metal, and pollutant exposure are known to induce the aberrant expression of miRNAs in a dose and stress-dependent manner. Remarkably, some miRNAs are commonly

responsive to all stresses. Gathering evidence, however, clearly shows that differential expression of certain miRNAs is dependent on the specific stress condition, even in the same species (Leung and Sharp, 2010b; Magenta et al., 2013).

Temperature raised to several degrees during embryonic and larval developmental stages of Atlantic cod (*Gadus morhua*) was shown to significantly alter the miRNA profile and 389 putative miRNA precursor loci, 120 novel precursor miRNAs, and 281 mature miRNAs were identified. Some miRNAs showed stage- or tissue-enriched expression and miRNAs, such as the miR-17 ~ 92 clusters, myomiRs (miR-206), neuromiRs (miR-9, miR-124), miR-130b, and miR-430 showed differential expression in different temperature regimes. Long-term effect of embryonic incubation temperature was discovered on expression of some miRNAs in juvenile pituitary (miR-449), gonad (miR-27c, miR-30c, and miR-200a), and liver (let-7 h, miR-7a, miR-22, miR-34c, miR-132a, miR-192, miR-221, miR-451, miR-2188, and miR-7550), but not in brain. Some of differentially expressed miRNAs in the liver were confirmed using LNA-based RT-qPCR (Bizuayehu et al., 2015).

miRNAs under hypoxic stress

Identification and Expression Profiling of microRNAs in the Brain, Liver, and Gonads of Marine Medaka (*Oryzias melastigma*) was studied in response to Hypoxia (Lau et al., 2014). It showed that a total of 223 distinct miRNA types were identified in marine medaka, with the greatest number expressed in brain tissue. The data suggested that 55 miRNA types from 34 families are common to all tested tissues, while some of the miRNAs are tissue-enriched or sex-enriched. Quantitative real-time PCR analysis further demonstrated that let-7a, miR-122, and miR-9-3p were downregulated in hypoxic female medaka, while miR-2184 was specifically upregulated in the testis of hypoxic male fish.

miRNAs expression under hypoxia was also studied in Atlantic salmon (*Salmo salar*). Using a novel high-throughput sequencing (RNA-Seq) method a total of 18 miRNAs were found significantly differentially expressed in exposed compared to control fish, 4 were down-regulated and 14 were up-regulated (Kure et al., 2013). Besides these, small RNA sequencing was done to determine the change in miRNA profile in the ovary of marine medaka (*Oryzias melastigma*) under hypoxic stress. A total of 509 miRNAs were found in the ovary of marine medaka, in which, 33 and 10 miRNAs were found to be

significantly upregulated and downregulated under hypoxia, respectively. Bioinformatics analysis highlighted that a large number of hypoxia-suppressed miRNAs that target a variety of steroidogenic enzymes including steroidogenic acute regulatory protein, aromatase, and 17-alpha-monooxygenase. Also, estrogen receptor 2 and androgen receptor were found to be targeted by hypoxia-responsive miRNAs (Lai et al., 2016).

miRNAs under osmotic stress

A recognized miR-429 binding site in the OSTF1 (osmotic stress transcription factor 1) mRNA was known by bioinformatics investigation. Interestingly, miR-429 is down-regulated in Nile tilapia (*Oreochromis niloticus*) upon osmotic stress, which is consistent with OSTF1 protein up-regulation. miR-429 directly regulates OSTF1 expression by targeting its 3'-UTR. Inhibition of miR-429 significantly increases OSTF1 level in tilapia *in vivo*. Moreover, miR-429 loss of function could stimulate the regulation of plasma osmolality and ion concentration responding to osmotic stress. Taken together, miR-429 is an endogenous regulator of OSTF1 expression, which participates in a regulatory circuit that permits rapid gene program transitions in response to osmotic stress (Yan et al., 2012b). The mir-8 family of miRNAs in zebrafish show role in osmoregulation by modulating the expression of nherf1, which regulates Na+/H+ exchange activity. It was established that miR-8 microRNAs control the response to osmotic stress in zebrafish embryos (Flynt et al., 2009). It was established that miR-30c, a kidney-enriched miRNA, appears as a vital osmoregulator in Nile tilapia. miR-30c loss of function leads to an incapability to respond to osmotic stress. HSP70 (Heat Shock Protein 70) was established as one of the direct regulatory targets of miR-30c. miR-30c directly regulates HSP70 by targeting its 3'-UTR, and inhibition of miR-30c substantially increases HSP70 mRNA level *in vivo*. Taken together, it was suggested that miRNAs take part in a regulatory circuit that allows rapid gene program transitions in response to osmotic stress. miR-30c may be established as a molecular marker to assist in breeding or genetically engineer salt tolerant species (Yan et al., 2012a).

miRNAs involved in oxidative stress

MicroRNAs (miRNAs) are known to regulate cellular processes such as apoptosis, differentiation, cell cycle, and immune functions, and their expression can be altered by distinct stress conditions, such as oxidative stress (Chen et al., 2005; Lai et al., 2016;

Leung and Sharp, 2010a; Li et al., 2011; Magenta et al., 2011). In immune systems of fish, vitamin E (VE) has a defined role as an antioxidant. Characterization of miRNAs expression helped to understand the molecular mechanism of vitamin E defending from oxidative stress juvenile Nile tilapia (*Oreochromis niloticus*). Results showed that VE-deficiency (0 mg/kg supplementation) decreased the activity of superoxide dismutase (SOD), and decreased the expressions of miR-223, miR-146a, miR-16, and miR-122, while excessive supplementation of VE (2500 mg/kg) decreased SOD activity and increased the expressions of eight miRNAs. The study confirmed that the differences in dietary VE affected the expression of hepatic miRNAs which may partly demonstrate the molecular mechanism of VE, and the novel idea of introducing miRNAs into research will provide the basic data for researchers of molecular nutrition (Tang et al., 2013).

1.1.6 Background study about primer design

Primer designing is one of the most important task to do before performing PCR. In general, primers can be 18–30 nucleotides in length and the amplicon length can vary nearly 50–1000 bp but longer products do not amplify as efficiently (Abd-Elsalam, 2003). This provides for practical annealing temperatures. Primers should be designed according to standard PCR guidelines (Primer design software programs, such as OligoPerfect™ designer and Primer ExpressR software and sequence analysis software, such as Vector NTIR Software can automatically evaluate a target sequence and design primers for it based on the criteria listed above).

Degeneracy in primer sequence should also be taken into consideration. Degenerate primers based on the nucleotide sequence of conserved regions were also used to search for members of a gene family (Wilks et al., 1989). Computer programs have also been developed specifically for degenerate primer design (Chen and Zhu, 1997).

They should be specific for the target sequence and be free of internal secondary structure. Primers should avoid stretches of homopolymer sequences (e.g., poly dG or repeating motifs, as these can hybridize inappropriately. Primer pairs should have compatible melting temperatures (within 5°C) and contain about 45-60% GC content (Abd-Elsalam, 2003; For et al., n.d.). Primers with high GC content can form stable imperfect hybrids. Conversely, high AT content depresses the Tm of perfectly matched hybrids. If possible, the 3′end of the primer should be GC rich (GC-clamp) to enhance

annealing of the end that will be extended. Analyze primer pair sequences to avoid complementarity and hybridization between primers (primer-dimers).

1.2Rationale

Bangladesh has made considerable progress in aquaculture sector in recent years and now it contributes 3.69% of GDP (Ministry of Fisheries and Livestock, 2015). Environmental pollution in Bangladesh is currently a major concern due to unorganized and increased industrial development and urban expansion. However, aquatic contaminants such as industrial effluents, domestic sewage, pesticides, agricultural run-off can impose various types of stresses specifically hypoxia, oxidative stress in fish that result in a serious problem in fish production (Davies, 1995; Ekambaram et al., 2014; Pasnik et al., 2010; Slaninova et al., 2009). Rapidly growing evidence suggests that miRNA regulation of mRNA expression may be affected by environmental stressors (Leung and Sharp, 2010a). Stress conditions can modify the biogenesis of miRNAs and the expression of miRNA targets (Leung and Sharp, 2010a). Understanding the effects of different stressors and the molecular responses to stress can contribute in upholding the current progress of the fisheries sector of Bangladesh. Manipulation of the miRNA (such as inhibition or upregulation) of those miRNAs could be a potential way to develop stress tolerant fish. These interventions would result in a pronounced effect in fish production sector in Bangladesh, which could be realized by scaling up of the extensive or semi-intensive culture system to intensive culture system in long run.

1.3Problem Statement

With increasing human interventions of the environment, there is increasing water pollution events that impose stress to fish and other aquatic organisms. Oxidative stress is a common magnitude of this pollution that has many adverse health effects in fish like DNA damage, RNA damage, cancer, necrosis, cell apoptosis, growth, reproduction etc. (Ekambaram et al., 2014; Kelly et al., 1998; Kong and Lin, 2010b; Livingstone, 2003). To cope with these problems and to reduce the impacts of these problems, identification and use of new molecular tools are necessary.

1.4Research Gap

During stress, the level of reactive oxygen species (ROS: peroxides, superoxide, hydroxyl radical, and singlet oxygen) can increase dramatically in living cells creating oxidative stress. ROS may cause significant damages to the cell structures (Devasagayam et al., 2004). In addition, ROS can interrupt translational process through the oxidative modification of RNA, either microRNAs (miRNAs) or messenger RNA (mRNA), hence producing less protein and/or defective protein leading to detrimental cellular function (Kong and Lin, 2010b). Stress conditions can alter the biogenesis of miRNAs, the expression of miRNA targets, and the activities of miRNA-protein complexes (Leung and Sharp, 2010a). There are some reports on the stress responsive miRNA found in some fishes like Medaka, Whitefish, Zebrafish, Salmon and Tilapia (Babiak, 2014; Brzuzan et al., 2010; Flynt et al., 2009; Hairul-islam et al., 2014; Lai et al., 2016; Lau et al., 2014; Yan et al., 2012a), but there is no such data available for the indigenous economically important cultured fishes of Bangladesh. This study deemed to identify oxidative stress responsive specific miRNAs in economically important fishes of Bangladesh.

1.5Objectives

The overall objective of this study is to identify specific stress-responsive miRNAs in economically important fishes of Bangladesh.

The specific objectives are:

1. Identification of potential conserved miRNAs by the computational approach and *in silico* method.
2. *In silico* analysis to make a stress-related miRNAs library and select specific potential candidate miRNA for experimental validation.
3. Design degenerate primers to identify pre-miRNAs in economically important fish of Bangladesh.
4. Identification of oxidative stress responsive pre-microRNAs in economically important fishes of Bangladesh.
5. Prediction secondary structures, mature miRNAs of identified pre-miRNAs.
6. *In silico* analysis to find the potential target genes and mRNAs of putatively identified miRNAs.

Materials and Method

2.1 Study design

The workflow for the study is given in below (Figure 2.1).

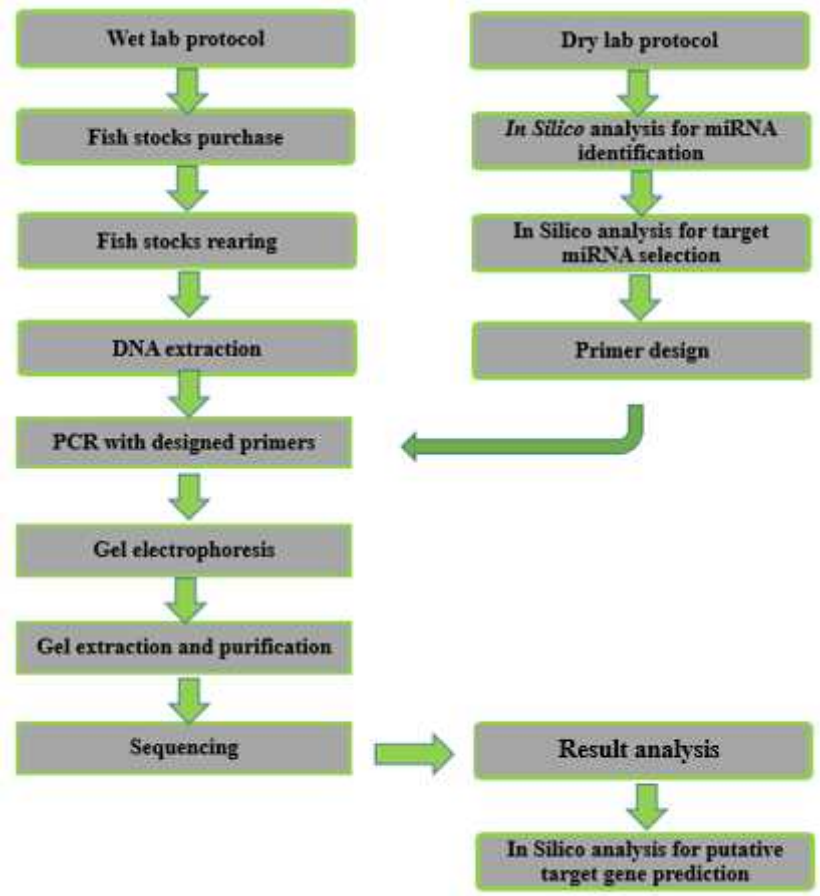

Figure 2. 1 Overall workflow for the present study

2.2 Experimental Organisms

Total seven fish; Zebrafish (*Danio rerio*), Rohu (*Labeo rohita*), Catla (*Catla catla),* Silver carp (*Hypophthalmichthys molitrix*), Climbing perch (*Anabas testudineus),* Hilsa (*Tenualosa ilisha*), and Gangetic mystus(*Mystus cavasius)* were selected based on their economic importance and research value. These are described below in brief,

2.2.1 Zebrafish (*Danio rerio*)

Scientific classification:

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Cypriniformes

Family: Cyprinidae

Genus: *Danio*

Species: *Danio rerio* (F. Hamilton, 1822)

Zebrafish (*Danio rerio*) is a tropical freshwater fish belonging to the minnow family (Cyprinidae) of the order Cypriniformes (**Figure 2.2**). The zebrafish is also an important and widely used vertebrate model organism in scientific research(Froese et al., 2016) and its genome sequence is already identified (http://www.sanger.ac.uk/Projects/D_rerio). The zebrafish is a potent model organism for the study of vertebrate biology and human disease modeling, being well suited to both developmental and genetic analysis as there are a large number of orthologous genes that encode similar proteins to those found in humans (Brown et al., 2012; Grunwald and Eisen, 2002)

Figure 2. 2 Zebrafish (*Danio rerio*), an excellent model organism for vertebrate research

2.2.3 Rohu (*Labeo rohita*)

Scientific classification:

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Cypriniformes

Family: Cyprinidae

Genus: *Labeo*

Species: *Labeo rohita*(Hamilton, 1822)

The Rohu is a freshwater fish of the carp family Cyprinidae that is also commonly called the Ruee, Rui or Tapra (**Figure 2.3**). This fish is considered to be a sanctified delicacy to be eaten at all special occasions in Bangladesh. The texture of the fish is white, non-oily and the bigger the size of the fish the tastier it is. Rohu isgreatly consumed throughout all areas in which they are found and prepared in a variety of ways. The rohu is especially sought after as a delicacy and praised for its unique flavors. In many cases, the roe is also considered sacred and is most often fried and served hot as an appetizer at special ceremonies.

Figure 2. 3 Rohu fish (*Labeo rohita*), commercially cultured fish in Bangladesh

2.2.4 Catla fish (*Catla catla)*

Scientific classification:

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Cypriniformes

Family: Cyprinidae

Genus: *Catla*

Species: *Catla catla* (F. Hamilton, 1822)

Catla (*Catla catla*), also known as the major (Indian) carp, is an economically important South Asian freshwater fish in the carp family Cyprinidae (**Figure 2.4**). It is commonly found in rivers and lakes in northern India, Nepal, Myanmar, Bangladesh, and Pakistan. Catla is a fish with large and broad head, a large protruding lower jaw, and upturned mouth. It has large, grayish scales on its dorsal side and whitish on its belly. It is a surface and midwater feeder. Adults feed on zooplankton, but young ones on both zooplankton and phytoplankton. Catla attains sexual maturity at an average age of two years.

Figure 2. 4 Catla fish (*Catla catla*), commercially cultured fish in Bangladesh

2.2.5 Silver carp (*Hypophthalmichthys molitrix*)

Scientific classification:

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Cypriniformes

Family: Cyprinidae

Genus: *Hypophthalmichthys*

Species: *Hypophthalmichthys molitrix*(Valenciennes, 1844)

The silver carp (*Hypophthalmichthys molitrix*) is a species of freshwater cyprinid fish, a variety of Asian carp native to China and eastern Siberia (**Figure 2.5**). By weight, more silver carp are produced worldwide in aquaculture than any other species of fish except for the grass carp. Silver carp are usually farmed in polyculture with other Asian carp, or sometimes with catla or other fish species. The species has also been introduced to, or spread by connected waterways, into at least 88 countries around the world. The reason for importation was generally for use in aquaculture, but enhancement of wild fisheries and water quality control have also been intended on occasion. In some of these places, the species is now considered an invasive species. The species is currently classified as near threatened in its original range, as its habitat and reproductive behavior are impacted by construction of dams, pollution, and overfishing.

Figure 2. 5 Silver carp (*Hypophthalmichthys molitrix*), cultured fish of Bangladesh

2.2.6Climbing perch (*Anabas testudineus)*

Scientific classification:

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Perciformes

Family: Anabantidae

Genus: *Anabas*

Species: *Anabas testudineus* (Bloch, 1792)

Anabas testudineus, the climbing perch (locally Koi fish), is a species of fish in the family Anabantidae, the climbing gouramis (**Figure 2.6**). This species is important as a food fish in Bangladesh, certain areas of India and in Southeast Asia, where its ability to survive out of the water for extended periods of time, provided it is kept moist, improves its marketability.

Figure 2. 6 Climbing perch (*Anabas testudineus*), indigenous fish of Bangladesh

2.2.7 Hilsa (*Tenualosa ilisha*)

Scientific classification:

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Clupeiformes

Family: Clupeidae

Subfamily: Alosinae

Genus: *Tenualosa*

Species: *Tenualosa ilisha* (F. Hamilton, 1822)

Tenualosa ilisha (ilish, hilsa, hilsaherring or hilsashad) is a species of fish in the herring family (Clupeidae), and a popular food fish in South Asia (**Figure 2.7**). Hilsa fish is a fish of saltwater. Thy live in a group in the coastal area of the sea. Hilsa is the national fish of Bangladesh. Among the total fish production of Bangladesh, about 30% is hilsa and the fish contributes about 1% of GDP in Bangladesh(Ministry of Fisheries and Livestock, 2015).

Figure 2. 7 Hilsa fish (*Tenualosa ilisha*), economically important fish in Bangladesh

2.2.8Gangetic mystus (*Mystus cavasius)*

Scientific classification:

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Siluriformes

Family: Bagridae

Genus: *Mystus*

Species: *Mystuscavasius*(Hamilton, 1822)

Mystuscavasius, the Gangetic mystus (locally Gulsatengra), is a species of catfish of the family Bagridae (**Figure 2.8**). In the wild, it is found in Indian Subcontinent countries such as India, Pakistan, Sri Lanka, Nepal, and Myanmar. Reports of this species from the Mekong basins, Malaysia, and Indonesia are misidentifications of the species Mystusalbolineatus or Mystussingaringan. Few populations occur in Thailand, but only in the Salween basin. It grows to a length of 40 cm. The pectoral spine of the species may give painful wounds and sometimes can be venomous. The population is known to be decreasing in recent past, due to catching, pet trading and habitat destruction.

Figure 2. 8*Mystus cavasius*, the gangetic mystus. An indiginious fish of Bangladesh

2.3 Fish stocks purchase and rearing

30 days old Zebrafish fries were purchased from Al-Amin fish hatchery in Mymenshing and reared in Departmental Aquatic Laboratory for 8 months maintaining optimum culture condition and feed (Reed and Jennings, 2011). Other fishes were collected from thenearby fish market during the experiment.

2.4*In Silico* analysis

2.4.1 Bioinformatics tools

Nucleotide BLAST

The program relates nucleotide or protein sequences to sequence databases and determines the statistical significance of matches. BLASTcan be used to understand functional and evolutionary relationships between sequences as well as identify members of gene families. In bioinformatics, BLAST for Basic Local Alignment Search Tool is an algorithmic system for comparing primary biological sequence information, such as the amino-acid sequences of different proteins or the nucleotides of DNA sequences (http://blast.ncbi.nlm.nih.gov). In this study, it was used to find homologous GSS and EST sequences by searching against fish miRNA sequences.

CLUSTAL W

CLUSTAL W increases the sensitivity of advanced multiple sequence alignment through sequence weighting, position-specific gap penalties, and weight matrix choice. The sensitivity of the commonly used progressive multiple sequence alignment methods has been significantlyupgraded for the alignment of divergent protein and nucleotide sequences. Firstly, individual weights are consigned to each sequence in a partial alignment in order to down weight near-duplicate sequences and up-weight the most divergent ones. Secondly, amino acid substitution matrices are varied at different alignment periods according to the divergence of the sequences to be aligned. Thirdly, residue-specific gap penalties and locally reduced gap penalties in hydrophilic regions encourage new gaps in potential loop regions rather than regular secondary structure. Fourthly, positions in early alignments where gaps have been opened locally reduced gap penalties to inspire the opening up of new gaps at these positions. These modifications are integrated into a new program, CLUSTAL W which is freely available (Thompson et

al., 1994).This tool was used to find similarity among miRNAs for degenerate primer designing and miRNAs analysis.

2.4.2Identification of potential conserved microRNAs and their target genes in selected fish through in silico analysis

2.4.2.1 Databases of miRNAs, EST, GSS, and mRNA sequences, and software

To search for potential miRNAs, total 1623 entries representing hairpin precursor miRNAs, expressing 1637 mature miRNA products of *Cyprinus carpio*, *Danio rerio*, *Fugu rubripes*, *Hippoglossus hippoglossus*, *Ictalurus punctatus*, *Oryzias latipes*, *Paralichthys olivaceus*, *Salmo salar*, *Tetraodon nigroviridisspecies* were obtained from miRBase (Release 21.0; http://microrna.sanger.ac.uk). These miRNAs were defined as a reference set of miRNA sequences. To skip the identification of overlapping miRNAs, repeated sequences were discarded and the remaining sequences were used as a miRNA reference.

2.4.2.2 Prediction of potential miRNAs and their precursors

All non-redundant mature miRNA sequences were used as BLAST queries against the EST and GSS databases of all selected fish except zebrafish individually (**Figure 2.9**). The initial candidate miRNAs with 0-4 mismatches in all known fish mature miRNA sequences were predicted using the local BLASTN 2.6.0+ (W. Li et al., 2015). Additionally, 80 bp of bilateral neighboring sequences from the candidate miRNAs in the ESTs and GSS were extracted and their stem-loop structures were predicted using the web-based Mfold 3.1 software (http://www.bioinfo.rpi.edu/applications/mfold/) (Zuker, 2003). A sequence was accepted to be a candidate miRNA precursor if the hairpin structure pleased the criteria described by (Fu et al., 2011): 1) the minimum free energy (ΔG) is $\leq$ -15 kcal/mole; 2) the stem region contains at least 80% mature miRNA; 3) the number of allowable errors in 1 bulge is $\leq$18 bp; 4) the hairpin is >53 bp long; 5) the loop region is <22 bp; and 6) the number of mismatches between the miRNA and the anti-stem sequence is $\leq$6 bp.

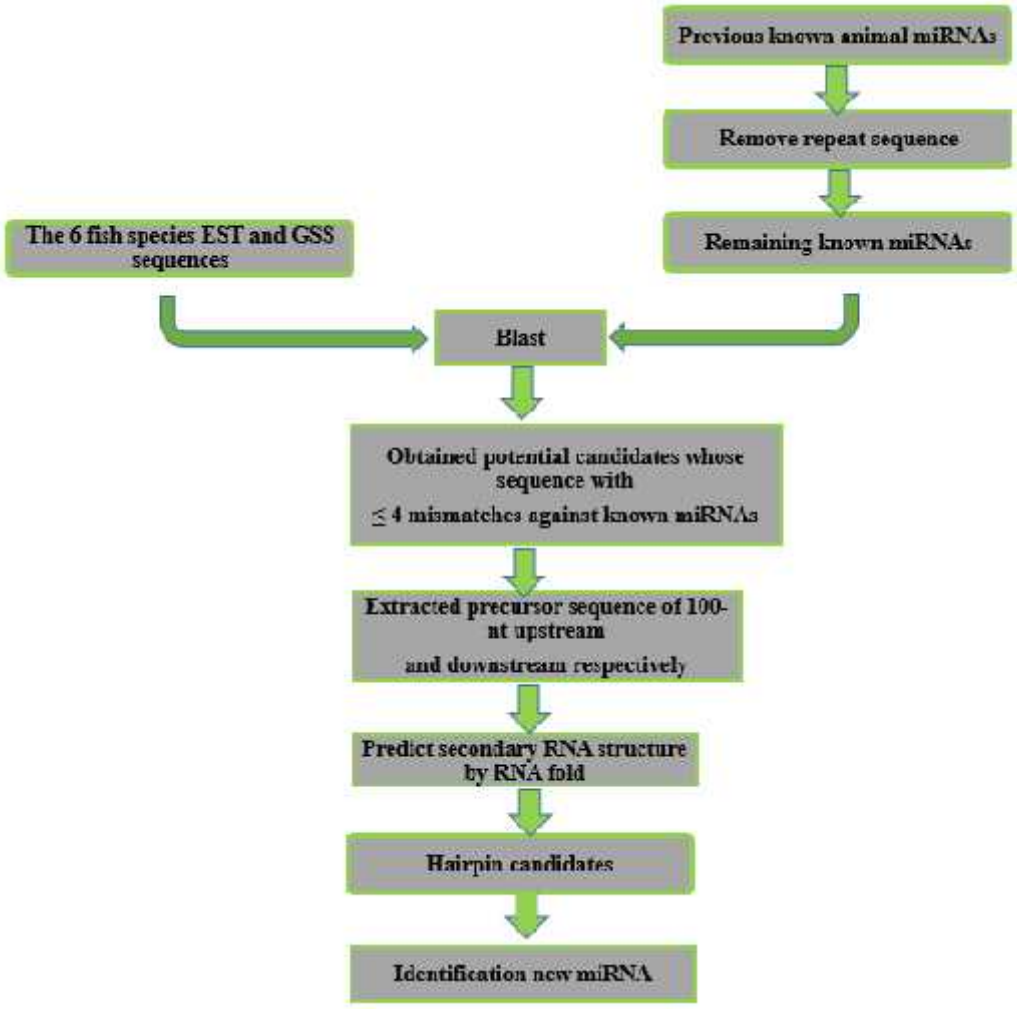

Figure 2. 9 Procedure for conserved miRNA identification.

2.4.4.3 Prediction of miRNA targets

The potential targets of computationally identified miRNA were predicted using the miRanda program, which is publicly available (http://www.microrna.org/microrna/home.do).The parameters of miRanda were set as follows: the score was set to ≥100 and the free energy was set to ≤ -20 kcal/mol (Details in **Appendix F**).

TargetScan Fish (http://www.targetscan.org/fish_62/), version 6.2 (June 2012) was also used for target prediction of all identified (Lewis et al., 2005). TargetScan Fish considers matches to annotated zebrafish UTRs (Ulitsky et al., 2012). It predicts regulatory targets of zebrafish microRNAs (miRNAs) by identifying mRNAs with conserved complementarity to the seed (nucleotides 2-7) of the miRNA. So the miRNAs targets

were predicted by searching for the presence of conserved 8 and 7-mer sites that match the seed region of each miRNA, lowest total context+ score and links to sites in 3' UTR regions (Garcia et al., 2011; Grimson, 2010).

2.4.5 Selection of stress related potential candidate miRNAs for experimental validation

By focusing on previous studies, a stress-responsive miRNAs library was constructed and among them, potential miRNAs candidates were cross-checked and validated using *in silico* tools (Mentioned under section **1.1.4.3**). After selecting some oxidative stress-related potential miRNAs by in silico analysis among them, miR-21 was considered for experimental validation in thewet lab.

2.5Confirmation the presence of pre-miRNA-21 in fish

After target miRNA selection, to confirm the presence of pre-miRNA-21 in selected fishes, PCR was conducted with degenerate primer.

2.5.1Primer for PCR

2.5.1.1 Design degenerate primer for pre-miRNA identification

As miRNAs in selected local fish were not identified yet and totally unknown, for this reason, degenerate primerswas to design to identify miRNA in target fish. Degenerate primer was designed as follows;at first miR-21 was selected as target miR based on literature review.Then to find homologous regions in precursor sequences of miR-21 family, pre-miRNA-21 from 14 known fish species were aligned by CLUSTAL W software (Lassmann et al., 2009; Lassmann and Sonnhammer, 2005; W. Li et al., 2015). But homologous conserved regions was too short to design primers. So further only 3 fish pre-miRNA-21 were aligned by Clustal Kalign to find maximum homologous regions(**Figure 2.10**). In this case >dre-mir-21-1 (Zebrafish), >ola-mir-21-2 (Medaka), >fru-mir-21(Japanese puffer) were aligned and two long conserved homologous region were found at 5' & 3' region (Sequences mentioned in **Appendix A**).

Figure 2. 10 CLUSTAL multiple sequence alignment of pre-miRNA-21 from three fish to find conserve regions.

5' conserved region:

RNA seq: AUAGCUUAUCAGACUGGUGUUGGCUGUUA

DNA seq: **ATAGCTTATCAGACTGGTG**TTGGCTGTTA

3' conserved region:

RNA seq: GGCGACAACAGUCUGUAGGCUGUCUGACA

DNA seq: GGCGACAACAGT**CTGTAGGCTGTCTGACA**

From the 5' conserved portion 17-22 nt was converted into DNA sequence by Nucleic Acid Sequence Massager (http://www.attotron.com/cybertory/analysis/seqMassager.htm). Then this DNA seq was analyzed in OligoAnalyzer 3.1 (http://sg.idtdna.com/calc/analyzer) by setting these following parameters (**Table 2.1).**

Table 2. 1 Considered parameters for primer designing

Parameters	Consideration
Target type	DNA
Oligo Concentration	0.25 µM
Na+ Concentration	50 mM
Mg++ Concentration	2 mM
dNTPs Concentration	0.2 mM
Nucleotide length	17-22
G,C in last 5 nt of 3' end	2-3
Melting temperature (Tm)	50-60
GC content	35-50 %
Delta G	> -7 kcal/mole
Self-Dimer	> -7 kcal/mole

In thecase of reverse primer, from 3' conserved portion 17-22 nts were converted into DNA seq as like as forward primer. Complement and reverse of this seqwere done by Nucleic Acid Sequence Massager and then analyzed this seq in OligoAnalyzer 3.1 as like forward primer designing.Hetero-Dimer was checked in OligoAnalyzer 3.1 by inputting forward and reverse primer. Hetero-Dimer should not be less than -7 kcal/mole.By considering mentioned criteria the degenerate primer set was designed for pre-miRNA-21 is in **Table 2.2**.

Table 2. 2 Primer set for pre-miRNA-21

Primer name	Sequence (5'-3')	Expected amplicon size
Pre-miR-21 forward primer	ATA GCT TAT CAG ACT GGT G	67 bp
Pre-miR-21 reverse primer	TGT CAG ACA GCC TAC AG	

2.5.1.2 PCR primers of a housekeeping gene

A housekeeping (also called reference) gene called elongation factor 1 alpha (Elfa 1) was selected as apositive control according to (McCurley and Callard, 2008). Then PCR primers (Forward and Reverse) for this reference gene were designed from 5' and 3' regions of the gene to amplify this gene from genomic DNA (Primer details in **Appendix A**). The designed primer set for the reference gene is given (**Table 2.3**).

Table 2. 3 Primers for reference gene

Primer name	Sequence (5'-3')	Expected amplicon size
Elfa 1 forward primer	CTTCTCAGGCTGACTGTGC	356 bp
Elfa 1 reverse primer	CCGCTAGCATTACCCTCC	

2.5.1.3 Primer purchase

The designed primer sets were ordered and purchased from Eurogentec, Belgium(Details in **appendix A**).

2.5.2 Genomic DNA extraction

To identify the target precursor miRNA in selected fishes, genomic DNAs were extracted from Zebrafish (*Danio rerio*), Rohu (*Labeo rohita*), Catla (*Catla catla),* Silver carp (*Hypophthalmichthys molitrix*), Climbing perch (*Anabas testudineus),* Hilsa (*Tenualosa ilisha*), and Gulsatengra (*Mystus cavasius)*. Genomic DNAs were isolated according to lab established protocol (Molecular Biology Lab, Department Biochemistry and Molecular Biology, University of Dhaka) based on (Meeker et al., 2007). The protocol was performed as follows, at first samples (Liver, eggs, and muscle) were collected from the fishes and grinded and homogenized with scissor, mortar, and pestle.Then sufficient amounts of thesamplewere taken in Eppendorf tube and genomic DNA extraction lysis buffer (500 µL) was added slowly allowing it to spread and wet on the surface of the whole sample and shake to submerge material.Samples were incubated at 55°C for at least 3 hrs (up to overnight) in awater bath with occasional gentle swirling. The solution was cooled to room temperature adding 500 µL phenol:chloroform:isoamyl

alcohol (25:24:1) and separated by phases by centrifugation at 13000 rpm for 15 mins at 4°C. Then supernatant (each sample separately) was collected as much as possible carefully using a wide-bore pipette. Chloroform:isoamyl alcohol was added as same amounts of supernatant and centrifugation was done again at 13000 rpm for 15 mins at 4°C. Then thesupernatant was collected. 500 µL isopropanol was added and centrifugation was performed again at 13000 rpm for 15 mins at 4°C. Then aqueous phase was removed and Eppendorf tube was dried in air. Final centrifugation was done at 13000 rpm for 15 mins at 4°C after adding 500 µL 70% ethanol alcohol and thesupernatant was discarded. Later Eppendorf tube was dried in theair about 10 mins. DNA was confirmed by observing clumped concentrated DNA in thebottom of thetube. Then concentrated DNA was dissolved in 40 µL Nano-pure water. The concentration of extracted DNA was determined using Nanodrop absorbance. (All used chemicals and apparatus are mentioned in **Appendix B).**

2.5.3 Polymerase Chain Reaction (PCR)

Amplification of the target pre-miRNA and reference gene in isolated DNAs was carried out by polymerase chain reaction (PCR) with designed primer sets. PCR has completely revolutionized the detection of RNA and DNA. Undoubtedly PCR (**Figure 2.11**) has been established, since its invention in the mid-1980s by Kary Mullis, as the most important technique in genetics and molecular biology study.

The parameters of three major steps of PCR reaction were used are stated below.

Denaturation: High-temperature incubation is used to “melt” double-stranded DNA into single strands and loosen secondary structure in single-stranded DNA. The highest temperature that the DNA polymerase can withstand is typically used (usually 95°C).

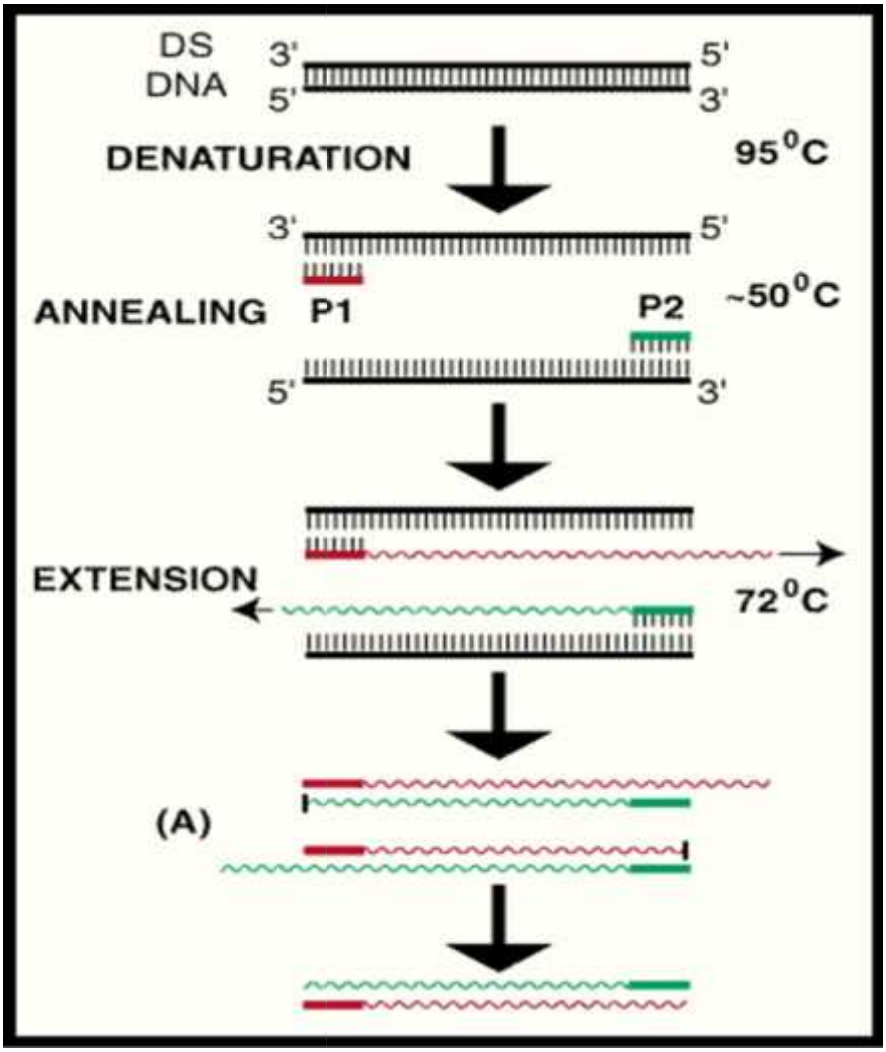

Figure 2. 11 Polymerase chain reaction. Double stranded DNA is denatured at 95°C, then primer annealed at 50-55°C, and finally strands are extended at 72°C

Annealing: During annealing, complementary sequences have an opportunity to hybridize, so an appropriate temperature is used that is based on the calculated melting temperature (Tm) of the primers (5°C below the Tm of the primer).

Extension: At 70-72°C, the activity of the DNA polymerase is optimal, and primer extension occurs at rates of up to 100 bases per second. When an amplicon in real-time PCR is small, this step is often combined with the annealing step using 60°C as the temperature.

2.5.3.1 Preparation of PCR Reaction Mixture

The reaction mixture for PCR was prepared by mixing the specific volume of the components in an appropriate sized tube (**Table 2.4**). For a large number of reactions, a

master mix without any template DNA was prepared and aliquoted into PCR tubes. At the end, thespecific template was added into a properly labeled PCR tube. The PCR tube containing reaction mixture and template DNA was capped properly followed by vortex and centrifugation briefly to mix gently and collect all components to the bottom of the tube respectively. In all PCR, a negative control that contained no DNA template but all other components of the reaction was included and in relevant cases, a positive control that contained known DNA template carrying known gene was also included. The PCR tubes were then placed in a thermal cycler (Veriti 96- Well Thermal Cycler, Applied Biosciences, USA) and the amplification parameters were set correctly.

Table 2. 4 Components of the reaction mixture for PCR

Components	Amounts per reaction
Nuclease-free water	6.7 µL
DMSO	3.9 µL
[illegible]	1.2 µL
Mg buffer	1.5 µL
dNTPs	0.3 µL
Commercial taq polymerase	0.3 µL
Forward Primer	0.5 µL
Reverse Primer	0.5 µL
Template DNA	01 µL
Total Reaction Volume	15 µL

2.5.3.2 PCR Conditions and PCR amplification

All the PCR tubes containing the appropriate mixtures were heated at 95 ºC for 5 minutes in the thermal cycler (Applied Biosystem, USA) to ensure denaturation of all DNA templates. Thirty (30) cycles of these segments were repeated with a final extension of 5 minutes at 72ºC. After this, PCR tubes were stored at -20 ºC until further analysis. The cycling profile for each primer: target combination was optimized accordingly as (**Table 2.5)**& (**Table 2.6)**

Table 2. 5 Thermocycling conditions of PCR for reference gene

Steps	Temperature and Time Profile
Denaturation	95 ºC for 30 seconds
Annealing	58 ºC for 40 seconds
Extension	72 ºC for 40 seconds

Table 2. 6 Thermocycling conditions of PCR for Pre-miRNA-21

Steps	Temperature and Time Profile
Denaturation	95 ºC for 50 seconds
Annealing	52 ºC for 60 seconds
Extension	72 ºC for 60 seconds

2.5.3.3 Bulk PCR

Bulk PCR (10 reactions at a time) was performed and then PCR products were electrophoresed onagarose gel and then extracted as pure DNA.

2.5.4 Analysis of the amplicons by agarose gel electrophoresis

The successful amplification of the desired genes was visualized by resolving the PCR products in agarose gel (1% for reference gene and 3% for target pre-miRNA) depending on the size of theamplicon. The specific amount of agarose (Sigma, USA) was added in 10X Tris-acetate-EDTA (TAE) buffer to prepare a desired final concentration of agarose in a final volume of 25 mL and was heated in a microwave oven for about 1:30 minutes to dissolve the agarose. The boiled mixture was allowed to cool to about 45°C and was added to a final concentration of 0.5 µg/mL. The gel was poured onto gel casing preset with well former (comb). The casing was then allowed to set on a flat surface for about 15 minutes. After solidification of the gel, the comb was removed and buffer (10x TAE) was poured into thetank to submerge the solidified gel. Samples were prepared by mixing 5µl PCR product with 2µl loading DNA dye. Samples and 1-kb plus DNA ladder were loaded into the wells formed in the gel. Electrophoresis was operated at 100 volts

for 100-120 minutes as per requirement. Then gel was stained in Ethidium Bromide (EtBr) solution for 10 mins following by 2 mins destaining in water and finally, thegel was viewed using Alpha Imager HP Geldocumentation system (Cell Bioscience, USA). The image was analyzed to compare the desired band size with DNA ladder's band.

2.5.5 Gel extraction and gel purification

After bulk PCR and gel electrophoresis, specific desired bands series from the gel was cut and extracted gel was purified to send for sequencing. The gel was extracted using FavorPrepTM gel extraction & Purification Kit purchased from theFavogen biotech crop (**Figure 2.12**). For extraction of DNA fragments from agarose gel,the agarose gel was excised with a clean scalpel and the extra agarose gel was removed to minimize the size of the gel slice. The gel slice was transferred into a microcentrifuge tube. The maximum volume of the gel slice is 300mg. 500 µL of FADF Buffer was added to the sample and mixed by vortex. For > 2% agarose gels, 1000 µL of FADF Buffer was added. Incubation was done at 55 °C for 5 ~10 minutes and vortex the tube every 2 ~ 3 minutes until the gel slice dissolved completely. During incubation, interval vortex can accelerate the gel dissolved. The gel slice has been dissolved completely before proceed the next step. After gel dissolved, the color of sample mixture is yellow. If the color is violet, added 10 µL of sodium acetate, 3M, pH 5.0. Mixed well to make the color of sample mixture turned to yellow. Then the sample mixture was cooled down to room temperature and placed an FADF Column into a Collection Tube.800 µL of the sample mixture was transferred to the FADF Column and centrifuged at 11,000 x g for 30 seconds, then discarded the flow-through. If the sample mixture is more than 800 µL, repeated this step for the rest of the sample mixture. 750 µL of Wash Buffer (with ethanol) was added to the FADF Column and centrifuged at 11,000 x g for 30 seconds, then discarded the flow-through. Ethanol (96-100 %) has been added to Wash Buffer when first use. Centrifugation was done again at full speed (~ 18,000 x g) for an additional 3 minutes to dry the column matrix. The residual liquid was removed thoroughly on this step. FADF Column was placed to a new microcentrifuge tube. 40 µl of Elution Buffer was added to the membrane center of the FADF Column and kept for 2 mins. Final Centrifugation was done at full speed (~ 18,000 x g) for 1 min to elute the DNA.Finally, thenucleic acid concentration of extracted gel was measured and gel extraction was confirmed through further PCR and gel run of extracted products.

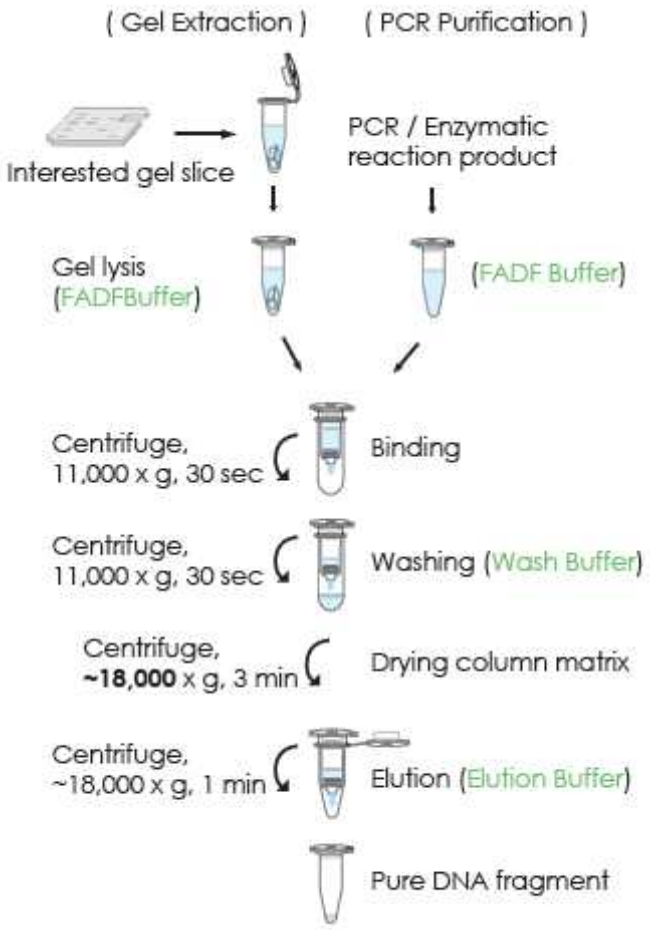

Figure 2. 12 Purification of gel extracts. Gel extracts were purified through sequential centrifugations with FavorPrepTM gel purification kit.

2.6 Sequencing

After confirmation the presence of pre-miRNA-21 in selected fishes by PCR, the Gel extracted products were sent to First BASE Laboratories Sdn Bhd (Malaysia) where cycle sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®, USA) according to manufacturer's instruction and extension product was purified followed by capillary electrophoresis using ABI Genetic Analyzer (Applied Biosystems®, USA). Bidirectional (5′ to 3′ and 3′ to 5′) sequences were done for all the 7 fish samples. The basic sequencing protocol involved amplification of target sequence by PCR and purification of thedesired amplicon followed by cycle sequencing reaction, cycle sequencing product purification, and capillary electrophoresis.

2.7 Analysis of sequence through chromatogram study

Sanger's Sequencing services were provided by FIRST BASE, Malaysia. Sequences results were visualized and cured by using software package Chromas LITE version 2.1. The sequencewas normally done using forward and reverse primers individually. After obtaining sequences, theCAP3 program (Huang and Madan, 1999) was used to assemble the forward and reverse sequences to produce a single contig. The insignificant nucleotide from 5' and 3' regions denoted as N were trimmed through chromatogram study (**Figure 2.13**).

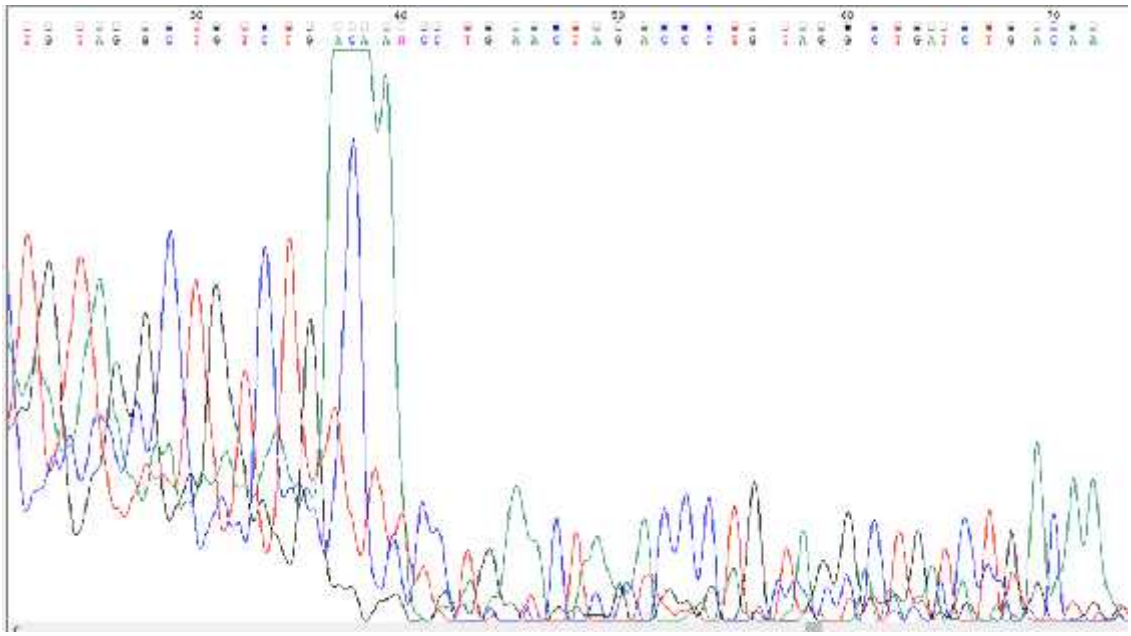

Figure 2. 13 Chromatogram study of identified miRNAs. Sequences were trimmed by chromatogram study using Chromas LITE software

2.8 Sequence validation and similarity checking

Then the assembled sequences were verified through the use of BLAST against NCBI Nucleotide Databases and miRBase databases. NCBI BLAST was done to check the similarity of sequences with fishes.

The sequences were also aligned with miRBase miRNAs database to check the conservatory behavior of miRNAs (Details in **Appendix E**).

2.9 Identification of hairpin structures and mature miRNAs regions

Secondary hairpin structures and possible mature miRNAs regions of these pre-miRNAs were identified using web bases Mfold software (Mentioned in section 2.3.3.2). A

sequence was considered to be a candidate miRNA precursor if the hairpin structure satisfied the criteria described by (Fu et al., 2011): 1) the minimum free energy (DG) is ≤ -15 kcal/mole; 2) the stem region includes at least 80% mature miRNA; 3) the number of allowed errors in 1 bulge is ≤18 bp; 4) the hairpin is >53 bp long; 5) the loop region is <22 bp; and 6) the number of mismatches between the miRNA and the anti-stem sequence is ≤6 bp (Details in **Appendix C**).

2.10 In Silico analysis for putative target gene prediction of the identified miRNAs

After successful identification of selected miRNAs in those fishes, their putative target mRNA or gene was predicted using NCBI BLAST and computational in silico tools such as TargetScan Fish. The TargetScan Fish software is based on zebrafish genes and its recorded miRNAs sequences. To find potential miRNAs targets, some parameters are considered like 7, 8 mer site, 6-8 nts seed match region at 5' end of miRNAs to 3' end of gene sequences (Details in **Appendix D**).

Chapter 3

Results

3.1. Putative conserved miRNAs from 6 fish species were identified

Based on homology searching and secondary configuration assessment approaches, this study displayed potential miRNAs in 6 economically important cultured fish species of Bangladesh. As the whole genome sequences of these fish species are not known yet, only available databases such as ESTs and GSSs sequences were used to identify potential miRNAs in these species. The EST and GSS databases of 6 selected fish species were obtained from the NCBI GenBank nucleotide databases. Total 4,798 EST sequences from Rohu (*Labeo rohita*), 1 EST from Catla (*Catla catla)* and climbing perch (*Anabas testudineus),* 65 GSS & 20 EST from Silver carp (*Hypophthalmichthys molitrix*) were found from NCBI, however, Hilsa (*Tenualosa ilisha*), and gangetic mystus(*Mystus cavasius)* have no EST and GSS sequences in NCBI GenBank.

After the manual curing of the redundant sequences of the genes and the protein-coding sequences, two potential miRNAs were identified from Silver carp GSSs only (**Table 3.1**). All of the precursors for those mature miRNAs fold into the distinctive secondary structure of miRNAs and they are hypothesized to be important authentication parameters for the miRNA genes predicted.

Table 3. 1 Two identified potential miRNAs in Silver carp

New miRNAs	Reference miRNA	sequences (5'-3')	Gene ID	LP	A+U (%)	ΔG
hmo-miR-2192	dre-miR-2192	AAAGUGAAAGGUG ACUGAGGC	JX499811.1 (GSS)	79	55.69	-28.40
hmo-miR-2293	bta-miR-2293	UGACUUUUGUUGU UUUGUAU	DQ136011.1 (GSS)	143	69.93	-34.10

* LP = Length of precursor, ΔG = Minimum free energy, A+U= Adenine + Uracil

There were 2 hits (**Figure 3.1**) found from *Labeo rohita*GSS database after blast against the created miRNA from mirBase database, but none of them satisfied the miRNAs requirements (**Figure 3.2**). Due to unavailability of data, no miRNAs were found in other fish (Details in **Appendix G**)

dre-miR-143 MIMAT0001840

Sequence ID: Query_116184 Length: 21 Number of Matches: 1

Range 1: 3 to 18 Graphics

Score	Expect	Identities	Gaps	Strand
30.1 bits(32)	0.001	16/16(100%)	0/16(0%)	Plus/Minus

```
Query  274  CTACAGTGCTTCATCT  289
            ||||||||||||||||
Sbjct  18   CTACAGTGCTTCATCT  3
```

dre-miR-144-3p MIMAT0001841

Sequence ID: Query_116155 Length: 20 Number of Matches: 1

Range 1: 1 to 19 Graphics

Score	Expect	Identities	Gaps	Strand
30.1 bits(32)	0.001	18/19(95%)	0/19(0%)	Plus/Plus

```
Query  127  TATAGTATAGATGATGTAC  145
            || ||||||||||||||||
Sbjct  1    TACAGTATAGATGATGTAC  19
```

Figure 3. 1 Blast of Rohu ESTs against fish miRNAs database. Two hits were found based on E value.

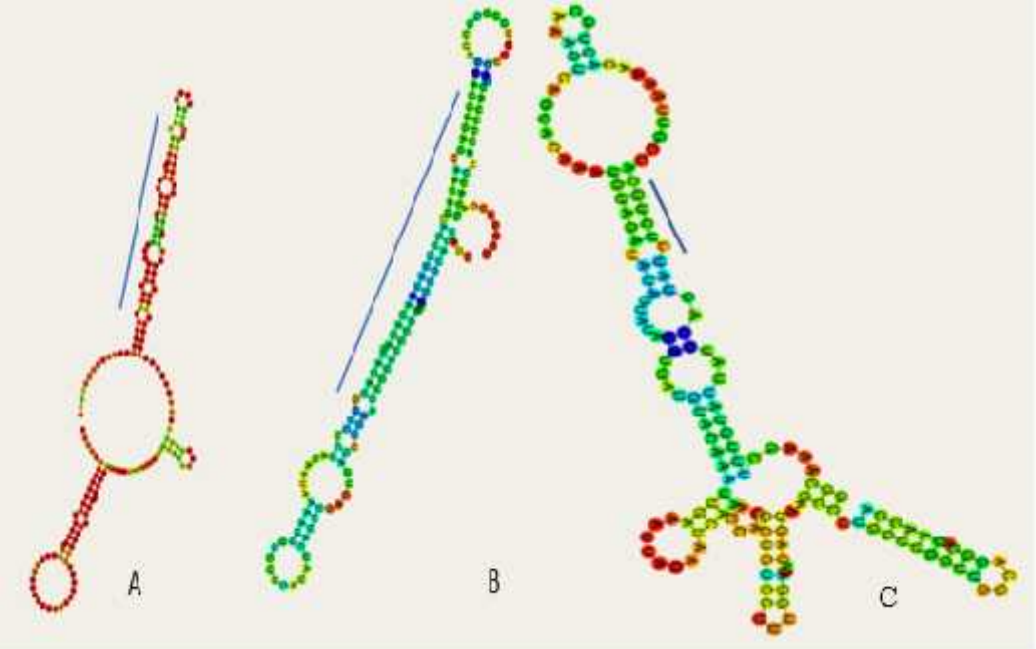

Figure 3. 2 Stem-loop structures of identified miRNAs. Long blue margin of hmo-miR-2192 (A) and hmo-miR-2293 (B) indicates possible mature miRNA regions but Rohu EST hit (C) has no significant stem loops.

3.2 Construction of stress-responsive miRNAs database

Stress-responsive miRNAs were sorted out by extensive review on miRNAs and its regulations in literature. By focusing on stress and stressors, all found miRNAs were retrieved and a stress-responsive miRNAs database was manually constructed. The local database includes more than 550 miRNAs which are responsive to different types of stresses in different animals and cell lines of some fishes like Atlantic salmon, Nile tilapia, Marine medaka, Zebrafish etc. (**Table 3.2**)

Table 3. 2 A stress-related miRNAs database

miRNAs name and sequence	**Organism**	**Stress type/stressor**	**Reference**
>rno-miR-181a-5p **AACAUUCAACGCUGUCGGUGAGU**	Rat	Hydrogen peroxide	(L. Wang et al., 2014)
>hsa-miR-200c-5p **CGUCUUACCCAGCAGUGUUUGG** **>hsa-miR-147a** **GUGUGUGGAAAUGCUUCUGC**	HUVEC	Hydrogen peroxide	(Magenta et al., 2011)
>hsa-miR-155-5p **UUAAUGCUAAUCGUGAUAGGGGU** **>hsa-let-7f-5p** **UGAGGUAGUAGAUUGUAUAGUU** **>hsa-miR-143-5p** **GGUGCAGUGCUGCAUCUCUGGU** **>hsa-let-7b-5p** **UGAGGUAGUAGGUUGUGUGGUU** **>hsa-miR-16-5p** **UAGCAGCACGUAAAUAUUGGCG**	Human	Hydrogen peroxide	(Simone et al., 2009)
>rno-miR-21-5p **UAGCUUAUCAGACUGAUGUUGA** **>rno-miR-499-5p** **UUAAGACUUGCAGUGAUGUUU**	Rat cardiomyocytes cell	Hydrogen peroxide	(J. Wang et al., 2014)
>dre-miR-9-5-3p **UAAAGCUAGAUAACCGAAAGU** **>dre-miR-30d-5p** **UGUAAACAUCCCCGACUGGAAGCU** **>dre-miR-49** **UUCACUGUGGCGGAAAUGACC** **>dre-miR-46** **AAGAGAAGAGUGAGCGAGUGA** **>dre-miR-30e-2-5p** **UGUAAACAUCCUUGACUGGAAGC** **>dre-miR-24-4-3p**	Adult Zebrafish	Cold shock	(Yang et al., 2011)

UGGCUCAGUUCAGCAGGAACAG >dre-miR-2191-3p UCACACCUACAAUCCCUGGCA >dre-miR-146a-5p UGAGAACUGAAUUCCAUAGAUGG >dre-miR-48 UGAGGAGUUUAGAGCAAGUAA >dre-miR-23 AGCUGGUGUCCUGCAGAGUUU >dre-miR-455-5p UAUGUGCCCUUGGACUACAUC			
>dre-miR-141-5p CAUCUUACCUGACAGUGCUUGG >dre-miR-429a UAAUACUGUCUGGUAAUGCCGU >dre-miR-200c-3p UAAUACUGCCUGGUAAUGAUGC >dre-miR-200a-5p CAUCUUACCGGACAGUGCUGGA >dre-miR-200c-5p CAUCUUACAAGGCAGUUUUGGA >dre-miR-429b UAAUACUGCCUGGUAAUGCCAU >dre-miR-200b-3p UAAUACUGCCUGGUAAUGAUGA >dre-miR-200b-5p CAUCUUACGAGGCAGCAUUGGA >dre-miR-200a-3p UAACACUGUCUGGUAACGAUGU >dre-miR-141-3p UAACACUGUCUGGUAACGAUGC	Zebrafish embryos	Osmotic stress	(Flynt et al., 2009)
>dre-miR-451 AAACCGUUACCAUUACUGAGUU	Zebrafish erythrocytes cells	Hydrogen peroxide	(Yu et al., 2010)
>oni-mir-429 UAAUACUGUCUGGUAAUGCCGU	Tilapia	Salinity	(Yan et al., 2012b)
18 miRNAs	Atlantic salmon	acidic aluminum-rich	(Kure et al., 2013)

		water	
509 miRNAs	Marine medaka	Hypoxia	(Lai et al., 2016; Lau et al., 2014)
miR-141-3p , miR-141, miR-200a, miR-200a	Human ovarian cell line	Hydrogen peroxide	(Mateescu et al., 2011)
miR-103	Human embryonic kidney cells	Hydrogen peroxide	(Xu et al., 2015)
miR-92a	Zebrafish cell line	Hydrogen peroxide	(Chen et al., 2015)
miR-21, miR-223, miR-146a, miR-125b, miR-181a, miR-16, miR-155 and miR-122	Nile tilapia	Vit-E	(Tang et al., 2013)
>let-7h **UGAGGUAGUAAGUUGUGUUGUU** **>let-7j** **UGAGGUAGUUGUUUGUACAGUU** **>miR-10c** **UACCCUGUAGAUCCGGAUUUGU** **>miR-10d** **UACCCUGUAGAACCGAAUGUGU** **>miR-16b** **UAGCAGCACGUAAAUAUUGGAG** **>miR-457a** **AAGCAGCACAUCAUUACUGGUA** **>miR-19d** **UGUGCAAACCCAUGCAAAACUG** **>miR-22b** **AAGCUGCCAGUUGAAGAGCUGU** **>miR-27c** **UUCACAGUGGUUAAGUUCUGC** **>miR-27e** **UUCACAGUGGCUAAGUUCAGU** **>miR-130c** **CAGUGCAAUAUUAAAAGGGCAUU** **>miR-135c** **UAUGGCUUUCUAUUCCUAUGUG** **>miR-301c** **CAGUGCAAUAGUAUUGUCAUA**	Marine medaka	Hypoxia	(Lai et al., 2016; Lau et al., 2014)

>miR-456 **CAGGCUGGUUAGAUGGUUGUCU** **>miR-458** **AUAGCUCUUUAAAUGGUACU** **>miR-460** **CCUGCAUUGUACACACUGUGC** **>miR-462** **UAACGGAACCCAUAAUGCAGCUG** **>miR-722** **UUUUGCAGAAACGUUUCAGAUU** **>miR-723** **AGACAUCAGAAAAAUCUGUGCU** **>miR-724** **UUAAAGGGAAUUUGCGACUGUU** **>miR-725** **UUCAGUCAUUGUUUCUGGUCGU** **>miR-727** **UUGAGGCGAGUUGAAGACUUCA** **>miR-728** **AUACUAAGUAUACUACGUUUAC** **>miR-730** **UCCUCAUUGUGCAUGCUGUGUG** **>miR-731** **AAUGACACGUUUUCUCCCGGAUU** **>miR-734** **UAAAUGCUGCAGAAUUGUGC** **>miR-737** **AAAUCAAAGCCUAAAGAAAAUA** **>miR-1388** **AUCUCAGGUUCGUCAGCCCAUG** **>miR-2184** **AACAGUAAGAGUUUAUGUGCUG** **>miR-2187** **UUACAGGCUAUGCUAAUCUGU** **>miR-2188** **AAGGUCCAACCUCACAUGUCCU**			

3.3 Selection of oxidative stress responsive candidate miRNAs

By using bioinformatics tool and *in silico* methods, total 11 oxidative stress responsive potential candidate conserved miRNAs were selected from the stress-responsive miRNAs database specially focusing on oxidative stress (**Table 3.3**)

Table 3. 3 Potential oxidative stress responsive miRNAs

miRNAs name	Sequence (5'-3')
>dre-miR-181a-5p	AACAUUCAACGCUGUCGGUGAGU
>dre-miR-200c-5p	CAUCUUACAAGGCAGUUUUGGA
>dre-let-7f	UGAGGUAGUAGAUUGUAUAGUU
>dre-miR-21	UAGCUUAUCAGACUGGUGUUGGC
>dre-miR-499-5p	UUAAGACUUGCAGUGAUGUUUA
>dre-mir-455-5p	UAUGUGCCCUUGGACUACAUC
>dre-miR-143	UGAGAUGAAGCACUGUAGCUC
>dre-miR-155	UUAAUGCUAAUCGUGAUAGGGG
>dre-miR-451	AAACCGUUACCAUUACUGAGUU
>dre-miR-200a-5p	CAUCUUACCGGACAGUGCUGGA
>dre-miR-141-5p	CAUCUUACCUGACAGUGCUUGG

3.4 miRNAs targets were identified by *in silico* analysis

The sorted potential candidate oxidative stress related miRNAs were cross checked by using a combination of BLAST and Targetscan fish online software (Details in **Appendix D**). Several oxidative stress related putative target genes of candidate miRNAs were identified using Zebrafish genome sequences, and these targets belong to a variety of gene families that has role in various biological and physiological functions (**Table 3.5**).

Table 3. 4 Target genes of potential stress related microRNA

miRNAs name	Name of target genes
>dre-miR-181a-5p	GPx8, cat
>dre-miR-200c-5p	Cat
>dre-let-7f	hspb6
>dre-miR-21	pdcd4b, hspb8
>dre-miR-499-5p	GPX8
>dre-mir-455-5p	-
>dre-miR-143	pdcd8, hspa8, hspe1, GPX2
>dre-miR-155	pdcd2
>dre-miR-451	pdcd4b
>dre-miR-200a-5p	Cat
>dre-miR-141-5p	Cat

Moreover, target genes of identified potentially conserved miRNAs in Silver carp were predicted using BLAST and RNA hybrid software.Result was summarized in **Table 3.5**.

Table 3. 5 Target of identified putative miRNAs

miRNA name	Target protein	Protein function	Gene ID
hmo-miR-2192	Glucose phosphate isomerase	Metabolism	337255732
	Copper/zinc superoxide dismutase	Metabolism	300087118
hmo-miR-2293	Lipoprotein lipase	Metabolism	253317430
	Putative interleukin-8 like protein	Immunoregulation	205278402

3.4 Confirmation the presence of miR-21 in economically important cultured fish species of Bangladesh

From the potential candidate miRNAs by in silico method, miR-21 was selected for the experimental validation. At first, as a model organism, zebrafish was selected for experimental validation of miR-21 and the miR-21 was confirmed in Zebrafish (Kolpa et al., 2013). Then miR-21 in our economically important indigenous fish species was confirmed by PCR with degenerate primer set and gel electrophoresis (**Figure 3.3**). This result showed that all our selected fish species have miR-21 and expected precursor lengths are about 67 nts. Here elongation factor elfa 1 (elfa 1) was used as PCR positive control (McCurley and Callard, 2008) (**Figure 3.4).**

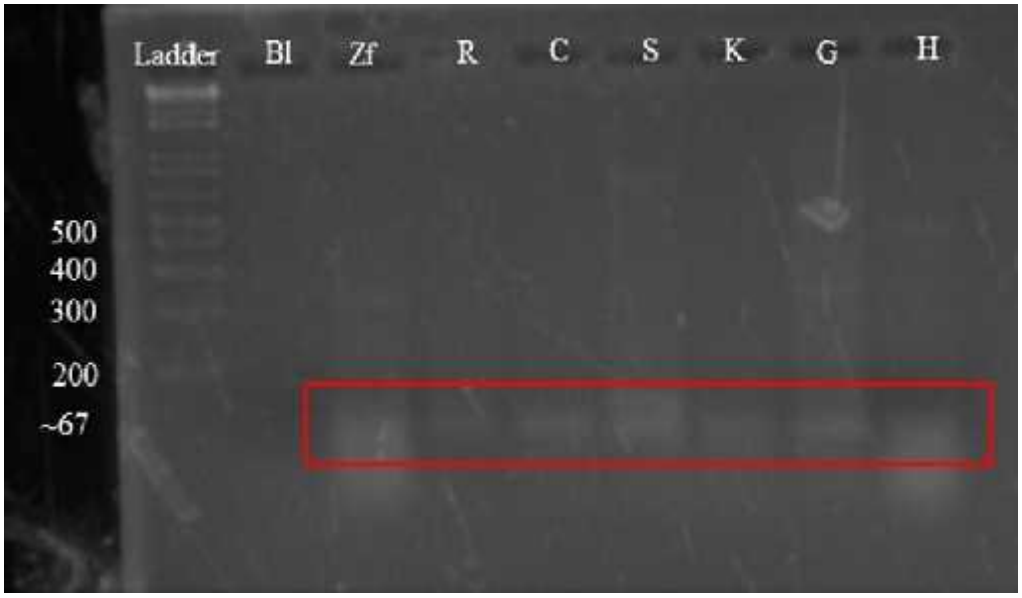

Figure 3. 3 Agarose gel electrophoresis of pre-miRNA-21 amplicons. White bands around 67 bp but no band in blank sample (Bl) that indicated the presence of pre-miRNA-21 in all selected fish (Zf- zebrafish , R-Rohu, C-catla, S-Silver carp, K-Climbing perch, G-Gulsa

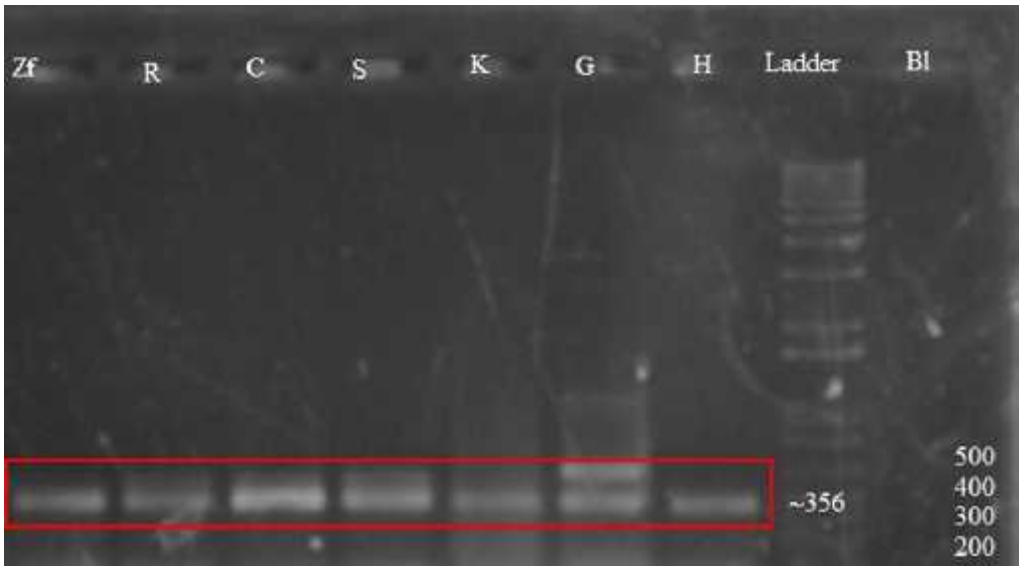

Figure 3. 4 Agarose gel electrophoresis of reference gene amplicons. White bands around 356 bp but no band in blank sample (Bl) that indicated the accuracy of PCR amplification for all fish (Zf- zebrafish , R-Rohu, C-catla, S-Silver carp, K-Climbing perch, G-Gulsa

3.5 Identification of miR-21 sequence from 7 selected fish

After being confirmed the presence of miR-21 in the studied these fish, sequencing was done from FIRST BASE, Malaysia. Sequences were analyzed using Chromas lite software version 2.01. The identified mir-21 sequences are enlisted in **Table 3.6**.

Table 3. 6 Sequencing results of putative mir-21 of the studied fish.

Pre-miRNAs name	Fish species	Identified Sequences (5'-3')
dre-mir-21	*Danio rerio*	UUAUGUGUCUUUAUUGGCGUGGAUAUAA GUCUUUCCCAGUGUGUCAGAUAGCUUAUC AGACUGGUGUUGGCUGUUACAUUCGCCCG GCGACAACAGUCUGUAGGCUGUCUGACAU UUUGGGCAUUUUCUUCUCCGAUUAAAAAU AUGA
lro-putative mir-21	*Labeo rohita*	CCUGUCUGAUAAGCUAUANCGGNAACUCG CACCGGCUCUGAUCCCGUAUA
cca-putative mir-21	*Catla catla*	GUACACCGUCUGAUAGCUAU
ate-putative mir-21	*Anabas testudineus*	AUGACGUCGUGACGACAAACGCUGUGUGA GAAGAUGAAAGGUGUGUAUACCUGUGUG AGA
hmo-putative mir-21	*Hypophthalmichthys molitrix*	CUGUAGGCUGUCAG
til-putative mir-21	*Tenualosa ilisha*	AACUGUAGGCUGCUCUUCACAA
mca-putative mir-21	*Mystus cavasius*	UACUUGUCUGAGAGCUUAGAGUGUAUAU

3.6Stem-loop structures and mature miRNAs regions were identified

Stem-loop structures of the sequenced miRNAs were predicted using Mfold software. Mature miRNAs regions of these miRNAs were in stem portions (**Figure 3.5**).

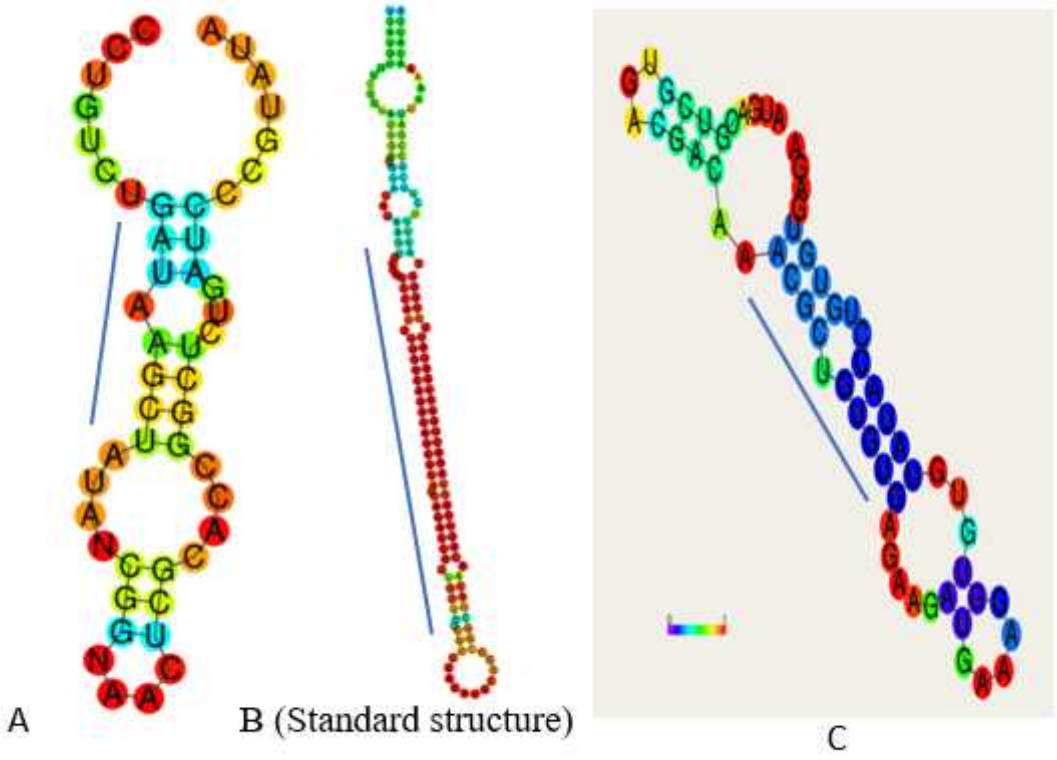

Figure 3. 5 Stem-loop structure of pre-miRNA-21 from (A) *Labeo rohita*, (B) *Danio rerio*, (C) *Anabas testudineus*.

3.7 Identified miRNAs were conserved among miR-21 family

Mature miRNA sequences, along with their corresponding precursor sequences, are highly conserved among distantly related animal species (Chen et al., 2012; Lee et al., 2007). This observation directs to investigate the evolutionary relationships of miRNAs belonging to the same families in different animal species. In this study, a comparison of the precursor sequences of the identified miRNAs was done with other members of the same family (miR-21 family) and it showed that identified miRNAs are similar and aligned well with other members of the miR-21 family (**Figure 3.6**).

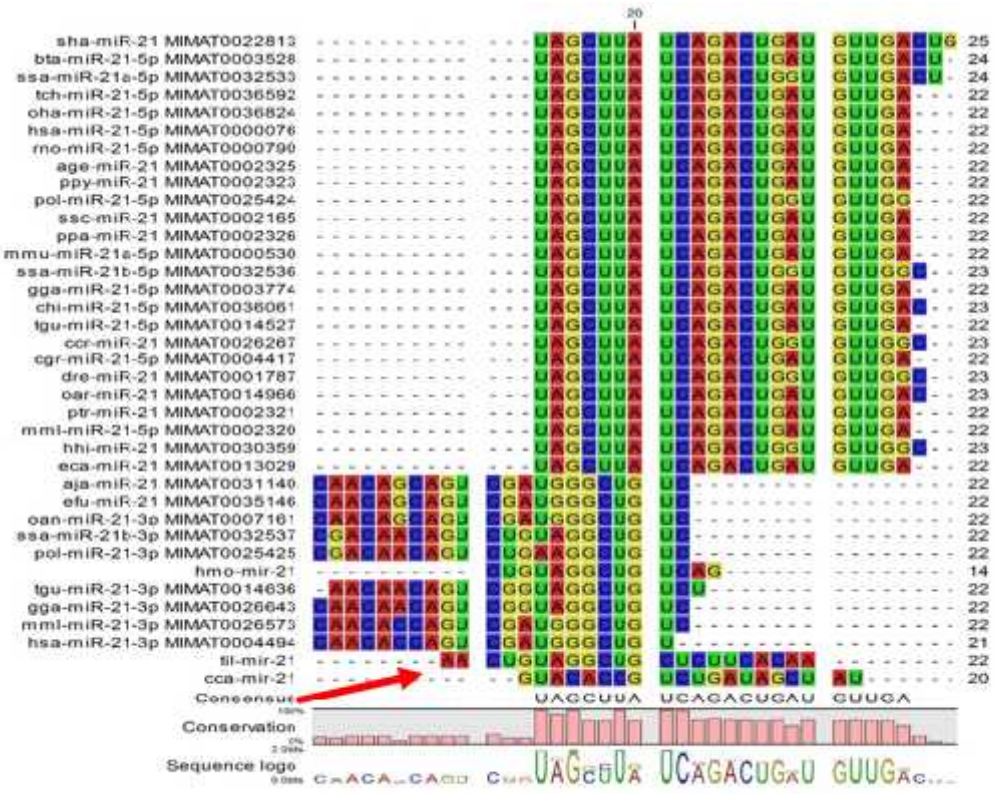

Figure 3. 6 Alignment of identified pre-miRNAs with miRBase data. That shows the conserveness of miRNA.

Chapter 4

Discussion

Due to rapid developmental expansion and environmental manipulation, aquatic pollution, a worldwide problem in the marine and freshwater environments, is likely to become more serious in present and in near future. Some previous reports have demonstrated that aquatic pollution can make anomaly in case of water chemistry and water quality in aquatic environment that creates various types of stresses (i.e. hypoxia, thermal shock, cold shock, osmotic imbalance, oxidative imbalance etc.) to aquatic living organism including fish (Barton, 2002; Lackner, 1998; Pasnik et al., 2010; Stoliar and Lushchak, 2012b).

Organisms face constantly changing environments in the wild, but homeostasis is achieved through biological processes associated with stress responses. Cellular antioxidant defense systems, when exposed to environmental pollutants, are depleted, but levels of these antioxidants in living organisms may rise also to redress the imbalance caused by the oxidative damage. When the system cannot cope with these challenges, homeostasis breaks down, which can result in diseases such as cancers, diabetes, neurodegenerative disorders, cardiovascular diseases, viral infections and many others. In some of these cases, homeostasis can be mediated via miRNAs (Umbach et al., 2008). Among the various regulatory mechanisms in a biological process, regulation of gene expression by microRNAs (miRNAs) is of critical importance. miRNAs are important for an extensive array of biological processes. Deceptively normal single miRNA mutant was shown to display phenotypic crisis in stress conditions (Rooij et al., 2007). miRNA was shown to involve in B-cell differentiation(Chen et al., 2004), adipocyte differentiation (Esau et al., 2006), cardiogenesis(Zhao et al., 2005), insulin secretion (Poy et al., 2004), antiviral defense (Lecellier et al., 2005), and in growth of cancer (Bushati and Cohen, 2007; Magenta et al., 2011). Definite role of some miRNAs expressed in zebrafish, medaka, atlantic salmon, rainbow trout, nile tilapia are reported such as miR-430 in brain formation (A. Giraldez et al., 2006), miR-126 in vascular development (Fish et al., 2008), miR-430 in embryonic development (A. Giraldez et al., 2006), osmoregulation (Flynt et al., 2009), tumor cell development (Huang et al., 2008), and in growth regulation (Huang et al., 2012) etc. With increasing risks of environmental stress and in the context of the importance of fisheries in Bangladesh; novel tools are required to uphold the increasing trends of this sector in Bangladesh. So far there is a huge gap in miRNA research in the context of fisheries sector of Bangladesh. This drives

the present study to explore and shed some light on the potentials of the miRNAs in economically important fishes of Bangladesh.

At first, this study explored the available data on the selected fish through*in silico* approach. Based on homology searching and secondary configuration assessment this study displays potential miRNAs in 6 economically important cultured fish species of Bangladesh. As whole genome sequences of the selected fish species are not known yet, firstly GSS and EST database were used to identify conserved miRNAs in these fishes. Recently, several studies reported a similar approach to identify conserve miRNAs in various species using anavailable database like EST, GSS, protein, and mRNA databases (Andreassen and Worren, 2013; Hairul-islam et al., 2014; X. H. Li et al., 2015; Xu et al., 2013).Rohu (*Labeo rohita*) EST database was found comparatively enriched followed by silver carp (*Hypophthalmichthys molitrix*) than the other studied fishes.However, this study has found two potentially conserved miRNAs from Silver carp. The secondary structure prediction of these putative miRNAs also showed stem-loop formation, which is a hallmark of microRNA. In the case of four *L. rohita* positive hits, none formed such secondary structure, hence rejected as true hits for miRNA. In general, the EST and GSS databases of the studied fishes are very low in sequence richness. In addition, hilsa (*Tenualosa ilisha*), and gengetic mystus (*Mystus cavasius)* have no EST and GSS sequences in NCBI GenBank. This observation, in fact, indicates the importance of research in this arena to establish the knowledge base of our economically important fish species.

Stress biology in fish is a growing concern and many studies are going on to decipher molecular regulator of stresses (Anderson, 2016; Barton, 2002; Power, 1997). miRNAs can alter many kinds of normal physiological processes, such as proliferation, apoptosis, and metabolic pathways under stress responses that can cause cancers and tumors and so many diseases. Many of which are regulated by miRNAs. A hallmark of cancer is the presence of stress phenotypes (Luo et al., 2009) has been recently established. Stress phenotypes, including those resulting from DNA damage/ replication stress, proteotoxic stress, mitotic stress, metabolic stress and oxidative stress, may not be responsible for initiating tumorigenesis, but cancer cells develop tolerance to these stresses, and, in turn, become dependent on stress response pathways to maintain their growth and survival. Dependency on these stress response pathways regulated by miRNAs thus provides new

avenues for therapeutic intervention by modulating miRNA levels or activities. miRNA regulatory impact is even more pervasive as a potential therapeutic tool because often miRNA regulate multiple mRNA targets that belong to the same signaling pathway or protein complexes at the same time (Tsang et al., 2010). The key is to identify which miRNAs and which targets are involved in each particular disease. High-throughput biochemical techniques have been developed to identify endogenous mRNA targets (Chi et al., 2009; Hafner et al., 2010; Zisoulis et al., 2010). Alternatively, multiple signaling molecules in stress responses modulate the transcription, processing, and stability of miRNAs. These provide further avenues for modulating the miRNA pathway. For example, a small molecule has been identified from a pilot screen for positive modulators of miRNA processing (Shan et al., 2008). This raises the possibility of restoring the global miRNA level in cancer cells to a level similar to their normal counterparts. As our understanding of these fundamental processes deepens, therapeutic possibilities will continue to rise. Therefore, the present study emphasizes to search for a specific stress-responsive miRNA and a potential stress responsive miRNAs database was prepared. The database contains 550-stress responsive miRNA from fish and from different species. From the database then oxidative stress responsive miRNAs from fish were further sorted and studied in more details. In total 11 oxidative stress responsive miRNAs in fish were found. The target gene analysis of these stress responsive miRNAs shows that each of them targets genes related to stress response except one (dre-mir-455-5p). This study has validated the presence of one specific oxidative stress-responsive miRNA (mir-21) in rohu, catla, silver carp, climbing perch, gangetic mystus, hilsa of Bangladesh to contribute in fisheries biology research of Bangladesh.

In this study, we selected miR-21 as our target because miR-21 is known stress responsive miRNA and miR-21 showed the regulatory behavior in many organisms (Alvarez-Garcia and Miska, 2005; Cheng et al., 2009; Kolpa et al., 2013; Tang et al., 2013). miR-21 acts as a pro-proliferative and anti-apoptotic factor in the context of kidney regeneration in fish that indicates the role of miR-21 in the kidney (Uribe et al., 2011). miR-21 can inhibit the expression of cytokines by negative regulation of TLR28 (Troll Like Receptor), thereby inhibiting the generation of excessive immunity and maintaining the balance of the body. It also can suppress cytokines by regulating the TLR signaling pathway in teleost fish, and also can provide some new ideas for the research of the regulation of miRNA and immune system in mammals (Bi et al., 2017).

A study revealed a novel mechanism for the therapeutic function of fish oil diet that blocks miR-21, thereby increasing PTEN (Phosphatase and tensin homolog protein) levels to prevent expression of CSF-1 (Colony stimulating factor-1) in breast cancer (Cai et al., 2004). The small regulatory RNA miR-21 plays a crucial role in a plethora of biological functions and diseases including development, cancer, cardiovascular diseases and inflammation (Kumarswamy et al., 2011). miR-21 participates in H_2O_2-mediated gene regulation and cellular injury response through Programmed cell death 4 gene (PDCD4) (Cheng et al., 2009; Kolpa et al., 2013). Furthermore, miR-21 is overexpressed in solid tumors of the lung, breast, stomach, prostate, colon, brain, head and neck, esophagus and pancreas that promotes cell transformation and acts as an oncogene by a mechanism that involves in translational repression of the tumor suppressor gene (PDCD4) (Lu et al., 2008). Recently, miRNAs in fish associated with biotic stress have been identified and it would be a novel biomarker to manage biotic stress in fish (Hairul-islam et al., 2014). Assaying antioxidant enzymes can offer an indication of the antioxidant status of the organisms during stressed condition and can serve as biomarkers of oxidative stress (Ekambaram et al., 2014; Hou et al., 2011). Further research about miR-21 and its responses to oxidative stress could help to develop miR-21 as a biomarker in oxidative stress.

Degenerative primers were designed based on the homology of the miR-21in fish. PCR amplification of the pre-miRNA from all of the fish samples showed an expected amplicon size of around 67 bp. Bulk PCR was performed to have more DNA to facilitate downstream purification of the DNA. pre-miRNA-21 amplicon from hilsa showed smear formation after the expected band size, might be due to degradation by DNase or could be due to the presence of contaminants. The Same pattern of band formation was also evident in zebrafish sample. However, the control PCR for housekeeping gene showed expected band size for all of the samples tested. High level of agarose (1 to 3%) gels was used to facilitate proper separation of the small size pre mi-RNAs. Putative mir-21 sequences were validated by sequencing. Analysis of the sequence for secondary structure could identify the mature regions of the putative mir-21 of *Labeo rohita*, *Danio rerio*, and *Anabas testudineus*. Due to poor sequence quality, it was not possible to find the secondary structure of the putative mir-21 from all studied fish. However, it showed that identified miRNAs are similar and aligned well with other members of the miR-21 family.Conservation among species is one of the most important properties of miRNAs.

So, this feature facilitates us to perform the computational search for miRNAs based on the highly conserved sequence in the mature miRNAs and long hairpin structures in miRNA precursors (Mishra and Lobiyal, 2011).

This study identified some miRNAs in our local fish through the computational approach and *in silico* analysis. Further study could be done to decipher the roles of the identified miRNAs in these fish. The identified miRNAs were highly conserved and predicted target genes were found to be involved in various biological processes such as cell development and stress biology. These findings suggested that computational tools are pertinent to screen-out miRNAs and their targets from non-model fishes.An iterative integration of both experimental and bioinformatic approaches can provide better insight into biogenesis, mechanisms, and functions of miRNAs. Bioinformatics approaches and their associated methodologies can be applied across a range of technologies, facilitating rapid identification of thenew target. In other words, bioinformatics can accelerate this silent race from RNA laboratory to theclinic.

In brief, the study on computational profiling and identification of oxidative stress-responsive miRNAs of zebrafish (*Danio rerio*), rohu (*Labeo rohita*), catla (*Catla catla),* silver carp (*Hypophthalmichthys molitrix*), climbing perch (*Anabas testudineus),* hilsa (*Tenualosa ilisha*), and gangetic mystus (*Mystus cavasius)* can serve as an important platform, not only for understanding fundamental biological processes but also provides new insight into the functional roles of miRNAs in responses to environmental stresses. Conceivably, changes in miRNA profile and specific miRNAs may be employed as biomarkers for transgenerational effects of environmental stress.

Chapter 5

Conclusions and Recommendations

5.1 Conclusions

Aquaculture is a rapid growing sector of Bangladesh. Aquatic pollution cause stress to an aquatic organism. Oxidative stress in fish is a general consequence of the environmental pollution. Molecular explanation of stress biology is necessary to develop tools for handling this problem. Recently it has been established that miRNAs are biological regulators that can alter protein coding gene expression during stress. Proper identification of stress-responsive miRNA could potentially contribute in handling the raising concern about aquatic pollution and fisheries sector of Bangladesh. This study will contribute to building the baseline knowledge on one aspect of the molecular mechanism of stress management in fish through tracing the oxidative stress responsive miRNA. These findings of miRNA will be helpful to understand the gene regulation concept in these fish species. Moreover, it shows a convenient approach for the prediction and analysis of miRNAs of those species whose genomes are not available.

5.2 Recommendations

- miRNAs play indispensable roles in stress responses. Yet, in many cases, the molecular mechanisms are still unclear and should be investigated further to elucidate these fundamental roles of miRNAs in controlling mRNA regulation during stress.
- Future experimental validation would determine how many of these predicted targets are targeted by miRNAs in these fish species *in vivo*.
- Moreover, if miRNAs expression and their target genes expression (up-regulation or down-regulation) can be identified specifically, then it would be possible to understand their mode of action and pathway.
- Future aspects of the study would be to study miR-21 expression in specific stress responses and to establish a novel PCR-based method to use microRNA as a biomarker of stress.
- Early warning of this effect of pollutants, particularly in spontaneously polluted areas can be predicted using a biomarker approach, including oxidative stress-responsive gene and miRNAs. Elucidations of complete mechanism of specific microRNA would provide a possible way for manipulation of the miRNA to produce a stress-tolerant variety of fish.

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Appendices

Appendix A: Primers and primer designing

Pre-mir-21 sequences for alignment

>dre-mir-21-1

UUAUGUGUCUUUAUUGGCGUGGAUAUAAGUCUUUCCCAGUGUGUCAGAUA
GCUUAUCAGACUGGUGUUGGCUGUUACAUUCGCCCGGCGACAACAGUCUG
UAGGCUGUCUGACAUUUUGGGCAUUUUCUUCUCCGAUUAAAAAUAUGA

>ola-mir-21-2

CCCGGCCUGUCAGAUAGCUUAUCAGACUGGUGUUGGCUGUUAAGAUUGCA
AGGCGACAACAGUCUGUAGGCUGUCUGACAUUUUGGG

>fru-mir-21

UGUCAAAUAGCUUAUCAGACUGGUGUUGGCUGUUAAGAUUGCAAGGCGAC
AACAGUCUGUAGGCUGUCUGACA

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DELPLACE brigitte	BELGIUM LOUVAIN-LA-NEUVE 1348

OLIGO NAME	BATCH	SCALE	CHEMISTRY	BASES
SK_21F	6108505	10 nmol	DNA	19

SEQUENCE (5' → 3') (*: Phosphorothioate linkage)

1	ATA-GCT-TAT-CAG-ACT-GGT-G

BACKBONE (BASES + LINKAGES)

A	5	GC (%)[1]	42.1	Quantity	(OD@260nm) 10.49
C	3	MW (g.mol^{-1})[1]	5842.9		(nmol) 55.6
G	5	Ext. coeff. (l.mol^{-1}.cm^{-1})[1]	188600		(µg) 325
U / T	0 / 6	Tm (°C)[1]	54.0		
Others	0				
Wobbles	0	[1] Theoretical values			

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OLIGO NAME	BATCH	SCALE	CHEMISTRY	BASES
Sk_21R	6108506	10 nmol	DNA	17

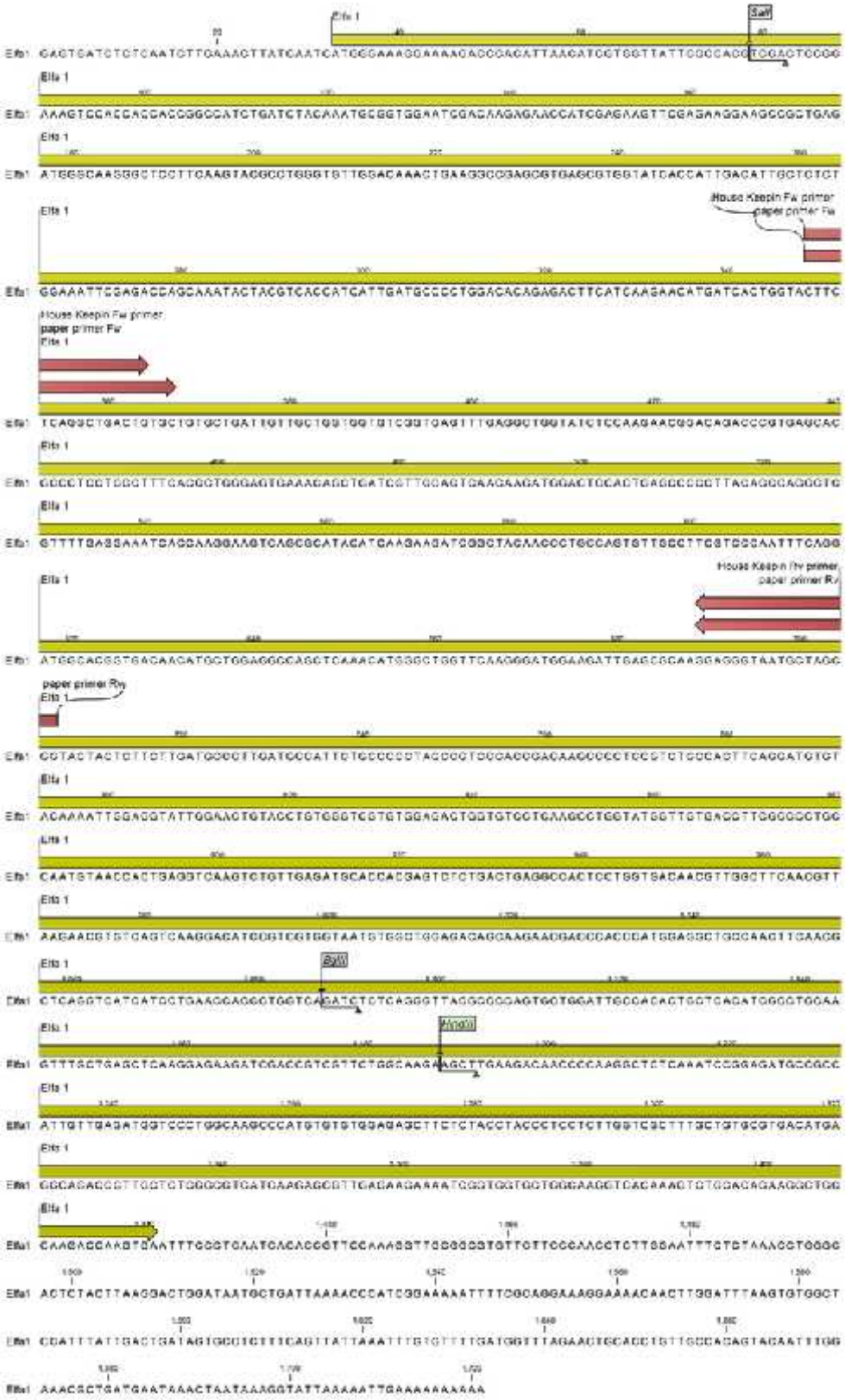
SEQUENCE (5' → 3') (*: Phosphorothioate linkage)

1	TGT-CAG-ACA-GCC-TAC-AG

BACKBONE (BASES + LINKAGES)

A	5	GC (%)[1]	52.9	Quantity	(OD@260nm) 9.35
C	5	MW (g.mol^{-1})[1]	5179.4		(nmol) 56.2
G	4	Ext. coeff. (l.mol^{-1}.cm^{-1})[1]	166300		(µg) 291
U / T	0 / 3	Tm (°C)[1]	52.0		
Others	0				
Wobbles	0	[1] Theoretical values			

Reference gene (elfa-1) and primers regions



Primers for Reference gene

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OLIGO NAME	BATCH	SCALE	CHEMISTRY	BASES
SI_HKE	6108507	10 nmol	DNA	19

SEQUENCE (5' → 3') (* Phosphorothioate linkage)

1 CTT-CTC-AGG-CTG-ACT-GTG-C

BACKBONE (BASES + LINKAGES)

A	2	GC (%)*	57.9	Quantity	(OD@260nm) 11.00
C	6	MW (g.mol^{-1})*	5770.6		(nmol) 66.6
G	5	Ext. coeff. (l.mol^{-1}.cm^{-1})*	166300		(µg) 384
U / T	0 / 6	Tm (°C)*	60.0		
Others	0				
Wobbles	0				

* Theoretical values

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DELPLACE brigitte	BELGIUM LOUVAIN-LA-NEUVE 1340

OLIGO NAME	BATCH	SCALE	CHEMISTRY	BASES
SK_UKE	[illegible]	10 nmol	DNA	19

SEQUENCE (5' → 3') (*: Phosphorothioate linkage)

1 CCG-CTA-GCA-TTA-CCC-TCC

BACKBONE (BASES + LINKAGES)

A	3	GC (%)*	61.1	Quantity	(OD@260nm) 9.10
C	9	MW (g.mol^{-1})*	5955.5		(nmol) 50.3
G	2	Ext. coeff. (l.mol^{-1}.cm^{-1})*	156200		(µg) 312
U / T	0 / 4	Tm (°C)*	58.0		
Others	0				
Wobbles	0				

* Theoretical values

Appendix B: Laboratory reagents

Reagents, which were used in carrying out different methods together with their sources, are mentioned below:

Lysis buffer

Components of lysis buffer
100 mM Tris-HCl pH 8.0
5 mM EDTA pH 8.0
2 % (w/v) SDS
400 mM NaCl

PCR chemicals

Polymerase chain reaction (PCR) reagents
Nuclease-free water
DMSO

Mg buffer
dNTPs
Commercial Taq polymerase
Forward Primer
Reverse Primer
Template DNA

Gel loading buffer:

Ingredients	Amount (g/L)
Sucrose	6.7
Bromophenol blue	0.04
Distilled water	Up to 1 L

Ethidium bromide solution

1.0g of ethidium bromide was dissolved in distilled in distilled water to a final volume of 100 ml. The container was wrapped in aluminum foil and stored at 4ºC.

Common Laboratory Apparatus Used In this Study

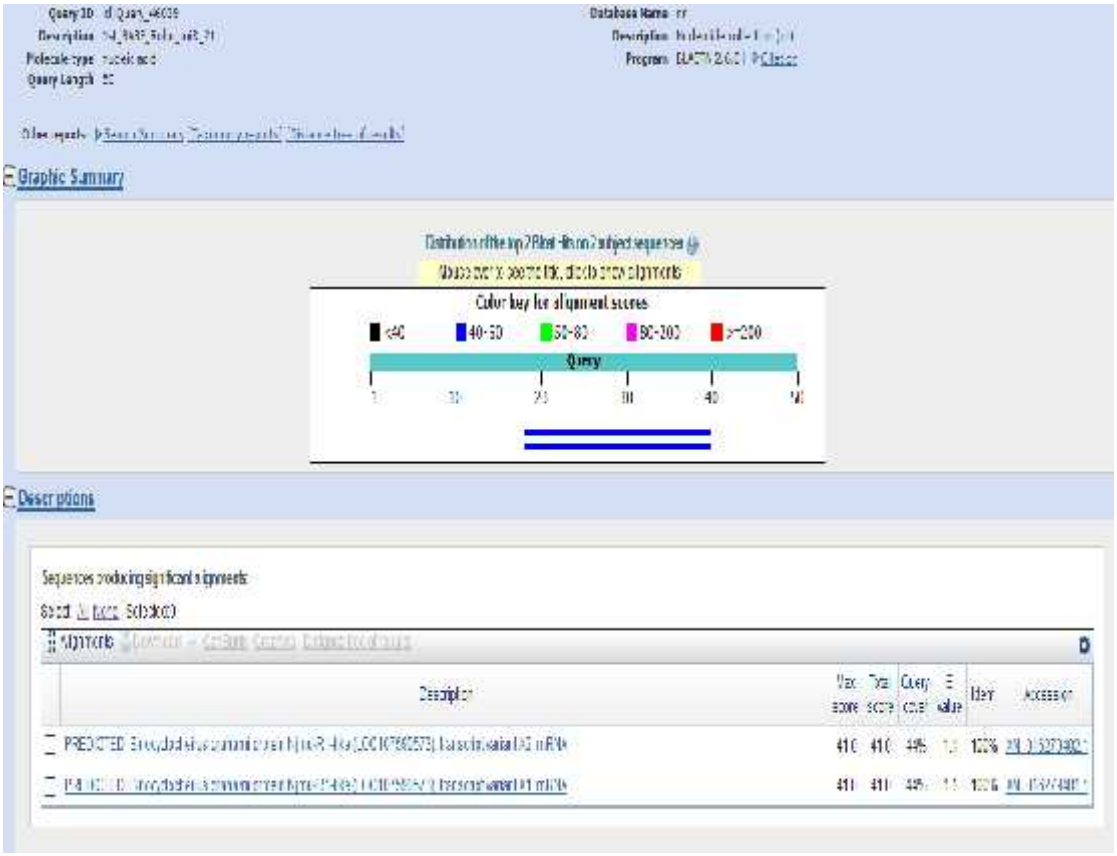
Name	Description	Source
Eppendorf tubes	Size 1.5 ml, colorless	Eppendorf, Germany
Micropipette tips	Vol. Range 0.5-200 µL, 200-1000 µL	Labsystems, Finland
PCR tubes	Size 0.2, 0.5 ml	USA
Gloves	Disposable	Labsystems, Finland
Autoclave machine	Model HL-42A	Japan
Micro-centrifuge	5415C	Germany
DNA thermal cycler		Proflex thermal cycler (Applied Biosystems)
Vortex	Model 1190-1	LabineInstruments, USa
Circulating water bath	Model 1260	Precision Scientific
UV trans illuminator	Model T2201	SIGMA Chemical CO., USA
pH meter		Orion Research, USA
Tube rack		SIGMA, USA
Balance machine		Mettler AE 100, Switzerland
Centrifuge tube		USA
Refrigerated super speed centrifuge	Model RC 5B	Sorvall
Refrigerator	-80 ºC	Emerson, Korea
Microwave		Japan
Nanodrop	Thermo Scientific Nanodrop 1000	Fischer Scientific, USA

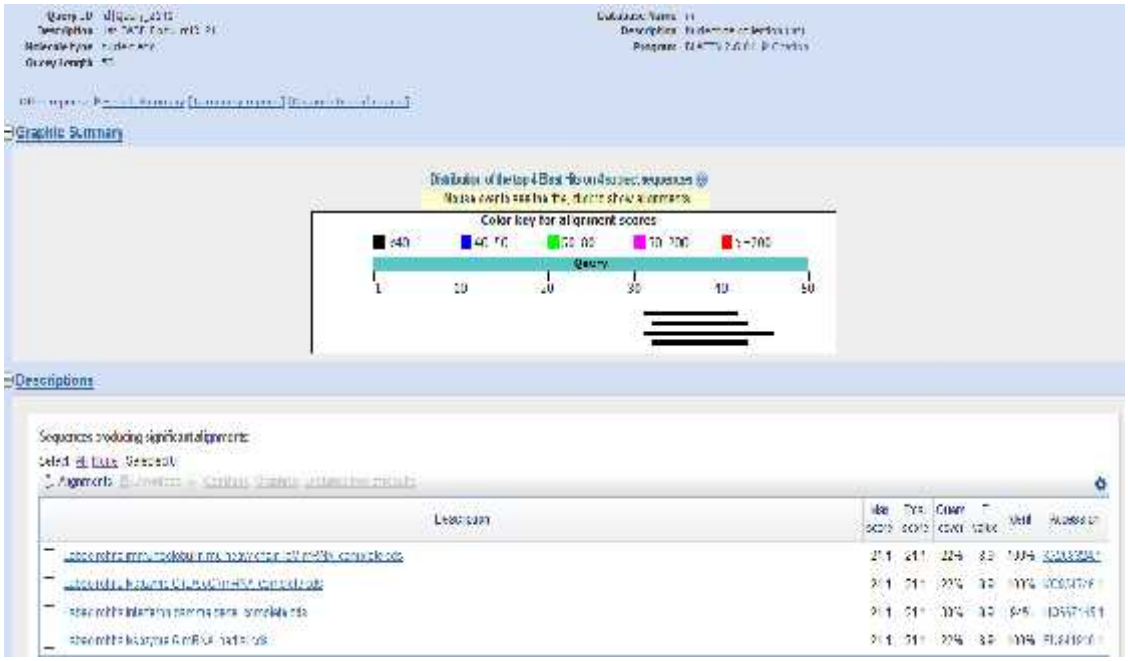
Appendix C: *In Silico* analysis for miRNA identification

Blast results for conserved miRNA identification

Blast for miRNA identification

Rohu pre-miR-21 BLAST





Appendix D: In Silico analysis for miRNA target prediction

Target prediction using TargetScan Fish software

Target sites

	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match	site-type contri- bution	3' pairing contri- bution	local AU contri- bution	position contri- bution	TA contribution	SPS contribution	context+ score	context+ score percentile
[illegible] dre-miR-181a	5' [illegible] 3' [illegible]	7mer-m8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match
Position 498-504 of GPX8 3' UTR dre-miR-181a	5' ...CAGAACACAUAAUAGUGAAUGUG... ||| ||||||| 3' UGAGUGGCUGUCGCA--ACUUACAA	7mer-m8
Position 485-492 of cat 3' UTR dre-miR-181a	5' ...UCGGUUCUCAUAAUAUGAAUGUA... ||||||| 3' UGAGUGGCUGUCGCAACUUACAA	8mer

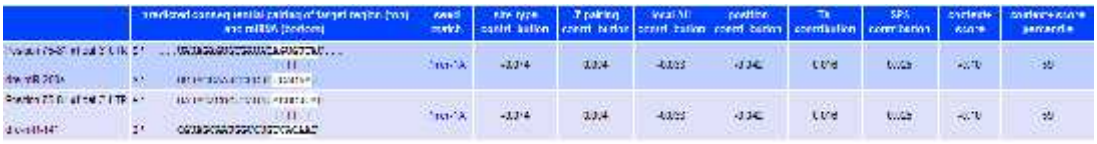
	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match	site-type contri- bution	3' pairing contri- bution	local AU contri- bution	position contri- bution	TA contribution	SPS contribution	context+ score	context+ score percentile
Position 485-492 of cat 3' UTR dre-miR-181a	5' [illegible] 3' [illegible]	8mer	[illegible]	[illegible]	0.073	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Position 705-711 of hspb6 3' UTR dre-let-7f	5' [illegible] 3' [illegible]	7mer-m8	-0.120	-0.007	-0.014	0.032	[illegible]	-0.026	[illegible]	[illegible]

	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match
Position 705-711 of hspb6 3' UTR dre-let-7b	5' ...GGCCUUACCCCACCUCUACCUCU... ||||||| 3' UUGGUGUGUUGGAUGAUGGAGU	7mer-m8
Position 705-711 of hspb6 3' UTR dre-let-7f	5' ...GGCCUUACCCCACCUCUACCUCU... ||||||| 3' UUGAUAUGUUAGAUGAUGGAGU	7mer-m8

	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match	site-type contri- bution	3' pairing contri- bution	local AU contri- bution	position contri- bution	TA contribution	SPS contribution	context+ score	context+ score percentile
Position 243-250 of hspb8 3' UTR dre-miR-21	5' ...GUCUAUAGAAGCUAAAUAAGCUA... 3' CGGUUGUGGUCAGACUAUUCGAU	8mer	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match
Position 243-250 of hspb8 3' UTR dre-miR-21	5' ...GUCUAUAGAAGCUAAAUAAGCUA... ||||||| 3' CGGUUGUGGUCAGACUAUUCGAU	8mer

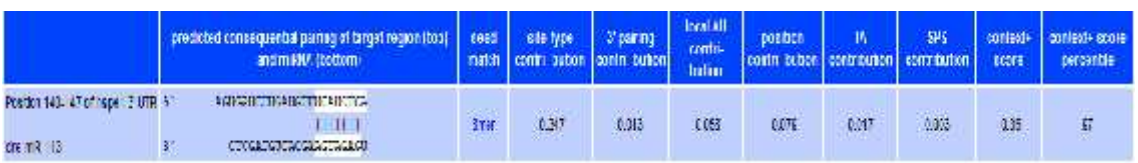
	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match
Position 205-212 of pdcd4b 3' UTR dre-miR-21	5' ...CUCCUUCUGACUCGUAUAAGCUA... 3' CGGUUGUGGUCAGACUAUUCGAU	8mer



	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match
Position 75-81 of cat 3' UTR dre-miR-200a	5' ...UAUAGAGUGUGAUAUAGUGUUAU... 3' UGUAGCAAUGGUCUGUCACAAU	7mer-1A
Position 75-81 of cat 3' UTR dre-miR-141	5' ...UAUAGAGUGUGAUAUAGUGUUAU... 3' CGUAGCAAUGGUCUGUCACAAU	7mer-1A

	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match
Position 322-328 of pdcd8 3' UTR dre-miR-143	5' ...GAGGUCAGUAAAUGUUCAUCUCU... 3' CUCGAUGUCACGA---AGUAGAGU	7mer-m8

	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match
Position 321-327 of hspa8 3' UTR dre-miR-143	5' ...GUCUUUUUACCUUUACAUCUCAA... 3' CUCGAUGUCACGAAGUAGAGU	7mer-1A



	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match
Position 85-92 of GPX2 3' UTR dre-miR-143	5' ...AGCAGCUCUACGGCA--UCAUCUCA... |||| ||||||| 3' CUCGAUGUCACGAAGUAGAGU	8mer

	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match	site-type contri-bution	3' pairing contri-bution	local AU contri-bution	position contri-bution	TA contribution	SPS contribution	context+ score	context+ score percentile
Position 270-[illegible] of [illegible] 3' UTR dre-miR-[illegible]	5' ...[illegible]... 3' [illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match	site-type contri-bution	3' pairing contri-bution	local AU contri-bution	position contri-bution	TA contribution	SPS contribution	context+ score	context+ score percentile
Position 578-584 of [illegible] 3' UTR dre-miR-[illegible]	5' ...[illegible]... 3' [illegible]	7mer-m8	[illegible]	0.003	[illegible]	-0.056	[illegible]	0.004	[illegible]	84

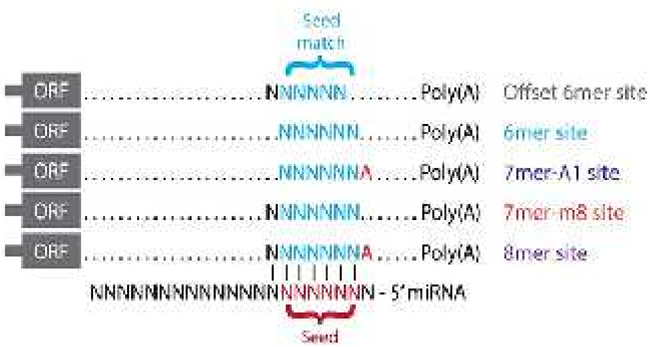
	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match	site-type contri-bution	3' pairing contri-bution	local AU contri-bution	position contri-bution	TA contribution	SPS contribution	context+ score	context+ score percentile
Position 615-621 of GPX8 3' UTR dre-miR-[illegible]	5' ...[illegible]... 3' [illegible]	7mer-m8	-0.123	0.003	[illegible]	[illegible]	[illegible]	[illegible]	-0.12	73

	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match	site-type contri-bution	3' pairing contri-bution	local AU contri-bution	position contri-bution	TA contribution	SPS contribution	context+ score	context+ score percentile
Position [illegible] of [illegible] 3' UTR dre-miR-[illegible]	5' ...[illegible]... 3' [illegible]	7mer-1A	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Position [illegible] of [illegible] 3' UTR dre-miR-[illegible]	5' ...[illegible]... 3' [illegible]	7mer-m8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

In addition to 7mer sites, TargetScan predicts 8mer sites defined as:

- An exact match to positions 2-8 of the mature miRNA (the seed + position 8) followed by an 'A'

Canonical site types



Appendix E: miRBase alignment of identified precursors

Sequence search results

See the BLAST help pages for detailed information about the meaning of the scores shown here.

Accession	ID	Query start	Query end	Subject start	Subject end	Strand	Score	Evalue	Alignment
[illegible]	[illegible]	[illegible]	31	11	[illegible]	[illegible]	[illegible]	[illegible]	Align

Alignment of Query to mature miRNAs

```
Query: [illegible]      [illegible]      score: [illegible]      evalue: [illegible]
      UsedSeq             [illegible]  [illegible]  31
      [illegible]         31  [illegible]  [illegible]
```

Search parameters

Search algorithm:	BLASTN
Sequence database:	mature
Evalue cutoff:	10
Max alignments:	100
Word size:	4
Match score:	+5
Mismatch penalty:	-4

Accession	ID	Query start	Query end	Subject start	Subject end	Strand	Score	Evalue	Alignment
[illegible]	[illegible]	[illegible]	[illegible]	4	21		65	[illegible]	Align
[illegible]	[illegible]	42	[illegible]	[illegible]	22	+	64	4.4	Align
[illegible]	[illegible]	49	69	1	21	-	60	9.6	Align

Alignment of Query to mature miRNAs

```
Query: 28-45      [illegible]: 4-21      score: 65      evalue: [illegible]
      UsedSeq             28  [illegible]  [illegible]
      [illegible]         21  [illegible]  4

Query: 42-61      [illegible]: [illegible]-22      score: 64      evalue: 4.4
      UsedSeq             42  [illegible]  61
      [illegible]         [illegible]  [illegible]  22

Query: [illegible]      [illegible]: 1-21      score: 60      evalue: [illegible]
      UsedSeq             [illegible]  [illegible]  [illegible]
      [illegible]         21  [illegible]  1
```

Search parameters

Search algorithm:	BLASTN
Sequence database:	mature
Evalue cutoff:	10
Max alignments:	100
Word size:	4
Match score:	+5
Mismatch penalty:	-4

Alignment of Query to mature miRNAs

```
Query: 24-39        dre-miR-21: 1-16          score: 71      evalue: 1.2
  lrc miR 21        24  ccugucugauaagcua  39
                        || ||||||||||||
  dre-miR-21        16  ccagucugauaagcua  1

Query: 24-39        fru-miR-21: 1-16          score: 71      evalue: 1.2
  lrc-miR-21        24  ccugucugauaagcua  39
                        || ||||||||||||
  fru miR 21        16  ccagucugauaagcua  1

Query: 24-39        tni-miR-21: 1-16          score: 71      evalue: 1.2
  lrc-miR-21        24  ccugucugauaagcua  39
                        || ||||||||||||
  tni-miR-21        16  ccaguctgauaagcua  1

Query: 24-39        ola-miR-21: 1-16          score: 71      evalue: 1.2
  lrc-miR-21        24  ccugucugauaagcua  39
                        || ||||||||||||
  ola miR 21        16  ccaguctgauaagcua  1

Query: 24-39        mmu-miR-21c: 1-16         score: 71      evalue: 1.2
  lrc-miR-21        24  ccugucugauaagcua  39
                        || ||||||||||||
  mmu-miR-21c       16  ccaguctgauaagcua  1

Query: 24 39        pol miR 21 5p: 1 16       score: 71      evalue: 1.2
  lrc miR 21        21  ccugucugauaagcua  36
                        || ||||||||||||
  pol miR 21 5p     16  ccaguctgauaagcua  1
```

See the BLAST help pages for detailed information about the meaning of the scores shown here.

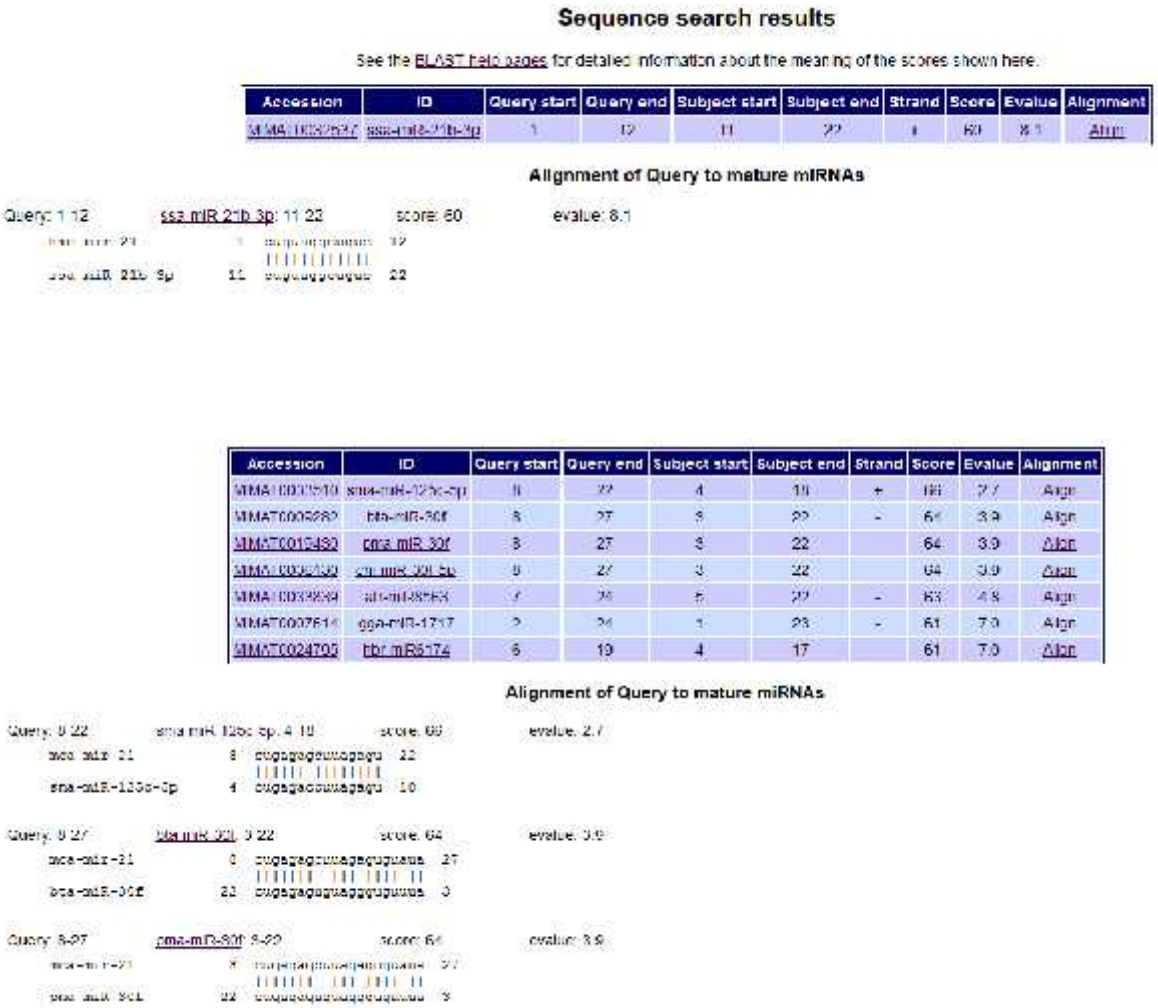
Accession	ID	Query start	Query end	Subject start	Subject end	Strand	Score	Evalue	Alignment
MIMA0001[illegible]	cin-miR-[illegible]-5p	56	74	3	21	-	10	6.7	Align

Alignment of Query to mature miRNAs

Query: 56-74 cin-miR-[illegible]-5p: 3-21 score: 69 evalue: 6.7

Search parameters

Search algorithm:	BLASTN
Sequence database:	mature
Evalue cutoff:	10
Max alignments:	100
Word size:	4
Match score:	+5
Mismatch penalty:	-4



Sequence search results

See the BLAST help pages for detailed information about the meaning of the scores shown here.

Accession	ID	Query start	Query end	Subject start	Subject end	Strand	Score	Evalue	Alignment
MIMAT0003927	ssa-miR-21b-3p	1	12	11	22	+	60	8.1	Align

Alignment of Query to mature miRNAs

Query: 1 12 ssa-miR-21b-3p: 11 22 score: 60 evalue: 8.1

Accession	ID	Query start	Query end	Subject start	Subject end	Strand	Score	Evalue	Alignment
MIMAT0015510	sma-miR-125c-5p	5	22	4	19	+	66	2.7	Align
MIMAT0003682	bta-miR-30f	5	27	5	22	-	64	3.9	Align
MIMAT0012480	gma-miR-30f	5	27	5	22		64	3.9	Align
MIMAT0003149	ptr-miR-30f-5p	5	27	5	22		64	3.9	Align
MIMAT0003649	ssc-miR-9783	7	26	5	22	-	63	4.8	Align
MIMAT0007614	gga-miR-1717	2	24	1	23	-	61	7.0	Align
MIMAT0024795	hbr-miR6174	5	19	4	17		61	7.0	Align

Alignment of Query to mature miRNAs

Query: 5 22 sma-miR-125c-5p: 4 19 score: 66 evalue: 2.7

Query: 5 27 bta-miR-30f: 5 22 score: 64 evalue: 3.9

Query: 5 27 gma-miR-30f: 5 22 score: 64 evalue: 3.9

Appendix F:

Identification of silver carp miRNA target using RNA hybrid software:

```
Version: RNAhybrid 2.2
Command line:/usr/bioapps/bin/RNAhybrid.bin -s 21 -q /var/bibiserv2/
searching
dataset: 1
mfe of hmo-miR-2192:  -23.700001
Individual hits
-----------------------------------------------------------

dataset: 1
target: JF958124.1
length: 2008
miRNA : hmo-miR-2192
length: 21

mfe: -20.3 kcal/mol
p value: 1.000000e+00

position  442
target 5' G        GGUAAA   A      G  3'
           GCUUCAGU      GCC  UCACU
           CCCACUCA      UCC  ACUCA
miRNA  3'          G        AA     AA 5'

-----------------------------------------------------------
```

Appendix G: Prediction of secondary structure by RNA web server