BREEDING BEHAVIOUR, EMBRYONIC DEVELOPMENT AND BARCODING OF THE ORNAMENTAL LOACHES (COBITIDAE: CYPRINIFORMES) OF TERAI REGION OF WEST BENGAL, INDIA.

Thesis Submitted to the University of North Bengal
For the award of
Doctor of Philosophy in Zoology

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This is to certify that the work embodied in the Thesis entitled "**Breeding Behaviour, Embryonic Development and Barcoding of the Ornamental Loaches (Cobitidae: Cypriniformes) of Terai Region of West Bengal, India"** submitted by Ms. Arpita Dey, M.Sc., and carried out under my Supervision in the Aquaculture and Limnology Research Unit, Department of Zoology, University of North Bengal, is based on the original investigative study. Neither this Thesis nor any part of it has been submitted for any Degree or any other Academic Awards anywhere before.

Miss Arpita Dey has fulfilled the requirements of the Degree of Doctor of Philosophy in Science (Zoology) of the University of North Bengal. She has carried out the work thoroughly. In character and disposition, Ms. Dey is fit to submit the Thesis for Ph. D Degree.

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Ms. Arpita Dey, had carried out a very small part of the work at the Fishery Unit, Uttar Banga Krishi Viswavidyalaya, Pundibari, Cooch Behar. This Ph. D. thesis is based on the original investigations done by her and that neither this thesis, nor any part of it has been submitted earlier for any other Degree or Academic Awards.

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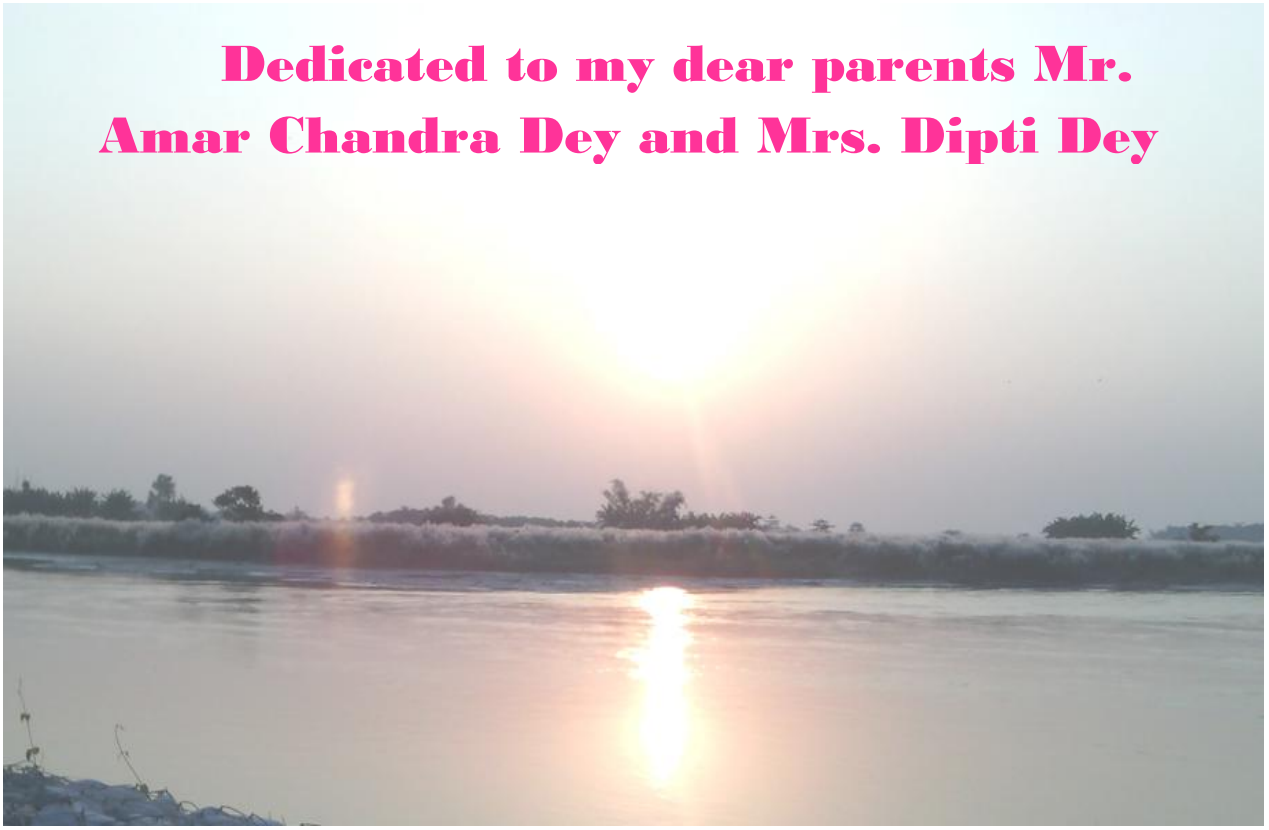
Dedicated to my dear parents Mr.
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Place: University of North Bengal

Dated**:* ***Arpita Dey

Abstract

Ornamental fish production globally is a multibillion dollar industry. India's overall ornamental fish trade was about 1.06 million US$ during year 2009. India has recorded at least 150 commercially important ornamental fish species and trade mainly indigenous freshwater species collected from rivers. Among them, *Botia* loaches classified as aquarium fish due to their beautiful colouration, small size, bright bands, blotches, peaceful nature and hardiness can be reared and bred in aquarium throughout their life span.

Botia almorhae (Grey), *Botia dario* (Hamilton-Buchanan), *Botia lohachata* (Chaudhuri) and *Botia rostrata* (Gunther) were selected for present study. They are highly demanding both as an ornamental and edible fish in the Terai region of Eastern Himalaya of West Bengal, India, a "Hot Spot" for fresh water fish biodiversity. With a view to rearing and breeding of loaches in captivity, which are Vulnerable and Endangered, their conservation and ichthyofaunal diversity of river Kaljani, Cooch Behar district a study was executed during the period August 2012 to July 2015.

In the present study, for both river Kaljani and Captive study water was soft, alkaline in nature with high Dissolved Oxygen and medium productive condition. The temperature was good for growth of the fishes of Kaljani river water and Laboratory water (Captive condition). TDS, Free Carbon Dioxide and average concentrations of ammonium-nitrogen (0.017 mg L^{-1}), nitrite-nitrogen (0.009 mg L^{-1}), nitrate-nitrogen (0.312 mg L^{-1}) and Phosphate-phosphorou**s** concentration in the Kaljani river and captive condition were also within normal range.

The average Gonado-somatic Index data of *Botia* species revealed to be *Botia almorhae* (11.96±10.29), *Botia dario* (8.34±5.4), *Botia lohachata* (13.86±11.50) and *Botia rostrata* (10.29±9.01). Among the *Botia* species, *Botia lohachata* had the highest GSI and *Botia rostrata* the lowest. Condition Factor or K- factor in Captive condition for

Abstract

Botia almorhae was 1.390, *Botia dario* was 1.788., *Botia lohachata* was 1.538 and *Botia rostrata* was 1.399. The values indicated good general condition of fish as 'K' was greater than 1.0.

Length - Weight relationship was calculated by the determination of Coefficient of Correlation (r). The Coefficient of Correlation of *Botia almorhae* was 0.811; *Botia dario*: 0.802; *Botia lohachata*: 0.753 and *Botia rostrata*: 0.936. *Botia* species indicated positive allometric growth and suggested that all fish grows in proportion to the length in Captive condition. Average fecundity of *Botia almorhae*, *Botia dario*, *Botia lohachata* and *Botia rostrata* were 18539, 22573, 18053 and18698 respectively and fertilization rate 90.03%, 82.09 %, 95.98 % and 67.60 % respectively

Fish collected from river water were first acclimatized in aquarium and induced bred using synthetic hormone WOVA-FH at a dose of 0.025ml/fish. The latency period was 4.30 to 6.00 hours. The embryonic development studied in *Botia* species was divided into eight stages namely Zygote, Cleavage, Blastula, Gastrula, Segmentation, Pharyngula, Hatching and Early larval period. Complete adult stage was obtained within one year. After captive breeding of wild *Botia* species, F_1 generation of *Botia* loaches were aqua-ranched into the natural environment of the river system (River Kaljani).

Spawning behaviour was observed during the night or afternoon in absence of light. At the time of spawning, they made loud cracking sound repeatedly. Six types of breeding behaviour were observed during spawning time like a) male hitting the female on snout, b) male hitting the female fish in vent the region more frequently, c) fighting between the males, d) male chasing the female, e) male and female fish were embraced together and swam and f) Cannibalism behaviour.

In the present study, good growth was observed in Tank-D (only minced snail or bivalve flesh fed) and lowest growth rate was observed in Tank-A (fed only commercial

fish feed). The growth rates were similar in Tank B (fed only live zooplanktons) and Tank C (fed with boiled minced meat).

The pre-spawning phase or developing phase of ovary was found to be during March to May and testis was found from April to May. Spawning phase of ovary of *Botia almorhae*, *Botia lohachata* and *Botia rostrata* were found to be during June to August and *Botia dario* was found to be during May to July. Post-spawning phase of ovary of *Botia* species was found to be during September to October. Spawning phase of testis of *Botia* species was found during May to September

Further, to confirm the *Botia* species, identification, barcoding study was done to reveal the evolutionary distances among *Botia* genus ranging from 0.002 to 0.112. The Barcode ID number of four *Botia* species was **SDP657007-17** (*Botia almorhae*), **SDP657005-17** (*Botia dario*), **SDP657002-17** (*Botia lohachata*) and **SDP657006-17** (*Botia rostrata*).

The present study permitted to study the Ichthyodiversity of river Kaljani, Cooch Behar district, West Bengal, 138 fish species were recorded which belonged to 31 families. Among the 138 species, 55 species had food value, 58 species ornamental value and 25 species both ornamental and food value. *Tenualosa toil, a* Chinese herring, was also found at Chhat Bhelakopa (Site-4) only during monsoon. The thesis provides baseline data on biodiversity of river Kaljani which may be helpful for conservation and management of the *Botia* loaches and also useful for fish breeders, aquarium keepers.

Keywords: Terai region of Eastern Himalaya, River Kaljani, *Botia almorhae*, *Botia dario*, *Botia lohachata*, *Botia rostrata*, Length-weight relationship, Condition factor, Gonado-somatic Index, Captive breeding, Fecundity, Breeding behaviour, Embryonic development, Histology, Barcoding.

Preface

Aquaculture and Fisheries are both industries serving not only as a source of providing essential nutrition but also, helping in the upliftment of livelihood of the greater human population and in earning foreign exchange. India, after China, is the second largest country in global fish production with aquaculture contributing about 5.68% (30,213 crores). India's share in ornamental fish trade is estimated to be less than 1% of the global trade (0.008%) and a domestic market of Rupees 10 crores. The State of West Bengal, in the forefront, has a share of around 90 percent of the total export earnings from ornamental fish.

The loaches belonging to the family Cobitidae and Balitoridae are classified as aquarium fish due to their beautiful colouration, small size, bright bands, blotches, peaceful nature and hardiness can be reared and bred in aquarium throughout their life span. *Botia* species are highly valued and demanding both as an ornamental and edible fish in the Terai region of Eastern Himalaya of West Bengal, India, a "Hot Spots" for fresh water fish biodiversity. In the Terai region, the existence of *Botia* species is very rare or their occurrence is low throughout the year. Endangered and Vulnerable status of the loaches in the Terai region of the studied area is mainly due to the water quality deterioration. Immediate conservation or rehabilitation of *Botia species* is therefore required from its extinction from the environment.

Thus, Captive breeding was conducted using synthetic hormone WOVA-FH at a dose of 0.025ml/fish in the present study. Barcoding was also done to identify of *Botia* species at molecular level. This technology is very useful for Endangered and Vulnerable species to survive in nature.

Hence, the present study, in the Eastern Himalaya region highlights the status quo of *Botia* species, the conservation steps and market value.

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List Of Abbreviations

A	Adenine
APHA	American Public Health Association
BLAST	Basic Local Alignment Search Tool
bp	Base pair
cm	Centimeter
CO-I	Cytochrome C oxidase subunit-I
Cyt-b	Cytochrome-b
C	Cytosine
^0C	Degree celcius
DO	Dissolved Oxygen
DDW	Double Distilled Water
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide tri phosphates
EDTA	Ethylene diamine tetra acetic acid
e.g.	For example
Etbr	Ethidium bromide
Fig.	Figure
FCO_2	Free Carbon dioxide
G	Guanine
HCl	Hydrochloric acid
i.e.	that is
IUCN	International Union for Conservation of Nature and Natural Resources
µl	Micro litre
mA	Milliampere
mg	Milligram
$MgCl_2$	Magnesium chloride

List Of Abbreviations

min	Minute
ml	Millilitre
mM	Millmolar
Mt DNA	Mitochondrial DNA
NaOAc	Sodium acetate
No.	Number
NCBI	National Center for Biotechnology Information
Ng	Nanogram
OD	Optical density
PCR	Polymerase Chain Reaction
pH	Hydrogen Ion Concentration
Pmoles	Pico moles
RNA	Ribonucleic acid
RNAse	Ribonuclease
rpm	Revolution per Minute
SDS	Sodium dodecyl sulphate
SPSS	Statistical Package for the Social Sciences
T	Thymine
TAE	Tris-Acetate EDTA
Taq	Thermus aquticus
TE	Tris-EDTA
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultra-Violet
V	Volt

INTRODUCTION

1. INTRODUCTION

Recent years have witnessed that both Aquaculture and Fisheries have developed into potential industries by providing nutrition for uplifting livelihood through foreign exchange. Aquaculture and Fisheries are both industries serving not only as a source of providing essential nutrition but also, helping in the upliftment of livelihood of the greater human population in the form of earning foreign exchange. India is the second largest country in global fish production with aquaculture contributing about 5.68% (30,213 crores) (**Goswami and Zade, 2015**). The total fish production during 2013-14 was 9.58 million metric tonnes with a contribution of 6.14 million metric tonnes from Inland sector and 3.44 million metric tonnes from Marine sector, respectively. The overall growth in fish production in 2013-14 was 5.9%, being mainly due to 7.3% growth in inland and 3.7% in marine fish production (**Handbook on Fisheries Statistics, 2014**).

The ornamental aquatic industry dealing with live animals and plants for aquaria or ponds, its associated equipment and feed is a worldwide business with a trade of approximately 15 million US $ (**FAO, 2013**) retailed value. India's share in ornamental fish trade is estimated to be less than 1% of the global trade (0.008%) and a domestic market of Rupees 10 crores with the state of West Bengal in the forefront with a share of around 90 percent of the export earnings from ornamental fish (**Dholakia, 2009**). In terms of money it comes to 23 million or two crores and thirty lakhs rupees.

Ornamental fishes are called 'living jewels' for their beautiful colour and playful behaviour and are typically small sized; attractive and bizarre shaped in appearance (**Dey, 1996**). Ornamental fish keeping being one of the most popular hobbies in the world is triggering a hike in aquarium fish trade globally. They are usually kept in aquarium and popularly known as aquarium fishes. It is a source of attraction for fish lovers and

aquarists all over the world. According to **Angami (2012),** Indian ornamental fishes with their brilliant colours and unique features need no introduction to the world market. The tropical ornamental fishes from North Eastern and Southern provinces of India are in great demand in the hobbyists market. Many attractive loaches, barbs, badis, zebra fishes, catfishes and glass fishes are indigenous to the lentic and lotic water ecosystems of India. Terai and Dooars regions of Eastern Himalaya are considered as "Hot Spots" for fresh water fish biodiversity (**Dey *et al.*, 2015f**). In these regions, ornamental fishes are dominant over the food fishes. Almost 85 % of the exportable ornamental fish are contributed by the North Eastern region of India (**Swain, 2004**). A great number of species have been reported from Cooch Behar district on fish biodiversity and 10 species of loaches are available in Cooch Behar district **(Dey *et al*., 2015a).** Among all the rivers flowing through the district of Cooch Behar, Kaljani is the richest in fresh water fish biodiversity. This river which is about 96 Km long originates from Gabaur Bachhra forest, lying in the borders of Bhutan and West Bengal, and outfalls into Shiltorsa in Cooch Behar. Biodiversity is essential for balancing ecosystem but it is drastically reduced by anthropogenic activity. The river water is used for agriculture, fisheries, residential and industrial developments, mining activity, navigation, power generation and variety of other activities including sand digging and disposal of industrial and domestic wastes and as such natural breeding is hampered.

The fishes of the family Cobitidae and Balitoridae are popularly known as 'Loach'. Balitorinae fishes are known as 'hill stream loaches' whilst, Cobitidae fishes are popularly known as 'Loaches'. These loaches are mainly found both in lentic and lotic water bodies. *Botia* species (subfamily Botinae) are less abundant, Endangered and Vulnerable whereas, other subfamily (Nemacheilinae, Cobitinae and Balitorinae) of loaches are abundant and Least Concern. Therefore, *Botia* species were found in the river

Kaljani selected as experiment fish for the present study. The *Botia* loaches are high demanding species having both ornamental and economical important food value and contribute to a major share of the world market for beautiful coloured indigenous ornamental fish. Among the loaches, *Botia dario* (Hamilton-Buchanan) commonly known as "Queen loach*"* or "Rani Mach*"*, *Botia rostrata* (Gunther), commonly known as "Ladder loach", are vulnerable fishes (**IUCN, 2010**) whereas, *Botia almorhae* (Grey), commonly known as "Almorha loach*"* and *Botia lohachata* (Chaudhuri), popularly known as "Y-loach*"* or "Tiger loach*"* or "Lohachata*"*, are endangered species (**IUCN, 2010**) are distributed widely in North-East India and Bangladesh. These *Botia* species are high demanding species of Terai region of West Bengal for aquarium fish .They lead a nocturnal life and remain buried in sand or silt for most of the time. The fishes are very colourful with bright bands, peaceful nature, lesser scales and barbles. Loaches are omnivores, and usually prefer *Daphnia*, earthworms, bloodworms, snails and animal proteins. The "Y-loach" is a scavenger but does not eat fish wastes.

Schistosomiasis, also known as "bilharzias", "bilharziosis" or "snail fever" is a serious disease affecting human and domestic animals as well as wild animals **(Dey *et al*., 2015c).** The intermediate host of the parasite which causes the disease is a species of freshwater snails. The snail eating loach is one of the many natural controls of the freshwater snail population and plays and an important role in controlling the disease. *Botia* loach makes a cracking sound. This is either produced by forcing air through the gills and may be connected with feeding on the surface of the water or alternately produced by specialized teeth in the throat of the fish that appear to aid in the extraction of snails muscle from their shells.

Fig. 1. The Botia species (a) *Botia almorhae* (b) *Botia dario* (c) *Botia lohachata* (d) *Botia rostrata*

Loaches are considered as Endangered. The Endangered status of the loaches are mainly because of the deterioration of the environment particularly, water quality which may be due to agricultural run-offs or pesticidal effect of tea gardens in the Terai and Dooars regions, and big-water bodies being fragmented into small water bodies; thus drying up the water. *Botia* species are regarded an excellent ornamental fish and highly preferred among villagers for fish ornamental farming. In the Terai region *Botia almorhae*, *Botia lohachata* and *Botia rostrata* are very rare or their occurrence is low throughout the year. The habitats of fish fauna are rapidly shrinking due to human activity and drought. Immediate rehabilitation of *Botia species* is important from its extinction from the environment. Therefore, rearing, captive breeding and embryonic development of *Botia species* are the vital recodes to be taken without delay before their extinction from this region. Captive breeding of any species helps to reintroduce then in their natural habitat. This technology is very useful for Endangered species to survive in nature.

Studies of breeding behaviour, embryonic development and reproductive biology of any fish is essential for evaluating the commercial potentialities of its stock, life history, cultural practice and actual management of small indigenous fishes (**Lagler 1956; Doha and Hye, 1970)**. The reproductive potential of a population is one of the basic exigencies to designate the individuals of that population in respect of their gonadal condition (**Jhingran and Verma, 1972).** Knowledge of gonadal development and the spawning season of species allow subsequent studies on spawning frequency of its

population which is very important for its management. The histological studies help in detecting the breeding season and establishing phenotypic characters of mature brooders for successful artificial propagation of a species. Hence, it is very important to assess the yearly breeding cycle of loaches to ensure success in culture practice. Development of captive breeding and seed production techniques for the indigenous fish species is also an approach solution for conservation. This can be achieved by restocking their natural habitat with hatchery-reared individuals (**Philippart, 1995; Poncin & Philippart, 2002** and **Montchowui *et al.*, 2011**); a viable alternative to capture fisheries in providing a sustainable source of protein for fishing communities and local populations.

Mitochondrial DNA (mtDNA) is a valuable marker in population genetics or phylogeographic studies because it is maternally inherited and recombination is absent **(Singh *et al.*, 2014)**. Mitochondrial DNA sequences being embedded in every cell are considered as genetic 'bar-codes'. The variation among DNA sequences is used to identify organisms **(Singh *et al.*, 2014).** DNA bar-coding is the use of a short DNA sequence or sequences from a standardized locus (loci) as a species identification tool (**Hebert *et al.*, 2003a**). The DNA barcode that is well established in animals is a sequence of a 655 base fragment of the 5' end of the mitochondrial Cytochrome C Oxidase 1 (COI or Cox1) gene. Fish mitochondrial DNA is small (16.5 kb approximately) double stranded, circular molecule composed of about thirty seven genes coding for twenty two tRNAs, two rRNAs and thirteen mRNAs. Mitochondrial DNA was used to examine the evolutionary and taxonomic relationships amongst taxa. The barcode sequence from each unknown specimen is then compared with a library of reference barcode sequences derived from individuals of known identity. A specimen is identified if its sequence closely matches one in the barcode library. Otherwise, the new record can lead to a novel barcode sequence for a given species (that is, new haplotype or

geographical variant), or it can suggest the existence of a newly encountered species (**Hajibabaei *et al.*, 2007**). The present study, therefore, also focussed to establish the evolutionary and taxonomic relationships amongst the species of genus *Botia* using mtDNA and to show the genetic distance between them. The study may thus contribute to some extent to the information database and conservation approach of the fish diversity in natural resources.

Therefore, a detailed survey of the Kaljani river of Cooch Behar district of North Bengal, West Bengal, India was carried out to select the *Botia* species and standardization of captive breeding, the barcoding of the selected species for understanding their taxonomic status and phylogenetic interrelationships among the species.

1.1. Objectives of the study

Based on the concept that *Botia spp* are Endangered, and to bring about its conservation a research project entitled **"Breeding behaviour, embryonic development and barcoding of the ornamental loaches (Cobitidae: Cypriniformes) of Terai Region of West Bengal, India"** was executed for a period of three years (August 2012 to July 2015) based on the following objectives.

1. To survey the water of river Kaljani; collect the loaches and estimate the limnochemistry of water.
2. To standardize breeding behavior and techniques in captivity and characterize embryonic development.
3. To standardize supplementary feed for growth of spawns.
4. To identify the species through molecular characterization using barcoding.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. An overall view of the importance of water

Water is the soul of nature and is one of the basic requirements of mankind. It is also the most essential environmental component for the well being of the living world**.** Life originated in water and most of the biological phenomenon takes place in liquid medium **(Dhamak , 2013).** About 70% of Earth's surface is water, of which 97 .5% is saline and 2.5% is fresh. Less than 1% of this 2.5% amount of freshwater is accessible.

According to **Postel *et al.*, (1996)** human population used 54 percent of all the fresh water contained in rivers, lakes, and aquifers and this percent is expected to climb by at least 70 percent in 2025. By 2025, 50 countries and more than 3.3 billion people will face water stress or scarcity and majority of these countries, 40 of them, are in the near East, North Africa, and sub -Saharan Africa (**Gardner-Outlaw and Engelman, 1997; UNFPA, 1997**).

In 1996, 734 species of endangered fish of the world, 84 percent are found in freshwater environments. Globally, over 20 percent of all freshwater fishes are endangered, vulnerable, or recently extinct (**Brautigam, 1999**).

Therefore, water is essential at all level, that is, from cellular to ecosystem and within the body of each living being. Water is the key substance for existence and continuity of life. Human beings depend on this resource for all their needs. For existence and survival of aquatic fauna like phytoplankton, zooplankton and fish which are the major elements of food chain in aquatic ecosystem this resource plays a key role **(Dhamak , 2013).**

2.1.2. Freshwater aquatic environment

Communities of plants and animals living in water are known as aquatic ecosystem. Aquatic or watery environment are divided in to fresh water and marine water (**Lerner *et al.*, 2001**). **Welch (1952)** defined Limnology as the branch of science which deals with biological productivity of inland waters and with all the causal influences which determine it and again **Wetzel (2001)** explained it as the study of the structural and functional interrelationships of organisms of inland waters as they are affected by their dynamic physical, chemical and biotic environments.

Flowing fresh water environment is called lotic ecosystem for obvious reason of unidirectional water movements along a slope in response to gravity (**Wetzel, 2001**). Lotic environments are fundamental components of regional and global biogeochemical cycles, acting as both transport pathways and sites of elemental transformations and storage and they act as sources of drinking water, fisheries resources, irrigation supplies, and waste removal systems (**Roy, 2014**). They are characterized by interactions among physical, chemical and biological processes, which reach a higher degree of complexity downstream (**Wehr and Descy, 1998**).

Due to high population densities and multiplicity of industrial and agricultural activities expose most hydrographic basins to heavy and rising environmental impacts especially to pollution by domestic and industrial waste residues (**Salomoni *et al.*, 2006**). River water finds multiple uses in every sector of development like agriculture, industry, transportation, aquaculture, public water supply and so on (**Kumar, 2010**). River pollution in India has now reached a point of crisis. Surface waters are most vulnerable to pollution due to their easy accessibility for disposal of wastewaters (**Patel and Minakshi, 2015**).

2.1.3. Water quality parameters

Fish are in equilibrium between potential disease organisms and their environment. Changes in this equilibrium such as deterioration in water quality (environment) can result in fish becoming "stressed" and vulnerable to disease and it is very important to know something of the water quality parameters that have influence on growth and survival of aquatic organism (**Lokare, 1989**). Water is known as blue gold, one of the most priceless gifts of Nature is also regarded as the life line of Earth, because evolution of life and development of human civilization could not have been possible without water and rivers (**Patel and Vaghanib, 2015**). **Yahya *et al.*, (2012**) stated that management and protection strategies had to develop for each water basin individually because, polluted water used for drinking, domestic purpose as well as for irrigation without assessing its suitability in different parts of the world.

Table.1. Different analytical water quality parameters Standards guideline values as per WHO, Indian standard and USEPA (Patil *et al.*,2012)

Sl. No.	Parameters	WHO	Indian Standards	US EPA
1	pH	6.5 to 9.5	6.5 to 9.5	6.5 to 9.5
2	Specific Conductivity ($\mu Scm^{-1)}$	-	-	2500
3	Total Dissolved Solids (mgl^{-1})	-	500	-
4	Dissolved Oxygen (mgl^{-1})	6	6	-
5	Free Carbon dioxide (mgl^{-1})	200	-	-
6	Total Alkalinity (mgl^{-1})	-	200	-
7	Total Hardness (mgl^{-1})	200	300	<200
8	Ammonium-N (mgl^{-1})	0.3	0.5	0.5
9	Nitrite –N (mgl^{-1})	3	45	0.5
10	Nitrate-N (mgl^{-1})	45	45	50
11	Phosphate-P (mgl^{-1})	-	5	-

2.1.3.1. Temperature

Temperature is one of the essential physical parameter of water quality to be measured because it influences the aquatic life by altering the dissolved oxygen concentration in the water making oxygen less available for respiration and metabolic activity of aquatic organisms (**Tank and Chippa, 2013; Jalal and Sanalkumar, 2012**). The air temperature is the resultant effect of several meteorological factors such as solar radiation, humidity, wind etc. and also the latitudinal and altitudinal position of the place under study **(Wetzel, 2001).**Water temperature plays a very important role in some physiological processes like release of stimulii for breeding mechanisms in fish, both under natural and artificial conditions **(Hora, 1945; Chaudhuri, 1964).**Water temperature is an affective factor to control the chemical reactions and its rate within the water body that determines the usefulness of the water (**Metcalf and Eddy, 2003**). The standard temperature for sustaining aquatic life varies between 28^{o}C to 30^{o}C (**Weldermeriam, 2013**). Temperature is a measure of how much heat is present in the water, and cold water holds more oxygen than warm water. Warm water enters the river, raises the temperature of the downstream area and changes the oxygen levels. These are forms of thermal pollution. Thermal pollution is one of the most serious ways humans affect rivers. Cutting down trees along the bank of a river or pond also raises the water temperature (**Lodh *et al.*, 2014; Mandal *et al.*, 2012**).

2.1.3.2. pH (Hydrogen Ion Concentration)

The pH an important parameter of water maintains the acidic or basic property of an aquatic ecosystem and determines the suitability of water for various purposes such as drinking, bathing, cooking, washing, agriculture and so on. The higher range of pH indicates higher productivity of water (**Gopalkrushna, 2011**) because availability of

carbonates and bicarbonates in water enhance free carbon dioxide level by dissociation and acts as a raw material for photosynthesis. According to **Boyd (1982),** at extremely high or low levels namely pH of 9 or 4, was unsuitable for most organisms and young fish and insects. The pH of natural water ranges between the extremes of <2 -12 **(Wetzel, 2001).** pH can vary from its normal levels (6.5 to 8.2) due to pollution from automobiles and coal-burning power plants (**Mandal *et al.*, 2012**). pH was positively correlated with electrical conductance and total alkalinity. Most of the similar studying suggested that water samples were slightly alkaline due to presence of carbonates and bicarbonates (**Tank and Chippa, 2013 and Gopalkrushna, 2011**). The pH level of water having desirable limit is 6.5 to 8.5 as specified by the BIS. Pure water is said to be neutral, with a pH of 7.0. Water with a pH below 7.0 is considered acidic while, with pH greater than 7.0 is considered as basic or alkaline. The pH acts as a pollution indicator of water and pH of natural water can provide important information about many chemical and biological processes and provides indirect correlations to a number of different impairments. The acidic pH may be due to the high organic load and decomposition. The rain water is responsible for neutralization and finally makes it alkaline **(Saha, 2014)**. **Mishra and Yadav (1978)** reported high pH value rivers and lakes in Central India.

2.1.3. 3. Specific Conductivity

The electrical conductivity represents the total concentration of soluble salts or mineral salts in water (**Trivedy and Goyal, 1986**), so making it sour and unsuitable for drinking **(Saha, 2014)**. Conductivity is a measure of the ability of water to pass an electrical current which is affected by the presence of inorganic dissolved solids such as chloride, nitrate, sulphate, and phosphate anions (ions that carry a negative charge) or sodium, magnesium, calcium, iron, and aluminum cations (ions that carry a positive charge) (**Mondal *et al.*, 2014)**. Organic compounds like oil, phenol, alcohol, and sugar

do not conduct electrical current very well and therefore have a low conductivity when in water. **Mondal *et al.*, (2014)** also reported that conductivity is also affected by temperature. Warmer water have higher conductivity than cooled water. The conductivity of rivers in the United States generally ranges from 50 to 1500 μmhos/cm and inland fresh waters indicate that streams supporting good mixed fisheries have a range between 150 and 500 μhos/cm (**Mondal *et al.*,2014)**. Specific conductance of north western Himalayan river water ranged from 20 to 468.2 μmho cm-1 and it increased upstream to downstream **(Singh, 1988)**. In glacier-fed Trishuli river system of Nepal it varied from .2 to 534.0 μmho cm-1 **(Thapa *et al.*, 2010)** and in the rivers of Bhutan Specific conductance ranged from 20-140 μmho cm-1 **(Dubey, 1978)**. Conductivity outside this range could indicate that the water is not suitable for certain species of fish or macro-invertebrates. Industrial waters can range as high as 1000μmhos/cm (**United States Environmental Protection Agency, 1986**). Electrical conductivity is directly related to concentration of ionized substances in water and may also be related to problems of excessive hardness.

2.1.3. 4. Total Dissolved Solids (TDS)

According to **Mandal *et al.*, (2012)** Total dissolved solids are measure of dissolved matter (salts, organic matter, minerals and so on) in water, and TDS can be toxic to aquatic life through increases in salinity or changes in the composition of the water, or it may include substances that are toxic to people or aquatic life. Most aquatic ecosystems involving mixed fish fauna can tolerate TDS levels of 1000 mgL^{-1} **(Boyd, 1999).** Total Dissolved Solids define the color and electrical conductivity of the water body and the amount of TDS in water indicates salinity of water and may also be used as an indicator for rapid plankton growth and sewage (**Tank and Chippa, 2013**). In natural

waters, salts are chemical compounds comprised of anions such as carbonates, chlorides, sulphates, and nitrates (primarily in ground water), and cations such as potassium (K), magnesium (Mg), calcium (Ca), and sodium (Na) and in ambient conditions, these compounds create a balanced solution if there are additional inputs (natural and anthropogenic source) of dissolved solids in the system, the balance is altered and detrimental effects may be seen **(Tiwari, 2015).**

2.1.3. 5. Dissolved Oxygen

Oxygen is the single most environmental parameter that exerts a tremendous effect on growth and production through indirect effect on feed consumption and metabolism and its direct effect on environment (**Lokare, 1989).** Dissolved oxygen in water is an indicator for water quality and diversity of living things. The reason behind the fact is the turbulence and oxygenation resulting from rainfalls and mixing up of gleaming aerated water **(Saha, 2014)**. The DO value indicates the degree of pollution in the water bodies (**Gupalkrushna, 2011)**. Deficiency of dissolved oxygen gives bad odour to water due to anaerobic decomposition of organic wasters **(Manivasakam, 1980).** Almost all plants and animals need dissolved oxygen for respiration. A good quality of water should have a solubility of oxygen from 7.0 to 7.6 mg/l at 30^{o}C respectively (**Kudesia, 1985**). **Hancock (1973)** and **(Welch, 1952)** reported that it was maximum in winter and minimum in summer. **Krishnamurthy (1990**) reported that greater qualities of Oxygen recorded during summer. In the progress of summer, dissolved oxygen decreased due to increase in temperature and also due to increased microbial activity (**Moss, 1972; Morrissette, 1978; Sangu, 1987; Kataria, 1996)**. In the rivers of north eastern Himalaya values of dissolved oxygen ranged from 3.6 to 15.4 mg L^{-1} (**Acharjee, 2013**). **Jhingran (1991)** reported average value of 9.4 mgL^{-1} in the river Brahmaputra.

Oxygen enters into the water by aerial diffusion and as a photosynthetic by-product of aquatic plants and algae (**Lodh *et al.*, 2014)**. The DO depends upon the temperature, salinity and pressure of the water. The aquatic life gets distressed when DO levels drops to 4 to 2 mgL^{-1} (**Francis-Floyd, 2003**) and as DO level falls, undesirable changes in odor, taste and color reduces the usefulness of water (**Tank and Chippa, 2013**). DO in correlation with water body gives direct and indirect information for example bacterial activity, photosynthesis, availability of nutrients, stratification and so on (**Premlata, 2009**). Addition of domestic sewage, municipality wastes, waste from market and hospital encourage the growth of micro organisms which use the dissolved oxygen for decomposition. The concentration thus gradually decreases (**Patra *et al.*, 2011**).

2.1.3.6. Free Carbon dioxide

Carbon dioxide (CO_2) is readily soluble in water but very little amount of CO_2 is present in simple solution because, small amount of CO_2 is present in the atmospheric air. Apart from this, decomposition of organic matter and the respiration of aquatic plants and animals contribute to the free CO_2 present. Water percolating through the vegetation and soil take up CO_2 released from the soil. Carbon dioxide combines chemically with water to form carbonic acid (H_2CO_3), dissociates partly to produce hydrogen (H^+) and bicarbonate (HCO_3^-) ions **(Faurie *et al.*, 2001)**. CO_2 can build up to significantly high levels in systems with large numbers of fish and relatively slow water turnover (**APHA, 2005**). **Thapa-Chhetry and Pal (2011)** reported free CO_2 to range from 4.15 to 5.92 mg L^{-1} in Nepal. **Biswas and Boruah (2002)** reported the range of free CO_2 to be from 1.9 to 12.3 mg L^{-1} in the river Brahmaputra with an average value of 5.2 mg L^{-1}. The free CO_2 found in the Relli river water was low and varied from 0.6 mg L^{-1} to 5.8 mg L^{-1} with a mean value of 3.1 mg L^{-1} (±1.4) **(Acharjee and Barat, 2012)**. The presence of carbonic

acid (H_2CO_3) in water may be good or bad depending on the water's pH and alkalinity **(Saha, 2014)**.

2.1.3. 7. Total alkalinity

Alkalinity is an indicator for a solution's capacity to react with acid and "buffer" its pH. Water is said to be **alkaline** when concentration of the hydroxyl ions exceeds that of hydrogen ions **(Trivedy and Goel, 1984)**. Total alkalinity is the sum of hydroxides, carbonates and bicarbonates. Increased dilution of river water may be responsible for lower values of alkalinity in rainy seasons (**Bhargava, 1982**).. Alkalinity is not a pollutant and it is measured in water that have "acid-neutralizing" ability, and pH measures the strength of an acid or base **(Saha, 2014)**. In rivers and streams of the north eastern Himalaya total alkalinity ranged from 12 to 207.7 mg L^{-1} (**Bhattacharjya *et al.*, 2002)**. **Biswas and Boruah (2002)** reported range of total alkalinity from 44.3 to 110.8 mg L^{-1} with an average value of 63.4 mg L^{-1}in the river Brahmaputra. **NWWFCC (2001)** recorded range of total alkalinity from 13 to 120 mg L^{-1} in the rivers of Bhutan.

2.1.3. 8. Total hardness

Total hardness is defined as the sum of calcium and magnesium hardness express in mgl^{-1} as $CaCO_3$. The anions responsible for hardness are mainly bicarbonate, carbonate, sulphate, chloride, nitrate and silicates etc **(Trivedy and Goel, 1984)**. According to **Swingle (1967a)**, a total hardness of 50 mg L-1 is the dividing line between soft and hard water. **Kannan (1991)** had classified water on the basis of hardness values in the following manner, 0- 60 mg l^{-1} soft, 61-120 mg l^{-1} moderately hard, 121-180 mg l^{-1} hard and above 180 mg l-1 very hard. Calcium hardness in freshwater is in the range of 10 to 250 mgl^{-1}, often double that of magnesium hardness (5 to 125 mgl^{-1}) and total hardness of 630 mgl^{-1} as $CaCO_3$ (**Pawari and Gavande, 2013)**.. A high concentration of

hardness may be due to leaching from of the soils or due to the high background concentration of the waters. The limit of total hardness value for drinking water is to be within 300 mgl^{-1} of CaCO3.Higher concentration of hardness was found to be due to natural accumulation of salt, surface runoff and water entering from direct pollution by human activities (**Pawari and Gavande, 2013)**.

2.1.3. 9. Ammonium-N

Ammonia is naturally present in surface water and groundwater and can be produced by the deamination of organic nitrogen containing compounds and by the hydrolysis of urea. The problem of taste and odour may arise when the ammonium-N (NH_4-N) level is greater than 2 mgl^{-1}. Greater than 10 mgl^{-1}, appreciable amounts of NO3-N may be produced from NH_4-N under suitable anaerobic conditions (**WHO, 1993** and **Kempster *et al.*, 1997**). **Ammonium ion (**NH_4 +) is also brought to natural waters by animal and human wastes. Even more than nitrates, it is preferentially absorbed by plants. It is also produced by fishes and zooplankton, which leads to very rapid development of phytoplankton in waters in which this chemical form of nitrogen cannot be detected by chemical analyses **(Faurie *et al*., 2001).** Distribution of ammonia in unpolluted rivers ranges from 0.005 to 0.040 mg L^{-1} with an average value of 0.018 mgL^{-1} **(Wetzel, 2001)**. **Lodh *et al.*, (2014)** found that ammonia level was higher than the prescribed value of BIS and average value is ranges between 0.06 to 3.20 mgl^{-1}.

2.1.3. 10. Nitrite-N

Nitrite (NO_2^-) is not abundant in water. They are rare because they constitute only a passing form between the ammonia and nitrate, during the processes of nitrification and denitrification. In natural waters, any concentration higher than 10 **µ**gL^{-1} indicates a dysfunction of microbial mechanisms involved in the nitrogen cycle (**Faurie**

***et al.,* 2001**) and water is not suitable for drinking purpose if the concentration is greater than 0.05mgl^{-1} (**WHO, 1992**). Nitrites are readily oxidized to nitrates and are seldom present in significant concentration in the surface waters (**Roy, 2014**). Nitrite nitrogen concentrations in the Himalayan Rivers ranged from traces to 0.035 mg L^{-1} **(Semwal and Akolkar, 2006)**. **Thapa *et al.,* (2010)** recorded range of NO_2-N and NO_3-N together in the river Trishuli of Nepal Himalaya ranged from nil to 0.138 mg L^{-1}. According to **Barat and Jha (2002)** NO_2-N ranged 0.002- 0.030 mg L^{-1} in the sub Himalayan Rivers Mahananda of North Bengal.

2.1.3. 11. Nitrate–N

Inorganic nitrogen present in water as Nitrate (NO_3-N) is the main nutrient that accelerates the growth of hydrophytes and algae. Nitrate occurs in water from various natural sources and human activities like food production, agriculture and manure disposal of domestic and industrial sewage. Welch (1952) opined that nitrate in natural waters will be in a continuously changing state due to the relation of nitrate with nitrifying bacteria and demand by nitrate consuming organisms such as phytoplankton and higher aquatic plants. Nitrate (NO3 -N) is one of the important nutrients in water body which is the common form of nitrogen in natural water **(Saha, 2014)**. High level of nitrates is found in rural areas because of extensive application of nitrogenous fertilizers in agriculture (**Lodh *et al.,* 2014)**. In urban areas sewage water rich in nitrates contaminate surface water thus increases the nitrate amount. (**Tank, 2013; Gopalkrushna, 2011)**. An average **NO3**-N concentration of nearly 100µg N L^{-1} is found in natural river waters **(Wetzel, 2001).** Range of **NO3**-N in Himalayan River varied from traces to 0.315 mg L^{-1}**(Semwal and Akolkar, 2006).** The concentration of nitrate-nitrogen was more during monsoon than during winter months in the river Brahmaputra

and ranged from 0.030 to 0.300 mg L^{-1}**(Jhingran, 1991).** A small amount of nitrate is common in all kinds of surface water. Most natural water are deficient in nitrate having a concentration usually below 5 mg/L, but certain polluted surface water and ground water may have substantially higher quantities.

2.1.3.12. Phosphate-P

Phosphate has a limited source in nature and also acts as a limiting factor for productivity of water body. Phosphate may occur in lake as a result of domestic waste, detergent and agricultural runoff containing fertilizer (**Gopalkrusna, 2011).** Phosphorous is an essential nutrient to living organisms and inorganic phosphate is the main ingredient of eutrophication in a water body **(Saha, 2014)**. PO_4-P averages about 10 μg L^{-1} worldwide among unpolluted rivers **(Meybeck, 1982).** Algae require only small amounts of phosphorus. Excess amounts of phosphorus can cause eutrophication leading to excessive algal growth called algal blooms (**WHO, 1992**). According to **Strokal *et al.*, (2016)** eutrophication increased day by day in Chinese rivers from direct discharge of manure. **Flura *et al.*, (2016)** worked on Meghna river and reported that water quality of Meghna river were safe for aquatic lives, but the continuous sewage disposal may create problems in the future. **Barat and Jha (2002)** reported that values of PO_4-P in the river Mahananda ranged from 0.060 to 0.0340 mg L^{-1}.

Some authors have been made enormous contribution on Indian rivers like **Mishra and Tripathi (2003)** on river Ganga; **Swer and Singh (2004, 2006)** on Meghalaya, **Singh and Gupta (2010)** on the Thoubal rivers; **Kumar *et al.*, (2014)** on Kali river; **Baitule *et al.*, (2015)** on Nag River at Nagpur city; **Patela and Vaghanib (2015)** on Par River Valsad, Gujarat and **Lamare and Singh (2016)** on Lukha River of Meghalaya.

Some pioneer work have been made on water quality of West Bengal rivers like, **Mondal *et al.,* (2010)** on North 24-Parganas; **Mandal and Das (2011)** on Torsha River; **Mandal *et al.,* (2012a)** on Karala river; **Mandal *et al.,* (2012b)** on the drinking water quality of Chamurchi tea garden of Darjeeling; **Saha (2014)** on Shutunga river; **Mozumder *et al.,* (2015)** on Mahananda river; **Chakrabarty and Nath (2015)** on tributary of Ganga.

2.2. Importance of Fish

Fish is an important group of the vertebrates which influences the life of human and is a good source of protein and occupying a significant position in the socio-economically fabric of South Asian countries **(Dhamak, 2013).** Fishes also provide several by- products such as fish meal, fish glue, fish oil and so on.

Fish diet provides different minerals like Ca, Mg, P, Na, Fe, I and rich source of protein in the form of simple proteins with different essential amino acids, fats, and traces of vitamin B Complex and so on along with non-protein nitrogenous forms. They have good taste and are easily digestible with growth promoting value **(Dhamak, 2013).** Fish provides nutrition and foreign money through ornamental fish.

Fish fat contains Omega-3 long-chain PUFA, including EPA and DHA, are dietary fats with an array of health benefits (**Su *et al.,* 2008**). EPA and DHA are incorporated in many parts of the body including cell membranes (**Lazzarin *et al.,* 2009**) and play a role in anti inflammatory processes and in the viscosity of cell membranes (**Smith *et al.,* 2011; Conquer *et al.,* 2000**). EPA and DHA are essential for proper fetal development and healthy aging (**Dunstan *et al.,* 2007**). DHA is a key component of all cell membranes and is found in abundance in the brain and retina (**Krauss-Etschmann *et al.,* 2007**).

According to **Rajender (2013)** the concept of entrepreneurship development through Ornamental fish farming is gaining popularity day by day. Ornamental fish farming is an important primary industry (**Lim and Wong, 1997**).The Ornamental fish trade plays an important role in the socio-economic upliftment of backward class and females in our country with little investment of money (**Rajender, 2013**).

In last the 25 years, unprecedented population growth and rapid industrialization coupled with intensive agricultural activities have exerted intolerable stress over the aquatic ecosystems. These developments have given rise to new management and conservation concepts based on basic research **(Dhamak, 2013).** The lack of information on the ichthyo-fauna is a big handicap for popularizing little known fish variety in a particular ecosystem. Thus, there is need to survey fish fauna associated with different fresh water habitats, which will help in planning methods for their production and effective exploitation **(Sharma and Nayak, 2001).**

2.2.1. Ornamental fish

Ornamental fishes are also called 'living jewels' for their beautiful colour and playful behaviour. Ornamental fishes are typically small sized; attractive and bizarre shaped in appearance **(Dey, 1996)**. Since ornamental fishes are usually kept in glass aquarium, these are popularly also known as aquarium fishes. Ornamental fishes are the most popular pets in the world **(Singh, 2005)**. Aquarium keeping has emerged as the second most popular hobby in recent years next to photography **(Chapman, 1997).**The ornamental fish market in the world for public aquaria is less than 1%, and 99% of the ornamental market is still for the hobbyist. Aquarium keeping of fish began in 1805 with the first public display aquarium at Regent's Park in England in 1853. Development of aquaria picked up and by 1928, there were 45display aquaria open to public, and at

present, there are over 500 aquaria functioning worldwide **(Handbook of Fisheries and Aquaculture, 2015)**.

According to **Kottelat and Whitten (1996)** India occupied eighth position in the world and third in Asia. **Mittermeier and Mittermeier (1997)** also stated, that India was the megabiodiversity country which occupied the ninth position in the world. About 21,730 species of fishes have been recorded in the world; of which about 11.7% are found in Indian waters. Out of the 2546 species so far listed, 73belonged to cold freshwater, 544 to the warm fresh water, 143 to the brackish water and 1440 to the marine ecosystem (**International Consultation on Biological Diversity, 1994**).

2.2.1.1. Ornamental fish diversity in India

In India, aquarium hobby is nearly 70 years old and dates back to pre-independence era **(Ayyappan *et al.,* 2006)**. India's overall ornamental fish trade was about 1.06 million US$ during the year 2009 **(UNCOMTRADE, 2014)**. India possesses rich resources namely, the lagoons and coral reefs of Lakshadweep and Minicoy Islands, Andaman and Nicobar islands, Gulf of Kutch complex, Coast of Kerala, Cape Comorin, Gulf of Mannar and Palk bay are abound with highly attractive and varied species of ornamental fishes. India has recorded at least 150 commercially important ornamental fish species and trade mainly indigenous freshwater species collected from rivers **(Madhu *et al.,* 2009)**. About 600 freshwater fish species of ornamental value have been reported worldwide from various aquatic environments. Indian waters possess a rich diversity of ornamental fishes with over 200 varieties of indigenous species (**Swain *et al.,* 2001**). Prominent among the fresh water Indian ornamentals are Loaches, Eels, Barbs, Catfish, and Goby **(Ayyappan *et al.,* 2006)**. About 90 % of ornamental fish is traded from Kolkata port followed by 8 % from Mumbai and 2 % from Chennai **(Ghosh**

***et al.,* 2003)**.400 species of ornamental fishes belonging to 175 genera and 50 families were reported in the Indian waters by **Satheesh (2002)**. Indian ornamental fish were primarily dependent on wild catch (85%) and a few artificially bred varieties (15%) of exotic fish **(Mahapatra *et al.,* 2006)**.

According to **Anna Mercy (2009)** out of the 300 species of fishes inhabiting the different river systems of the Western Ghats, 155 were considered as potential ornamental fishes. Among them 120 species were endemic to the Western Ghats and most of them belonged to the categories of barbs, loaches, danios, killifishes, hill trout and catopras; again **Raju Thomas** and **John (2009)** observed 102 species of ornamental fishes from the inland waters of Kerala.

Several workers reported on ornamental fish diversity in India like **Usha *et al.,* (2013)** 12 ornamental species, **Rahana *et al.,* (2014)** found 30, **Sirajudheen and Biju (2014)** recorded 138 species; **Sureshkumar *et al.,* (2014)** reported 20 species of indigenous ornamental fishes from Western Ghats. **Rao *et al.,* (2013)** reported 58 species from Andhra Pradesh; **Kumar *et al.,* (2013)** reported 100 from Mahanadi, Bhubaneswar; **Rao (2014)** reported 53 species from Andhra Pradesh and **Patel *et al.,* (2014)** reported 61species;

In North- East India, **Chakravartty *et al.,* (2012)** reported 67 species; **Baro *et al.* (2014**) recorded 49 species; **Khomdram *et al.,* (2014)** recorded 139 species from Manipur; **Goswami and Zade (2015)** reported 81 species of fishes and **Biswas *et al.,* (2015)** reported 109 species.

2.2.1. 2.Ornamental fish diversity in West Bengal

Several investigators worked on different rivers of West Bengal on fish diversity like **Mandal *et al.,* (2012)** who identified 67 species from Sundarban; **Basu *et al.* (2012)** reported 41 ornamental fish species from Cooch Behar district; **Baro *et al.* (2014**)

recorded 49 ornamental fishes from the Sankosh river; **Mahapatra and Lakra (2014)** reported 41 ornamental fish from East Kolkata Wetlands and **Acharjee and Barat (2014a)** reported 20 species of loaches from Terai and Dooars region of North Bengal; **Pal (2015)** listed 58 species from North Bengal.

Recent studies done in North Bengal by **Das (2015)** recorded 53 ornamental from Torsa river; **Dey *et al*. (2015a)** reported 58 ornamental out of total recorded 138 fishes from Kaljani river; **Debnath (2015)** recorded 46 ornamental out of 73 recorded fishes from Gadadhar river; **Dey and Sarkar (2015)** recorded 55 ornamental fishes out of 107 recorded species from Torsa river; **Dey *et al*. (2015e)** reported 46 ornamental fishes from Ghargharia river; **Sarkar *et al*., (2015)** recorded 24 and 26 ornamental fish species from the river Torsa and Ghargharia respectively whereas, **Paul and Das (2016)** reported 52 species of indigenous ornamental fish from Cooch Behar district of West Bengal.

2.3. Fish Biology Study

Information of biology of all types of fishes is a must, not only, for conservation of biodiversity and ecosystem but also, for commercialization of food production on sustainable basis through farming or enhancement of fish production in the natural habitat by stocking. For efficient planning of fisheries development, it is imperative to have complete information on the biology of fishes and ecology of water bodies (**Sarma, 2008).**

2.3.1. Fish growth parameters

2.3.1.1. Gonado-Somatic Index (GSI)

Gonado-somatic Index (GSI) is the measure of the relative weight of the gonad with respect to total or somatic weight (**King, 1996**). Usually, the percentage of body weight of fish that is used for egg production is determined by the Gonado-somatic Index

(**Agbugui, 2013**). It increases in fish with its maturation, being maximum during the peak period of maturity and declining abruptly after spawning **(Khanna and Pant, 1967).** Total spawners are said to produce a large number of smaller sized eggs which are deposited over a short period of time while, the multiple spawners produce fewer and larger eggs with a longer breeding period which may last throughout the year, wherein only a proportion of the eggs ripe in the gonad at one spawning (**Lowe-McConnell, 1987**), though total spawners are said to have a higher GSI than multiple spawners (**Wootton, 1990**).

Many researchers have worked on the Gonado- Somatic Index of different fishes and reported that high GSI indicates peak breeding season of fishes and this statement was supported by the following authors **Sathyanesam (1962**) on *Mystus seenghala*; **Khanna and Sanwal (1971**) on *Channa gachua*; (**Raizada, 1971**) on *Rasbora daniconius;* **Bisht (1972**) on *Schizothorax richardsonii;* **Arockiaraj *et al.*, (2004**) found five developmental stages of gonad in *Mystus montanus;* **Joshi and Pathani (2009)** worked on *Botia almorhae* from Kumaun Himalaya; **Bandpei *et al.*, (2011)** studied *Rutilus frisii kutum* from Southern Part of Caspian Sea, Iran; **Islam *et al.*, (2012)** investigated *Sillaginopsis panijus* from the Meghna River Estuary, Bangladesh; **Shinkafi and Ipinjolu (2012**) on *Auchenoglanis occidentalis* from River Rima, North-Western Nigeria; **Ghanbahadur and Ghanbahadur (2012)** on *Cyprinus carpio;* **Amtyaz *et al.*, (2013)** on *Pomadasys stridens* from Karachi Coast, Pakistan; **Ghanbahadur *et al.*, (2013)** on *C. gachua;* **Nandikeswari and Anandan (2013)** on *Terapon puta* from the Bay of Bengal, Pondicherry; **Ashwini *et al.*, (2013)** on *Channa bleheri* ; **Agbugui (2013)** on *Pomadasys jubelini*; **Tiwari *et al.*, (2014)** on *Channa marulius* ; **Wagle (2014)** on *Schizothorax richardsonii* collected from Nallu River of Lalitpur district; **Roy and Manda (2015)** reported on *Labeo bata* ; **Oliveira *et al.*,**

(2015) on *Hemiramphus brasiliensis* from Brazil*;* **Chakrabarti and Banerjee (2015)** on *X. cancila;* **Jan and Ahmed (2016)** on snow trout, *Schizothorax plagiostomus;* **Pal and Mahapatra (2016)** on *Amblypharyngodon mola;* **Sales *et al.,* (2016)** on *Hypostomus francisci*; **Dey *et al.,* (2016)** on *Barilius barila* and **Mahmud *et al.,* (2016)** on *Channa striata.*

2.3.1.2. Condition Factor

Condition factor or K- factor or Ponderal Index is used to compare the 'condition', 'fatness' or well being of fish, and it is based on the hypothesis that heavier fish of a given length are in better condition **(Bagenal & Tesch,1978)**. Condition factor has been used as an index of growth and feeding intensity **(Fagade, 1979).** According to **Le Cren (1951**), 'Kn' greater than 1 indicates good general condition of fish. Fish with high value of 'Kn' are heavy for its length, while with low 'Kn' are lighter (**Bagenal and Tesch, 1978; Froese, 2006**). According to **Brody (1945)** and **Lagler (1952**) the growth of fishes obeys the Cube law when fish stay an ideal environmental condition. It is also a useful index for the monitoring of feeding intensity, age and growth rates in fish **(Oni *et al.,* 1983)**. It provide external measures of overall health of the fish (**Naeem *et al.,* 2011**). The K value indicates the size at which the fish matures and the variation in the value in relation to size may attribute spawning and feeding intensity due to availability of select food or absence of food (**Mohanraj, 2008)**. The Length-Weight relationship parameters and Condition factor have been found very useful to evaluate the well-being of populations, their biology for scientific management of fisheries in stock assessment (**Ujjania *et al.,* 2012**). It is also important to note that the Physio-chemical parameters of water influence vertical and horizontal migrations of fishes in aquatic ecosystem, their distribution and feeding pattern (**Imam *et al.,* 2010** and **Dar *et al.,* 2012**). Higher value of Condition factor was reported in matured fish (**Olurin and Aderibigbe, 2006;**

Telvekar *et al.,* 2006). The value of 'K' usually shows fluctuations which may due to sample size, different stages of maturity, spawning on the parts of females or difference in weight of food content in the stomach (**Dars *et al.,* 2010**). Variations in the condition factor of many fishes were observed in relation to their reproductive cycle (**Narejo *et al.,*2002**).

According to **Arockiaraj *et al.,* (2004**), Condition factor of *Mystus montanus* was ranged from 4 to 6 and 6 to 9, respectively. **Deka and Gohain (2015**) reported relative condition factor of *Rita rita, Pangasius pangasius* and *Chitala chitala* were ranged 0.78 to 1.55, 0.85 to 1.30 and 0.79 to 1.24 respectively from Brahmaputra river system of Assam, India. (**Jyrwa *et al.,* 2015**) reported condition factor ranged 1.43 to 2.19 of *Neolissochilus hexagonolepis* from Meghalaya. Recently, **Rahman *et al.,* (2016)** studied on *M. vittatus* and reported that minimum and maximum Condition factor was 0.95 and 1.32, respectively, with a mean value of 1.14±0.09 indicating, that mature female *M. vittatus* stock was in good condition in the Padma River. **Lal *et al.,* (2016)** reported condition factor of *M. armatus* to be 0.22 (SD=0.07) and 2.84 (SD=0.28) for *Etroplus suratensis.*

2.3.1.3. Length-Weight Relationship

Length-weight relationship of fish is an important fishery management tool because they allow the estimation of the average weight of the fish of a given length group by establishing a mathematical relationship between the two **(Beyer, 1987)**. The length and weight relationship indicates the gonad development of fish **(Le Cren, 1951**). The study of length-weight relationship also helps in setting up yield equations (**Beverton and Holt, 1957; Ricker, 1958)** for estimating the numbers of fish landed, and in comparing populations in time and space (**Pandey *et al,* 1974**). The deviation from the general Cube Law governing the length weight relationship have been utilized by fishery

biologists to determine the condition and general well being of fish and serves as a useful index of the nutritional and biological cycle of the species (**Jhingran, 1972).** As length and weight of fish are among the important morphometric characters, they can be used for taxonomy and ultimately in fish stock assessment. While attempting a study of the biology of a fish, it is usual to analyze the mathematical relationship between its length and weight. This analysis reveals the extent to which the two variables, length and weight are related to each other and thereby help one to calculate with ease one variable when the other is known (**Chandrika & Balasubramonian, 1986**). The study of length-weight relationship of fishes have two objectives, (i) to determine the type of mathematical relationship between two variables so that if one variable is known the other could be computed and (ii) to know the well being of fish and also type of growth, that is, whether isometric or allometric (**Kumar *et al*., 2005).**

The literature available on length-weight relationship have been given by **Jhingran (1952); Chatterji (1980); Chatterji *et al.*, (1980); Choudhary *et al.*, (1982); Malhotra (1982, 1985); Mohan and Sankaran (1988); Kurup (1990); Pandey and Sharma (1998); Sarkar *et al.*, (1999); Sunil(2000); Mercy *et al.*, (2002); Kumar *et al.* (2005)** and **Prasad *et al.*, (2007).**

Recently, some important work has been carried out on length –weight relationship of fishes by **Joshi and Pathani (2009)** on *Botia almorhae;* **Vaitheeswaran *et al.*,(2012)** on *Panulirus versicolor;* **Islam *et al.*, (2012)** on *Sillaginopsis panijus* from the Meghna River Estuary, Bangladesh; **Jan *et al.*, (2014)** studied on *Schizothorax plagiostomus* collected from the river Jhelum, Kashmir; **Rejitha and Pillai (2015)** on six coral reef fishes, *Chaetodon octofasciatus, Lutjanus decussatus, Lutjanus rivulatus* ,

Lutjanus lutjanus, Lutjanus johnii and *Apolemichthys xanthurus* and **Lal *et al.*, (2016) on** 57 freshwater fish species from three diverse ecological regimes in India.

2.3.2. Fecundity

Fecundity has been considered as the number of ripening eggs in the female prior to spawning (**Begenal and Braum, 1968**). It is described as the number of matured eggs, filled with yolk or all vitellogenic oocytes found in the ovary immediately before the reproduction process (**Bagenal, 1963**). Fecundity is important in the estimation of abundance and reproductive potential (**Gupta, 1967**). **Begenal (1967)** also stated, that in most fishes, the number of eggs does not change significantly as the season progresses. Fecundity appears to bear some broad relationships to the care or nurture accorded to the eggs (**Lagler *et al.*, 1967**). **Franz (1910)** and **Clark (1934)** have observed that fecundity in the fishes, they studied, increased in proportion to square of length. Similarly, the existence of straight line relationship between fecundity and weight of fish has been reported by several workers (**Benegal, 1957; Pillay, 1958; Kandhar, 1959; Bridger, 1961; Varghese, 1973).** They found that the relationship between fecundity and weight to be curvilinear which indicates that length is also an important factor for determining the fecundity of fish as has been reported by **Smith (1947), Begenal (1967) and Manooch (1976)**.Fecundity studies have been considered useful in tracing the different stocks or populations of the same species of fish in different areas (**Gupta, 1967**). Knowledge about fecundity of a fish is essential for evaluating the commercial potentialities of its stock, life history, practical culture and actual management of the fishery (**Lagler, 1956; Doha and Hye, 1970**). Knowledge of fecundity is also important for understanding the life history and for modelling population dynamics of a species (**Bruch *et al.*, 2006**) as fecundity varies from one species to another, depending on the

environmental conditions, length, age and so on **(Jacob 2013).** Fecundity has no relation with fish age **(Simpson, 1951; Bagenal, 1957** and **Sarma, 2008).**

The literature available on fecundity have been given by **Guar and Pathani (1996)** on *Barilius vagra*; **Islam *et al.*, (2012)** on *Sillaginopsis panijus* from the Meghna River Estuary, Bangladesh; **Shinkafi and Ipinjolu (2012)** on *Auchenoglanis occidentalis* from River Rima, North-Western Nigeria; **Agbugui (2013)** on *Pomadasys jubelini*; **Nandikeswari and Anandan (2013)** on *Terapon puta*; **Jan *et al.*, (2014)** on *Schizothorax plagiostomus;* **Wagle (2014)** on *Schizothorax richardsonii* ; **Oliveira *et al.*, (2015)** on *H. Brasiliensis*; **Dey and Barat (2015)** on *Botia almorhae*; **Dey *et al.*, (2015d, 2015c** and **2015b)** on *Botia dario, Botia lohachata* and *Botia rostrata*; **Dey *et al.*, (2016)** on *Barilius barila.* **Silva *et al.*, (2016)** on *Cynoscion leiarchus* from Southern Brazil; **Gomez-Marquez *et al.*, (2016)** on *Poecilia sphenops* is a batch breeder, from Mexico; **Pal and Mahapatra (2016)** on *Amblypharyngodon mola*; **Rahman *et al.*, (2016)** on *Mystus vittatus*; **Sales *et al.*, (2016)** on *Hypostomus francisci* from Brazil and **Jan and Ahmed (2016)** on *Schizothorax plagiostomus* in river Lidder, Kashmir Himalaya.

2.3.3. Captive breeding of ornamental fish

According to **Minckley and Deacon (1991)** captive breeding is one of the proven techniques of saving endangered species from extinction and increase its population size with the help of sound breeding techniques under controlled conditions. Captive breeding is one of the major steps in the conservation programme of a species. It also can provide critical life history information, as well as helping supplement of existing or restoring populations and allows discovery of important behavioural or life history characteristics that may constrain reproduction of rare species in altered natural habitats (**Rakes *et al.*,**

1999). Such ecosystems are declared as national parks, biosphere reserves and sanctuaries but, the constraints in in-situ conservation are the need for large investment of finance, trained manpower, reorientation and modification of development project like river-valley project which have to be earned out (**Padhi, 1987**).

Although the merits of supplemental breeding are debated **(Hutchins *et al*., 1997),** this approach has become a common management technique for many fish species (**Brown *et al.,* 2000**). Captive raised specimens can be re-introduced is the natural habitat thereby protecting the species from extinction. Supplemental breeding is an intensive population management strategy wherein, adults are captured from nature and spawned in controlled settings, and the resulting offsprings are later released into the wild (**Fiumera *et al.,* 2004**). It is also a good conservation method for endangered freshwater fish species (**Philippart, 1995; Poncin and Philippart, 2002**). In ex-situ conservation, the fish species are conserved outside its natural habitat. This includes (1) Live Gene Bank where the threatened species are reared in captivity and bred therein and (2) Gamete/Embryo Bank where cryopreservation of milt, eggs and embryos is carried out (**Pandey and Das, 2002**).

Bindu (2006) reported three caveats for employing seed production and stocking method for commercially farmed aquatic species. Firstly, many species are reticent to mature and spawn in captivity. It is often difficult to simulate in captivity, the environmental conditions, cues and triggers that are necessary for successful reproduction, particularly the unique river habitat situations essential and conducive for spawning. Successful development of captive breeding requires a sound understanding of general biology and reproductive characteristics of the species. Secondly, small size of the fish species and low fecundity of some of the species render development of artificial

breeding techniques difficult (**Poncin & Philippart, 2002**). In high fecund species where large numbers of eggs are produced by the spawning female, high mortality of early life history stages such as the fertilized egg, larvae, post larvae, fry and fingerlings in captivity is another problem. This might also sometimes lead to a more serious situation of artificial selection. Thirdly, the high costs of keeping aquatic organisms in hatchery systems might encourage the use of small broodstock populations and this might lead to inbreeding related problems and negative consequences in the released stocks. Conservation of these threatened stocks can be done either through in-situ or ex-situ methods and threatened fish species are conserved by protecting the ecosystem in which they occur naturally or the habitat restoration is done **(Thumpy, 2009**).

Kiyzhanovsky (1949) reported five types of spawning of fish like i) Lithophils : Fishes which spawn on hard, stony surface, ii) Phytophils : Fishes which lay eggs among aquatic plants, iii) Psammophils : Fishes which deposit eggs in sandy surface, iv) Ostracophils : Fishes which deposit eggs inside a bivalve and v) Pelagophils : Fishes which spawn freely in column of water and the eggs float.

Hypophysation is the technique of breeding of fishes in confined waters with the injection of pituitary extracts for the production of seeds. A recent development in induced breeding is the stimulation of endogenous gonadotropin release from the pituitary of the treated fish using synthetic analogue of gonadotropin releasing hormone (GnRH). Hypophysation technique was first reported by Brazilians (**Von Ihering, 1937**) and this technique was used for Indian carps by **Chaudhuri & Alikunhi (1957)** and then Blue Revolution occured in seed production of exotic and Indian Major Carp. **Varghese *et al.* (1976)** and **Varghese and Rao (1976),** used pituitary of marine catfish (*Tachysurus* spp.) for carp induced breeding. **Chondar (1985)** used HCG for spawning

of carp except silver carp. **Peter *et al.,* (1988)** reported that LH-RH analogue combine with pituitary or HCG to get the desired spawning success. In 1990, used LH-RH and its analogues for induced breeding of carp (**Tripathi and Khan, 1990**). Ovaprim is a mixture of the analogue of salmon gonadotropin releasing hormone (SGnRHa) and a dopamine antagonist domperidone. Ovaprim hormone was more effective than mammalian releasing hormone (**Sherwood *et al.,* 1983**). Then **Nandeesha *et al.* (1990)** reported the Ovaprim, a synthetic hormone, which saves time and reducing post-spawning mortality. In 1997, Ovatide launched which cantain salmon gonadotropin releasing hormone (SGnRHa) and a dopamine antagonist and was succefully tested by the Central Institute of Fisheries Education (CIFE). WOVA-FH was manufactured by Syndel laboratory, Canada in 1988, used for induced breeding of carp and other fishes along with shrimp. It contained salmon gonadotropin releasing hormone (SGnRHa), propylene glycol and a domperidone. WOVA-FH was easy to use than Ovaprim (**Dey *et al.,* 2015b, 2015c and 2015d**). **Das *et al.,* (2016)** used three different types of inducing agents like Ovaprim, Ovatide and WOVA-FH for breeding threatened fish, *Osteobrama belangeri* and reported that fertilization rate was significantly higher ($P < 0.05$).

Some important work on captive breeding of ornamental fish that have been done are *Danio rerio* **(Kimmel *et al.*,1995);** *Ompok bimaculatus* (**Sridhar *et al.,* 1998**); *C. batrachus* (**Mahapatra *et al*., 2000; Dhawan and Kaur, 2004**), *Heteropneustes fossilis* (**Alok *et al*., 1998;Vijayakumar *et al*., 1998; Sreedhar and Haniffa, 1999; Pandian *et al*., 2001**), *Ompok malabaricus* (**Haniffa *et al.,* 2001**), *Ompok pabo* (**Mukherjee and Das, 2001**); *Etroplus suratensis* **(Bindu, 2006);** Cherry barb (*Puntius titteya*) (**Sundarabarthy *et al*.,2004);** *Macrognathus aculeatus* (**Das and Kalita, 2003**); *Devario aequipinnatus* (**Kharbuli *et al*., 2004**); *Puntius gelius* **(Sarma,2008**); *Macrognathus aral* and *M. pancalus* (**Singh, 2011**); *Anabas testudineus* (**Kumar *et al*.,**

2010 and Pius, 2010); *Ompok pabda* (**Purkayastha *et al*., 2012**); *Puntius sarana* (**Udit *et al.*, 2014**); *Devario aequipinnatus (***Dey *et al*., 2014**); *Anabas testudineus* (**Sarkar *et al*., 2015)**; *Ompok bimaculatus* (**Malla and Banik, 2015**); *Botia almorhae* (**Dey and Barat, 2015**); *Botia rostrata* (**Dey *et al.*, 2015b**); *Botia lohachata* (**Dey *et al.*, 2015c**); *Botia dario* (**Dey *et al.*, 2015d**); *Barilius barila* (**Dey *et al.*, 2016**) and *Sahyadria denisonii* (**Sajeevan *and* Anna Mercy, 2016**).

2.3.4. Behaviour study

The patterns of reproductive behaviour among teleost fishes range from the simple release of gametes in the proximity of conspecifics to complex sequences which may include defense and preparation of a nest site or territory, pair formation, and spawning (**Liley & Stacey, 1984**). Reproductive strategies have already been categorized in cyprinid fish (**Balon, 1984; Turner, 1986**). In some groups, fertilization is internal and results in the release of fertilized eggs, larvae, or sexually mature offspring. The term reproductive behaviour can be used in general to encompass all activities involved in reproduction. Sexual behaviour is restricted to any behavioural interaction between the sexes leading to the union of gametes (**Liley & Stacey, 1984**). Knowledge of behavioural patterns and characteristics of spawning aggregation is important to fully understand the function of group spawning in a species (**Shapiro *et al*, 1993**).

According to **Erisman and Allen (2006),** fish breeding behavioural pattern was thirteen type (Table.2). In the case of externally fertilizing species, it is important to distinguish between pre-spawning and spawning behaviours. Pre-spawning behaviour includes sexual activities, often referred to as courtship, involved in the search for, and attraction and excitation of, a potential sexual partner (**Jacob, 2013**). The male fish dressed up in overall reddish tinge and the depth of the colour varied. Based on maturity status of the gonad and spawning, is the complete removal of ovulated eggs from the

ovary, is accomplished by the accompanying of the male holding behaviour. In this behaviour, male hold the female by curving its body in a semi- circle fashion along the dorsal side. During this behaviour, it brings the genital regions to a close proximity. Each male holding lasted approximately one second. **(Jacob, 2013)**

Table 2. Different behavioural patterns shown during courting and spawning based on (Erisman and Allen, 2006)

Behaviour	Occurrence	Description
1) Female display	Courtship	A gravid female hovers motionless in the water column or adjacent to the substrata, using a fanning motion of the pectoral and caudal fins to maintain position; body is relatively straight with the head angled upwards at $20–60^0$.
2) Lateral display	Courtship	Sexually active male shows its colourful flanks in a special way towards the female along with tail quivering.
3) Hover	Courtship	A courting male hovers in close proximity to a female
4) Rubbing	Courtship	A male approaches a gravid female from the side or from underneath; the snout, operculum and dorsal portion of the male's body make physical contact with the lower abdomen of the female as he swims past.
5) Nipping	Courtship aggression	One individual will nip or bite the posterior flank or caudal region of another individual; often occurs during courtship chase and aggressive chase events; during courtship, male nipping behaviour is often followed by female darting behaviour.
6) Bumping	Courtship aggression	The snout or operculum of a male makes physical contact with the lower abdomen of the female for several seconds; sometimes resulting in displacing the location and orientation of the female.
7) Following	Courtship	The ripe male swims behind a gravid female, without

		making physical contact with the female; common during early courtship periods.
8) Courtship chase	Courtship	Ripe male swims rapidly after a gravid female
9) Darting	Courtship	A female swims in an erratic manner, constantly changing directions while swimming away rapidly from chasing males; usually it ends in hiding in safer places available in the tank.
10) Female colour change	Courtship	The overall body became more silvery, the spots becoming less distinct
11) Male colour change	Courtship	The overall body became more reddish, the spots becoming less distinct. As the courting progresses the red colour deepens.
12) Male holding	Spawning	The male hold the female across her body through dorsal side in such a way that both the genital openings come close; it lasts for one second.
13) Pausing	Spawning	This is the part of the holding behaviour, lasts for a fraction of second, during which the release of gametes occur.

According to **Paray *et al.,* (2013)** breeding behaviour or courtship behaviour is a very important act in fish breeding and it varies from simple swimming of the breeders along the side of each other to elaborate act of nest building and intense male competition inherent in group spawning. The absence of breeding behaviour from any of the breeders often results in spawning failure (**Marimuthu *et al.,* 2001**). Several factors like body size, pigmentation, age, and social dominance, environmental conditions, mating history, female reproductive state, male dominance and aggression are known to affect the mating behaviour of fishes in many species (**Deaton, 2008; Marimuthu *et al.,* 2001; Arockiaraj *et al.,* 2004**).

Few workers have studied on breeding behaviour of fishes. As for information, **Thakur (1976**) on behaviour of *Heteropeneustes fossils;* **Hutchings *et al.,* (1999)** on *Gadus morhua* in Nova Scotia, Canada; **Arockiaraj *et al.,* (2003**) on *Mystus montanus;* **Anna Mercy *et al.,* (2003)** on *Pristolepis marginata;* **Haniffa *et al.,* (2004)** on *Channa punctatus;* **Prado *et al., (*2006**) on *Hoplias malabaricus*; **Graya and Kinnon (2006)** on Telmatherinidae; **Veerappan *et al.,* (2009)** on sound producing behaviour of *Kathala axillaris*; **Jones (1940)**; **Padmanabhan (1955); Norman and Greenwood (1963)**; **Pal and Southwick (1965)**;**Indira (1986) and Chandran *et al.,* (2013)** studied on breeding behaviour of *Pseudosphromenus cupanus;* **Paray *et al.,* (2013)** on breeding behavior of the *Channa striatus* and **Behera *et al.,* (2016)** observed pairing and chasing courtship behaviour of climbing perch, *Anabas testudineus* from West Bengal.

2.3.5. Embryonic development

Embryonic development of suitable protocols for the mass rearing of larval fish represents one of the important barriers for the successful propagation of most of the freshwater species **(Thakur, 1980). Blaxter (1974**) suggested, that fish eggs and larvae are useful for estimating the fish stock. Different modes of larval development are evident in fishes. It depends on the fact to which taxa it belonged to. The development seen in egg scatterers is different from fishes showing parental care. The mouth brooders release very few eggs with big size. The live bearers produce juveniles directly by passing the larval stage (**Wourms, 1981**).

Embryonic and larval developmental studies provide sufficient information regarding the successful rearing of larvae (**Mathew *et al.,* 1996**). Most authors accept the division of fish development into five periods; embryo, larva, juvenile, adult and senescence (**Kovac and Copp, 1996**).Refining the techniques of larval rearing is very

important for practical and commercial applications (**Liao, 1993**). Great variation in size exists among species in young fishes before they become free living forms [free from yolk sac after 3-5 days] (**Diwan and Dhakad, 1995**). Apart from the academic interest, experimental based information on these early stages is required for progress in the advanced fields of fish culture (**Thampy, 2009**). Despite the success in artificial propagation of *Garra surendranathanii,* by induced spawning it is necessary to understand the developmental biology of the species which is a prerequisite for successful rearing of the species and commercialization of the induced breeding techniques (**Thampy, 2009**).

Jacob (2013) Studying on the evolution of development showed large scale comparisons of diverging taxa in a known phylogenetic context to uncover the developmental pathways of animals. **Galis and Sinervo (2002)** explained the similarity of early embryos resulting from a variety of developmental constraints names Teratogens, and studied on the early life stages of fishes which are important because, the requirement of young fish changes rapidly as a function of age. The embryonic and larval development studies provide useful information for the successful rearing of larvae.

Noakes (1991) gave a detailed review of developmental stages, hatching, feeding, social behaviour and parental-young interactions in cichlid larvae. Several authors worked on embryonic development of different fishes like **Kimmel *et al.,* (1995)** on Zebra fish; **Arockiaraj *et al.,* (2003)** on *Mystus montanus* **; Schmidt and Starck (2004**) on Zebra fish; **Dirisu and Innocent (2004)** on *Clarias gariepinus* from Nigeria; **Bindu, (2006)** on two ornamental fishes *Etroplus suratensis* and *Horabagrus brachysoma* from Western Ghats; **Mohammadi *et al.,* (2012)** on Yellow fin sea bream (*Acanthopagrus latus*) from south of Persian Gulf , Iran; **Bindu and Padmakumar (2012)** on *Etroplus*

maculates; **Purkayastha *et al*., (2012)** on *Ompok pabda;* **Udit *et al.*, (2014)** on *Puntius sarana;* **Sarkar *et al*.,(2015)** on *Anabas testudineus;* **Dey and Barat (2015)** on *Botia almorhae;* ***Dey et al*.,(2015c, 2015d and 2015b)** on *Botia lohachata*, *Botia dario* and *Botia rostrata ;* **Anna Mercy *et al.*, (2015)** studied on embryonic development of *S. denisonii;* **Samuel Moses *et al.*, (2016)** on *Pangasianodon hypophthalmus* from Tamil Nadu; **Guralp *et al.*, (2016)** on Pike perch (*Sander lucioperca*) from Vodnany**; Yang *et al.*, (2016)** on Yellowtail Kingfish, *Seriola lalandi* from Korea and **Dey *et al.*, (2016)** on hill stream fish *Barilius barila.*

2.3.6. Supplementary feed for larval rearing in captive breeding

Nikolsky (1963) categorized fishes according to their extent of variation and types of food consumed by them such as a) Euryphagic: feeding on varieties of food. b) Stenophagic: feeding on few selected types of food and, c): Monophagic: feeding on single type of food. The main problems arising in larval fish rearing is the relatively smaller size of the mouth and limited yolk reserves of the larvae **(Shirota, 1970).** The rearing of early life stages by fish culturists is important because the requirement of young fish change rapidly with every hour or days. Working definitions of developmental stages for aquaculture are, therefore, practically very useful **(Haylor, 1992 and Thampy, 2009).**

The success in the hatchery production of fish fingerlings for stocking in the grow-out production system is largely dependent on the availability of suitable live food organisms for feeding fish larvae, fry and fingerlings. The availability of large quantities of live food organisms such as, marine rotifer (*Brachionus plicatilis* and *Brachionus rotundiformis*) and *Artemia* nauplii to meet the different stages of fry production has contributed to the successful fry production of at least 60 marine finfish species and 18

species of crustaceans **(Dhert, 1996).** Many of the modern larviculture technologies used in marine food fish hatcheries could be adapted for application in the freshwater ornamental fish production. Some of the possible applications have been reported in by **Dhert *et al.*, (1997).**

According to **Lima *et al.*, (2003),** the industrial development of freshwater ornamental fish culture has been hampered by the lack of suitable live feeds for feeding the fish at the various production stages. Currently, inert food items such as egg yolk suspension, milk powder or powdered feeds and natural plankton bloom induced by artificial fertilisation of water are used in larval feeding, and *Moina* and Tubifex that are cultured in water enriched with organic manure are fed to bigger fish or brooders. There is also no suitable live feed for feeding early fish larvae with small mouth. These traditional practices not only limit the fish stocking density, but also adversely affect fish quality. Many freshwater ornamental fish farmers have shifted from Moina to the cleaner Artemia nauplii for feeding their young fish.

Recently, some work have been done on larval rearing in captivity by **Vishwas Rao and Ajith Kumar (2014)** on captive breed of *Pterapogon kauderni* and comparative high growth rate, when larvae were fed with algal enriched *Artemia* than the Poly Unsaturated Fatty Acid (PUFA). The larvae were reached the marketable size within 45 days. Brood fish were fed with boiled clam meat, octopus, oyster, squid and trash fish thrice a day. **Afroz *et al.*, (2014)** studied on larval rearing of spiny eel, *Mastacembelus pancalus* in captivity and reported that larvae started to feed with *Artemia* nauplii when 5-10 days old, *Moina* sp at 11-20 days old, blood worm at 21-35 days old, tubifex species and blood worm (*Chionomid* sp.) at 35-50 days old and small shrimp after 50 days. **Dey *et al.*, (2015d, 2015c and 2015b)** and **Dey and Barat (2015)**

studied on *Botia* loaches namely *Botia dario, Botia lohachata, Botia rostrata* and *Botia almorhae* and reported that at first larvae were fed with *Paramecium* sp. and then *Artemia* after 3days. The larvae also consumed small sized zooplanktons.

Larval rearing in captivity of many fishes have been studied by several workers. **Karim and Hossain (1972)** on *Mastacembelus pancalus;* **Serajuddin and Mustafa (1994)** on *Mastacembelus armatus*; **Umezawa *et al.* (1991)** on *Anguilla rostrata*; **Ignatius *et al.*, (2001)** on *Amphiprion sebae;* **Das and Kalita (2003)** on *Macrognathus aculeatus*; **Suresh *et al.*(2006)** on *Macrognathus pancalus;* **Williot *et al.*, (2009)** on *Acipenser sturi;* **Oliveira and Hable (2010)** on *Macrognathus pancalus*; **Rahman *et al.* (2011)** on *Mastacembelus pancalus;* **Rahman and Awal (2016)** on *Channa striatus* and **Ghosh *et al.*, (2016)** on Clownfish, *Amphiprion Clarkii*.

2.4. Histological study of the gonads

Studies on the gonad developmental cycle and histological examination of gonads are appropriate to determine the precise spawning period and frequency in a breeding season (**Conover 1992**). The knowledge about different stages of fish gonadal maturation provides important information necessary to prohibit fishing during the reproduction period; allowing the fishery stock to recover (**Noble and Jones 1993**).

The fishes reproduce in their natural environment to produce offspring and continue their progeny. Both environmental and hormonal factors are extremely important in regulating reproductive behaviour and spawning in fishes (**Chakrabarti and Choudhury, 2015)**. Various central mechanisms translate environmental cues into chemical messengers which function to activate and maintain the reproductive organs. In this regard, the functional relationship between the hypothalamus and pituitary gland is important (**Chakrabarti and Choudhury, 2015)**. The pituitary has a central role in

controlling gonadal activity and the identification and distribution of the cell types in the pituitary gland of the different teleosts. These have attracted some investigators from the histochemical, ultrastructural and immunocytochemical techniques (**Ali, 2003; Al-Absawy, 2004; Chatterjee and Chakrabarti 2014**). The spermatogenesis is a very active process and the testicular cycle in a majority of freshwater teleosts, which are seasonal breeders, undergo remarkable changes during various periods of the season (**El-Boray, 2001; Suwanjarat *et al.*, 2005 and Ahmed *et al.*, 2013**).

Reproduction in most of the tropical and subtropical fish species is periodic, and the peak reproductive event. Spawning, occurs in the most suitable time of the year to ensure maximum survival and growth of the young. Annual fluctuation in photoperiod and its dependant variable temperature are considered as the primary environmental factors regulating reproductive cycle of fish. (**Garg and Sundararaj , 1985; Davis et al., 1999; Bhattacharyya and Maitra 2006**). Fish reproduction, especially teleost has achieved more attention among fisheries scientists during recent few years due to economic interest and nutritional requirements among increasing population **(Roy and Mandal, 2015).**

Reproductive development in fishes is well understood by histological techniques which are the most reliable method to determine the reproductive state of fishes **(West 1990).** The ovarian histological pattern of teleosts is described according to the division of ovarian tissues into seven or eight (**Crim and Glebe 1990**) or five (**Brown-Peterson *et al.*, 2011**) stages of maturity based upon the presence of dominant gametogenic cell types. In the case of synchronic oogenesis, all the oocytes develop at the same time, ovulation also being simultaneous. According to **Dopeikar *et al.*, (2015)** group synchronous ovary consists of at least two populations of the oocytes at different

developmental stages; teleosts with this type of ovary generally spawn once a year and have a relatively short breeding season. In the case of asynchronic ovulation, different development stages of the oocyte maturation and ovulation in groups may be found within the ovaries (**Nagahama 1983, Nejedli *et al.*, 2004**).

The testicular maturity can be judged by visual observation on the morphology and histological survey (**Rath *et al.*, 2000**). However, many have classified development stages of testes as Stage I as resting phases, Stage II as late immature phase, Stage III as maturing phase, Stage IV as mature phase and Stage V as spent phase. Testes are composed of a large number of seminiferous tubules or lobules, which are closely bound together by a thin layer of connective tissue (**Rath *et al.*, 1984**). During breeding season, seminferous tubules are filled with sperm and a few numbers of spermatogonia are seen. After breeding season, empty and collapsing seminiferous tubules are seen, some of which contain residual or unexpelled sperm (**Sanwal and Khanna, 1972**). **Das (2002)** studied testicular maturity of *Anabas* and identified three phases of testes that is spawning, post spawning and preparatory .

Some of the earlier works on gonad histology on different fishes by authors like **Arockiaraj *et al.*, (2004**), who found five developmental stages of gonad in *Mystus montanus;* **Agbugui, (2013)** reported six developmental stages of gonad in *Pomadasys jubelini* namely, immature, quiescent, maturing, mature, running and spent from Nigeria; **Amtyaz *et al.*, (2013)** oberserved seven developmental stages of gonad in *Pomadasys stridens* from Karachi Coast, Pakistan; **Oliveira *et al.*, (2015)** investigate six phases of oocyte development and four developmental phases of the spermatogonia in *H. brasiliensis*: spermatogonia, spermatocytes, spermatids and spermatozoa of *H. Brasiliensis;* **Chakrabarti and Banerjee (2015)** found five stahes of spermatogonia of

X. cancila; **Behera *et al.,* (2015)** observed two testicular cyclicity of *Anabas testudineus.* According to **Dopeikar *et al., (*2015)** five gonad developmental stages namely Stage I (never spawned), Stage II (Early developing; developing; ovaries and testes beginning to develop, but not ready to spawn), Stage III (Spawning capable; fish were developmentally and physiologically able to spawn), Stage IV (Regressing; cessation of spawning) and Stage V (Regenerating; sexually mature, reproductively inactive) of *Barbus lacerta* from Bibi-Sayyedan River, Tigris basin.

Recently, gonad histology of many fishes have been studied by several workers viz **Roy and Manda (2015)** on *Labeo bata*; **Chakraborty and Choudhury (2015)** on *Notopterous notopterous;* **El-Nasr (2016)** on *Gerres filamentosus* from Hurghada Red Sea, Egypt; **Mahmud *et al.,* (2016)** on *Channa striata* from Bangladesh; **Silva *et al.,* (2016)** on *Cynoscion leiarchus;* and **Sales *et al.,* (2016)** on *Hypostomus francisci* from Brazil.

2.5. Molecular characterization of DNA barcoding

DNA barcode is a new tool for taxon recognition and classification of biological organisms based on sequence of a fragment of mitochondrial gene, Cytochrome Oxidase I (COI). In view of the growing importance of the fish, DNA barcoding for species identification, molecular taxonomy and fish diversity conservation is essential **(Nagpure *et al.,* 2012).** Molecular taxonomy is now used in harmony but in addition to other classical morphological data to delimit species (**Tautz *et al.,* 2002**). Although it has been well accepted that DNA taxonomy can solve many taxonomic problems but still it has not got a central role in it. Presently, scientists are working on phylogeny and phylogeography of different species using the DNA as the central theme of their analysis. Although the morphological attributes are going to play the major role in the taxonomic

description, DNA can be given a better position than what it has today. We believe the best way to give DNA its fair chance in taxonomy will be to implement "DNA barcoding" as an international unit for identification of species **(Kalyankar , 2012).**

Different molecular markers, such as allozymes, mitochondrial DNA, RAPD have been used to observe genetic variation and evolutionary relationship amongst the different taxa.DNA barcoding is a species level identification system based on mitochondrial DNA. Mitochondrial DNA was used to examine the evolutionary and taxonomic relationships amongst taxa. The DNA barcoding is based on a small sequence of about 655bp of mitochondrial gene Cytochrome oxidase subunit I (COI) with universal primers **(Hebert, 2003a)**. An international interest in fisheries sparked to launch the "Barcode of Life Project (iBOL)". **Hebert (2003b)** determined that mtDNA cytochrome oxidase subunit 1 (CO1) was a suitable gene marker for fish species identification due to the fast evolution of the mtDNA, its maternal inheritance and haploid condition **(Moore,1995).** The use of COI gene for barcoding is a suitable marker for discriminating between closely related species of fishes.

Some important molecular work for species identification at gene level were done by few worker, **Ward *et al.*, (2008)** barcoded 15 fish species from Northern (Atlantic and Mediterranean) and Southern (Australasian) Hemisphere waters and found that 13 species had low genetic variation and two species namely, *Lepidopus caudatus* showed 2.75% and *Zeus faber* showed 7.44% genetic distance between northern and southern clades. **Lakra *et al.*, (2011)** working on 115 marine fish species covering Carangids, Clupeids, Scombrids, Groupers, Sciaenids, Silverbellies, Mullids, Polynemids and Silurids representing 79 Genera and 37 families from the Indian Ocean have been barcoded for the first time using Cytochrome c oxidase I gene (COI) of the mtDNA. The

average Kimura two parameter (K2P) distances within species, genera, families, orders were 0.30%, 6.60%, 9.91%, 16.00%, respectively; **Nagpure *et al.*, (2012)** developed a Fish Barcode Information System (FBIS) for Indian fishes and the database contained 2334 sequence records of COI gene for 472 aquatic species belonging to 39 orders and 136 families, collected from available published data sources and this system also contained information on phenotype, distribution and IUCN Red List status of fishes. **Krishna *et al.*, (2012)** were barcoded 10 species from River Krishna region, Andhra Pradesh, India and recovered a new species; **Chandra *et al.*, (2012)** worked on Cytochrome Oxidase I (COI) DNA barcodes for the identification of two commercially important coldwater species of Genus *Schizothorax* (Snow trout) and mean intra-specific nucleotide sequence divergence was 1.75% (range 0.00-3.50%) and inter-specific divergence of *S. richardsonii* was 0.00% (range 0.00040-0.00080%) and *S. progastus* was 0.00% (range 0.00036-0.000206), respectively; **Sarkar *et al.*, (2013)** worked on 58S and 28S ribosomal RNA genes of *Pangio pangia* from Ganga river basin ; **Rahman *et al.*, (2013)** barcoded three genera and ten species of mullets (Family : Mugilidae) (*Liza macrolepis, Liza parsia, Liza planiceps, Liza subviridis, Liza tade, Liza vaigiensis, Mugil cephalus, Valamugil cunnesius, Valamugil seheli, Valamugil speigleri*) and among 10 species the sequence for *Valamugil speigleri* was not available in the GenBank and all species were clearly differentiated with COI gene sequences.

Recently some work on barcoding of fish by **Vij *et al.*, (2014)** were collected. Asian seabass from Western and Eastern Coastline of India, Andaman and Nicobar Islands, Bangladesh and Australia, Malaysia, Indonesia, Thailand and Singapore and barcoded all the sequences analyzed concordantly point to the existence of at least two distinct species one representing the Indian subcontinent plus Myanmar, and a second, representing Southeast Asia (Singapore, Malaysia, Thailand and Indonesia) plus

Northern Australia; **Ambili *et al*, (2014)** identified three *Tor* species from Southern Western Ghats, Kerala with the help of COI and *Tor khudree, Tor malabaricus* and *T. mussullah* was confirmed through DNA barcoding, morphometric and meristic characters. **Jegatheesh *et al.*, (2014)** studied six endemic fishes of the Western Ghats belonging to *Dawkinsia* genus and reported that all these six species are genetically distinct; five haplotypes within *filamentosa* group indicated that high level of genetic diversity found within COI sequences of studied species and *Dawkinsia* exhibited very close genetic similarity with *D. filamentosa*. **Bineesh *et al.*, (2014)** confirmed four species of elasmobranch, *Rhynchobatus australiae* , *Dasyatis microps, Himantura granulate and Aetomylaeus vespertilio* from Arabian Sea coast of India with the help of barcoding. **Kannan *et al.*, (2014)** using Cyt b and COI of mitochondrial DNA of *Dawkinsia tambraparniei* from Southern Western Ghats, India and recovered that Cyt *b* gene sequences showed that all populations belonged to a single haplotype and no nucleotide variation was observed between populations and in the case of COI gene sequences, two haplotypes were found and the pair wise genetic distance between *D. tambraparniei* and *D. arulius* was low; **Kannan *et al.*, (2014)** using Cyt b and COI of mitochondrial DNA of *Dawkinsia tambraparniei* from Southern Western Ghats, India and recovered that Cyt *b* gene sequences showed that all populations belonged to a single haplotype and no nucleotide variation was observed between populations and in the case of COI gene sequences, two haplotypes were found and the pair wise genetic distance between *D. tambraparniei* and *D. arulius* was low.

2.6. Ichthyofauna diversity in different rivers of India

According to **Cohen (1970)** approximately, there are 19650-21535 living species of fish. These one third are freshwater and two-third are marine. **Nelson (2006)** reported about 27,977 species of fish of which 11,952 were freshwater and 16,025 were marine and diadromous.

According to **Kottelat and Whitten (1996),** India occupied eighth position in the world and third in Asia. **Mittermeier and Mittermeier (1997)** stated, that India was a megabiodiversity country which occupied the ninth position in the world. About 21,730 species of fishes have been recorded in the world; of which about 11.7% are found in Indian waters. Out of the 2546 species so far listed, 73belong to cold freshwater regime, 544 to the warm fresh water domain, 143 to the brackish water and 1440 to the marine ecosystem (**International Consultation on Biological Diversity, 1994**).

Other important work done in India on Ichthyodiversity were by **Menon (1962)** who listed 218 species from Himalayan; **Mahanta (2006)** 258 freshwater fish species; **Ghosh and Lipton (1982)** reported 172 species; **Talwar and Jhingran (1991)** stated 930 fish species; **Tamang (1993)** reported 48 species from Sikkim and **Sinha (1994)** listed 230 species; **Choudhury (2005)** 297 and **Goswami *et al.*, (2012)** 422 species from North-Eastern India.

Many authors who worked on fish diversity of different rivers of West Bengal, **Sanyal *et al.*, (2012)** reported 207 species from Sundarban;. **Mahapatra *et al.*, (2015)** reported 190 native freshwater fish species; **Paul and Chanda (2015)** listed 44 indigenous fish species and **Mistry (2016)** recorded 47 species. Earliest study of fish diversity in West Bengal was done by British surveyors **Shaw and Shebbeare (1937)** who first reported that there were 131 fish species from North Bengal; **Hora and Gupta**

(1940) reported 58 species of fishes from Kalimpons ; **Mukherjee *et al.*, (2002)** recorded 39 local Endangered species present in West Bengal; **Patra and Dutta (2010)** recorded 31 species of Cypriniformes fish from River Karala; **Acharjee and Barat (2013a)** reported 65 from Teesta river; **Acharjee *et al*., (2014a)** reported 20 species of loaches from Darjeeling Himalaya; **Dey *et al*. (2015a)** reported 138 species from Kaljani river and **Dey *et al.*, (2015f)** reported 141 species three district of East Himalayan region; **Dey and Sarkar (2015)** recorded 107 from Torsa river; **Das (2015)** recorded 105 Torsa and **Debnath (2015)** recorded 73 Gadadhar river.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

With a view to fulfilling the objectives of the study a research was executed during the period 2012 to 2015 both in the Aquaculture and Limnology Research Unit, Department of Zoology, University of North Bengal and Fishery Laboratory in Uttar Banga Krishi Viswavidyalaya, Cooch Behar, as per the Experimental Design given in **Fig.2.**

3.1. Experimental Design

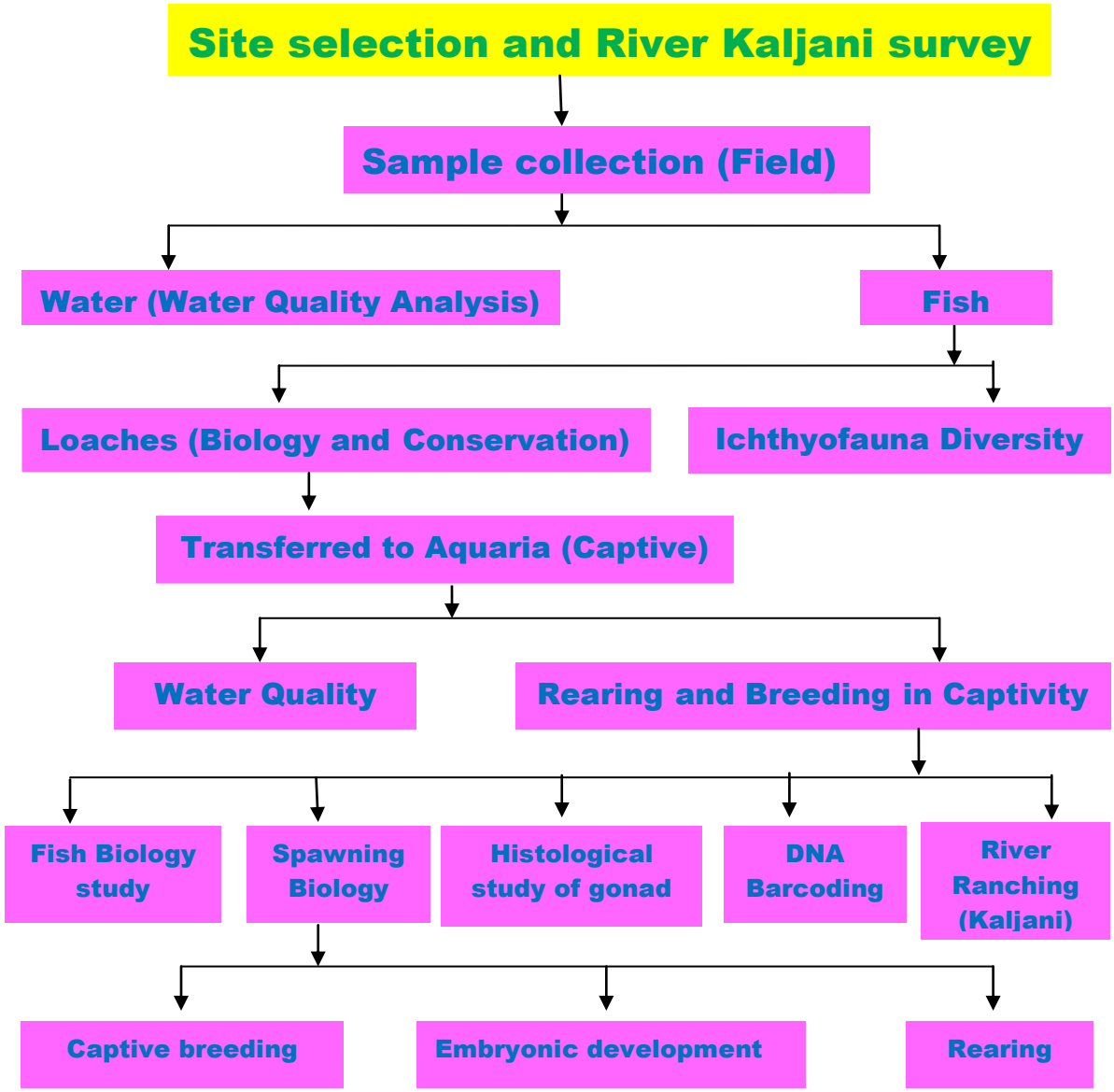


Fig.: 2. Experimental design of the research work done in the Field (Kaljani river) and in Captivity (Laboratory)

3.2. Study area and sampling sites

The present study was executed in the Cooch Behar district situated on the foothills of Eastern Himalaya, lying between 25^0 57′ to 26^0 36′ North latitude and between 89^0 54′ to $88^0$47′ East longitude. The district having a total water area of approximately 6121 ha includes hill stream rivers, ponds and beels. The aquaculture resource of the district is very rich with fishes being the major living components of these water bodies. The Eastern Himalaya with rich biodiversity is under immediate threat of species extinction and habitat destruction due to tremendous pressure from demotechnic growth and natural environmental changes. With a view to rearing and breeding of loaches in captivity, which are Vulnerable and Endangered, their conservation and ichthyofaunal diversity of river Kaljani, Cooch Behar district a study was executed during the period August 2012 to July 2015.

River Kaljani situated in Cooch Behar district covers a stretch of about 9 Km upto the lower reaches of the river, that is, from Amlaguri in the north to Chhat Bhelakopa in the south. The Kaljani river, has a total length of about 96 km., and runs down through the districts of Alipurduar and Cooch Behar, originating from Gabaur Bachhra forest lying in the borders of Bhutan and West Bengal and outfalls into Shiltorsa in Cooch Behar. The sampling areas which were divided into four sites and having a distance of 3 km between them included Amlaguri (26^0 34’ N latitude and 89^0 58’ E longitude), Chhatoa (26^0 32’ N latitude and 89^0 58’ E longitude), Jaigir Chilakhana (26^0 31’ N latitude and 89^0 58’ E longitude), and Chhat Bhelakopa (26^0 29’ N latitude and 89^0 58’ E longitude).

Fig.3. Physical map showing the sampling sites of river Kaljani (Source: Google map)

Fig. 3a: Site 1

Fig. 3b: Site 2

Fig. 3c: Site 3

Fig. 3d: Site 4

Fig.3a -3d: Photographs showing four different sampling sites (Amlaguri, Chhatoa, Jaigir Chilakhana and Chhat Bhelakopa) of River Kaljani

3.3. Collection Procedure

3.3.1. Water (Wild and Captive)

Water samples were collected from both natural water system (Kaljani river) and glass aquariums (Dimensions: 3′ × 1.5 ′ × 1.5′) with aeration in captive system (Laboratory) during the entire period of study in the early morning (6am to 8am). For analysis, water samples were transported to the Aquaculture and Limnology Research Unit, Department of Zoology, University of North Bengal, taking all precautions.

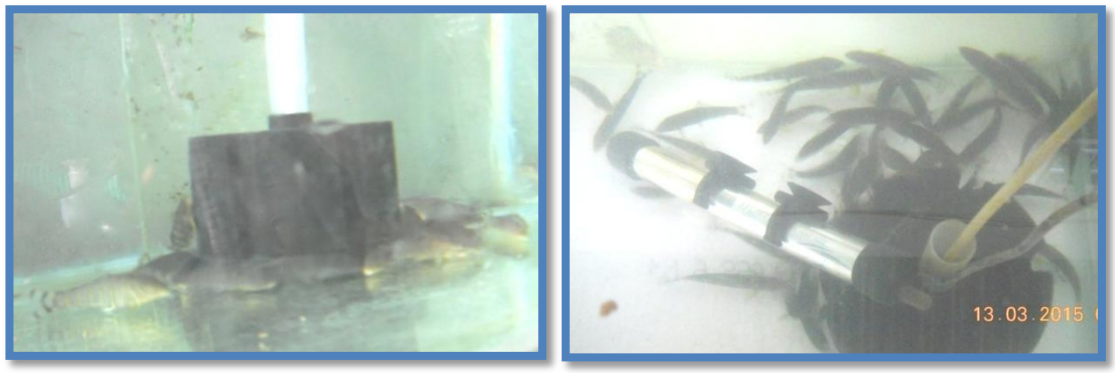


Fig. 3e and 3f: Glass aquariums showing live fishes in captive condition

3.3.2. Fish (Wild)

Fishes were collected from different sites of the river Kaljani during the months of September, October, April and May of respective years with the help of fishermen using different types of gears namely, gill nets, cast nets, dip nets, drag nets and other locally designed fishing gears like *Katal* fishing gear. In *Katal* fishing technique, some area of the river was temporarily fenced off by bamboo and *Eichhornia* or *Pistia* sp. After a few days, these areas were covered by nets and the fishes caught by cast net. This method was applied throughout the year except monsoon.

Botia species are less abundant, Endangered and Vulnerable whereas other genus of loach is abundant and Least Concern. Therefore, *Botia* species are selected as experiment fish of the present study. These loaches are high demanding species having both ornamental and food value. The fishes are very colourful with bright bands, barbles, peaceful nature, and small cycloid scales are usually immersed in mucous. *Botia* loaches consume different types of snails and therefore control naturally of the freshwater snail population. Four *Botia* loaches namely *Botia almorhae*, *Botia dario, Botia lohachata* and *Botia rostrata* were selected for the research study. Live fishes of *Botia* (*Botia almorhae*, *Botia dario, Botia lohachata* and *Botia rostrata*) were collected from different sampling sites of Kaljani river and immediately oxygen packed in sterile polythene bags and transported in cartons to the laboratory in the University.

3.4. Examination Procedures

3.4.1. Water Quality

Water samples for physico-chemical analysis of water were collected from the experimental areas at monthly interval during the study period. All standard procedures described in **APHA (2012); Trivedy and Goel (1984)** and **Chattopadhyay (1998)** were followed. Water samples were intensively studied during the breeding periods of selected fishes *Botia almorhae, Botia dario, Botia lohachata and Botia lohachata* in captivity.

3.4.1.1 Temperature

Temperature is an important parameter for growth of fish. Air and water temperatures were recorded at all sampling sites by standard mercury Celsius (^{o}C) thermometer. Air temperature was also taken at the sampling sites avoiding direct sunlight. Water temperature was recorded immediately after collection of water.

3.4.1.2. pH (Hydrogen Ion Concentration)

pH, the concentration of hydrogen ions (H+) present is a measure of the acidic or basic property. pH of the water was measured by portable pH meter(Eutech). For measuring pH, water sample was taken in a beaker and pH meter was immersed in the beaker to record the pH.

3.4.1.3. Specific Conductivity

Specific conductivity, expressed in μS, is a measure of the ability of a solution to carry an electric current. Specific conductivity was determined by using portable Specific Conductivity meter (Eutech).Water sample was kept in a glass-beaker and conductivity meter was immersed in beaker to record the reading.

3.4. 1.4. Total Dissolved Solids (TDS)

Total dissolved solids (TDS) is the total amount of mobile charged ions, including minerals, salts or metal dissolved in water. TDS of the natural water body mainly depends upon the nature of the bedrocks. Solids are composed mainly of carbonates, bicarbonates, chlorides, phosphates and nitrates of calcium, magnesium, sodium, potassium and manganese, organic matter, salt and other particles. Total dissolved solid was determined by using portable meter (Eutech).Water sample was kept in a glass-beaker and TDS meter was immersed in the beaker to record the readings.

3.4. 1.5. Dissolved Oxygen

Dissolved Oxygen is the most important water quality parameter for respiration of fish and other aquatic organisms. Dissolved oxygen either diffuses from air into water or enters water with the help of photosynthesis by algae and plants. Dissolved oxygen was

measured by modified Winkler's Iodometric method. Water samples were collected in BOD bottles 300ml capacity avoiding air bubbles. 2ml of Winkler's A (Manganous sulphate) and Winkler's B (Alkaline potassium iodide) reagents were added to immediately fix the samples. A brown coloured precipitate appeared which was later dissolved by 2 ml concentrated Sulphuric acid. The sample was then titrated against N/40 Sodium thiosulphate ($Na_2S_2O_3$) solution using starch (1%) as an indicator. The concentration of dissolved oxygen (mg l^{-1}) was calculated using the following formula,

$$\textbf{Dissolved Oxygen} = \frac{\textbf{Volume of } Na_2S_2O_3 \textbf{ (ml) consumed} \times 0.025 \times 8 \times 100}{\textbf{Volume of the sample titrated}} \ \textbf{mgl}^{-1}$$

Where,

Molecular weight of Oxygen=8, Volume of the sample to be taken for titration =101.35 ml (Calculated as shown below), $\text{Volume} = \frac{100 \times 300}{(300-4)}$ ml

Where, Original sample to be taken as per procedure = 100ml, Volume of the BOD bottle = 300ml, Total volume of Winkler's A and Winkler's B = 4ml

3.4. 1.6. Free Carbon Dioxide

Carbon dioxide is the end product of organic carbon degradation in almost all aquatic environments. Carbon dioxide remains in water in three closely related forms, namely (a) Free CO_2 (b) HCO_3 and (c) CO_3. Each remains in the water based on pH. Natural waters contain low concentration of free CO_2 (< 6.0mgl^{-1}). Carbon dioxide can be determined by titrating the sample using a strong alkali N/44 NaOH to pH 8.3. At this pH all the free CO_2 is converted into bicarbonates. The concentration of free CO_2 (mg l^{-1}) in 100ml sample water was analyzed using 5 drops of 50% Alcoholic Phenolphthalein

indicator and N/44 NaOH as titrant as described by **Trivedy and Goel (1984).** The concentration was calculated using the following formula,

$$\textbf{Free Carbon dioxide} = \frac{\textbf{Volume of } \frac{\textbf{N}}{\textbf{44}} \textbf{NaOH solution consumed} \times \textbf{1000}}{\textbf{Volume of the sample titrated}} \textbf{ mg l}^{-1}$$

3.4. 1.7. Total Alkalinity

Alkalinity is a measure of the buffering capacity of the water which considerably maintains the pH by absorbing excess H+ ions and protects the water body from pH fluctuation. Alkalinity of water is due to the presence of hydroxides, carbonates, phosphate, nitrates and bicarbonates. Total alkalinity, that is, carbonate and bicarbonates can be estimated by titrating the sample with strong acid (HCl or H_2SO_4), first to pH 8.3 with alcoholic Phenolphthalein as an indicator and then further using Methyl Orange as an indicator (pH maintained at 4.2 and 5.4). Total alkalinity is the value of the Phenolphthalein alkalinity (PA) and Bicarbonate alkalinity (BA). Low water alkalinity, that is, less than 20mg l^{-1} of $CaCO_3$ is very vulnerable to fluctuation of pH due to low buffering capacity.

Total alkalinity present in the 100 ml water samples were estimated as mg l^{-1} using 5 drops alcoholic phenolphthalein and 2 drops of Methyl Orange as indicator and then titrating against $\frac{N}{50}$ H_2SO_4 (**Golterman *et al.*, 1978**) and Phenolphthalein alkalinity was estimated only when free carbon-dioxide was found to be absent (**Trivedy and Goel, 1984**). The concentration (mg l^{-1}) of each alkalinity (carbonate and bicarbonate) was calculated by using the following formula,

Concentration of Total Alkalinity

$$= \frac{\textbf{Amount of titrant solution (ml) consumed} \times \textbf{1000}}{\textbf{Volume of sample used}} \textbf{ mg l}^{-1}$$

3.4. 1.8. Total Hardness

Total Hardness is caused by the Calcium and Magnesium ions of a water body. This is because to Ca^{2+} and Mg^{2+} are bound to the alkalinity bases bicarbonate and carbonate. Usually the concentrations of the total alkalinity and total hardness are similar because the calcium magnesium, bicarbonate and carbonate are derived from the solution of limestone. However, greater fish production occurs from the higher concentration of phosphorus and other essential elements that increase along with alkalinity and hardness. Calcium and magnesium form a complex wine red colour with Erichrome Black T indicator at pH of 10.0 ± 0.1. The EDTA has got a stronger affinity towards Ca^{++} and Mg^{++} ions and therefore by addition of EDTA the former complex is broken down and a new complex is formed.

Ethylene diamine tetra acetic acid (EDTA) method (**APHA, 2012**) was followed to estimate total hardness of 100ml water samples and expressed as mg $^{-1}$. It was estimated by titrating the water sample against EDTA after adding 0.5 ml ammonium buffer and 6 drops Eriochrome Black T as indicator. The end point was indicated by blue colour. The total hardness was calculated by using the following formula,

$$\textbf{Total Hardness} = \frac{\textbf{Amount of EDTA (ml) consumed} \times \textbf{1000}}{\textbf{Volume of sample taken}} \ \textbf{mg l}^{-1}$$

3.4. 1.9. Ammonium-N

Ammonium is the second most important water quality parameter for fish production. The source of ammonia in water is organic, inorganic and through air deposition. The most important ammonia contamination in water is excessive use of ammonia rich fertilizer, excretion of nitrogenous wastes from animals and sewage contamination in aquatic environments. Ammonia is required for life, but it is toxic to

aquatic organisms when the normal level exceeds. Ammonium concentration in water exists in two forms, (a) Unionized ammonium (NH_3) and (b) Ionized ammonium (NH_4). NH_3 is more toxic than NH_4 to fish. The amount of total NH_3 depends on pH and temperature. Recommended safe level of NH_3 –N is 0.025mg l^{-1} and 1.0 mgl^{-1} for total ammonium.

Ammonium-nitrogen (NH_4-N) of 25ml filtered water sample was estimated by Phenol-Hypochlorite Method (**APHA, 2005**). 1ml of Alcoholic phenol solution, Sodium Nitropruside solution and oxidizing solution (10ml alkaline citrate solution with 2.5 ml hypochlorite) were added to the sample. The sample was then kept for one hour at room temperature and wrapped with aluminium foil. A blue colour appeared which was stable for 24 hours. The blue compound, indophenol, is formed by the reaction of ammonium, sodium oxidising solution and alcoholic phenol catalysed by Sodium Nitropruside. The NH_4-N concentration of the sample was directly estimated through the double beam UV-Visible spectrophotometer (Ray Leigh UV-2601) at 640nm wavelength. A standard curve was prepared by using stock ammonium chloride solution (0.3819g anhydrous NH_4Cl in 100ml DW) to estimate the Ammonium-N concentration of the water sample.

3.4. 1.10. Nitrite-N

Nitrite is the intermediate product of nitrification. Nitrite-nitrogen is an unstable product, formed during nitrogen cycle (nitrification and denitrification process). High amount of nitrite present in water bodies will indicate pollution. Nitrite is present in low concentration in water and culture systems. A safe level of nitrite toxicity to fish is 0.02 to 1.0 mg l^{-1}. Nitrite-N of 50 ml filtered sample was estimated by α-Napthalamine and Sulphanilic Acid Method (**APHA, 2012**). 1ml of EDTA, Sulphanilic acid and α-Napthalamine hydrochloride were added to the sample. A pink colour appeared after 10

minutes. The Nitrite-N concentration of the sample was directly estimated through the double beam UV-Visible spectrophotometer (Ray Leigh UV-2601) at 520 nm wavelength. A standard curve was prepared by using $NaNO_2$ to estimate the Nitrite-N concentration of water sample.

3.4. 1.11. Nitrate–N

Inorganic nitrogen present in water is Nitrate-N. It is the main nutrient that accelerates the growth of hydrophytes and algae (**Lodh *et al.*, 2014**). Human activities like food production, agriculture and manure disposal of domestic and industrial sewage contribute to nitrate-N. High level of nitrate is found in rural areas because of extensive application of nitrogenous fertilizers in agriculture. In urban areas, sewage water rich in nitrate contaminates surface water and thus increasing the nitrate amount (**Tank, 2013; Gopalkrushna, 2011**).

To estimate the concentration of Nitrate-N, Brucine Method (**Trivedy and Goel, 1984**) was followed. Filtered sample was taken to remove residual chloride from the sample by adding one drop of Sodium Arsenite solution. 10 ml of sample was placed in a cool water bath and 2ml of 30% NaCl solution was added.10ml H_2SO_4 solution was added after mixing the contents thoroughly swirled by hand. 0.5 ml Brucine reagent was added and the sample was placed in hot water bath for 20 minutes. After cooling, the concentration of Nitrate-N was estimated by double beam UV-Visible spectrophotometer (Ray Leigh UV-2601) at 410 nm wavelength. A standard curve was prepared by using KNO_3 to estimate the Nitrate-N concentration of water sample.

3.4. 1.12. Phosphate-P

Phosphate is in a limited source in nature and important factor for productivity of water body. Phosphate may occur in lake as a result of domestic waste, detergent and agricultural runoff containing fertilizer (**Gopalkrusna, 2011**). The Phosphate concentration of water sample was determined by Stannous Chloride method (**APHA, 2005**). 2ml Ammonium Molybdate solution (25.0 g Ammonium Molybdate + 280ml concentrate H_2SO_4 + 1000ml distilled water) and 5 drops Stannous Chloride in glycerol were subsequently added to the properly filtered 50 ml water samples. A blue colour appeared in the sample. The readings were recorded within 10-12 minutes. The Phosphate-P concentration of the samples was directly estimated through the double beam UV-Visible spectrophotometer (Ray Leigh UV-2601) at 690 nm wavelength. A standard curve was prepared by using known concentration of Phosphate-P solution (10mg P/1ml) to estimate the Phosphate-P concentration of water sample.

3.4.2. Study of the Fish Biology Parameters in Captivity (Glass Aquarium)

The *Botia* loaches are high demanding ornamental fishes which have very beautiful bright bands, colour and barbles. Four *Botia* loaches namely *Botia almorhae*, *Botia dario*, *Botia lohachata* and *Botia rostrata* were studied for the biology in captivity. To study the fish biology of *Botia* species in aquarium, juvenile fish (length 3 to 4 cm; weight 2 gm) were collected from the river sites and reared for 7 to 9 months to produce broodstock fish. In the rearing tanks, temperature of 27^0 to 32 C^0 was maintained with the help of regulated water heaters (Thermostat). This temperature was good for optimum growth of loach and also gonad maturation. Broodstock can be managed in aquarium to promote gonad development. In the present study, the broodstock were fed with blood

worm, tubifex and commercially available fish food at 5 to 10% of the total body weight per day. Excess feeds were removed regularly twice in a day to prevent water contamination. The fishes were monitored regularly for morphological indicators of maturation.

3.4.2.1. Study of fish growth parameters

For this study Gonado-somatic index (GSI), gonad length with body length, body length versus body weight of the fish were determined.

3.4.2.1.1. Gonado-Somatic Index (GSI)

Gonad weight gives an easy measured quantitative record of changes in gonad condition. Thus, GSI is an indirect method of estimating spawning season of a fish species. The Gonado-somatic Index values of fishes indicate cyclical change during growth, maturation, spawning, post-spawning and resting phases of the gonads. The season change in gonad weight is more profound in female than male. To determine the relation between gonad weight and body weight it is calculated by Gonado-somatic index (GSI). Gonado-somatic Index was expressed according to the method of **Vlaming (1982).** This Index is calculated as

$$\textbf{Gonado-somatic Index (GSI)} = \frac{\textbf{Gonad weight X 100}}{\textbf{Total body weight}}$$

3.4.2.1.2. Condition Factor

Condition of fish means robustness (fitness) of a cultivated fish with respect to the same species taken from other water bodies or to other species of fish taken from the same water body. The robustness is calculated by Condition Factor or "K- factor" or "Ponderal Index". According to **Mir *et al.*, (2012),** the Condition Factor is used for comparing the condition, fatting or well-being of fish, based on the assumption that

heavier fish of a given length are in better condition. Difference in the Condition Factor have been interpreted as a measure of histological events such as fat reservation, adaptation to the environment and gonadal development (**Le Cren., 1951**).The Coefficient of Condition (K) was calculated using **Fulton (1904)** expression.

$$\textbf{Condition Factor or Coefficient of condition (K)} = \frac{\mathbf{W \times 100}}{\mathbf{L^3}}$$

where, W= Weight in gram, L=length in cm, and 100 is a factor to bring the value of K near unity (**Froese, 2006**).

3.4.2.1.3. Length-Weight Relationship

The Length-Weight relationship is a standard method providing authentic biological information. It helps to calculate weight from length of fish; it is direct method of converting logarithmic growth rat into weight and provides taxonomic differences and events in the life history. To determine the Length-Weight relationship the Method of Least Squares is applied, that is, the relation $\mathbf{W = aL^b}$ is considered where, W = fish weight in g, L = fish length in cm; 'a' is a constant being initial growth and 'b' is the growth coefficient which is equal to or greater than 3 (**Le Cren, 1951**).The relationship $W = aL^b$ or $W = aL^3$ (Cube Law) when converted into the logarithmic form gives the expression for the straight line Y= a + bX (Linear Regression). The Correlation of Cofficient (r) that is the degree of association between the length and weight was computed from the linear regression analysis:

$$\mathbf{LogW = Loga + b\ LogL}$$

where, W is the weight, 'b' represents the slope of the line and 'a' is a constant.

3.4.2.2. Study of fecundity and fertilization rate of the selected fishes

3.4.2.2.1. Fecundity

The fecundity of a fish is defined, as the number of ova found in the ovary of a female fish prior to spawning. There are different methods for the estimation of fecundity. In this study, eggs were collected from three regions of the gonad like anterior, middle and posterior as the distribution pattern was not uniform. The Absolute Fecundity was calculated according to the method of **Hartman and Conkle (1960)** using the expressions

$$\textbf{Fecundity (F)} = \frac{\textbf{n x G}}{\textbf{g}}$$

where, F is Fecundity; n is mean numbers of eggs in the sub-samples, G is weight of ovaries and g is weight of sub-samples.

3.4.2.2.2. Fertilization rate

After 1 hour of spawning 2 litres of water and eggs were collected from the hatchery and continued for 4 hours. Counting of the fertilized and unfertilized eggs were done. Fertilization rate was estimated by using the following formula (**Udit *et al*., 2014**).

$$\textbf{Fertilization rate (\%)} = \frac{\textbf{Fertilized eggs x 100}}{\textbf{Total number of eggs in sample}}$$

3.4.2.3. Standardization of breeding protocol of *Botia* species

For induced breeding of loaches, initially, 2- phenoxy ethanol @ 2ml in 20 lit. of water was used to anesthetize the fishes (Fig.3g) for easy handling prior to injection of the fish . This also prevented the fish from getting stressed. Thirty two (32) pairs of each group of matured fish were injected (Fig.3h) with different doses of synthetic hormone WOVA-FH (Biostadt India limited, Mumbai). The breeding trail was done twice. A total

of 64 pairs of fish were taken (8 pairs of *Botia almorhae*, 8 pairs of *Botia dario*, 8 pairs of *Botia lohachata* and 8 pairs of *Botia rostrata*) for the breeding experiment.

Fig.3g. Anesthetized fishes

Fig.3h. Hormone being injected at pectoral fin site

Tab. 3: Summary of the protocols of experimental Set-up and design for induced breeding of Genus *Botia* in100 litre tanks with running water system.

No. of Set-up	Species name	Sex ratio	Number of Fish	Dose of hormone (WOVA-FH)
Set-up-01	*B. almorhae*	1:1	2 pairs	0.5ml/kg
Set-up-02	*B. almorhae*	1:1	2 pairs	0.25ml/kg
Set-up-03	*B. almorhae*	1:1	2 pairs	0.025ml/kg
Set-up-04	*B. almorhae*	1:1	2 pairs	0.0125ml/kg
Set-up-05	*B. dario*	1:1	2 pairs	0.5ml/kg
Set-up-06	*B dario*	1:1	2 pairs	0.25ml/kg
Set-up-07	*B. dario*	1:1	2 pairs	0.025ml/kg
Set-up-08	*B. dario*	1:1	2 pairs	0.0125ml/kg
Set-up-09	*B. lohachata*	1:1	2 pairs	0.5ml/kg
Set-up-10	*B. lohachata*	1:1	2 pairs	0.25ml/kg
Set-up-11	*B. lohachata*	1:1	2 pairs	0.025ml/kg
Set-up-12	*B. lohachata*	1:1	2 pairs	0.0125ml/kg
Set-up-13	*B. rostrata*	1:1	2 pairs	0.5ml/kg
Set-up-14	*B. rostrata*	1:1	2 pairs	0.25ml/kg
Set-up-15	*B. rostrata*	1:1	2 pairs	0.025ml/kg
Set-up-16	*B. rostrata*	1:1	2 pairs	0.0125ml/kg

Insulin syringe of 1 ml, normally divided into 40 parts, that is, 1 part is 1/40 =0.025ml, was used. The fish were injected at the base of the pectoral or pelvic fin and then released within an hour's in running water of breeding tanks of 100 litres capacity. After injection of hormone WOVA-FH to the fish and the fish were transferred in the tanks. The detailed of the different Set-up of experimental protocols followed are given in **Tab.3.**

3.4.2.4. Pattern of behaviour of *Botia* species prior to breeding

Behaviour study is difficult in loach species because, *Botia* loaches are bottom feeders. In aquarium or tank they always try to escape to the sand or stone bottom. *Botia* species prefer to take rest in covered areas where sun or light does not reach. Behaviour was monitored continuously from morning to night. During breeding, in an aquarium male and female fishes were allowed to stay together and photographs were taken very quickly as they move fast to escape. At the breeding time, *Botia* species feel uneasy to spawn with interference of sound and light and are afraid to see moveable objects.

3.4.2.5. Embryonic development

Egg samples on hatching were examined hourly and the developing stages were documented through microphotograph. Eggs were collected as the fish were spawning. Initially photographs were continuously taken for the first two hours to capture the egg getting fertilized and the zygote stage undergoing cell division in the different stages and time frame. The standardization of rearing and culture of larvae and spawn to get healthy fingerlings were aquaranched in natural habitat. The yolk sac absorbed fry were harvested and stocked in well prepared cemented tanks for further rearing. Feed was given twice daily. The monthly total length, body depth and body weight were taken.

Before breeding season the F1 generation of captive bred adult fishes were aquaranched in different rivers of Terai, West Bengal, India for conservation of loach species.

3.4.2.6. Supplementary feed for larval rearing of *Botia* species

Four glass aquariums, (Tank A, Tank B, Tank c and Tank D) containing 50 litres water capacity was used for feeding experimental trails (**Tab. 4**). Juveniles from the same parents were stocked in different experimental tanks with same stocking density (20 fishes per tank). Among the 20 fishes, 5 numbers of healthy juveniles of *Botia dario*, *Botia almorhae, Botia lohachata* and *Botia rostrata* were taken in four different aquariums for feeding experiment. In aquarium marked **Tank-A** fish were fed only commercial fish feed (Fig.4a); **Tank-B** with different types of live zooplanktons (Fig.4b); **Tank-C** only boiled minced meat (Fig.4c) and **Tank-D** only minced snail or bivalve flesh (Fig.4d) which were available in the natural water bodies. The fish were fed with experimental diet thrice a day for 45 days. Excess feed were removed regularly twice in a day to prevent water contamination. Biological filter was used in all the experimental tanks. In the rearing tanks temperature of 27^0-32^0 C was maintained with the help of regulated water heaters (Thermostat). Absolute growth rate of fish were calculated by using the formula given by **Rao** and **Kumar (2014).**

$$\textbf{Absolute Growth Rate} = \frac{\textbf{Final weight} - \textbf{Initial weight}}{\textbf{Days of Experiment}}$$

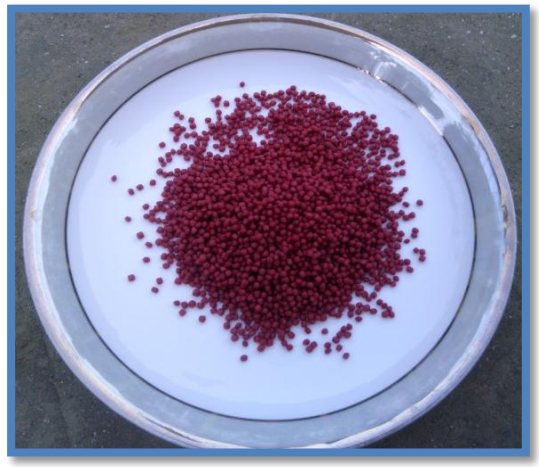


Fig.4a. Commercial fish feed

Fig.4b. Zooplanktons (*Daphnia*)

Fig.4c. Boiled meat

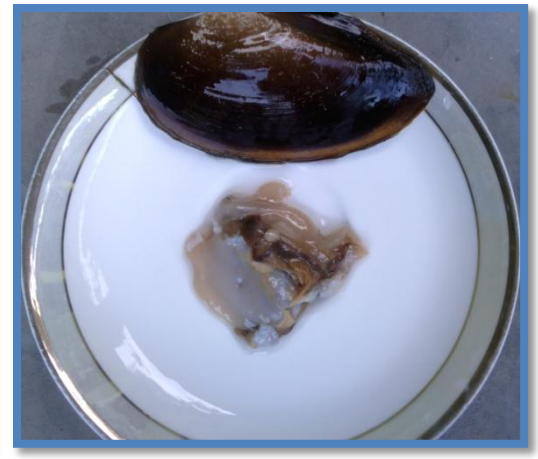


Fig.4d. Bivalve flesh

Fig: 4a-4d: Plate shows different types of supplementary feeds given to *Botia* spp.

Tab. 4: Summary of the protocols of Experimental Set-up of larval rearing of *Botia* spp.

Tank No.	Water capacity	No. of fish	Feeding time	Types of fed
Tank-A	50 litres	20 fish	Thrice a day	Commercial fish feed
Tank-B	50 litres	20 fish	Thrice a day	Zooplanktons
Tank-C	50 litres	20 fish	Thrice a day	Boiled minced meat
Tank-D	50 litres	20 fish	Thrice a day	Minced snail or bivalve flesh

3.4.3. Histological study of the gonads of selected fish

Fishes exhibit periodic or cyclic reproductive behaviour. A thorough knowledge of maturation cycle and depletion of gonad help to understand and predict the annual changes that population undergoes. This, therefore, involves morphological and histological examination of the gonads of the fishes to be studied. For histological study, the development stages of germ cells of the testes and the oocytes in ovary were studied by the following methods (**Agarwal, 1996**).

3.4.3.1. Collection and fixation of tissue

For histological study, a live fish was dissected and gonad removed from the fish (Fig.5a and 5b). The tissues were trimmed into small size for better penetration of fixatives into it. The tissues were kept in Formaldehyde Saline (**Baker, 1944**) for 24 to 48 hours as per size of tissues for fixation.

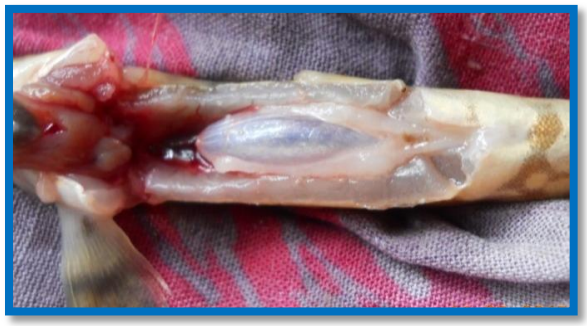


Fig. 5a. Collection of ripe testes from live matured male *Botia* spp.

Fig.5b. Collection of ripe ovary from live matured female *Botia* spp.

3.4.3.2. Post fixation treatment

3.4.3.2.1. Washing and Dehydration

The tissue (testes and ovary) were removed from the fixatives and subjected to overnight washing with flowing clear tap water until the formaldehyde odour had disappered. Water was removed from the tissue through ascending series of alcohols, starting from 30%, 50%, 70%, 90% and Absolute alcohol (100%) for 15 minutes each.

3.4.3.2.2. De-alcoholization and Infiltration

After dehydration the tissue were transferred to xylene for one hour to clear tissue from alcohol. For better impregnation of wax into the tissue, the xylene penetrated into the tissue to make it transparent and make the material to float to the top (**Behera *et al.*, 2015**). In the main time, Paraffin wax kept at 60 °C and allowed to melt for infiltration of the tissue. Three changes of wax (45 min each) were made to make the tissue xylene free and then kept in the incubator overnight at 60°C.

3.4.3.2.3. Embedding

For the preparation of blocks, pure paraffin wax was melted in water bath in between 58-60 °C. Metal 'L' moulds were adjusted according to the size of blocking materials. The melted paraffin was taken from the water bath and the block disc filled with it. A layer of wax was allowed to be solidified at the bottom of the disc. The completely infiltrated tissues were then carefully taken from the paraffin wax and put inside the different blocking disc according to their size. Care was taken so that the wax on the top of the disc did not solidify during keeping the material in the block disc. For this reason, a heated needle or forcep was put at the upper portion or inside the wax of the disc. After proper positioning of the tissues, the wax inside the disc was allowed to solidify. The 'L' moulds were removed from the wax block after a few minutes (**Behera *et al.*, 2015**)

3.4.3.2.4. Trimming and sectioning

The paraffin blocks were trimmed carefully by sharp blades. The trimmed blocks were firmly fixed to a holder and sectioning was done using a microtome. The sections

were cut at 5µm thickness. The ribbons were placed on clear glass slide smeared with egg albumin with the help of a fine brush.

3.4.3.2.5. Spreading and fixing

As the **Behera *et al.*, (2015),** Glass slides were cleaned properly by chromic-acid solution, soap and finally tap water. After cleaning, the slides were air-dried and a thin layer of glycerin egg albumin was smeared over it. The ribbons were then spread over the clean glass slides with a drop of water, the thin tissues were made wrinkle free and allowed to fix on slides by keeping them on a hot plate (30 °C) for 2 to 5 minutes.

3.4.3.2.6. De-waxing and staining

Dried slides were dipped in xylene to remove the wax and rehydrated in descending order of alcohol (100%, 90%, 70%, 50% and 30%) for 5 minutes at each step. The sections were stained with aqueous Haematoxylin for 1minute and the slides then washed with tap water to remove excess stain. The slides were dehydrated through ascending order of alcohol 30%, 50% and 70% (each step for 5 minutes). The slides were then dipped in Eosin for one minute and again dipped in 70% alcohol. The slides were then transferred to 90% and 100% alcohols for 5 minutes. At the end, slides were cleared in xylene.

3.4.3.2.7. Mounting

One or two drops of DPX (mountant) were put on the dried slide which was ready for mounting. Then, a cover slip or slide was slowly lowered over it when the mountant flowed ahead of the descending glass without trapping any air bubble between the cover slip and slide. The excess mountant on the slides was removed with xylene soaked in cotton. After mounting, the slides were allowed to dry. The excess mountant on the slides

were removed with xylene soaked in cotton wool. Dried slides were observed under optical microscope at 10X and 40X magnifications. Photographs were taken by camera (Nikon, Coolpix L24).

3.4.4. Molecular characterization of DNA barcoding and Evolutionary relationship among *Botia* species

For taxonomic identification of *Botia* species, some fish samples were fixed in ethanol and some live fish were oxygen packed in sterile polythene bags and kept in cartons for transport to the laboratory of Molecular Biology and Biotechnology Division, National Bureau of Fish Genetic Resources (Indian Council of Agriculture Research), Canal Ring Road, Dilkusha, Lucknow. Firstly all samples were tagged with the help of tagging gun (**Fig.6a**). Tagged fish image were displayed as in **Fig.6b**. The fins and muscles were then collected from the sample (**Fig.6c**) without skin as the skin of the fish had chance of microbial contamination.

Fig.6a. Tagging gun

Fig. 6b. Tagging fish

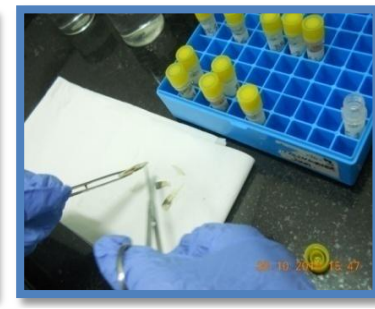


Fig. 6c. Collection of tissue

3.4.4.1. DNA extraction

Total genomic DNA was isolated from approximately 50 mg of pectoral or pelvic fins and muscle tissue following standard phenol/chloroform method (**Sambrook *et al.*, 1989**), which removed proteins and other cellular components from the nucleic acids and pure genomic DNA was obtained. Precipitated DNA was resuspended in TE buffer

(10mM tris –HCl, 0.1 mM EDTA, pH 8) with a final concentration of 100 ng⁄ µl using Nanodrop 2000 (Thermo Scientific, USA), for all samples.

Fig. 6d. Lysed sample for DNA isolation

Fig. 6e. Hood used to avoid contamination

Fig. 6f. Ultra centrifuge used for DNA isolation

3.4.4.2. Determination of quality and quantity of isolated DNA

Estimation of the DNA concentration was done on 0.7% agarose gels in submarine gel casting units (BIO-RAD). The qualitative and quantitative estimation was done by observing the bands in ultraviolet light on UV transilluminator with UV shield and UV protective goggles. The DNA was diluted to get a final concentration of 50 ng/µl.

Fig. 6g. Gel electrophoresis apparatus

Fig. 6h.Gel Documentation system

Fig. 6i. Nanodrop

3.4.4.3. Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is known as revolution method developed by **Kary Mullis (1990)**. It is a powerful tool precisely because it can be done using as little as a single or few copies of template DNA (**Hofreiter *et al.*, 2001**).The universal set of primers

FishF1 –TCAACCAACCACAAAGACATTGGCAC

FishR1- TAGACTTCTGGGTGGCCAAAGAATCA.

were used to amplify the mitochondrial gene cytochrome C oxidase I (COI) (**Ward *et al.*, 2005**).

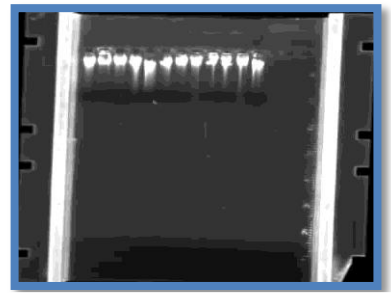


Fig. 6j. COI gene PCR amplified products of *Botia* species

A 20 µl PCR amplification of mitochondrial gene COI was performed with 2 µl of each template DNA. The reagents procured from Bangalore Genei (Bangalore) were used in PCR reactions containing 2 µl of 10X Taq polymerase buffer, 0.8 µl of $MgCl_2$ (25 mM), 0.8 µl of dNTP (2.5 mM each), 0.4 µl of each primer (10 mM) and 0.4µl of *Taq* polymerase (3U/µl). Veriti Thermal Cycler (Applied Biosystems) was used for PCR amplification. The PCR products were visualized on 1.2% agarose gels documented using Gel Documentation system (UVP, GelDoc-It™310 Imaging System). Products with concentration between 50 to 100 ng per µl were selected for sequencing.

3.4.4.4 Sequencing

Once target sequences were selected and successfully amplified, sequence reactions were performed. Sequencing was performed following the dideoxynucleotide chain termination method (**Sanger *et al.*, 1977**), using automated techniques in 3500 Genetic Analyzer (Thermo Fisher Scientific) sequencer.

3.4.4.4.1. Sequencing PCR

Products were labeled using the BigDye Terminator V.3.1 Cycle sequencing Kit (Applied Biosystems, Inc). Reagent quantity for one sequencing PCR reaction cocktail was Terminator Ready Reaction Mix (2.5X) 8.0 µl, BigDye Sequencing buffer (5X) 4.0 µl, PCR product (50ng/µl) 1 µl, Primer (3 µM) 1.0 µl (forward in one PCR tube and reverse in other tube), deionized water 6.0 µl, to make the total volume to 20 µl. Cycle sequencing PCR conditions was 96^0 C for 1 min and 25 cycles of 96^0C for 10s, 50^0C for 5s, 60^0C for 4 min.

Fig. 6k. 3500 Genetic Analyser used to analyse DNA structure

Fig. 6l. Working mode of sequencer, sequencing the DNA

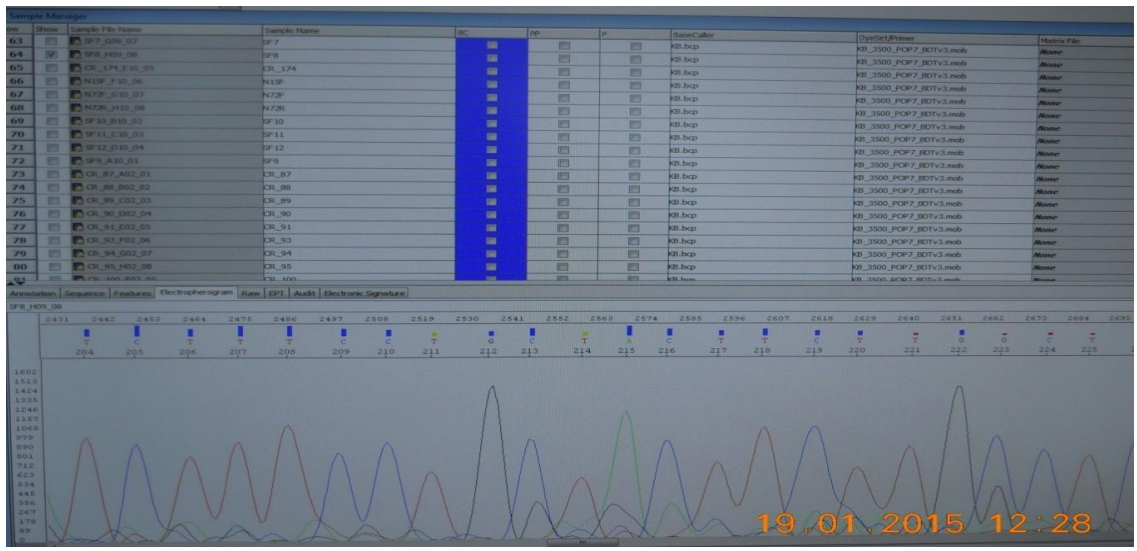


Fig. 6m. Working mode of sequencer, sequencing the DNA

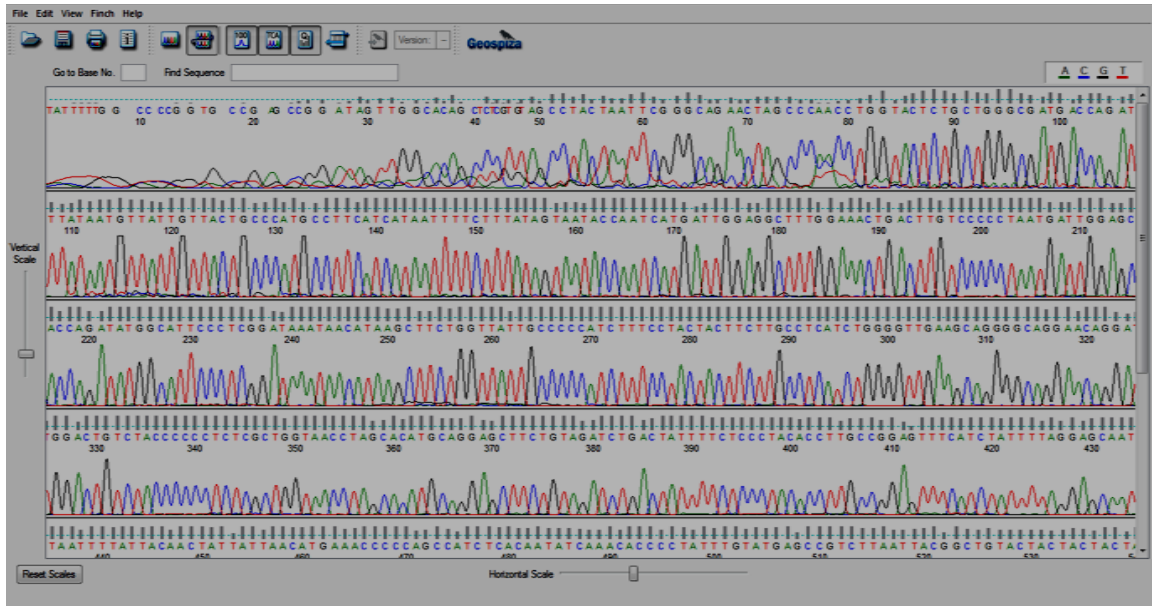


Fig. 6n. Chromatogram showing the forward sequence stand

3.4.4.4.2. Sequence Editing

Both Forward (light) and Reverse (heavy) strand sequences were obtained for the Cytochrome oxidase I gene. Forward strand sequences were generally better than reverse strand. Reverse strand sequences were inverted (reversed and complimented) and aligned with the forward strand sequence. Ambiguities were referenced against the sequencing chromatograms and corrected as per necessity. Full length sequences were made from

forward and reverse strands for all samples of species and aligned using CLUSTALW (**Thompson *et al.*, 1994**). The edited sequences were blasted in NCBI Genbank for the nearest similar sequence matches and submitted to Genbank.

3.4.4.5. Genetic distances and Phylogenetic or Evolutionary analysis

To analyze the evolutionary isolation of six species and the level of divergence within species, K2P distance was calculated by averaging pair wise comparisons of sequence difference across all individuals by the Kimura 2-Parameter method (**Saitou and Nei, 1987**) under Gama distribution estimated in MEGA 5.1(Molecular Evolutionary Genetics Analysis) software (**Tamura *et al*.,2011**).

The first phylogenetic tree was constructed by Neighbor-Joining method (**Saitou and Nei, 1987**) based on bottom-up clustering and distance between each pair of taxa was also calculated. The second phylogenetic tree was inferred by Maximum-Likelihood Tamura-Nei model (**Tamura and Nei, 2011**) using the Maximum Composition Likelihood approach.

3.4.4.6. DNA Barcoding of four *Botia* loaches

Barcode of Life Database Systems was created and is maintained by University of Guelph, Ontario, Canada. It offers researchers with a way to collect, manage and analyze DNA barcode data. DNA barcoding involves a four step uploading process through BOLD systems (Barcode of Life Data Systems) Version 3. A steps involve 1) to upload specimen data, 2) to upload image of the specimen, 3) to upload primers name and traces of the DNA sequence and 4) to uploaded FASTA sequence of the *Botia* species in BOLD systems. All the barcode sequences are deposited in the BOLD. The aim of this database

is to establish a large scale reference sequence database against which new or unknown sample sequence can be queried for species identification.

The International Nucleotide Sequence Database Collaborative (INSDC) is a partnership among the Genbank of **NCBI** in USA, the Nucleotide Sequence Data (NSD) of European Molecular Biology Lab (**EMBL**) in Germany and DNA Data Bank of Japan (**DDBJ**). They have recognized "CBOL" data standards for DNA Barcode records (**Singh *et al.*, 2014**).

3.4.5. Survey the Ichthyofauna diversity of river Kaljani

Besides selected loaches (*Botia* sp.), other fishes were also harvested and preserved in 10 % solution of formaldehyde for identification to study the fish biodiversity of river Kaljani. Fish photographs were taken from fresh samples by camera (Nikon, Coolpix L24) and were identified following their general body form, morphometric and meristic characteristics according to **Talwar and Jhingran (1991)**, **Jayaram (1999)** and **Vishwanath *et al*. (2011),** Conservation status of fish is given as per Conservation Assessment and Management Plan **(CAMP, 1998)** and International Union for Conservation of Nature (**IUCN, 2010**).

3.5. Statistical analysis

3.5.1. Study of correlation between body weight, gonad weight, body length, gonad length, fecundity and Gonado-somatic index

To establish the mathematical relationship between body weight, gonad weight, body length, gonad length, fecundity and Gonado-somatic Index the values of Correlation Coefficient (r) were established by using the statistical formula using MS Excel. The Scatter Diagram of different growth parameters will showed a linear relationship by using the linear regression equation Y = a+ bx, where, 'a' and 'b' are

constants and X and Y are the variables. In order to determine the strength of obtained data between length – weight relationship, Correlation analysis was performed. To show the linearity between Gonado-somatic Index among male and female, gonad length and body length, fecundity and body weight and body weight and gonad weight linear regression analysis was executed. All significant differences studied at 0.05 and 0.01 levels. Mean and Standard deviation were performed for Physico-chemical parameters to show the average and variation among the data. For the computation, software package using Microsoft Office Excel.

Fig.6o and 6p: Length and depth of fishes being measured using calipers

Fig.6q and 6r: Total weight and gonad weight of the fishes being measured

RESULTS

4. RESULTS

4.1. WATER QUALITY

Water is essential for all living organisms like human beings, animals and plants. It is one of the most valuable natural resource. However nowadays, due to human activity it is being polluted. The water quality parameters are very important for the rearing and breeding of both the ornamental and edible fish species. For the present study, it is therefore essential an eco-friendly environment is maintained so as to generate a baseline data of water quality change over a period of time.

4.1.1. Temperature

During the study period, coving from 2013- 2014, the air temperature over the river Kaljani ranged from 22^0C to 36^0C with mean value 30.5^0C (± 3.5); and in captivity at room temperature 20^0C to 34^0C with mean value 28^0C (± 4.53). Monthly variation of air temperature observed at both the sampling sites had an increasing trend.

The Kaljani river water temperature ranged from 26^0C to 31^0C with a mean value of 28.1^0C (± 2.3). Lowest river water temperature was recorded as 26^0C during March to April 2014 and highest 31^0C during August 2014. During this study period, water temperature in captivity ranged from 27^0C to 32^0C which was maintained with the help a Thermostat.

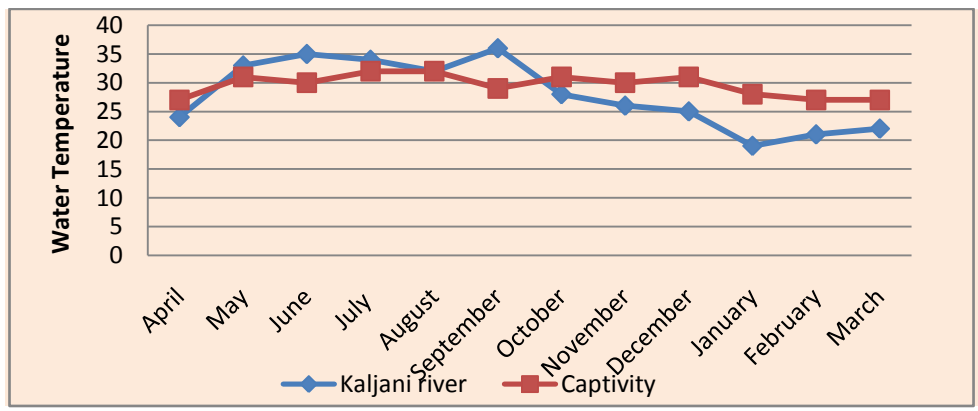


Fig.7a: Monthly variation of water temperature during the study period in Kaljani river and Captivity

4.1.2. Hydrogen ion concentration (pH)

Hydrogen ion concentration (pH) of the Kaljani river water ranged from 7.2 to 8.2 with a mean value of 7.82 (± 0.31). Lowest pH was recorded as 7.2 in April 2014 and highest 8.2 during August 2014. This highest value was found during the rainy season from June to October 2014. The pH value of the captive condition water was however maintained from 7.5 to 8.5 with mean value of 7.88 (± 0.34).

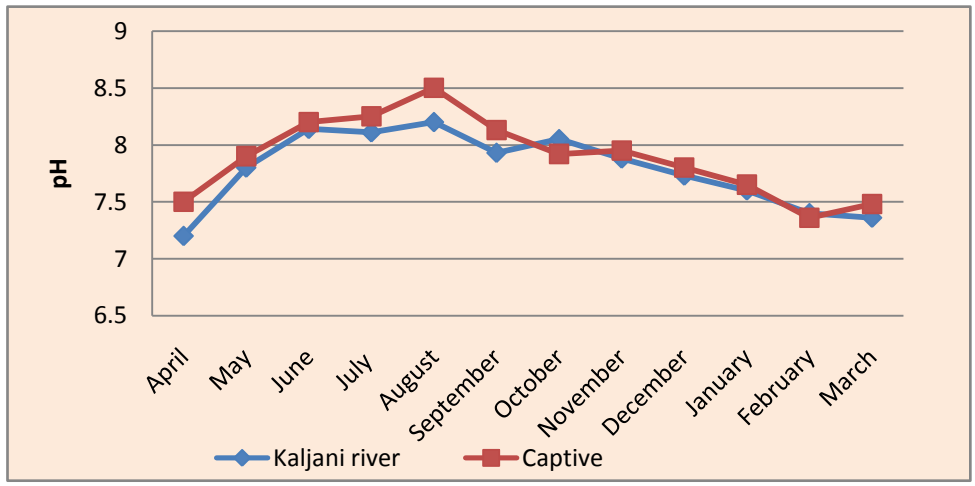


Fig.7b: Monthly variation of pH during the study period in Kaljani river and Captivity

4.1.3. Specific Conductivity

Specific Conductivity of the river water ranged from 110 µS cm^{-1} to 180 µS cm^{-1} with a mean value 151.27 (± 22.30). Lowest value of specific conductivity was recorded as 110 µS cm^{-1} in June 2014 and highest 180 µS cm^{-1} during November 2014. The specific conductivity in the captive condition ranged from 240 µS cm^{-1} to 250 µS cm^{-1} with mean value 246.14 µS cm^{-1} (± 3.18). Lowest value of specific conductivity of breeding tank was recorded as 240 µS cm^{-1} in July 2014 and highest value 250 µS cm^{-1} during April and August 2014.

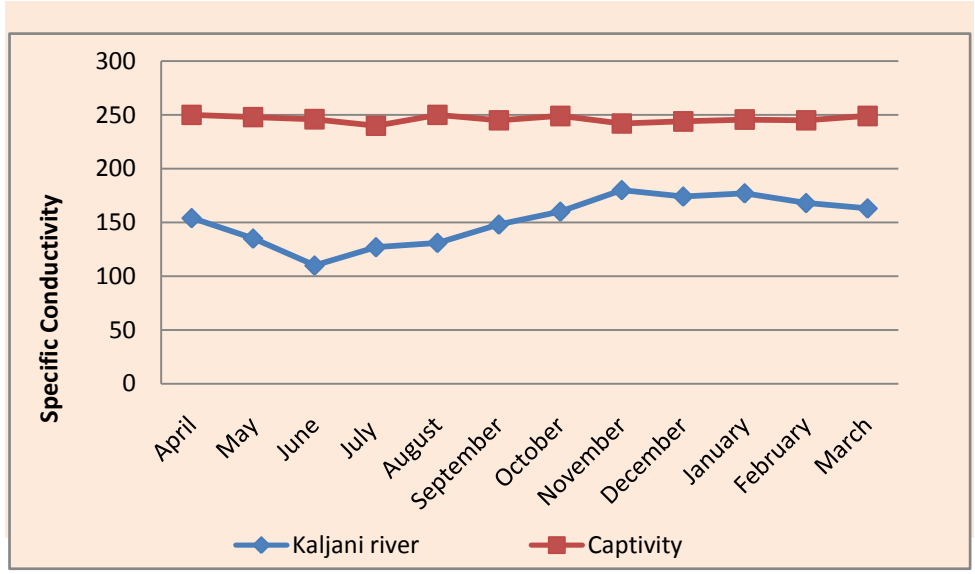


Fig.7c: Monthly variation of Specific Conductivity during the study period in Kaljani river and Captivity

4.1.4. Total Dissolved Solids (TDS)

Total dissolved solids are a measure of dissolved matter (salts, organic matter, minerals and so on) in water. Inorganic constituents comprise most of the total concentration of total dissolved solids. Total dissolved solids are naturally present in water or is the result of mining, oil and gas drilling or some industrial or municipal total dissolved solids. It can be toxic to aquatic life through increase in salinity or changes in the composition of the water, or it may include substances that are toxic to people or aquatic life. Most aquatic ecosystems involving mixed fish fauna can tolerate total dissolved solids levels of 1000 mg L^{-1}. (**Boyd, 1999**). The present study showed that the average value of total dissolved solids of river Kaljani varied from 80 to 120 mg L^{-1} with mean value of 135 mgl^{-1} (± 31.96 mgl^{-1}). Lowest value of total dissolved solids was recorded as 80 mg L^{-1} in September 2014 and highest value of 165 mg L^{-1} during May 2014. Total dissolved solids of breeding tank water were 250 to 260 mg L^{-1} with mean value 255 mg L^{-1} (± 3.97 mgl^{-1}).

4.1.5. Dissolved Oxygen

The amount of dissolved oxygen of the Kaljani river water which ranged from 9.46 mg L^{-1} to 12.4 mg L^{-1} with mean value 10.98 (± 0.914) was quite adequate and characteristic of hill stream. Lowest value of dissolved oxygen was recorded as 9.46 in September 2014 and highest value was recorded as 12.4 during January 2015. During the study period, dissolved oxygen in captivity was maintained at 6.3 mg L^{-1} to 7.6 mg L^{-1} with the help of aeration. This level of dissolved oxygen seemed significant for the survival and activity of *Botia* species.

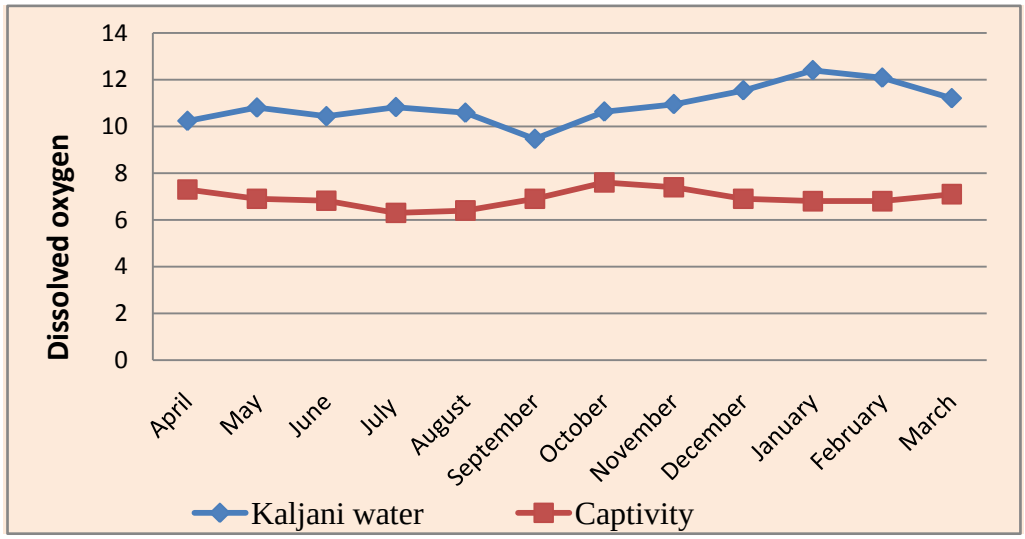


Fig.7d: Monthly variation of Dissolved Oxygen during the study period in Kaljani river and Captivity

4.1.6. Free Carbon Dioxide

The presence of carbonic acid in water may be good or bad depending on the water pH and alkalinity. Carbon dioxide in a water body may be derived from the atmosphere, biotic respiration, inflowing ground water which seep into the pond, decomposition of organic matter due to bacteria and may also be from within the water body itself in combination with other substances namely calcium, magnesium and others (**Abir, 2014**). In the present study, the free carbon dioxide concentration of river water

varied from 2.3 to 4.6 mg L^{-1} with a mean value of 3.76 mg L^{-1} (± 0.801). Free carbon dioxide of breeding tank water varied from 6.0 to 8.0 mg L^{-1} with mean value 6.67 mg L^{-1}(± 0.519 mg L^{-1}).

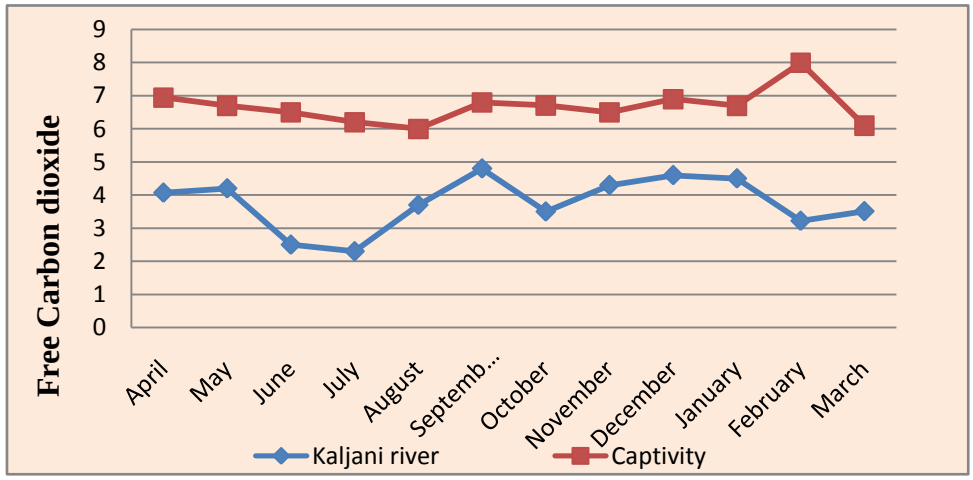


Fig.7e: Monthly variation of Free CO_2 during the study period in Kaljani river and Captivity

4.1.7. Total Alkalinity (TA)

Total alkalinity of the river water ranged from 48.0 mg L^{-1} to 86.2 mg L^{-1} with mean value 69.78 mg L^{-1} (± 12.28). Lowest total alkalinity was recorded as 48.0 mg L^{-1} in March 2015 and highest as 86.2 mg L^{-1} during October 2014. The total alkalinity of the captive condition water ranged from 36.0 mg L^{-1} to 72.0 mg L^{-1} with mean value 54.68 mg L^{-1} (± 14.40). Lowest total alkalinity of breeding tank was recorded as 36.0 mg L^{-1} in January 2015 and highest as 72.0 mg L^{-1} during July 2014.

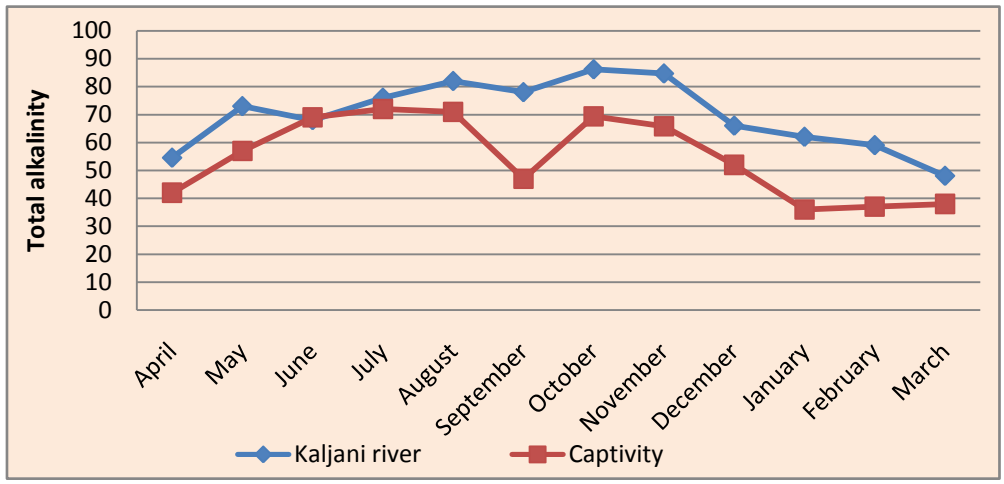


Fig.7f: Monthly variation of Total alkalinity during the study period in Kaljani river and Captivity

4.1.8. Total Hardness

Total Hardness in water is caused by the presence of cations like Ca^{+2} and Mg^{+2}. This property of water helps to precipitate soap by forming a complex with calcium and magnesium present in water. Total hardness of the river water ranged from 18.0 mg L^{-1} to 30.0 mg L^{-1} with mean value 24.0 mg L^{-1}(± 4.03). Lowest total hardness was recorded as 18.0 mg L^{-1} in July 2014 and highest value recorded as 30.0 mg L^{-1} during September 2014. The total hardness of the captive condition of water ranged from 26 mg L^{-1} to 30 mg L^{-1} with mean value 27.29 mg L^{-1}(± 1.29).

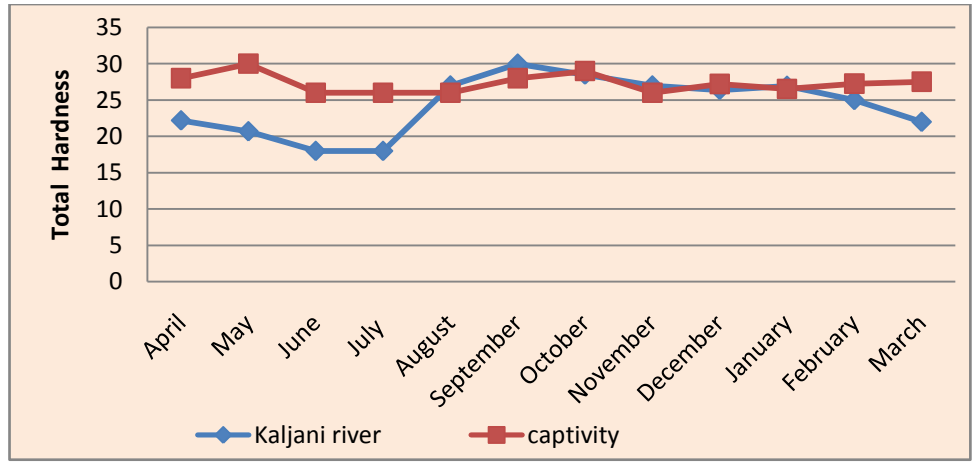


Fig.7g: Monthly variation of Total Hardness during the study period in Kaljani river and Captivity

4.1.9. Ammonium-nitrogen (NH_4-N)

Ammonium-nitrogen concentration of the river water ranged from 0.001 mg L^{-1} in April 2014 to 0.035 mg L^{-1} ; the highest being in June 2014. The average value was 0.0179 mg L^{-1} (± 0.0084). The ammonium-nitrogen concentration in the captive condition ranged from 0.00 mg L^{-1} to 0.007 mg L^{-1} with mean value 0.002 mg L^{-1} (± 0.002). Lowest ammonium-nitrogen of Kaljani river was recorded as 0.001 mg L^{-1} in April 2014, highest of 0.035 mg L^{-1} during June 2014.The low concentrations of ammonium-nitrogen in both the systems revealed no toxic effects.

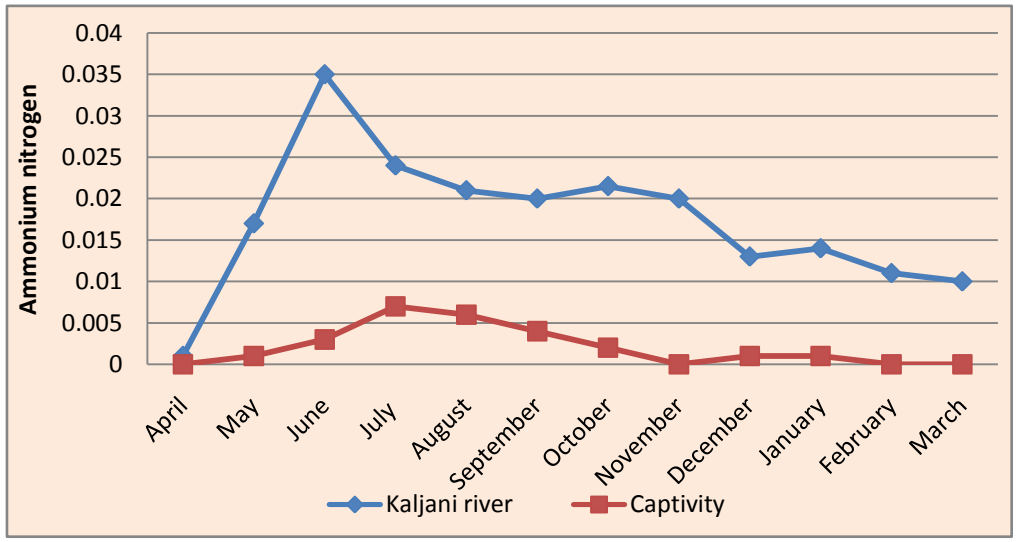


Fig.7h: Monthly variation of Ammonium nitrogen during the study period in Kaljani river and Captivity

4.1.10. Nitrite- nitrogen (NO_2-N)

Nitrite-nitrogen concentration of the river water ranged from 0.001 mg L^{-1} to 0.027 mg L^{-1} with mean value 0.009 mg L^{-1} (± 0.110). Lowest nitrite-nitrogen was recorded as 0.001 mg L^{-1} in April 2014 and highest as 0.027 mg L^{-1} during June 2014. The nitrite -nitrogen concentration of water in the captive condition also revealed a similar low trend 0.001mg L^{-1} to 0.008 mg L^{-1} with mean value 0.003 mg L^{-1} (± 0.003).

Lowest nitrite-nitrogen of breeding tank recorded was 0.001 mg L^{-1} in May 2014 and highest as 0.008 mg L^{-1} during July 2014. Thus toxicity due to Nitrite-nitrogen was negligible.

4.1.11. Nitrate– nitrogen (NO_3-N)

Similar to the low concentration of Ammonium-nitrogen (NH_4-N) and Nitrite-nitrogen(NO_2-N), Nitrate-nitrogen(NO_3-N) concentration of the river water was also low and ranged from 0.149 mg L^{-1} to 0.686 mg L^{-1} with mean value 0.312 mg L^{-1} (± 0.220). Lowest nitrate -nitrogen was recorded as 0.149 mg L^{-1} in June 2014 and highest 0.686 mg L^{-1} during July 2014. The nitrate -nitrogen concentration in the captive condition ranged from 0.085 mg L^{-1} to 0.316 mg L^{-1} with mean value 0.215 mg L^{-1}(± 0.086). Lowest nitrate-nitrogen of breeding tank was recorded as 0.085 mg L^{-1} in September 2014 and highest as 0.316 mg L^{-1} during June 2014.

4.1.12. Phosphate-phosphorous (PO_4-P)

Phosphate-phosphorous (PO_4-P) concentration of the river water ranged from 0.012 mg L^{-1} to 0.197 mg L^{-1} with mean value 0.101 mg L^{-1}(± 0.060). Lowest PO_4-P was recorded as 0.012 mg L^{-1} in August 2014 and highest 0.197 mg L^{-1} during June 2014. The Phosphate-P concentration in the captive condition ranged from 0.110 mg L^{-1} to0.318 mg L^{-1} with mean value 0.172 mg L^{-1} (± 0.078). Lowest Phosphate-P of breeding tank was recorded 0.110 mg L^{-1} in June 2014 and highest 0.318 mg L^{-1} during May 2014.

Tab.5: Summary of water quality parameters in River Kaljani (Natural environment) and Aquaria (Captivity)

Water quality parameters	Kaljani river			Aquaria		
	Min	**Max**	**Mean ± SD**	**Min**	**Max**	**Mean ± SD**
Air Temperature (^{0}C)	23.0	38.0	30.5 ± 5.79	20.0	34.0	28 ± 4.53
Water Temperature (^{0}C)	19.0	36.0	28.45± 5.8	27.0	32.0	29.54±2.42
pH	7.2	8.2	7.82± 0.31	7.36	8.5	7.88± 0.34
Specific Conductivity (µS cm^{-1})	110.0	180.0	151.27± 22.30	240.0	250.0	246.14± 3.18
Dissolved Oxygen (mg L^{-1})	9.46	12.4	10.98±0.914	6.3	7.6	6.93 ± 0.37
Total Dissolved Solids (mg L^{-1})	80.0	165.0	135± 31.96	250.0	260.0	255.5± 3.97
Free Carbon dioxide (mg L^{-1})	2.3	4.6	3.76± 0.80	6.0	8.0	6.67± 0.51
Total Alkalinity (mg L^{-1})	48.0	86.0	69.78± 12.28	36.0	72.0	54.68±14.40
Total Hardness (mg L^{-1})	18 .0	30.0	24 ± 4.03	26	30.0	27.29 ± 1.29
Ammonium-N (mg L^{-1})	0.001	0.035	0.017±0.008	0.00	0.007	0.002±0.002
Nitrite-N (mg L^{-1})	0.001	0.004	0.009±0.110	0	0.008	0.003±0.003
Nitrate–N (mg L^{-1})	0.149	0.686	0.312±0.22	0.085	0.316	0.215±0.086
Phosphate-P (mg L^{-1})	0.012	0.197	0.101±0.06	0.110	0.318	0.172±0.078

4.2. PROTOCOLS OF FISH BIOLOGY OF *BOTIA* SPECIES

To study the fish biology of *Botia* species in captivity, enough broodstock was procured. Broodstock was maintained in aquarium to promote gonad development. Morphological indicator of matured females was the development of enlarged belly which was lacking in males. During maturation, it was observed that males grew smaller in size than females but matured earlier than females. A common secondary sexual character was the brighter body colour of the male than that of the female fish. The dark bands on the skin of male were deep black during the breeding season, while such colour was absent in female.

4. 2.1. FISH GROWTH PARAMETERS

4.2.1.1. Gonado-Somatic Index (GSI)

Gonado-somatic index (GSI) is the ratio of fish gonad weight to body weight , it is particularly helpful in identifying days or seasons of spawning as the ovaries of gravid females rapidly increase in size just prior to spawning. At maturity stage fish had maximum Gonado-somatic Index value and after spawning the value declined. During the breeding season *Botia* showed maximum Gonado-somatic Index value and after spawning it got reduced. Gonado-somatic Index increased from April to August and declined from September to February. The Index was higher in female than male. In *Botia almorhae* for female the index was 21.36 and for male it was 2.62. The average Gonado-somatic Index of *Botia almorhae* was 11.96±10.29. Gonado-somatic Index of *Botia dario* for female was 13.21 and for male it was 3.4. The average Gonado-somatic Index of *Botia dario* was 8.34±5.4. The Gonado-somatic Index of *Botia lohachata* for female was 24.46 and for male 3.2. The average Gonado-somatic Index of *Botia lohachata* was 13.86±11.50.In *Botia rostrata* Gonado-somatic Index for female was 18.75 and for male 1.82.The average Gonado-somatic Index of *Botia rostrata* worked out

to be 10.29±9.01. Among the *Botia* species, *Botia lohachata* had the highest GSI than other species. Details of Gonado-somatic Index (GSI) of *Botia* species are given below in **Tab. 6**.

Tab.6: Details of Gonado-somatic Index of *Botia* species in Captive condition

Species name	GSI of male	GSI of female	Average of GSI	SD value
B. almorhae	2.62	21.36	11.96	10.29
B. dario	3.4	13.21	8.34	5.4
B. lohachata	3.2	24.46	13.86	11.50
B. rostrata	1.82	18.75	10.29	9.01

4.2.1.2. Condition Factor

Condition factor or Ponderal Index of fish expressed by K Factor, is an index used to monitor feeding intensity and growth rate (**Oni *et al.*, 1983**), and is based on the hypothesis that heavier fish for a given length are in better condition (**Bagenal and Tesch, 1978**). According to **Le Cren (1951),** "K" greater than 1.0 indicates a good general condition of fish. Fish with high values of 'K' are heavy for its length, while with low K are lighter **(Bagenal and Tesch, 1978).** However, in the present investigation it was found that the Relative Condition Factor (K) is interestingly similar in all three fishes studied. Condition Factor were *Botia almorhae* (1.390), *Botia dario* (1.788.), *Botia lohachata* (1.538) and *Botia rostrata* (1.399).

4.2.1.3. Length-Weight Relationship

Length- weight relationship has an important role to play in fish biology, physiology, ecology and fisheries resource management. In biological studies, Length-weight relationship helps in the seasonal variation in fish growth to be followed and the

calculation of Condition Indexes. Length- weight relationship gives us the history and morphological comparisons between different fish species or between different fish by the Least-Square Method from logarithmic data, and the association of degree between Weight-Length variables can be calculated by the determination of Coefficient of Correlation (r).

The Coefficient of Correlation (r) from length-weight relationship of *Botia rostrata* was more significant than other species. The Coefficient of Correlation of *Botia almorhae* was 0.811 (**Fig. 8a**); *Botia dario* 0.802 (**Fig. 8b**); *Botia lohachata* 0.753 (**Fig. 8c**) and *Botia rostrata* 0.936 (**Fig. 8d**). The Coefficient of Correlation (r) showed significance at $p \leq 0.01$. Length –weight relationship of *Botia* species are expressed as follows:

Botia almorhae : LogW =24.49 + 4.027 logL

Botia dario : LogW =23.87 + 4.005 logL

Botia lohachata : LogW =16.28 + 3.006 logL and

Botia rostrata : LogW =17.31 + 3.138 logL

The theoretical value of "b" (regression coefficient) in length-weight relationship is reported to be 3 when the body form of fish remains constant at different length, that is, the growth is isometric (**Allen, 1938**). If this value is less than or more than 3 it indicates that growth is allometric (**Bagenal and Tesch, 1978**).

Tab.7: Details of growth parameters of *Botia* species in Captive condition

Species name	Gonado-somatic Index (GSI)	Coefficient of Correlation (r)	Condi-tion factor (K)	Environ-ment condition for growth	Regression correlation (b)	Growth pattern
B. almorhae	11.96	0.811	1.390	Good	3.006	Allometric (+)
B. dario	8.34	0.802	1.788	Good	4.027	Allometric (+)
B.lohachata	13.86	0.753	1.538	Good	4.005	Allometric (+)
B. rostrata	10.29	0.936	1.399	Good	3.138	Isometric/ allometric (+)

From the length-weight relationship of *Botia* species, it was found that value of b was greater than 3 and therefore could be said to have a positive allometric growth. However, b<3 showed a negative allometric growth, or isometric growth when b=3. Further, when the value of 'b' is less than 3 it indicated that the fish became more slender as they increased in length **(Grover and Juliano, 1976).** During the present investigation, the value of 'b' was greater than 3. This Indicated that growth pattern of fish population was allometric but *Botia lohachata* growth pattern was isometric (b=3.006). *Botia lohachata* growth was slightly higher than isometric growth but present value showed positive allometric growth pattern in captivity. *Botia almorhae* (b=4.027), *Botia dario* (b=4.005) and *Botia rostrata* (b=3.138) indicated positive allometric growth because all values

were greater than 3.0. These results suggest, that all species show positive allometric growth and that the fish grows in proportion to the length in captive condition.

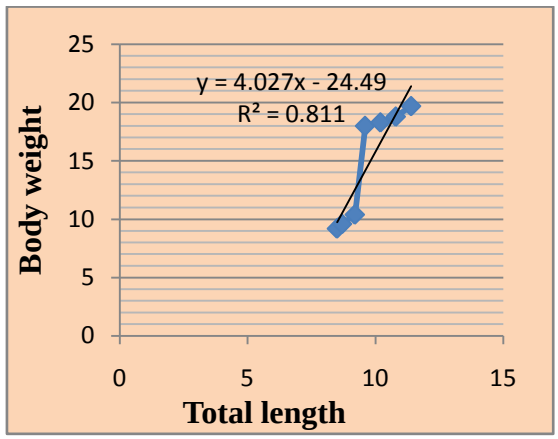


Fig. 8a: Length weight relationship of *Botia almorhae*

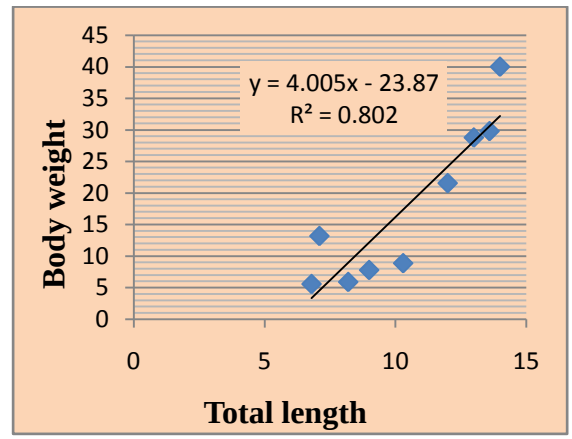


Fig. 8b: Length weight relationship of *Botia dario*

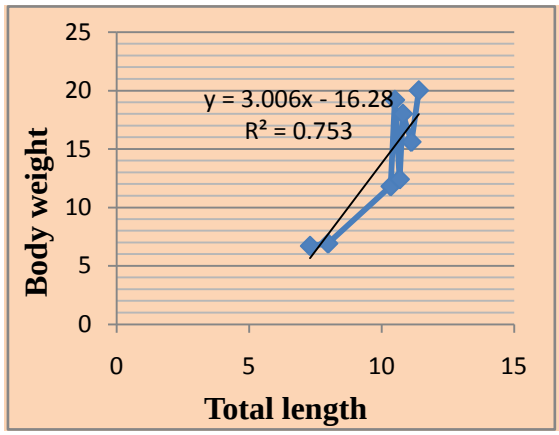


Fig. 8c: Length weight relationship of *Botia lohachata*

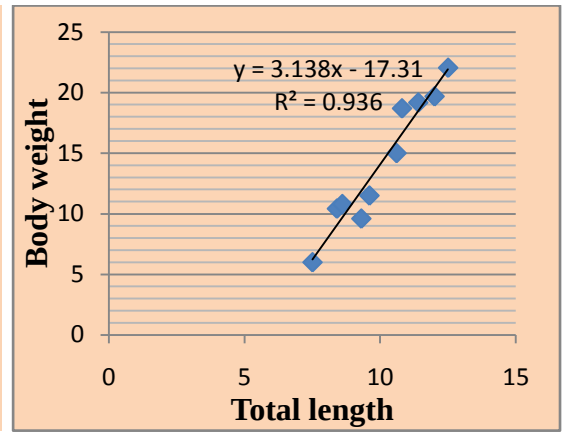


Fig. 8d: Length weight relationship of *Botia rostrata*

4.2.2. Study of fecundity and fertilization rate of the selected fishes

During the breeding period, the ripe male oozed out milt when slight pressure was applied on the vent. Eggs also oozed out with slight pressure on the belly of ripe female. The fecundity and fertilization rate were estimated by random sampling method (**Tab.8**).

4.2.2.1. Fecundity and fertilization rate of *Botia almorhae*

Fecundity of *B. almorhae* showed 12632 to 22456 (18539 ± 3828) numbers. The percentage of fertilization depended on the quality of brood stock. The fertilization rate was found to be 94.44% after one hour of spawning, 91.17% after two hours of spawning; 88.15% after three hours of spawning and 86.36% after four hours of spawning. The average fertilization rate was found to be 90.03%.

4.2.2.2. Fecundity and fertilization rate of *Botia dario*

The fecundity of *Botia dario* ranged from 13880 to 27510 (22573 ± 4949). The fertilization rate was found to be 93.75%, 80.55%, 78.20% and 75.86% after one, two, three and four hours respectively. The average fertilization rate was found to be 82.09 %.

4.2.2.3. Fecundity and fertilization rate of *Botia lohachata*

The fecundity of *Botia lohachata* ranged from 3731 to 23120 (18053 ± 7331). The fertilization rate was found to be 100% after one hour of spawning, 90% after two hours of spawning, 97.26% after three hours of spawning and 96.66% after four hours of spawning. The average fertilization rate was found to be 95.98%.

4.2.2.4. Fecundity and fertilization rate of *Botia rostrata*

The fecundity of *Botia rostrata* ranged from 14103 to 21352 (18698 ± 2772). The fertilization rate was found to be 89.28%, 72.41%, 65.67% and 43.05% after each hour respectively. The average fertilization rate was found to be 67.60%.

Tab.8: Details of Fecundity and fertilization rate of *Botia* species in Captive condition

Sl. No.	Species name	Fecundity			Fertilization rate		
		Lowest	Highest	Average	Lowest	Highest	Average
1	*B. almorhae*	12632	22456	18539	86.36	94.44	90.03
2	*B. dario*	13880	27510	22573	75.86	93.75	82.09
3	*B. lohachata*	3731	23120	18053	96.66	100	95.98
4	*B. rostrata*	14103	21352	18698	43.05	89.28	67.60

4.2.3. Standardization of breeding protocol of *Botia* species

Before hormonal induced breeding, male and female fish were kept in separate glass tanks for at least 2 days. Four different doses of WOVA-FH hormone (0.5 ml/kg as1st dose, 0.25 ml/kg as 2nd dose, 0.025ml/Fish as 3rd dose and 0.0125 ml/fish as 4th dose) were used. The best response to reproduction was obtained from the dosage of WOVA-FH of 0.025 ml/ fish (**Fig.8f**). Higher fertilization, hatching and survival rates were found in fish injected with 0.025 ml/fish in Set-ups: 3, 7, 11 and 15(**Tab.9**). Breeding trails were done twice by applying different doses of hormone in each breeding trial, and the same dose of WOVA-FH hormone was injected to both male and female.

Injected fishes were released in tanks and observed after 4-5 h when they started spawning simultaneously. Spawning was observed in Set-up-3, 7, 11 and 15 but there

was no spawning in others Set-ups. All fishes died in Set-ups-1, 5, 9 and 13 within 2days, whereas, in Set-up-2, 6, 10 and 14 all died within 4 days. In Set-up-4, 8, 12 and 16 all the fishes were most active and fed properly, but spawning was not observed (**Fig.8g**). The present study demonstrated the successful breeding of three species of genus *Botia* in captive condition with small dose of 0.025ml/fish WOVA-FH.

Fig.8e. Cuddling of fishes in a corner after injecting WOVA-FH at 0.5 ml/kg

Fig.8f. Active movement and spawning observed after WOVA-FH (0.025 ml/fish) injection

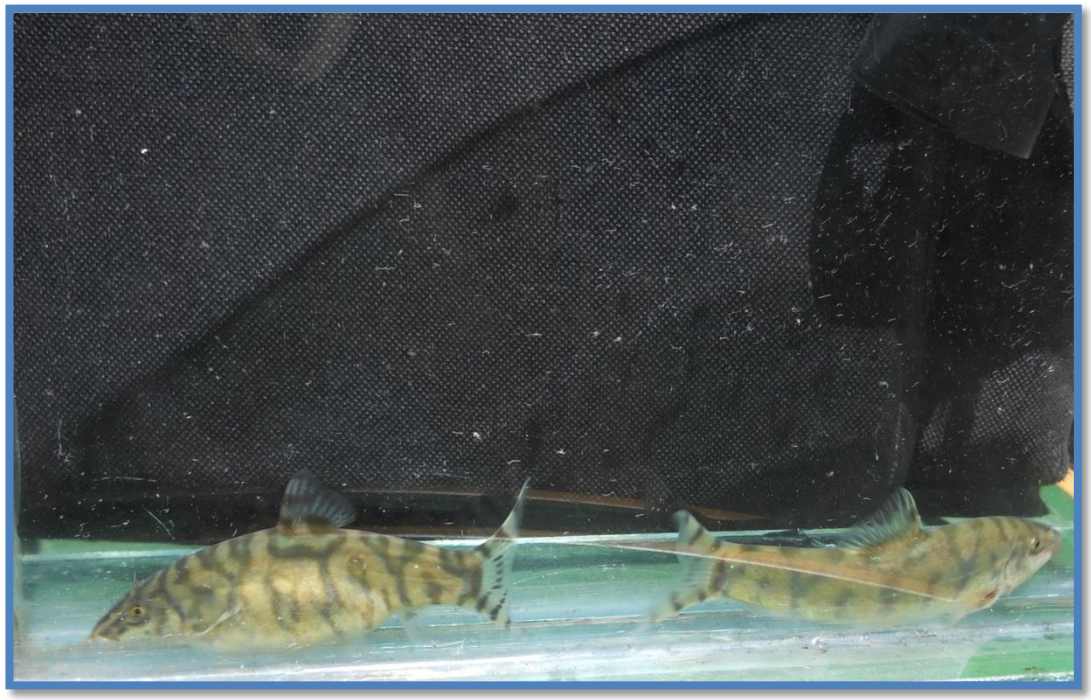


Fig.8g. Fishes most active but spawning not observed after WOVA-FH (0.0125 ml/ fish) injection

The latency period is described as the time interval between injection of hormone on a female fish and the starting of spawning (egg released by the female). The latency period of *Botia almorhae* was between 05.00 to 05.30 hours in fish injected with 0.025ml WOVA-FH per fish; for *Botia dario* it was 5 to 6 hours in fish injected with 0.025ml WOVA-FH per fish; *Botia lohachata* it was 4 to 5 hours in fish injected with 0.025ml WOVA-FH per fish and in *Botia rostrata* it was between 4.30 and 05.00 hours in fish injected with a dosage of 0.025ml WOVA-FH per fish.

Tab. 9: Summary of the different stages of breeding of Genus *Botia* in the different Set-ups.

Time counting after hormone injection	Set-up -1(No 5, 9 and 13) (0.5ml/Kg)	Set-up - 2(No 6,10 and10) (0.25ml /Kg)	Set-up -3(No 7,11 and 15) (0.025ml /fish)	Set-up – 4 (No 8, 12 and 16) (0.0125 ml/fish)
18:00	Transferred to tank	Transferred to tank	Transferred to tank	Transferred to tank
20:00.	Black patch appeared and cuddled in a corner	Some males active	All fishes were active	All fishes were active
21:00	Some males died	Some males active	Fishes were swimming against flow of water	Males were active
22:00	Females swelled up	Females swelled up	Females were being chased by the males at the same time the males were fighting with each other	Females swelled up and males started chasing.
23:00	All fish cuddled in a corner	Some males were chasing the females; no spawning.	Spawning had started; the paired fishes were swimming with the current	Males started chasing the females; no spawning.
24:00	All fish cuddled in a corner	No spawning	Spawning continued	No spawning
03:00	All fish cuddled in a corner	No spawning	Spawning continued	No spawning
04:00	All fish cuddled in a corner	No spawning	Spawning ceased	No spawning
05:00	Not spawning	No spawning	Fishes moved actively	No spawning
After 1 day	Eggs came out on pressing the abdomen	Eggs came out on Pressing the abdomen	Eggs no came out on Pressing the abdomen	Eggs came out on pressing the abdomen
After 2 days	Most of the fish died	Feeding ceased	All fishes took feed and moved actively	All fishes took feed and moved actively
After 4days	All fishes died	Almost all fishes died	All fishes took feed and moved actively	All fishes took feed and moved actively

4.2.4. Behaviour study of *Botia* loaches

Botia species stay together when in shoals or school. They took food in group and swam in a shoal. Naturally, this fish are bottom feeders but in captivity they consume food from the surface of water. They produce a loud cracking sound when feeding. *Botia* species liked to take rest in covered area where sun light did not penetrate. In aquarium or tank they always tried to hide in the sand or beneath the stones. Sometimes, they enjoyed staying under large stones parts and the gap between two stones under the water arranged like a comb. They also preferred to stay in pipe-like structure.

Fig. 9a: *Botia* species stay together in shoals

Fig. 9b: Feed being taken by group of *Botia* sp. from surface water

Fig.9c: Shoal of *Botia* sp. taking shelter in a plastic pipe

Fig. 9d: Group of *Botia* sp. hiding in the gap of two stones arranged like a comb

4.2.4.1. Breeding behaviour

Spawning behaviour was observed during the night or afternoon in absence of light. Male fishes were more actively involved in spawning. At the time of spawning, they made loud cracking sounds repeatedly. Six types of breeding behaviour were observed during the spawning time.

(1) Male hitting the female on the snout region and other body parts.

(2) Male fish was more active than female fish and the male hit the female fish in vent the region more frequently.

(3) Fighting was observed between the males when spawning occurring. Males were attacking each other on the head, tail and fins.

(4) The dominant male chased the female and later the spawning weaker male chased the females.

(5) Most important spawning activity, male and female fish were joined together and swam for about 30 seconds. They were attached together with help of spine that lies below the eye. The spine is a defence organ which causes a painful wound but not venomous. Male fish bent its tail region with female fish and formed "X" shape in tail region. Always male fishes were trying to keep the anal regions close to each other. This activity continued for 3 hours. Male released the sperm and female the egg and fertilization was external.

(6) Cannibalism behaviour was observed after spawning. Some male fishes ate fresh eggs immediately after spawning.

Fig. 10a: Male hitting the female on snout region

Fig. 10b: Male hitting the female on ventral region

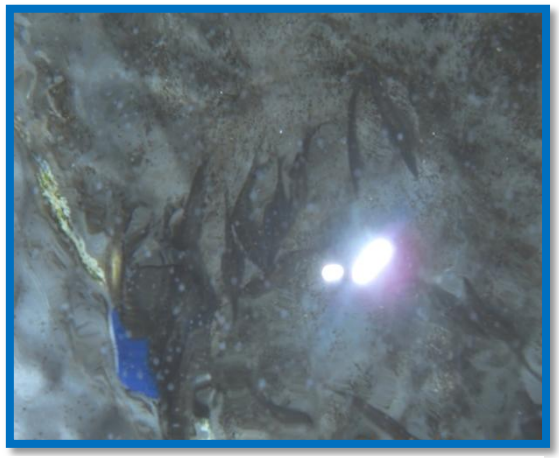


Fig. 10c: Males fighting with each other

Fig. 10d: Dominant male chasing the female and weaker male following them

Fig. 10e: Male and female fish clasping each other and swimming

Fig.10f: Fresh eggs being eaten by *Botia* male

4.2.5. Embryonic development of *Botia* species

The embryonic development of *Botia* species were divided into eight stages- Zygote, Cleavage, Blastula, Gastrula, Segmentation, Pharyngula, Hatching and Early larval period. These observed embryonic development stages are described below.

4.2.5.1. Zygote

The fertilized eggs of *Botia* species were non-adhesive, whitish in colour and optically transparent. Fertilization activated the cytoplasmic movements and the yolkfree cytoplasm began to stretch towards the animal pole gradually segregating the blastodisc from the vegetal cytoplasm. The diameter of the zygote was recorded to be 0.9 to 1mm (**Fig. 11: A1 to A2**).

4.2.5.2. Cleavage

The first cleavage occurred 24 to 28 minutes after fertilization. The two blastomeres rounded in shape just after first cleavage. The blastomeres observed at the animal pole were only half the size of the original cell. After the first cleavage, the blastomeres divided synchronously at an interval of 4 to 10 minutes. Cleavage period was observed to be from 24 min. to 1.15 hour in which the 64 cell stage was completed (**Fig. 11: A3 to A8**).

4.2.5.3. Blastula

The blastula stage with the 128-cell stage ended with the commencement of the gastrula. Blastula stage was observed to be linear from 1.15 to 3.10 hours and it completed 30% of the epiboly stage (**Fig. 11: A9 to A14**).

4.2.5.4. Gastrula

In the gastrula period, extensive cell movement was observed including involution, convergence and extension, producing the three primary germ layers and the embryonic axis. Gastrulation began with cell involution at around 50% epiboly and

completed the bud stage (**Fig. 11: A15-18**). In the bud stage (**Fig. 11: A18**), epiboly ended as the blastoderm completely covered the yolk plug. The tail bud then appeared as a distinct thickening at the caudal end of the embryo near the site of yolk plug closure. Early polster was seen. Gastrula period was observed to range from 3.10 to 6.36 hours. Epiboly continued, in addition the morphogenetic cell movements of involution, convergence and extension occurred producing the primary germ layers and the embryonic axis. As a consequence, within 15 minutes of reaching 90%-epiboly (**Fig. 11: A21**) a thickened marginal region termed the germ ring (**Fig. 11: A22**) appeared, nearly simultaneously all around the blastoderm. Convergence movements then, nearly as rapidly, produced a local accumulation of cells at one position along the germ ring, the so-called embryonic shield (**Fig. 11: A19**). During these events, epiboly temporarily got arrested, but after the shield formation, epiboly continued. The margin of the blastoderm advanced around the yolk cell to cover it completely. The advancement occured at a nearly constant rate, over an additional 15% of the yolk cell each hour, and provided a useful stage index during most of gastrulation (**Fig. 11: A23**). Gastrulation began with cell involution at around 50% epiboly (**Fig. 11: A18**) and completed at the bud stage. In the bud stage (**Fig. 11: A23**) epiboly ended as the blastoderm completely covered the yolk plug. The tail bud then appeared as a distinct thickening at the caudal end of the embryo near the site of yolk plug closure.

4.2.5.5. Segmentation

The segmentation period was characterized by the sequential formation of the somites, and lasted just prior to hatching. During this period, the embryo elongated along the animal pole axis, the tail bud developed longer and rudiments of the primary organs became visible. Somites, formed in bilateral pairs as the developing embryo, extended

posteriorly. Segmentation period was observed to be from 6.46 to 14.30 hours (**Fig. 11:A24** to **A34**).

4.2.5.6. Pharyngula

During this period the embryo were bilaterally organized, with a well-developed notochord and a newly completed set of somites that extended to the end of a long post-anal tail. Body axis straightened from its early curvature. The yolk sac, circulation, pigmentation, and fins development continued. The nervous system was hollow. The head straightened out and lifted to the dorsal side. The brain was prominently sculptured. The blood flow was visible. Pigment formation began in cells of the pigmented retinal epithelium. The embryo continued to exhibit spontaneous side-to-side contractions involving the trunk and tail and the rate of contractions increased in bursts till the embryo hatched out of the chorion (**Fig. 11: A35-42**). The C-shaped embryo elongated and gradually differentiated into a head and tail. The body formed into a C-shape (**Fig. 11: A42**). The yolk was attached between the tail and head. Myotomes development was observed. The embryo started occasional movement. At the twitching stage the tail got completely detached from the yolk. The yolk sac was restricted to the head region. The number of myotomes increased and the embryo became active and exhibited continuous twitching movement.

4.2.5.7. Hatching

Just after hatching from the chorion the larva at 14.30 to 14.45 hours measured 2.5 mm (**Fig. 11: A44**). Head was slightly bent on the yolk, the eyes were large, yolk sac was present on the anterior-ventral side of the body and the heart and optic vesicle were seen. They were responsive to stimulus and settled in the substrate. During this stage (**Fig. 11: A44 to A45**) the embryo continued to grow at about the same rate as earlier.

Morphogenesis of many of the organ rudiments were now rather complete and slowed down considerably, with some notable exceptions including the gut and its associated organs. However, these endodermal structures are difficult to visualize in the living embryo because of their deep position, and thus are not considered here. Pectoral fin development continued to be a useful feature for staging, especially during the early part of the hatching period. At the onset of the period, the paired fin rudiments were like elongated buds, each already containing centrally located mesenchymal condensation that formed the girdle cartilages.

4.2.5.8. Larval development

The mouth appeared to be opened and slit-like. After 22 h of hatching larvae started swimming and feeding. At first larvae, *Paramecium* sp. then *Artemia* were fed after 3days. The larvae consumed small sized zooplanktons (**Fig. 11:A46- A48**). There was complete resorption of the yolk sac and minute pectoral fins were observed. The eyes were well set in the optic sockets.

Tab. 10: Summary of the embryonic development of *Botia* species

Species name	Hormone dose	Latency period	Incubation period
Botia almorhae	0.025ml WOVA-FH/Fish	5 to 5.30 hours	14.40 hours
Botia dario	0.025ml WOVA-FH/Fish	5 to 6 hours	14.40 hours
Botia lohachata	0.025ml WOVA-FH/Fish	4 to 5 hours	14.30 hours
Botia rostrata	0.025ml WOVA-FH/Fish	4.30 to 5 hours	14.45 hours

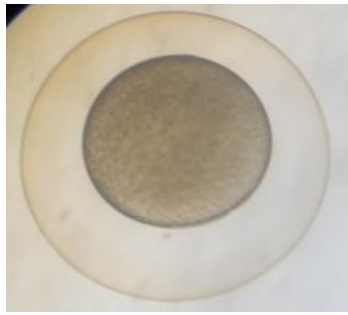
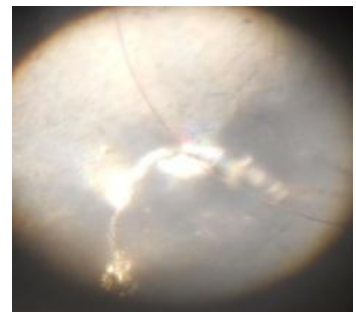
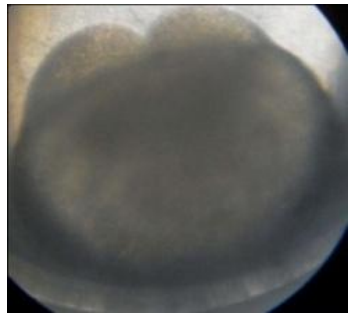
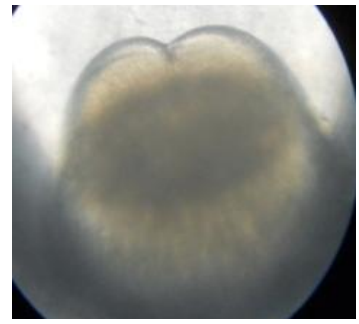
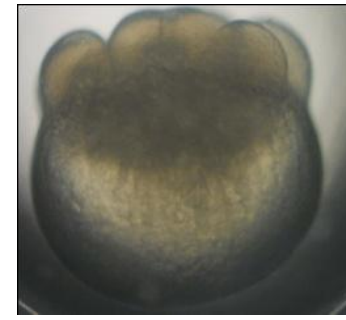
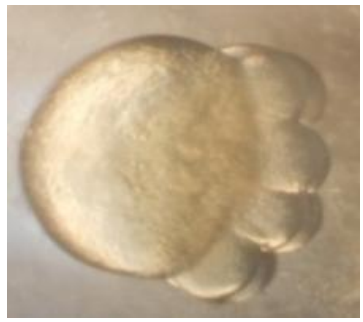
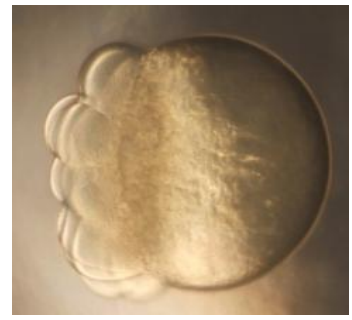



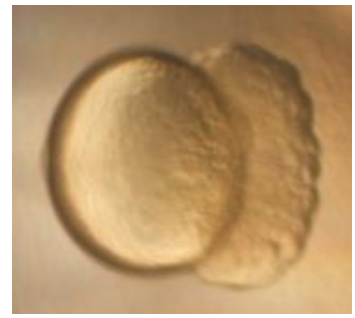
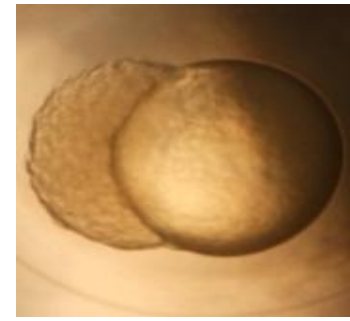

Fig. 11: A1 = Single cell A2 = Fertilization process A3 =Fertilized eggs

A4= 2-cell stage A5=4-cells stage A6= 8-cells stage


A7= 16 -cells stage A8= 32-cells stage A9 =64-cells stage

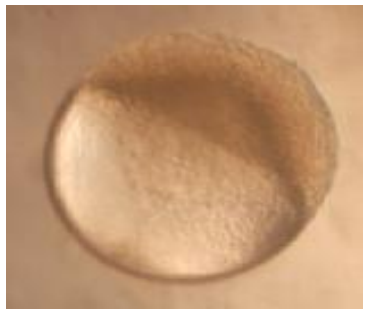




A10=128-cells stage A11= 256-cells stage A12= 512-cells stage

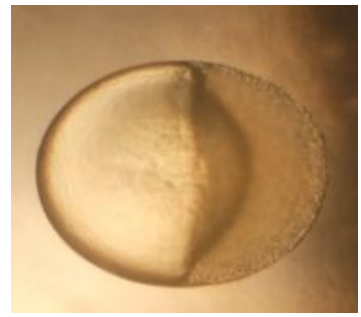
A13= 1,K cells stage

A14= Oblong stage

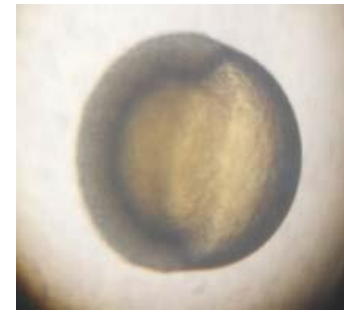
A15= Sphere stage



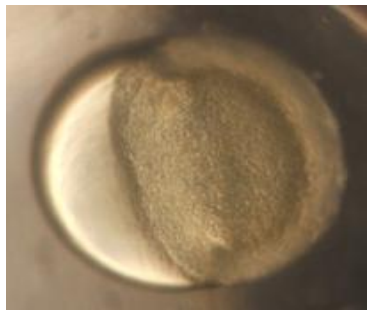
A16 = Dome stage



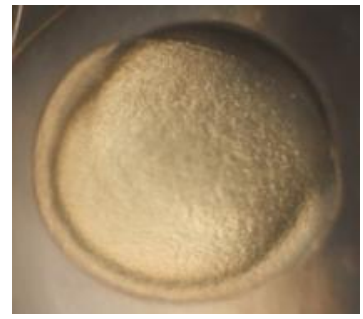
A17 = 30% epiboly



A18 =50% epiboly

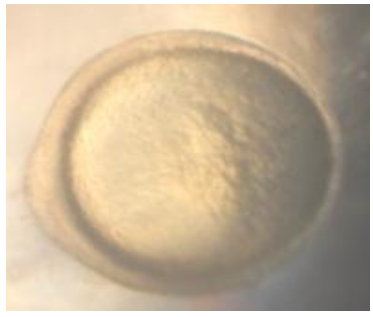


A19= embryonic shield stage



A20=75% epiboly

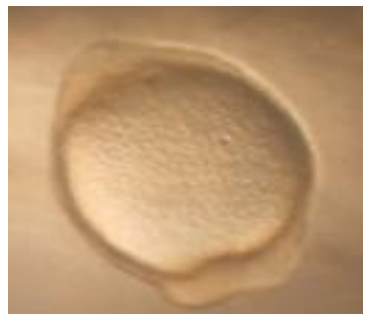
A21= 90% epiboly



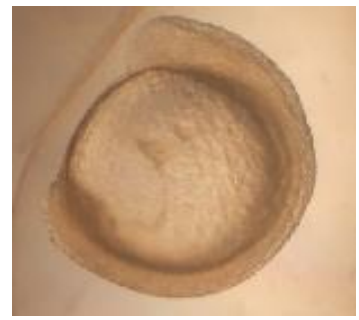
A22= Germ ring stage

A23= Bud stage

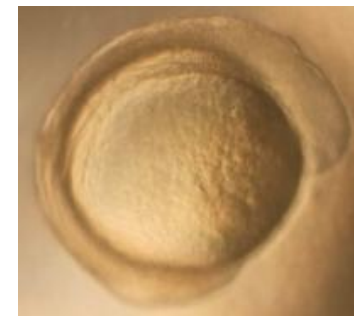
A24 = 1, somite stage



A25=2,somites stage



A26= 3, somites stage



A27= 4, somites stage

A28 = 7, somites stage

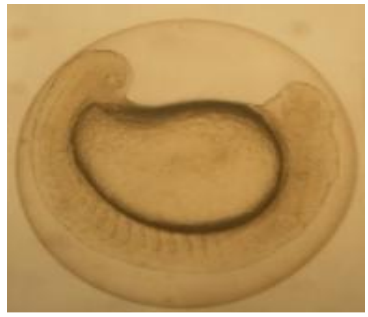
A29 = 8, somites stage

A30=12, somites stage

A31=14, somites stage

A32=16, somites stage

A33=18, somites stage

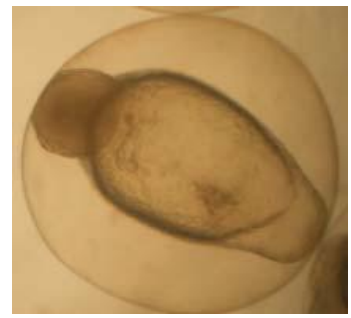


A34=20, somites stage

A35= Pharyngula stage

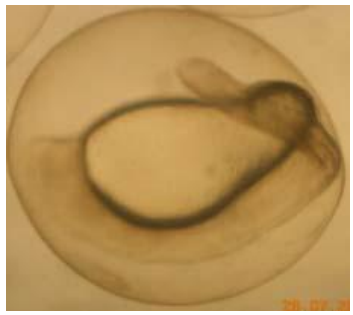
A36 = Pharyngula stage

A37= Pharyngula stage

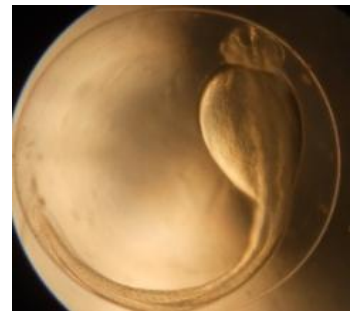


A38= Pharyngula stage

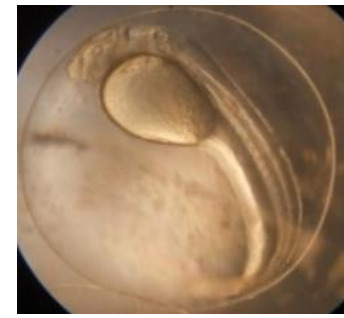
A39= late pharyngula stage



A40= Twitching stage



A41=C-shaped embryo

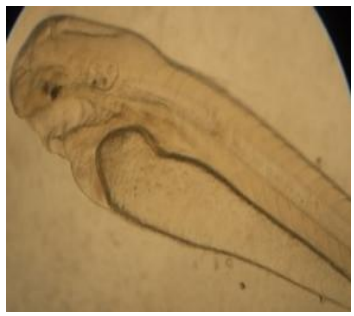


A42=C-shaped embryo

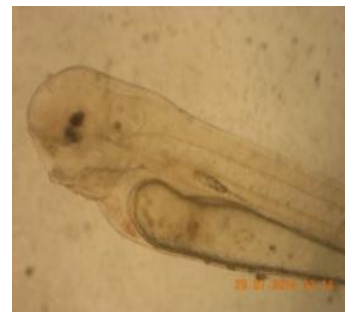
A43=Before hatching

A44= Hatching stage

A45=Newly hatched larva

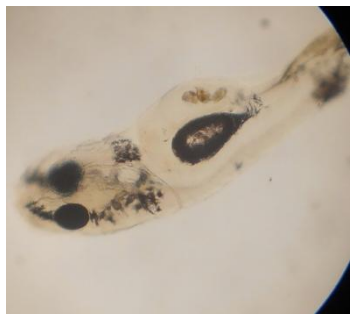


A46=larva take Paramecium

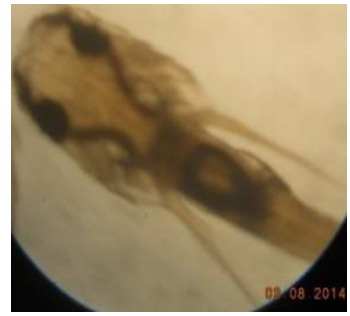


A47= larva take Artemia

A48= larva take zooplankton



A49= *Daphnia* inside the stomach of larva



A50= 6 day old larva

A51=7,day old larva

4.2.6. Supplementary feed for larval rearing of *Botia* species

Four glass aquariums containing 50 litres of water were used for feeding in experimental trials. Juveniles from the same parents were stocked in different experimental tanks with same stocking density (20 fishes per tank). Absolute Growth Rate in Tank-A was 0.037, Tank-B: 0.081, Tank-C: 0.086 and Tank-D: 0.117. In the present study, good growth was observed in Tank-D where only minced snail or bivalve flesh fed. The other experimental tanks, namely Tank-A, was fed only commercial fish feed, Tank-B: live zooplanktons and Tank-C: boiled minced meat. The growth rates were similar in Tank B and Tank C. Lowest growth rate was observed in Tank-A.

4.3. Histological study of the gonads of *Botia* species

4.3.1 Histology of the gonad of *Botia* species

Gonad maturation was a complicated process in *Botia* species and took one year time. Ovary developed slowly during its primary growth phase and then rapidly with the incorporation of yolk protein during its secondary growth phase, and finally attained maturation with the germinal vesicle breakdown. On the basis of size, morphology and cytoplasmic inclusion the growing oocytes were categorised into three stages and testes

were divided into two stages. Gonad development of *Botia almorhae, Botia dario* and *Botia lohachata* were similar. In *Botia rostrata,* gonad maturation took more than one year in captive culture. The size of gonad was too small, because *Botia rostrata* size was small than other *Botia* species. The distinctive features of each stages are given below.

4.3.1.1 Pre-spawning stage

Immature ovary of *Botia* species were small, elongated, thread like and translucent without visible oocytes. Immature stage of ovary of *Botia* species was found from February to March **(Fig.12a)**. The distinctive features of immature stage were presence of Chromatin nuclear oocytes (CNO) and Perinuclear oocytes (PNO). Chromatin nuclear oocytes are characterized by a nucleus containing a single nucleolus, surrounded by chromatin threads. In Perinucleolar oocytes, nucleolus was found in the periphery of nuclear membrane. Ovary occupied only a small portion of the abdominal cavity.

The pre-spawning phase or developing phase of ovary was found to be during March to May. Matured ovary was larger than immature ovary, pale yellow in colour and oocytes were visible to naked eye. Characteristics of early maturity stages of ovary were presence of Perinucleolar oocytes (PNO) and Cortical alveoli (CA) or Yolk vesicle Oocytes **(Fig.12b and 12c)**. Perinucleolar oocytes were round in shape and large in size than Chromatin and contained more than 15 peripheral nucleoli. Cortical alveoli possessed a large round nucleus which contained dispersed chromatin and numerous small nucleoli. Distinct zona granulosa (ZG) and zona radiata (ZR) layers appeared around the oocytes of CA. Yolk deposition stage was found to be slightly large in size and cytoplasm of oocytes was basophilic.

The testes were white in colour, thin, slender, thread-like, translucent and showed gradual increase in their volume and weight. They extended to about two third the length of abdominal cavity. No spermiation occurred after applying slight pressure on the abdomen. Developing stage of testis of *Botia* species were found from April to May. Histological features of developing testis were the presence of spermatids (ST), spermatocyts (SC) and spermatogonia (SG) (**Fig.12d**).

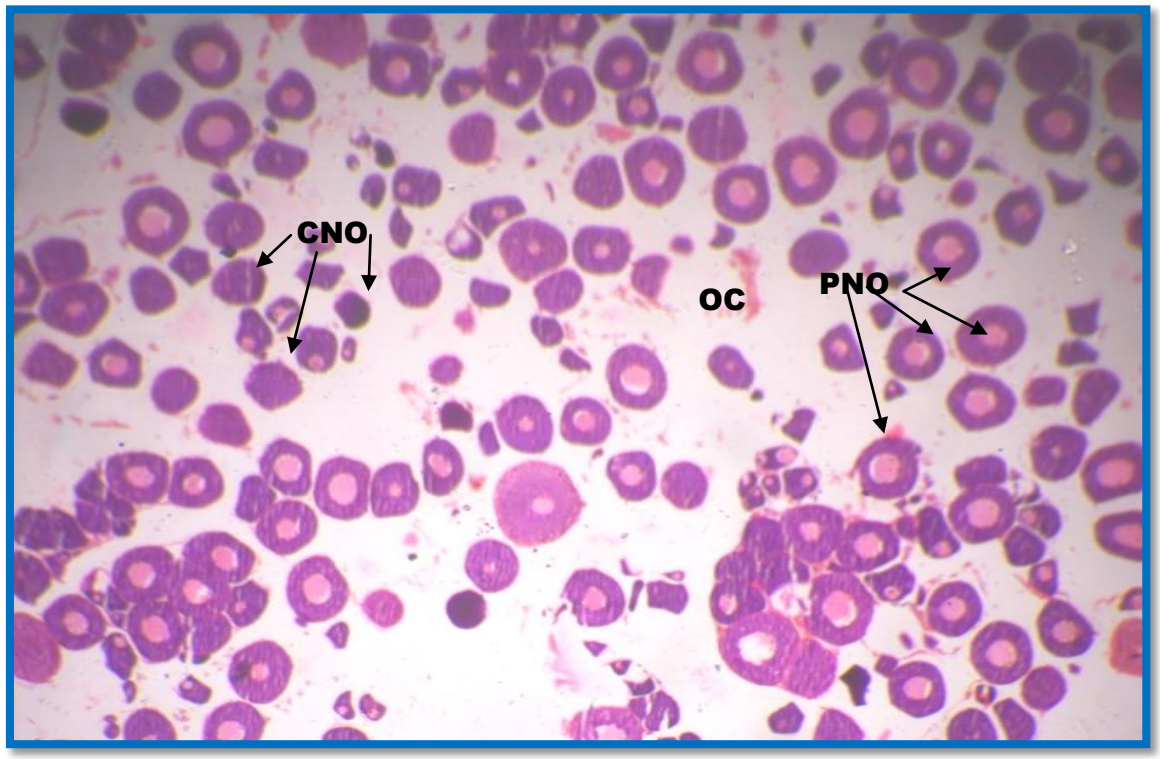


Fig.12a: Transverse section of primary growth phase of ovary of *Botia* species showing Chromatin nuclear oocytes (CNO) and Perinuclear oocytes (PNO)

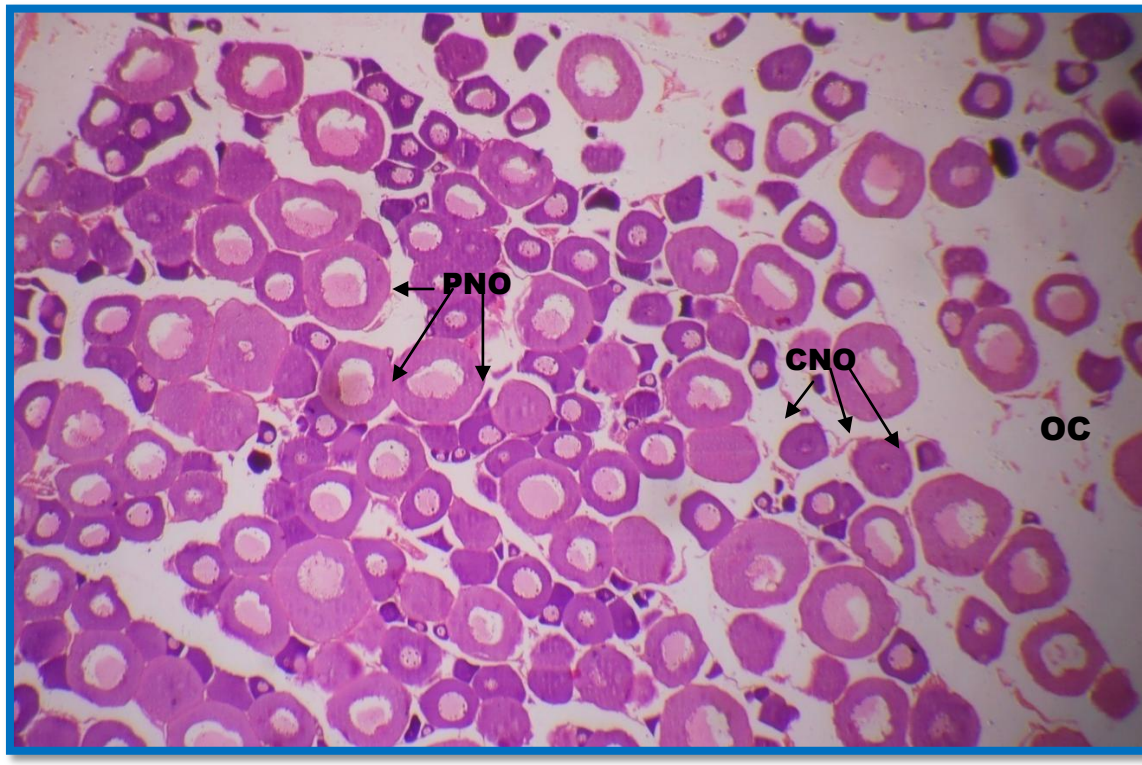


Fig.12b: Transverse section of primary growth phase of ovary of *Botia* species showing Chromatin nuclear oocytes (CNO), Perinuclear oocytes (PNO) and peripheral nucleus (PN)

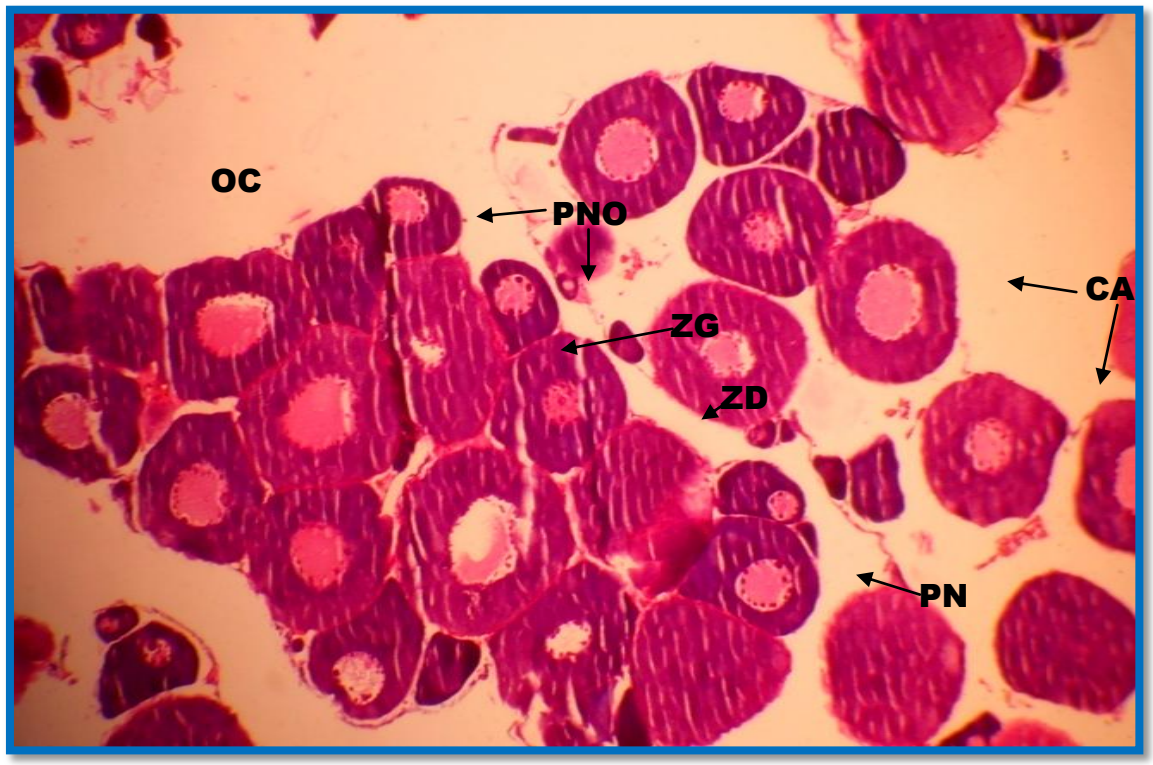


Fig.12c: Transverse section of pre-spawning phase of ovary of *Botia* species showing Perinuclear oocytes (PNO), peripheral nucleus (PN) and Cortical alveoli with zona granulosa (ZG) and zona radiate (ZR)

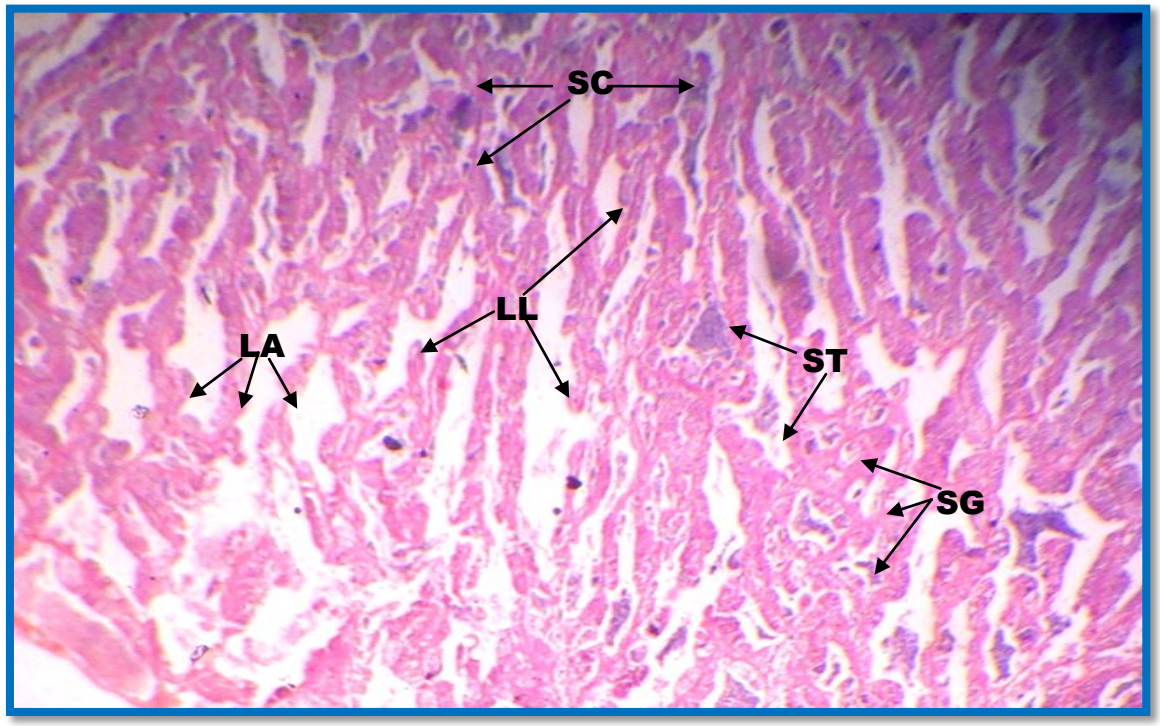


Fig.12d: Transverse section of developing stage of testis of *Botia* species showing Spermatogonia(SG), Spermatids (ST), Spermatocyts (SC), Lobule lumen (LL) and Lobule anastomose (LA)

4.3.1.2 Spawning stage

Spawning phase of ovary of *Botia almorhae, Botia lohachata* and *Botia rostrata* were found to be during June to August and *Botia dario* was found to be during May to July. Matured ovary was enlarged in size and yellow in colour. The oocytes were visible to the naked eye. Spawning phase of ovary was characterized by the occurrence of Cortical alveoli (CA) and vitellogenic oocytes (YGO) but post-ovulatory follicles were not present (**Fig.12e and 12f)**. The yolk globules increased in number, lipid droplets enlarged and was scattered between the yolk granules. At the running stage of ovary, germinal vesicle collapsed and migration of germinal vesicle occurred in Yolk globule oocytes and migration nucleus (MN) started to move to the animal pole.

The testes were milky white, long and flat, narrower behind, ribbon-like and increased in size. Spawning phase of testis of *Botia* species was found during May to September. Spawning stage was characterized by the onset of spermiation on applying

slight pressure to the abdomen. Testes extended to the entire length of the visceral cavity. Histological features of matured testis were the presence of spermatozoa (SP), spermatids (ST), spermatocyts (SC) and spermatogonia (SG) (**Fig.12g**).

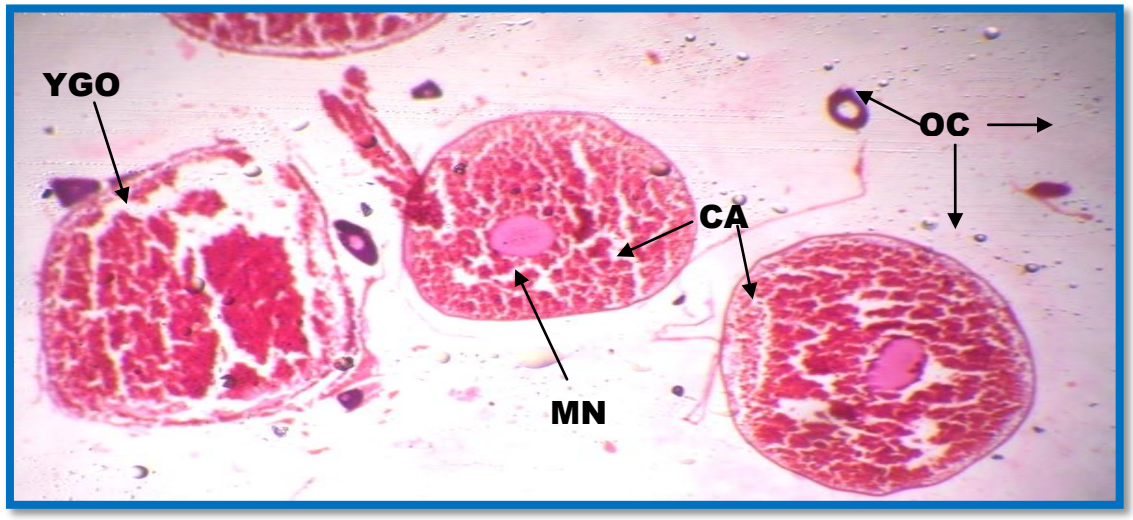


Fig.12e: Transverse section of spawning phase of ovary of *Botia* species showing Cortical alveoli (CA), Yolk globule oocyte (YGO) and Migratory nucleus (MN)

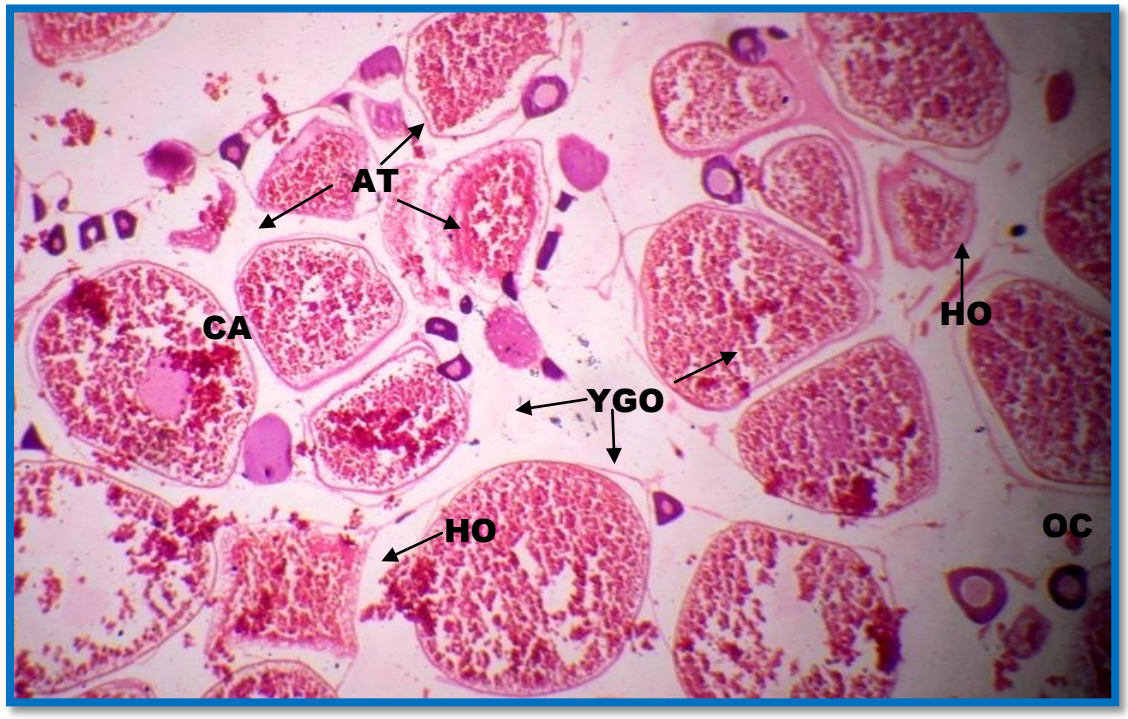


Fig.12f: Transverse section of running phase of ovary of *Botia* species showing Cortical alveoli (CA), Yolk globule oocyte (YGO), Hyaline oocytes (HO)and Atresia (AT)

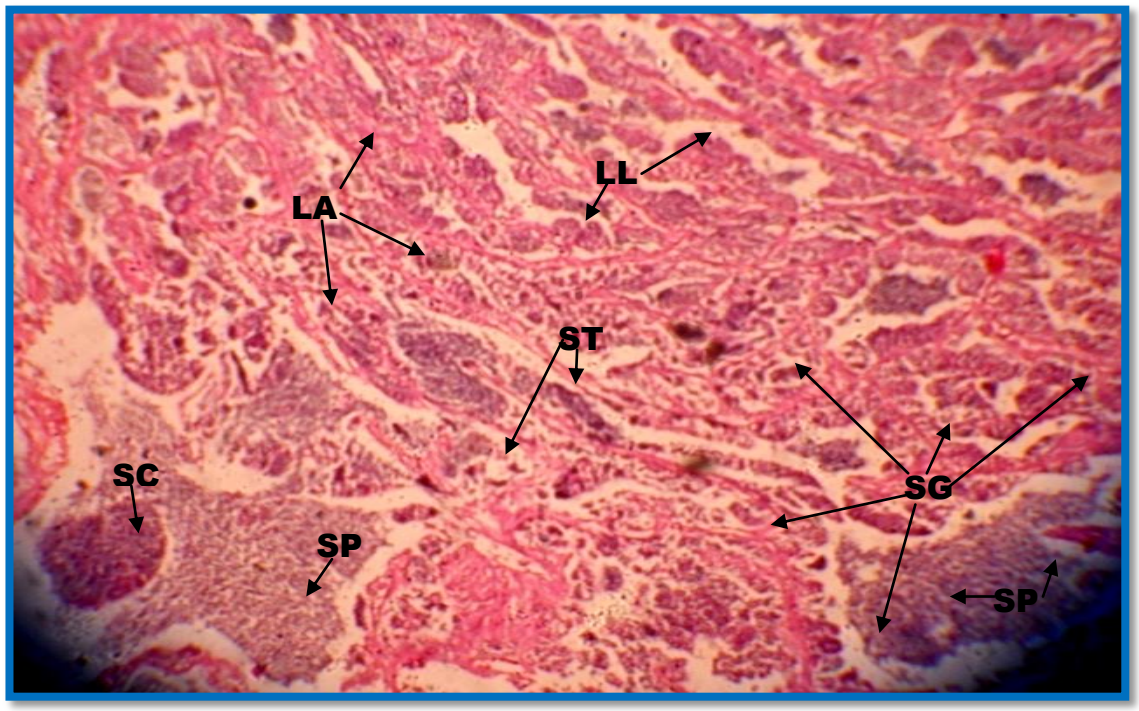


Fig.12g: Transverse section of spawning phase of testis of *Botia* species showing Spermatozoa (SP), Spermatogonia(SG), Spermatids (ST), Spermatocyts (SC), Lobule lumen (LL) and Lobule anastomose (LA)

4.3.1.3 Post-spawning stage

Post ovulated ovary of *Botia* species were elongated, thread like and transparent. Post-spawning phase of ovary of *Botia almorhae*, *Botia lohachata* and *Botia rostrata* were found to be during September to October. Post ovulated ovary of *Botia dario* was found to be during August to October. The ovaries were flat whitish with wrinkled membranes and residual eggs were occasionally visible through the ovarian walls. The weight of the ovary also decreased. Follicular cells of the atretic follicles were hypertrophid and the average oocyte diameter sharply declined. Characteristic features of post ovulated ovary were convoluted ovigerous folds containing large number of ruptured atretic follicles (AT), perinucleolar oocytes (PNO), post ovulated follicle (POF) and hyaline oocytes (HO) (**Fig.12h**). The unovulated mature oocytes (UOMO) became highly vacuolated, collapsed and shrunken inward, during the post-spawning period.

The testes were white in colour, thin, slender, and gradually decreased in their volume and weight. No spermiation occurred after applying slight pressure on the abdomen. Post spawning stage of testis of *Botia* species were found from October to February **(Fig.12i)**. Histological features of post spawning stage of testis were the presence of Spermatogonia (SG) and Residual spermatozoa (RSP).

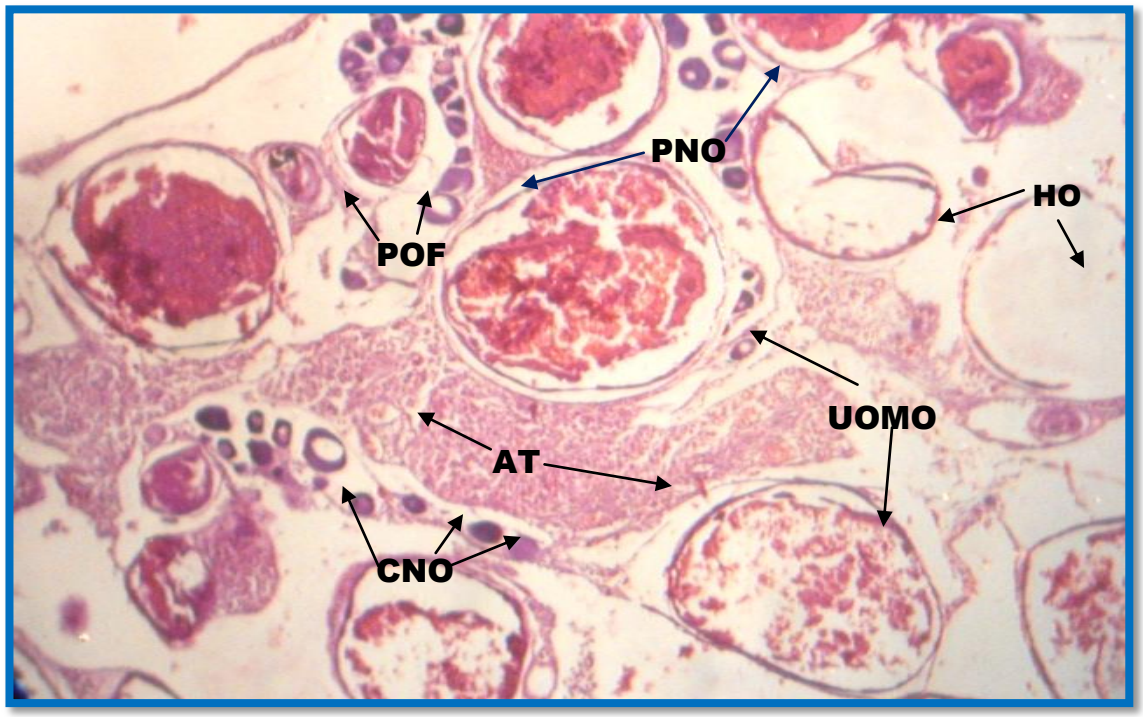


Fig.12h: Transverse section of Post-ovulated ovary (Spent stage) of *Botia* species showing Unovulated matured oocyte (UOMO), Hyaline oocytes (HO), Atresia (AT), Chromatin nuclear oocytes (CNO), Perinuclear oocytes (PNO) and Post ovulatory follicle (POF)

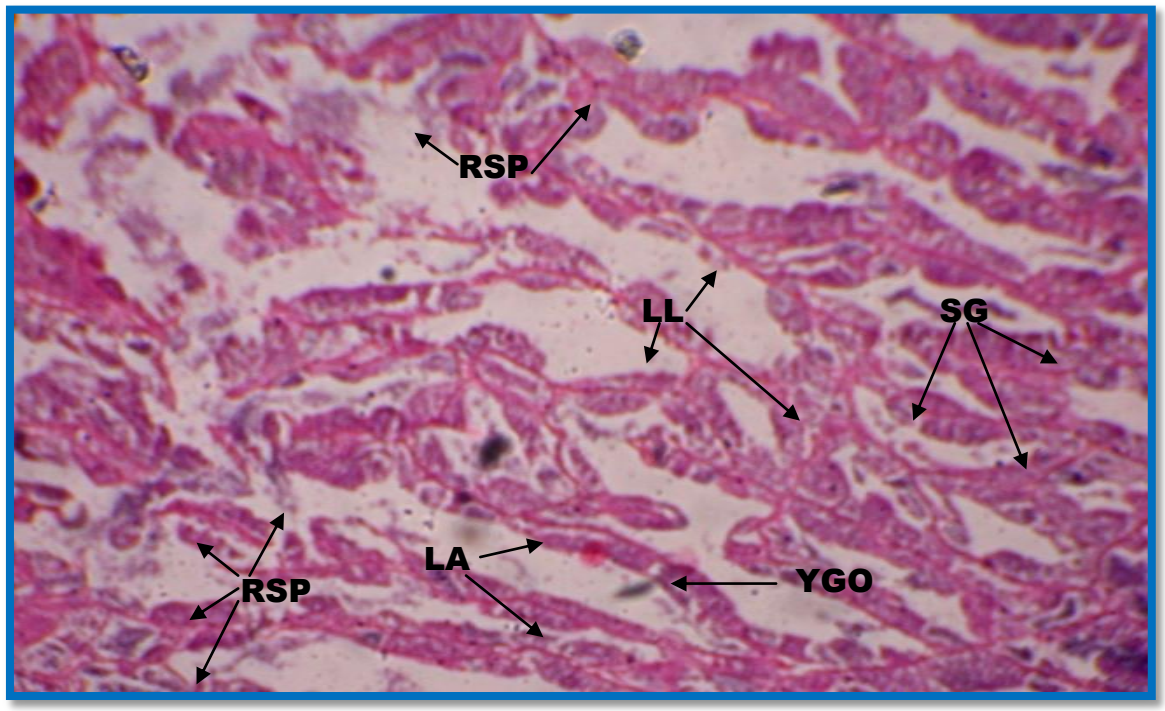


Fig.12i: Transverse section of post-spawning phase of testis of *Botia* species showing Spermatogonia(SG), Residual spermatozoa(RSP), Lobule lumen (LL) and Lobule anastomose (LA)

4.4. Molecular characterization of DNA barcoding and evolutionary relationship among *Botia* species

4.4.1. DNA sequence (FASTA sequence)

FASTA provides sequence similarity searching of query sequence against nucleotide and protein databases using FASTA programmes. The sequences in FASTA formatted files are preceded by a line starting with a ">" symbol. FASTA files containing multiple sequences are just same, with one sequence listed right after another. This format is accepted for many multiple sequence alignment programs. Ten FASTA sequences of COI of *Botia* species were obtained from the present study. FASTA sequences of Botia species are given below.

>Botia rostrata BR1

CCTATATCTCGTATGTGGTGCCTGAGCCGGAATAGTGGGCACGGCCATCAGC
CTTTTAATTCGGGCTGAACTCAGCCAACCTGGGTCCCTTCTAGGTGATGATCA
AATTTATAATGTTATCGTCACTGCACATGCCTTTGTTATGATTTTCTTTATAGT
AATACCAATCCTTATTGGGGGATTCGGGAACTGGCTTCTTCCACTTATGATTG
GAGCCCCTGATATAGCATTCCCTCGAATAAATAATATAAGCTTTTGACTTCTA
CCCCCATCTTTTCTTCTTCTCCTAGCATCCTCTGGAGTTGAAGCCGGAGCCGG
AACTGGGTGAACAGTATATCCTCCACTTGCTGGCAACTTAGCCCACGCAGGA
GCATCCGTAGACTTAACTATTTTCTCATTACATTTAGCAGGAGTTTCATCCAT
TTTAGGAGCAATTAATTTTATTACCACATCCATTAATATGAAACCCCCAGCAA
TTTCTCAATACCAAACACCATTATTTGTATGAGCCGTACTTGTAACGGCAGTT
CTACTGCTTTTATCCCTACCCGTACTGGCCGCCGGAATTACAATGCTGTTAAC
AGACCGTAATTTAAACACAACATTCTTCGACCCCGCTGGAGGAGGAGACCCA
ATCCTTTATCAACATTTATTC

>Botia rostrata BR2

CCTTTATCTCGTATGTGGTGCCTGAGCCGGAATAGTTGGCACGGCCCTCAGCC
TTTTAATTCGGGCTGAACTCAGCCAACCTGGGTCCCTTCTAGGTGATGATCAA
ATTTATAATGTTATCGTCACTGCACATGCCTTTGTTATGATTTTCTTTATAGTA
ATACCAATCCTTATTGGGGGATTCGGGAACTGGCTTCTTCCACTTATGATTGG
AGCCCCTGATATAGCATTCCCTCGAATAAATAATATAAGCTTTTGACTTCTAC
CCCCATCTTTTCTTCTTCTCCTAGCATCCTCTGGAGTTGAAGCCGGAGCCGGA
ACTGGGTGAACAGTATATCCTCCACTTGCTGGCAACTTAGCCCACGCAGGAG
CATCCGTAGACTTAACTATTTTCTCATTACATTTAGCAGGAGTTTCATCCATTT
TAGGAGCAATTAATTTTATTACCACATCCATTAATATGAAACCCCCAGCAATT
TCTCAATACCAAACACCATTATTTGTATGAGCCGTACTTGTAACGGCAGTTCT
ACTGCTTTTATCCCTACCCGTACTGGCCGCCGGAATTACAATGCTGTTAACAG
ACCGTAATTTAAACACAACATTCTTCGACCCCGCTGGAGGAGGAGACCCAAT
CCTTTATCAACATTTATTC

>Botia rostrata BR3

CCTATATCTCGTATGTGGTGCCTGAGCCGGAATAGTTGGCACGGCCCTCAGC
CTTTTAATTCGGGCTGAACTCAGCCAACCTGGGTCCCTTCTAGGTGATGATCA
AATTTATAATGTTATCGTCACTGCACATGCCTTTGTTATGATTTTCTTTATAGT
AATACCAATCCTTATTGGGGGATTCGGGAACTGGCTTCTTCCACTTATGATTG
GAGCCCCTGATATAGCATTCCCTCGAATAAATAATATAAGCTTTTGACTTCTA
CCCCCATCTTTTCTTCTTCTCCTAGCATCCTCTGGAGTTGAAGCCGGAGCCGG
AACTGGGTGAACAGTATATCCTCCACTTGCTGGCAACTTAGCCCACGCAGGA
GCATCCGTAGACTTAACTATTTTCTCATTACATTTAGCAGGAGTTTCATCCAT
TTTAGGAGCAATTAATTTTATTACCACATCCATTAATATGAAACCCCCAGCAA
TTTCTCAATACCAAACACCATTATTTGTATGAGCCGTACTTGTAACGGCAGTT
CTACTGCTTTTATCCCTACCCGTACTGGCCGCCGGAATTACAATGCTGTTAAC
AGACCGTAATTTAAACACAACATTCTTCGACCCCGCTGGAGGAGGAGACCCA
ATCCTTTATCAAC

>Botia rostrata BR4

CCTATATCTCGTATTTGGTGCCTGAGCCGGAATAGTTGGCACGGCCCTCAGCC
TTTTAATTCGGGCTGAACTCAGCCAACCTGGGTCCCTTCTAGGTGATGATCAA
ATTTATAATGTTATCGTCACTGCACATGCCTTTGTTATGATTTTCTTTATAGTA
ATACCAATCCTTATTGGGGGATTCGGGAACTGGCTTCTTCCACTTATGATTGG
AGCCCCTGATATAGCATTCCCTCGAATAAATAATATAAGCTTTTGACTTCTAC
CCCCATCTTTTCTTCTTCTCCTAGCATCCTCTGGAGTTCAAGCCGGAGCCGGA
ACTGGGTGAACAGTATATCCTCCACTTGCTGGCAACTTAGCCCACGCAGGAG
CATCCGTAGACTTAACTATTTTCTCATTACATTTAGCAGGAGTTTCATCCATTT
TAGGAGCAATTAATTTTATTACCACATCCATTAATATGAAACCCCCAGCAATT
TCTCAATACCAAACACCATTATTTGTATGAGCCGTACTTGTAACGGCAGTTCT
ACTGCTTTTATCCCTACCCGTACTGGCCGCCGGAATTACAATGCTGTTAACAG
ACCGTAATTTAAACACAACATTCTTCGACCCCGCTGGAGGAGGAGACCCAAT
CCTTTATCAACATTTATTC

>Botia lohachata BL1

CCTTTATCTAGTATGTGGTGCCTGAGCCGGAATAGGTGGCACGGCCCTCAGC
CTTTTAATTCGGGCTGAACTTAGCCAACCTGGGTCCCTTCTAGGTGACGATCA
AATTTACAATGTTATCGTCACTGCACATGCCTTTGTTATGATTTTCTTTATAGT
AATACCAATCCTTATTGGAGGATTCGGAAACTGGCTTCTTCCACTTATGATTG
GAGCCCCTGACATAGCATTCCCCCGAATGAATAATATAAGTTTTTGACTTCTG
CCCCCATCTTTTCTTCTTCTCTTAGCATCCTCTGGAGTTGAAGCCGGAGCCGG
AACTGGTTGAACAGTTTATCCCCCACTTGCTGGTAATTTGGCCCACGCAGGA
GCATCCGTAGACTTAACTATTTTCTCACTACATTTAGCAGGAGTCTCATCCAT
TTTAGGGGCAATTAATTTTATTACCACATCCATTAATATGAAACCTCCAGCAA
TTTCTCAATACCAAACACCATTATTTGTATGAGCCGTACTTGTAACGGCAGTT
CTACTGCTTTTATCCTTACCAGTACTAGCCGCCGGAATTACAATGCTGTTAAC
AGATCGTAATTTAAACACAACATTCTTCGACCCCGCTGGAGGAGGAGACCCA
ATCCTTTATCAACATTTATTC

>Botia almorhae BL3

CCTATATCTAGTATGTGGTGCGTGAGCCGGAATAGTTGGGACGGCCCTCAGC
CTTTTAATTCGGGCTGAACTTAGCCAACCTGGGTCCCTTCTAGGTGACGATCA
AATTTACAATGTTATCGTCACTGCACATGCCTTTGTTATGATTTTCTTTATAGT
AATACCAATCCTTATTGGGGGATTCGGAAACTGGCTTCTTCCACTTATGATTG
GAGCCCCTGACATAGCATTCCCCCGAATGAATAATATAAGTTTTTGACTTCTG
CCCCCATCTTTTCTTCTTCTCTTAGCATCCTCTGGAGTTGAAGCCGGAGCCGG
AACTGGTTGAACAGTTTATCCCCCACTTGCTGGTAATTTGGCCCACGCAGGA
GCATCCGTAGACTTAACTATTTTCTCACTACATTTAGCAGGAGTCTCATCCAT
TTTAGGGGCAATTAATTTTATTACCACATCCATTAATATGAAACCTCCAGCAA
TTTCTCAATACCAAACACCATTATTTGTATGAGCCGTACTTGTAACGGCAGTT
CTACTGCTTTTATCCCTACCAGTACTAGCCGCCGGAATTACAATGCTGTTAAC
AGATCGTAATTTAAACACAACATTCTTCGACCCCGCTGGAGGAGGAGACCCA
ATCCTTTATCAACATTTATTC

>Botia modesta BM1

CCTATATCTAGTATGAATTGGGTGAGCCGGAATAGTTGGGACTGCCCTCAGC
CTTTTAATTCGAGCTGAACTAAGCCAACCCGGATCACTTCTGGGCGATGATC
AAATCTACAATGTTATCGTTACTGCACATGCTTTCGTAATAATTTTCTTTATA
GTAATACCAGTCCTTATTGGAGGGTTTGGAAATTGACTCCTCCCGCTAATAAT
TGGAGCCCCAGACATAGCATTTCCGCGAATAAATAATATGAGTTTTTGACTC
CTACCCCCCTCTTTCCTACTACTCCTGGCCTCTTCCGGAGTTGAAGCAGGCGT
CGGAACAGGGTGAACAGTGTACCCGCCACTTGCGGGAAACTTAGCCCATGCA
GGCGCATCCGTAGACTTAGCTATCTTTTCTCTACACTTAGCAGGTGTATCCTC
CATCCTAGGCGCTATTAATTTTATCACCACCTCTATTAATATAAAACCCCCAG
CCATCACCCAATATCAAACTCCCCTATTTGTATGAGCTGTACTTGTAACAGCC
GTTCTTTTACTGCTATCCCTACCTGTTTTAGCCGCTGGAATTACAATGCTGTTA
ACAGACCGTAACTTAAATACAACATTCTTTGACCCCGCAGGAGGAGGAGACC
CAATTCTTTACCAACACCTATGC

>Botia macracanthus BMC1

CCTATATCTAGTATTTGGTGCCTGAGCCGGAATAGTTGGCACTGCCCTCAGTC
TCTTAATTCGAGCTGAACTTAGCCAACCTGGTTCACTTCTAGGTGACGATCAA
ATCTATAACGTTATCGTAACTGCACATGCCTTTGTTATAATTTTCTTTATAGTA
ATACCAATCCTCATCGGAGGATTCGGAAATTGACTTCTTCCATTAATAATTGG
AGCCCCCGATATAGCATTCCCCCGAATAAACAATATAAGCTTCTGACTCCTG
CCCCCATCATTCCTTCTACTTTTAGCCTCCTCTGGAGTTGAAGCAGGAGCTGG
AACGGGATGAACTGTTTATCCGCCACTCGCGGGTAACTTAGCCCACGCAGGG
CCATCCGTAGACCTAACTATCTTCTCACTACATTTGGCGGGTGTTTCATCAAT
TTTAGGGGCAATTAATTTTATCACCACCTGCATTAATATGAAACCTCCAGCCA
TTTCTCAATATCAAACACCTTTATTTGTATGAGCCGTCCTTGTAACGGCTGTC
CTGTTATTATTATCTTTACCAGTATTAGCTGCTGGAATTACAATACTTTTAAC
AGACCGTAATCTTAACACAACATTCTTTGACCCCGCAGGAGGAGGTGACCCA
ATCCTTTATCAACATTTATTT

>Botia dario BD1

ACTAGATTTAGTATATGATGCCTGAGCCGGCATCGGTGGGACAGCCCACAGC
CTTATATTACGAGCTGTACTCAGCCAACCTGGGTCCCTCCTAGGTGATGATCA
AATTTACAACGTTATCGTCACTGCCCATGCTTTCGTTATAATTTTCTTTATAGT
AATACCAATCCTTATTGGGGGATTCGGGAACTGACTCCTTCCACTTATAATTG
GAGCCCCTGACATAGCATTCCCTCGAATAAATAATATAAGCTTTTGACTTCTC
CCACCATCTTTTCTTCTCCTTTTAGCATCCTCTGGTGTCGAAGCTGGGGCCGG
AACTGGTTGAACAGTATACCCACCACTTGCTGGCAACTTAGCCCACGCAGGA
GCATCCGTAGACTTAACTATTTTTTCACTACACTTAGCAGGAGTTTCATCTAT
TTTAGGAGCAATCAATTTTATTACCACATCCATCAACATGAAACCCCCAGCTA
TTTCTCAATACCAAACACCATTATTCGTGTGAGCTGGACTTGTAACAGCAGTC
CTATTACTTTTATCCCTACCAGTGCTAGCTGCCGGAATTACAATACTGTTAAC
AGACCGTAATTTAAATACAACATTCTTTGACCCCGCCGGAGGAGGTGACCCA
ATCTTTTACCAACACTTATTC

>Botia dario BD2

ACTAGATTTAGTATATAGTGCGTGAGCCGGCATATGTGGCACAGCCCTCAGC
CTTTTAATTCGAGCTGAACTCAGCCAACCCGGGTCCCTTCTAGGTGATGATCA
AATTTACAACGTTATCGTCACTGCACATGCTTTCGTTATAATTTTCTTTATAGT
AATACCAATCCTTATTGGGGGATTCGGGAACTGACTCCTTCCACTTATAATTG
GAGCCCCTGACATAGCATTCCCTCGAATAAATAATATAAGCTTTTGACTTCTC
CCACCATCTTTTCTTCTCCTTTTAGCATCCTCTGGTGTCGAAGCTGGGGCCGG
AACTGGTTGAACAGTATACCCACCACTTGCTGGCAACTTAGCCCACGCAGGA
GCATCCGTAGACTTAACTATTTTTTCACTACACTTAGCAGGAGTTTCATCTAT
TTTAGGGGCAATCAATTTTATTACCACATCCATCAACATGAAACCCCCAGCTA
TTTCTCAATACCAAACACCATTATTCGTGTGAGCTGTACTTGTAACAGCAGTC
CTATTACTTTTATCCCTACCAGTGCTAGCTGCCGGAATTACAATACTGTTAAC
AGACCGTAATTTAAATACAACATTCTTTGACCCCGCCGGAGGAGGTGACCCA
ATTCTTTACCAACACTTATTC

4.4.2. DNA sequence variation analysis

Mitochondrial DNA 655bp Cytochrome Oxidase Subunit I (COI) gene were successfully amplified from individuals of *Botia dario, Botia rostrata, Botia almorhae, Botia lohachata, Botia macracanthus* and *Botia modesta* and sequences were submitted to Genbank databases (**Tab.11**). Simplicity and un-ambiguity were observed among all the sequences, and no insertions, deletions or stop codons were observed in any of the sequences. Some sequences were also derived from NCBI. Out of 655 positions in the COI gene sequences analyzed in 10 specimens, 196 positions were variable, and 172 were parsimoniously informative. The average base composition [Thymine/Uracil (T/U); Cytosine (C); Adenine (A) and Guanine (G)] over all the four codon positions is 31.5, 25.7, 25.8 and 17.0, respectively. The transition/transversion rate ratios are k1 =4.836 (purines) and k2 = 6.772 (pyrimidines). The overall transition/transversion bias is R = 3.084(**Tab.12**).

Tab. 11: The mitochondrial COI sequences of genus *Botia* with the accession number

Sl. No.	Species name	Genbank Accession Number	Authors
1	*Botia almorhae*	KF738184	NCBI
2	*Botia almorhae*	KF738185	NCBI
3	*Botia almorhae*	KF738183	NCBI
4	*Botia almorhae*	KT781504	PRESENT STUDY
5	*Botia lohachata*	KT781505	PRESENT STUDY
6	*Botia lohachata*	KF742423	NCBI
7	*Botia kubotai*	KF738178	NCBI
8	*Botia kubotai*	KF738179	NCBI
9	*Botia kubotai*	KF738180	NCBI
10	*Botia kubotai*	KF738181	NCBI
11	*Botia rostrata*	KT781497	PRESENT STUDY
12	*Botia rostrata*	KT781498	PRESENT STUDY
13	*Botia rostrata*	KT781499	PRESENT STUDY
14	*Botia rostrata*	KF738189	NCBI
15	*Botia rostrata*	KF738190	NCBI
16	*Botia rostrata*	KF738191	NCBI
17	*Botia rostrata*	KT781500	PRESENT STUDY
18	*Botia rostrata*	KF738192	NCBI
19	*Botia striata*	KF738186	NCBI
20	*Botia striata*	KF738187	NCBI
21	*Botia striata*	KF738188	NCBI
22	*Botia dario*	KT781502	PRESENT STUDY
23	*Botia dario*	KT781503	PRESENT STUDY
24	*Botia dario*	JX105475	NCBI
25	*Botia dario*	KF511556	NCBI
26	*Botia dario*	JX105468	NCBI
27	*Botia dario*	JX105477	NCBI
28	*Botia dario*	JX105478	NCBI
29	*Botia macracanthus*	KT781506	PRESENT STUDY
30	*Chromobotia macracanthus*	KF738204	NCBI
31	*Chromobotia macracanthus*	KF738207	NCBI
32	*Chromobotia macracanthus*	KF738205	NCBI
33	*Chromobotia macracanthus*	KF738206	NCBI
34	*Botia modesta*	KT781501	PRESENT STUDY
35	*Botia modesta*	JQ346170	NCBI
36	*Glyptothoraxbrevipinnis*	EU637829	NCBI

Table 12: Molecular characterization information content of the mtDNA COI region of analyzed *Botia*

No. of bases analyzed	Nucleotide composition				Invariable Sites	Polymorphic informative Sites	Parsimony informative Sites	Estimated tv/ts bias (R)
	%A	%G	%T	%C				
655	25.8	17	31.5	25.7	196	164	172	3.084

Table 13: Evolutionary divergence between Genus *Botia*

	B. dario	***B. lohachata***	***B. almorhae***	***B. macracanthus***	***B. modesta***	***B. rostrata***	***B. kubotai***	***B. striata***
B.dario		0.015	0.015	0.017	0.018	0.013	0.015	0.014
B.lohachata	0.112		0.002	0.017	0.020	0.009	0.009	0.014
B.almorhae	0.107	0.004		0.017	0.020	0.008	0.009	0.014
B.macracanthus	0.169	0.137	0.140		0.020	0.018	0.018	0.018
B. modesta	0.186	0.200	0.199	0.198		0.019	0.019	0.019
B. rostrata	0.094	0.045	0.040	0.151	0.193		0.008	0.012
B. kubotai	0.112	0.047	0.049	0.158	0.192	0.035		0.013
B. striata	0.109	0.093	0.097	0.155	0.199	0.074	0.088	

4.4.3. Evolutionary distances

Intra-species pair wise distances of *Botia* genus is highlighted in Table 3. The COI sequence pair of *Botia* evolutionary distances ranged from 0.004 to 0.200. The interspecies Kimura's 2- parameter pair-wise distance was highest (0.200) between *B. modesta* and *B. lohachata* and lowest (0.004) for *B. almorhae* and *B. lohachata* (**Tab.13**). Best fit models for COI dataset was Hasegawa-Kishino-Yano (HKY+ I) model for different population of *Botia* and closely related species such as *B. lohachata* and *B. almorhae*. 500 bootstrap re-sampling strategy was used to assess the reliability of a

phylogenetic tree. All the populations of *Botia* clearly separated from each other in the phylogenetic tree (**Fig.13**).

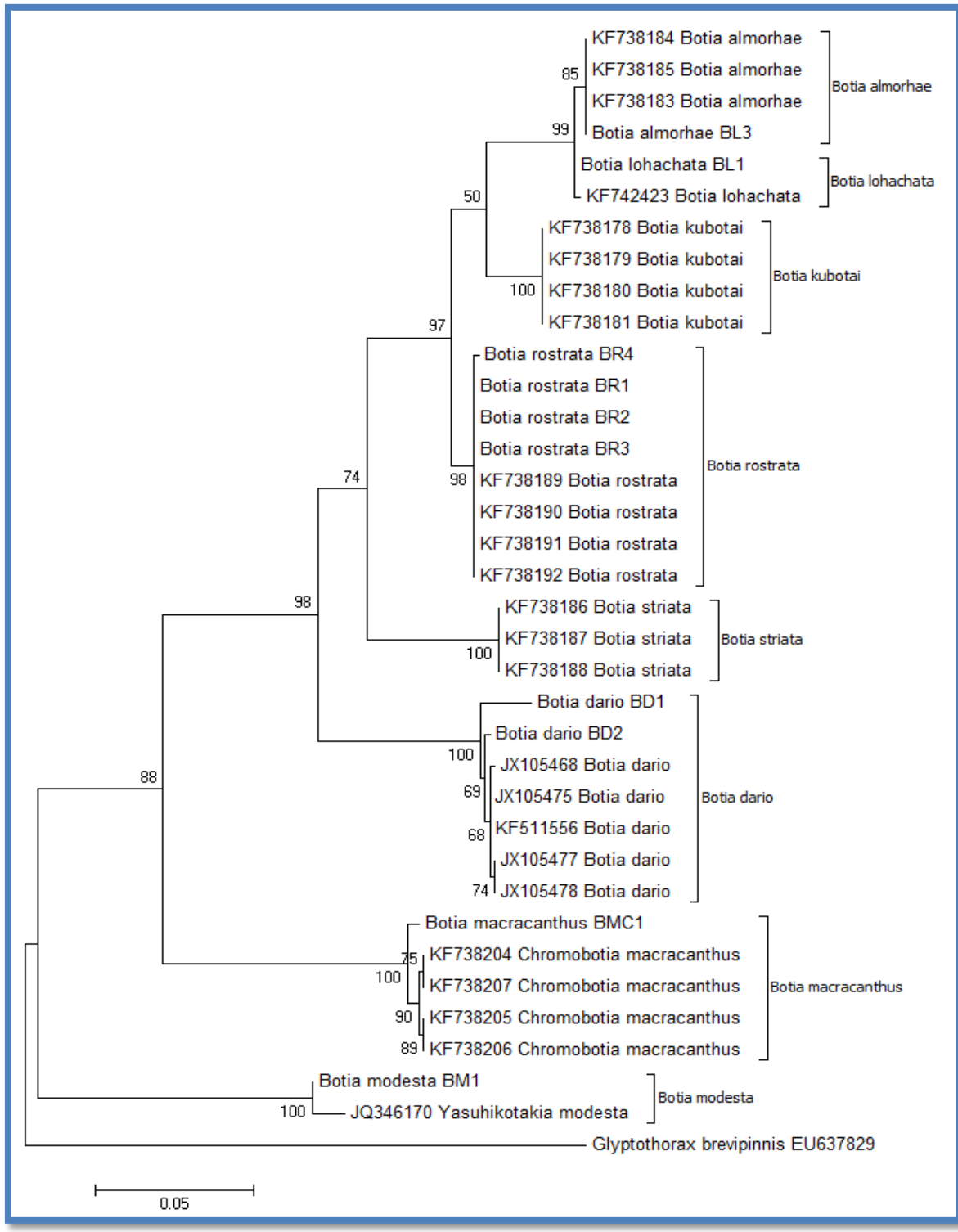


Fig. 13: Molecular Phylogenetic Analysis by Maximum Likelihood Method

4.4.4. Phylogenetic analysis

The nucleotide sequences of COI gene were aligned in order to determine the phylogenetic relationship among 6 species of *Botia*. The topology of ML and NJ tree were estimated. The phylogenetic tree showed that *B. almorhae* and *B. lohachata* formed a monophyletic group (supported by 100% bootstrap value) and then constituted one clade with *Botia kubotai*. Other Asian species, *Botia rostrata, Botia striata, Botia dario, Botia modesta and Botia macrocanthus* also contributed to this clade but were distant to native *Botia* species.

The evolutionary history was inferred by using the Maximum Likelihood Method based on the Hasegawa- Kishino-Yano model (**Hasegawa *et al.*, 1985**). The tree with the highest log likelihood (-2520.3362) is shown in Figure 8. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour- Join and BioNJ algorithms to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior loglikelihood value. The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 0.0010% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 36 nucleotide sequences. Codon positions included were 1st+2nd+3rd. All positions contained gaps and missing data were eliminated. There were a total of 592 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 **(Tamura *et al.*, 2011).**

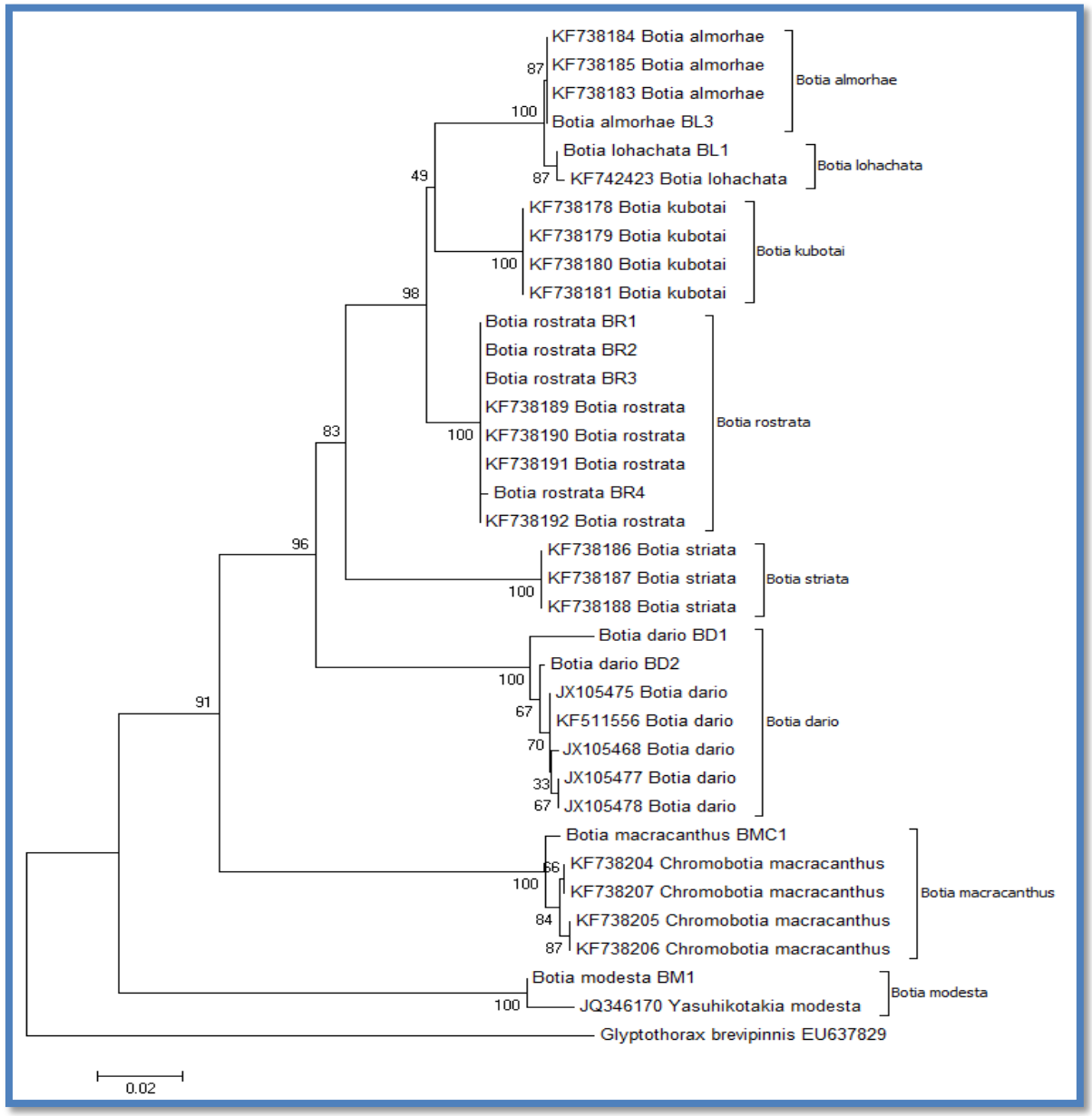


Fig. 14: Evolutionary relationships of taxa by Neighbour-Joining method

The evolutionary history was inferred using the Neighbour- Joining method. The optimal tree with the sum of branch length = 0.60289640 is shown in **Fig.14**. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method

and are in the units of the number of base substitutions per site. The analysis involved 36 nucleotide sequences. Codon positions included were 1st+2nd+3rd. All positions containing gaps and missing data were eliminated. There were a total of 592 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

4.4.5. DNA Barcoding of four *Botia* loaches

The Barcode ID number was generated from the Barcode of Life Data Systems Version 3. The Barcode ID number of four *Botia* species were **SDP657007-17** (*Botia almorhae*), **SDP657005-17** (*Botia dario*), **SDP657002-17** (*Botia lohachata*) and **SDP657006-17** (*Botia rostrata*).

Fig.14a: Barcode view of *Botia almorhae* in BOLD system

Fig.14b: Barcode view of *Botia dario* in BOLD system

Fig.14c: Barcode view of *Botia lohachata* in BOLD system

Fig.14d: Barcode view of *Botia rostrata* in BOLD system

4.5. Statistical analysis

In *Botia* species the mathematical relationship between body weight and gonad weight, body length and gonad length, body weight and fecundity and Gonado-somatic Index of male and female, Coefficient of Correlation (r) was done using MS- Excel.

4.5.1. Correlation study among growth parameters of *Botia almorhae*

The Coefficient of Correlation of *Botia almorhae* among gonad weight and body weight was 0.819, gonad length and body length was 0.611, fecundity and body weight was 0.848 and female and male Gonado-somatic Index was 0.87. The Coefficient of Correlation (r) among all relationships showed significance at $p \leq 0.01$.Correlation between the different parameters of *Botia almorhae,* Gonado-somatic Index of male and female were more significant than other parameters. The regression equation Y= 0.418X

+ 3.824 for body weight and gonad weight (**Fig. 15a**), Y= 0.364X – 0.735 for body length and gonad length (**Fig. 15b**), Y= 764.1X – 5812 for body weight and fecundity (**Fig. 15c**), Y= 9.155X + 2.734 for Gonado-somatic Index of male and female (**Fig. 15d**) showed a linear relationship by using the linear regression equation Y = a+ bx.

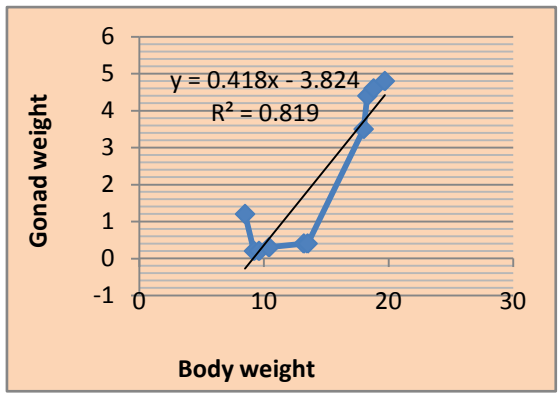


Fig. 15a: Correlation among gonad weight and body weight of *B. almorhae*

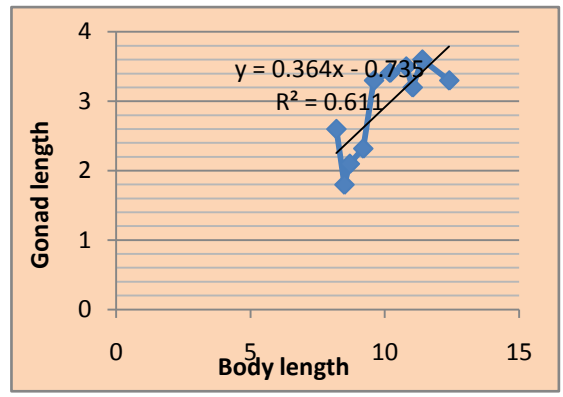


Fig. 15b: Correlation among gonad length and body length *B. almorhae*

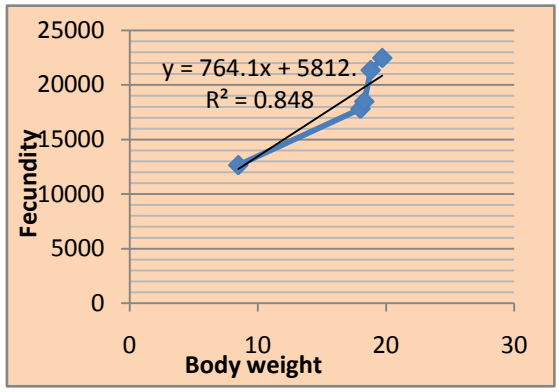


Fig. 15c: Correlation among fecundity and body weight of *B. almorhae*

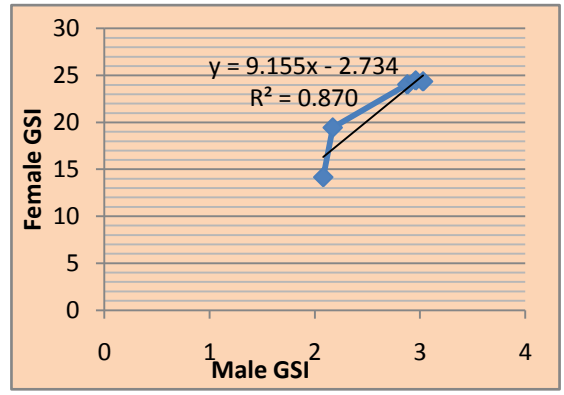


Fig. 15d: Correlation among female and male GSI of *B. almorhae*

4.5.2. Correlation study among growth parameters of *Botia dario*

The Coefficient of Correlation of *Botia dario* among gonad weight and body weight was 0.948, gonad length and body length was 0.410, fecundity and body weight was 0.676 and female and male Gonado-somatic Index was 0.99. The Coefficient of Correlation (r) among all relationships showed significance at $p \leq 0.01$.Correlation between the different parameters of *Botia dario*, Gonado-somatic Index of male and female was more significant than other parameters. The regression equation Y= 0.178X + 1.209 for body weight and gonad weight (**Fig. 16a**), Y= 0.208X – 1.377 for body length and gonad length (**Fig. 16b**), Y= 523.5X – 7806 for body weight and fecundity (**Fig. 16c**), Y= 3.613X + 0.655 for Gonado-somatic Index of male and female (**Fig. 16d**) showed a linear relationship by using the linear regression equation Y = a+ bx.

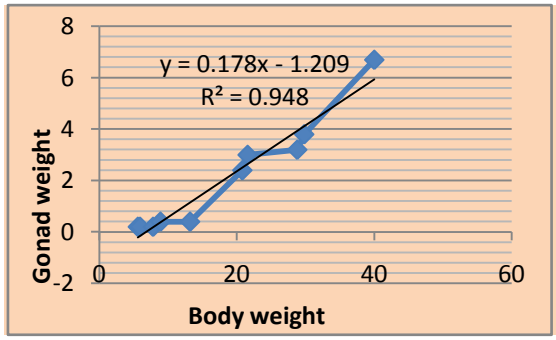


Fig. 16a: Correlation among gonad and body weight of *B. dario*

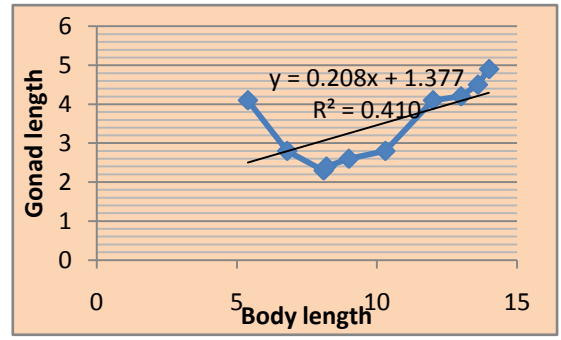


Fig. 16b: Correlation among gonad and body length of *B. dario*

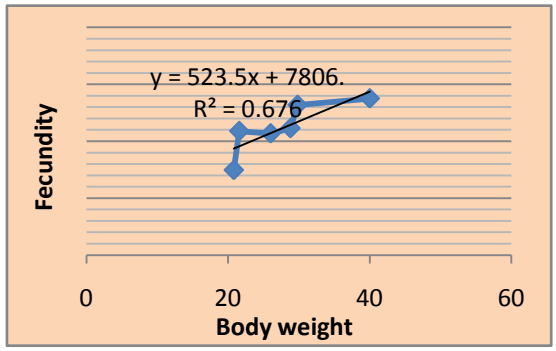


Fig. 16c: Correlation among fecundity and body weight of *B. dario*

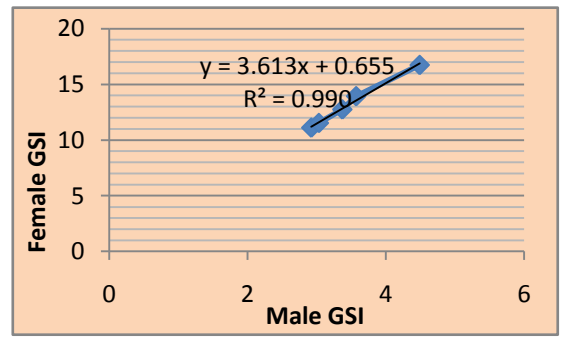


Fig. 16d: Correlation among female and male GSI of *B. dario*

4.5.3. Correlation study among growth parameters of *Botia lohachata*

The Coefficient of Correlation of *Botia lohachata* among gonad weight and body weight was 0.70, gonad length and body length was 0.865, fecundity and body weight was 0.832 and female and male Gonado-somatic Index was 0.949. The Coefficient of Correlation (r) among all relationship showed significance at $p \leq 0.01$.Correlation between the different parameters of *Botia lohachata*, Gonado-somatic Index of male and female was more significant than other parameter. The regression equation Y= 0.194X + 0.727 for body weight and gonad weight (**Fig. 17a**), Y= 0.386X – 0.754 for body length and gonad length (**Fig. 17b**), Y= 1237X – 1610 for body weight and fecundity (**Fig.17c**), Y= 3.434X + 15.48 for Gonado-somatic Index of male and female (**Fig.17d**) showed a linear relationship by using the linear regression equation Y = a+ bx.

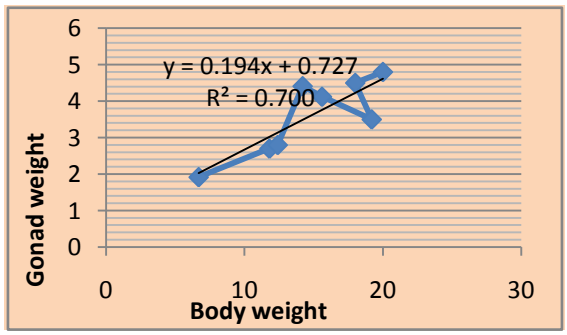


Fig. 17a: Correlation among gonad and body weight of *B. lohachata*

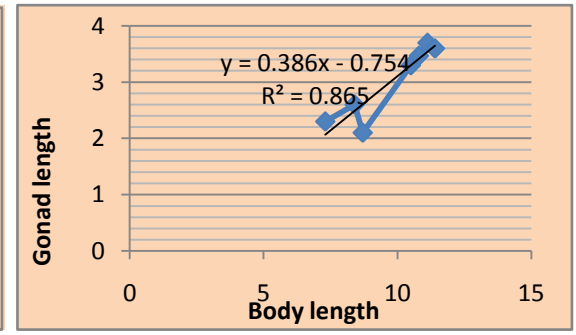


Fig. 17b: Correlation among gonad and body length of *B. lohachata*

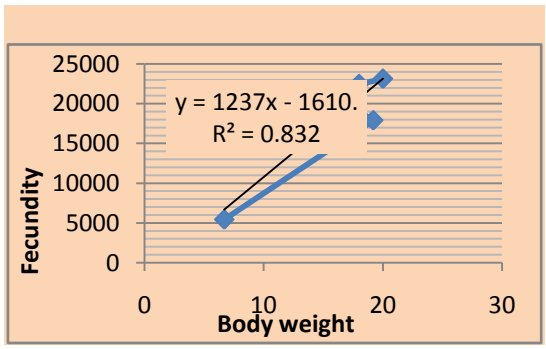


Fig. 17c: Correlation among fecundity and body weight of *B. lohachata*

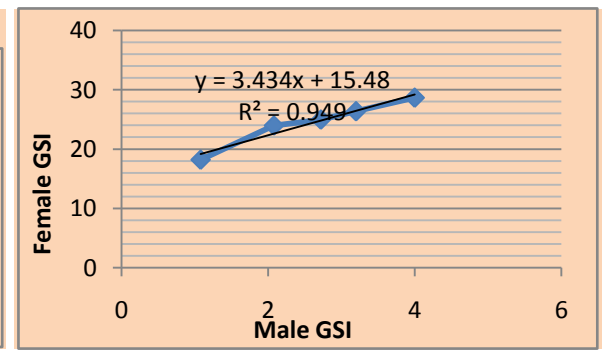


Fig. 17d: Correlation among female and male GSI of *B. lohachata*

4.5.4. Correlation study among growth parameters of *Botia rostrata*

The Coefficient of Correlation of *Botia rostrata* among gonad weight and body weight was 0.889, gonad length and body length was 0.949, fecundity and body weight was 0.956 and female and male Gonado-somatic Index was 0.817. The Coefficient of Correlation (r) among all relationship were showed significance at $p \leq 0.01$.Correlation between the different parameters of *Botia rostrata*, fecundity and body weight was more significant than other parameter. The regression equation Y= 0.284X + 2.032 for body weight and gonad weight (**Fig. 18a**), Y= 0.359X – 0.855 for body length and gonad length (**Fig. 18b**), Y= 1067X – 1498 for body weight and fecundity (**Fig. 18c**), Y= 10.04X + 0.420 for Gonado-somatic Index of male and female (**Fig. 18d**) showed a linear relationship by using the linear regression equation Y = a+ bx.

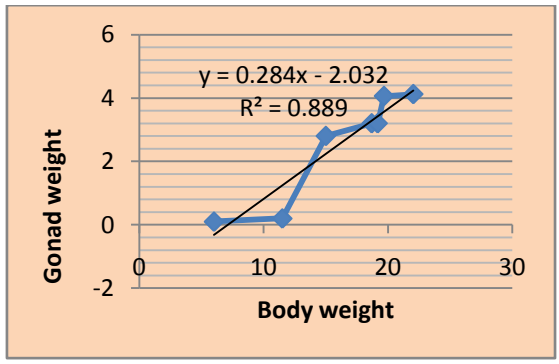


Fig.18a: Correlation among gonad and body weight of *B. rostrata*

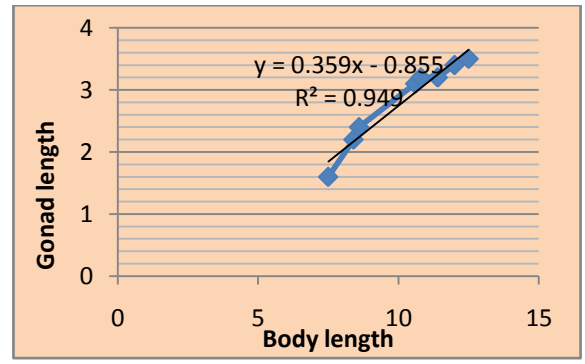


Fig. 18b: Correlation among gonad and body length of *B. rostrata*

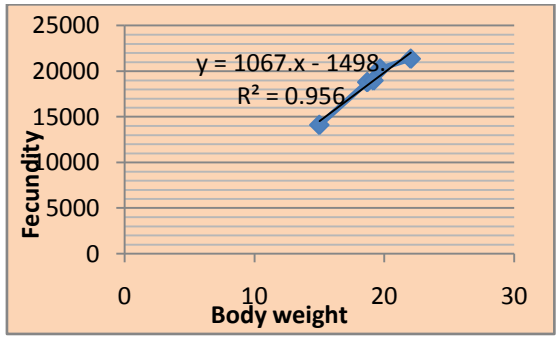


Fig. 18c: Correlation among fecundity and body weight of *B. rostrata*

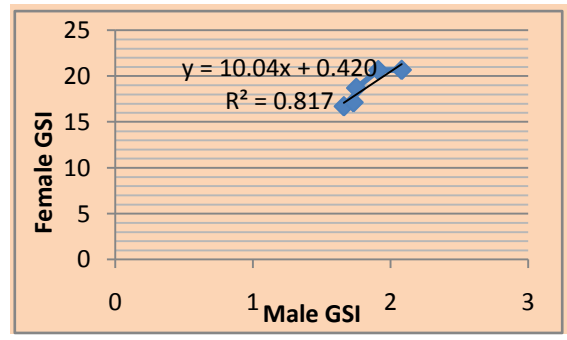


Fig. 18d: Correlation among female and male GSI of *B. rostrata*

4.6. ICHTHYOFAUNA DIVERSITY OF RIVER KALJANI

River Kaljani has the richest fish diversity among all other rivers of Cooch Behar district, originates from Gabaur Bachhra forest lying in the Bhutan ghat hills of Eastern Himalaya and outfalls into Shiltorsa in Cooch Behar district, West Bengal, India. Ornamental fish are dominant over the food fish in the Kaljani river.

About 138 fish species belonging to 31 families were recorded in the present study (**Tab.14**). As seen from **Fig. 19**, the most dominant fish families contributing to the study were Cyprinidae: 50 species and Sisoridae: 14 species. The less dominant family than Cyprinidae was Bagridae contributing 11 species and Cobitidae: 8 species. The families Belontiidae, Channidae, and Schilbeidae contributed 6 species. Mastacembelidae represented 4 species and Balitoridae, Badidae and Siluridae represented 3 species. Ambassidae, Amblycipitidae,Clupeidae and Notopteridae contributed 2 species.Other families Anabantidae, Anguillidae, Aplocheilidae. Belonidae, Chacidae, Clariidae, Engraulididae, Gobiidae, Heteropneustidae, Mugilidae, Nandidae, Ophichthidae, Pangasiidae, Synbranchidae, Syngnathidae and Tetradontidae all contributed 1 species each. Among the 138 species, 53 species had food value, 60 species ornamental value and 25 species both ornamental and food value **(Tab.14).** Therefore, in the present study, an attempt had been made to explore the available indigenous ornamental fish fauna including *Botia* species of River Kaljani, northern part of West Bengal. It was observed, ornamental fishes were dominant over the food fishes. All the three types of feeding habit of fishes like carnivorous, omnivorous and herbivorous were available in this region. About 97 species of fishes were carnivorous, 28 species were omnivorous and 13 species were herbivorous (**Tab.14**). According to IUCN (International Union for Conservation of Nature) and CAMP (Conservation Assessment

and Management Plan), the conservation status of the fishes listed are as, 1 species as Critically Endangered, 13 species as Endangered, 41 species as Vulnerable, 35 species as at Lower Risk Near Threatened, 41 species as Lower Risk Least Concerned, 4 species as Data Deficient and 3 species as Not Evaluated.

The evaluation of conservation status of the fishes and the results of the present study revealed that 25.36% of the fishes belonged to lower risk near threatened (LRnt); 29.71% vulnerable (VU); 29.71% lower risk least concern (LRlc); 2.17% not evaluated (NE); 9.42% endangered (EN); 0.72% critically endangered (CEN) and 2.89% data deficient (DD) category (**Fig-20)**. Month wise availability of fish species were high in the months of November (2012) to April (2013) and September (2013). Chhat Bhelakopa (Site -4) had the richest diversity than the other sites. *Pangasius pangasius* is a critically endangered species, found in this region. *Tenualosa toli* was also found at Chhat Bhelakopa (Site-4) only during monsoon. The above data showed that 11 species such as *Puntius ticto, Puntius sophore, Puntius conchonius, Puntius chola, Brilius barila, Barilius bendelisis, Cirrhinus mrigala, Mystus tengra, Channa punctatus, Mystus vittatus* and *Channa marulius* were abundant in the system and were collected from all locations throughout the year. The highest demandable ornamental species present are *Pseudambassis ranga, Chanda nama, Colisa lalia, Botia dario, Ctenops nobilis, Danio devario, Botia almorhae, Badis badis, Botia lohachata, Botia rostrata, Botia histrionic, Oreichthys casuatis, Oreichthys crenuchoides, Osteobrama cotio, Danio devario, Hara hara* and *Microphis deocata*. The present study showed that 20 endemic species were found in this region. According to **CAMP (1998),** India has 191 endemic species. Eastern Himalayan rivers represented many endemic species like *Badis badis, Badis bengalensis, Badis assamensis, Ctenop nobilis, Chaca chaca, Conta conta, Olyra longicaudata* and so on. The Eastern Himalayas is an area of considerable endemicity in

its freshwater ichthyofauna. Much of this endemicity stems from the presence of numerous hill stream species with highly restricted distributions for example, many members of the Balitoridae and Sisoridae. All known species of the genus *Aborichthys* are endemic to Brahmaputra drainage in northern Bengal, Meghalaya and Arunachal Pradesh.

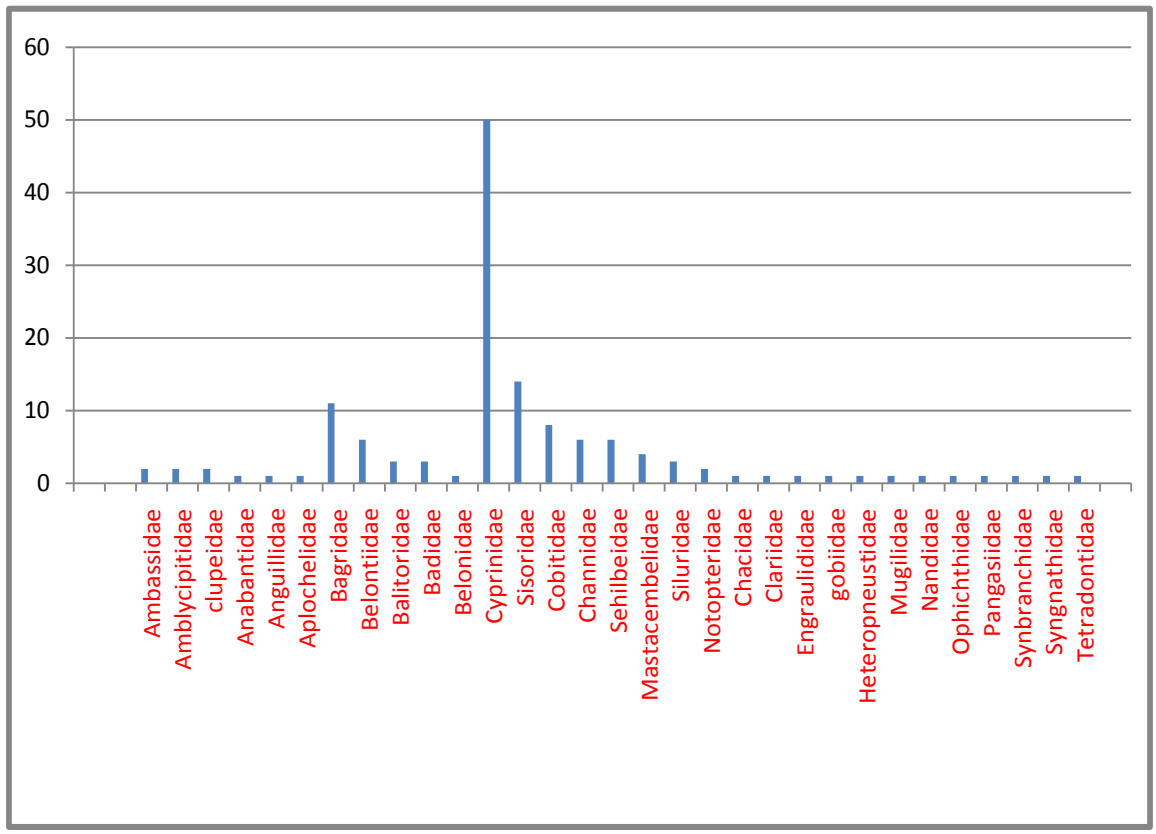


Fig. 19: Bar diagram showing the family wise distribution of fishes in the river Kaljani

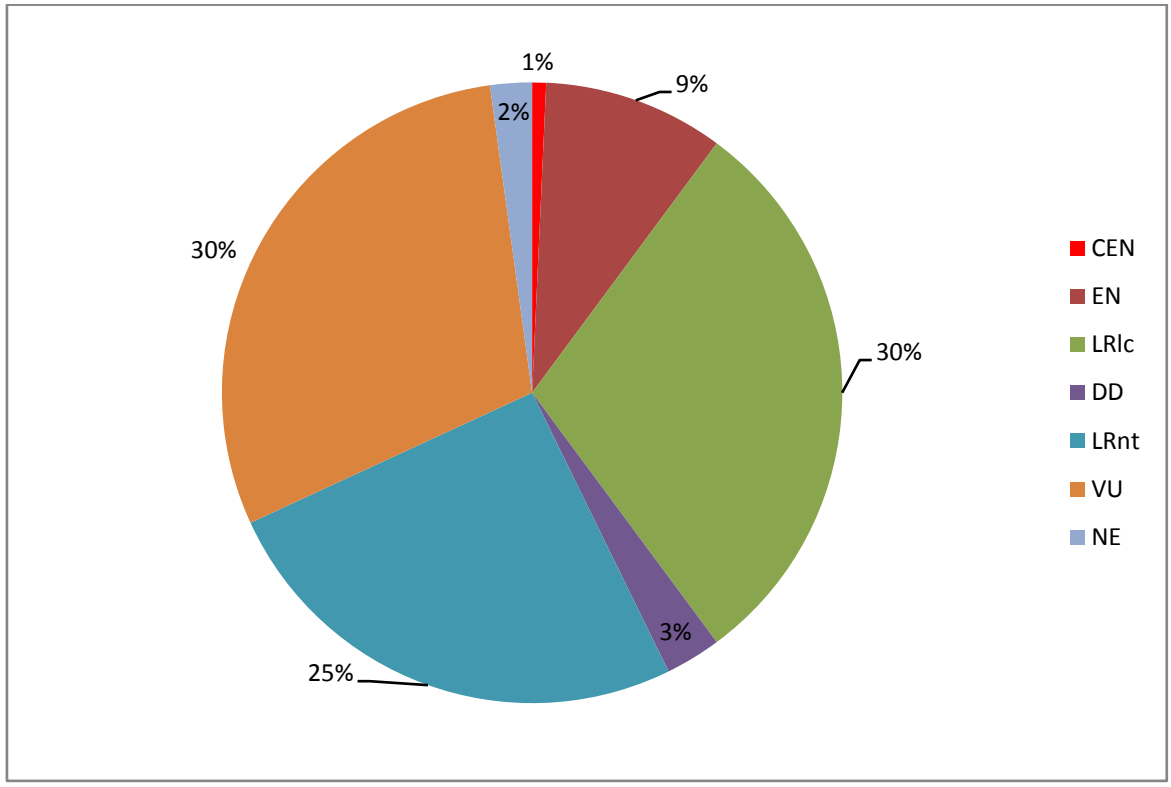


Fig. 20: Sector diagram showing the percentage of conservation status of fishes in river Kaljani recorded during the period 2012-14

Tab. 14: Ichthyofauna Diversity of River Kaljani in Cooch Behar District

Sl. No.	Scientific name	Family	Conser-vation status	Econ-omic value	Food habit
1	*Anabas testudineus* (Bloch)	Anabantidae	VU	Fd	C
2	*Pseudambassis ranga* (Hamilton-Buchanan)	Ambassidae	LRnt	Or	C
3	*Chanda nama* (Hamilton-Buchanan)	Ambassidae	LRnt	Or	C
4	*Amblyceps mangois* (Hamilton-Buchanan)	Amblycipitidae	EN	Or	C
5	*Amblyceps tuberculatum* (Linthoingambi and Vishwanath)	Amblycipitidae	LRlc	Or	C
6	*Anguilla bengalensis*(Gray)	Anguillidae	EN	Fd	O

7	*Aplocheilus panchax* (Hamilton)	Aplocheilidae	LRlc		O
8	*Mystus bleekeri* (Day)	Bagridae	VU	Fd/Or	C
9	*Mystus carcio* (Hamilton)	Bagridae	LRlc	Fd/Or	C
10	*Mystus cavasius* (Hamilton	Bagridae	LRnt	Fd/Or	O
11	*Mystus tengara* (Hamilton)	Bagridae	LRlc	Fd/Or	C
12	*Mystus gulio* (Hamilton)	Bagridae	LRlc	Fd/Or	C
13	*Mystus vittatus* (Bloch)	Bagridae	VU	Fd/Or	C
14	*Sperata aor* (Hamilton)	Bagridae	VU	Fd	C
15	*Sperata seenghala* (Sykes)	Bagridae	VU	Fd	C
16	*Batasio affinis* (Blyth)	Bagridae	LRnt	Fd/Or	C
17	*Rita rita* (Hamilton -Buchanan)	Bagridae	VU	Fd/Or	C
18	*Balitora brucei* (Gray)	Balitoridae	VU	Or	O
19	*Schistura fasciata*(Lokeshwar and Vishwanath)	Balitoridae	NE	Or	C
20	*Schistura tirapensis*(Kottelat)	Balitoridae	LRlc	Or	C
21	*Xenentodon cancila* (Hamilton)	Belonidae	LRlc	Or	C
22	*Badis assamensis* (Ahl)	Badidae	DD	Or	C
23	*Badis badis* (Hamilton)	Badidae	LRlc	Or	C
24	*Badis bengalensis* (Hamilton)	Badidae	LRlc	Or	C
25	*Ctenops nobilis* (McClelland)	Belontiidae	LRnt	Or	O
26	Colisa fasciatus (Schneider)	Belontiidae	LRnt	Or	C
27	*Colisa labiosus* (Day)	Belontiidae	LRlc	Or	C
28	*Colisa lalia* (Hamilton - Buchanan)	Belontiidae	LRlc	Or	C
29	*Colisa sota*(Hamilton-Buchanan)	Belontiidae	LRlc	Or	C
30	*Colisa chuna*(Hamilton)	Belontiidae	LRnt	Or	C
31	*Chaca chaca*(Hamilton-Buchanan)	Chacidae	EN	Or	C
32	*Channa striata* (Bloch)	Channidae	LRlc	Fd	C
33	*Channa bleheri* (Vierke)	Channidae	LRnt	Fd/Or	C
34	*Channa gachua* (Hamilton)	Channidae	LRlc	Fd/Or	C
35	*Channa marulius* (Hamilton)	Channidae	LRnt	Fd	C

36	*Channa punctatus* (Bloach)	Channidae	LRlc	Fd	C
37	*Channa barca*(Hamilton)	Channidae	DD	Fd/Or	C
38	*Clarius batrachus* (Linnaeaus)	Clariidae	VU	Fd	C
39	*Gudusia chapra*(Hamilton-Buchanan)	Clupeidae	EN	Fd	O
40	*Tenualosa toil* (Valenciennes)	Clupeidae	VU	Fd	C
41	*Botia dario* (Hamilton)	Cobitidae	VU	Fd/Or	C
42	*Botia rostrata* (Gunther)	Cobitidae	VU	Or	C
43	*Botia lohachata* (Chaudhuri)	Cobitidae	EN	Or	C
44	*Botia almorhae* (Grey)	Cobitidae	EN	Or	C
45	*Pangio pangio*(Hamilton)	Cobitidae	VU	Or	C
46	*Cantophrys gongota* (Hamilton)	Cobitidae	EN	Or	C
47	*Lepidocephalichthys berdmorei* (Blyth)	Cobitidae	LRlc	Or	C
48	*Lepidocephalichthys manipurensis* (Arunkumar)	Cobitidae	LRlc	Or	C
49	*Oreichthys casuatis*(Hamilton-Buchanan	Cyprinidae	LRlc	Or	C
50	*Oreichthys crenuchoides* (Schäfer)	Cyprinidae	DD	Or	C
51	*Chagunius chagunius*(Hamilton)	Cyprinidae	EN	Fd/Or	O
52	*Osteobrama belangeri* (Valencienes	Cyprinidae	LRnt	Fd	C
53	*Osteobrama cotio*(Hamilton	Cyprinidae	LRnt	Fd	C
54	*Tor putitora* (Hamilton)	Cyprinidae	EN	Fd	O
55	*Tor tor* (Hamilton)	Cyprinidae	EN	Fd	O
56	*Amblypharyngodon mola* (Hamilton-Buchanan)	Cyprinidae	LRlc	Fd/Or	H
57	*Cirrhinus reba* (Hamilton)	Cyprinidae	VU	Fd	O
58	*Crossocheilus burmanicus* (Hora)	Cyprinidae	VU	Fd	O
59	*Garra kempi* (Hora)	Cyprinidae	LRlc	Fd	H
60	*Garra gotyla* (Gray)	Cyprinidae	VU	Fd	H

61	*Garra lamta* (Hamilton)	Cyprinidae	LRlc	Fd	H
62	*Barilius barila* (Hamilton)	Cyprinidae	VU	Fd	O
63	*Barilius tileo* (Hamilton)	Cyprinidae	VU	Fd	O
64	*Barilius vagra* (Hamilton)	Cyprinidae	VU	Fd	O
65	*Barilius dogarsinghi* (Hora)	Cyprinidae	EN	Fd	O
66	*Barilius ngawa* (Vishwanath and Manojkumar)	Cyprinidae	LRlc	Fd	O
67	*Barilius bendelisis* (Hamilton)	Cyprinidae	VU	Fd	O
68	*Barilius barna* (Hamilton)	Cyprinidae	VU	Fd	O
69	*Aspidoparia morar* (Hamilton)	Cyprinidae	VU	Fd/Or	C
70	*Devario devario*(Hamilton)	Cyprinidae	LRnt	Or	C
71	*Devario assamensis* (Barman)	Cyprinidae	VU	Or	C
72	*Rasbora daniconius* (Hamilton)	Cyprinidae	LRlc	Or	C
73	*Rasbora rasbora* (Hamilton)	Cyprinidae	LRlc	Or	C
74	*Raiamas bola* (Hamilton)	Cyprinidae	VU	Fd/Or	C
75	*Salmophasia bacaila* (Hamilton)	Cyprinidae	LRnt	Fd/Or	C
76	*Psilorhynchus sucatio* (Hamilton)	Cyprinidae	LRlc	Or	O
77	*Psilorhynchus balitora* (Hamilton)	Cyprinidae	VU	Or	O
78	*Psilorhynchus homaloptera* (Hora and Mukherji)	Cyprinidae	VU	Fd	O
79	*Psilorhynchus brucei* (Gray)	Cyprinidae	LRnt	Or	O
80	*Schizothorax labialus* (McClelland and Griffith)	Cyprinidae	LRnt	Fd/Or	C
81	*Labeo rohita* (Hamilton - Buchanan)	Cyprinidae	LRnt	Fd	H
82	*Labeo calbasu* (Hamilton)	Cyprinidae	LRlc	Fd	H
83	*Labeo gonius* (Hamilton)	Cyprinidae	VU	Fd	H
84	*Labeo dyocheilus* (McClelland)	Cyprinidae	VU	Fd	H
85	*Labeo bata* (Hamilton)	Cyprinidae	LRnt	Fd	H
86	*Labeo boga* (Hamilton)	Cyprinidae	LRnt	Fd	H
87	*Labeo pangusia* (Hamilton)	Cyprinidae	LRnt	Fd	H

88	*Catla catla* (Hamilton-Buchanan)	Cyprinidae	VU	Fd	H
89	*Cirrhinus mrigala* (Hamilton-Buchanan)	Cyprinidae	LRnt	Fd	O
90	*Puntius chola* (Hamilton-Buchanan)	Cyprinidae	LRlc	Or	C
91	*Puntius conchonius* (Hamilton)	Cyprinidae	LRlc	Or	C
92	*Puntius phutunio* (Hamilton)	Cyprinidae	LRlc	Or	C
93	*Puntius sarana* (Hamilton)	Cyprinidae	VU	Fd	C
94	*Puntius sophore* (Hamilton)	Cyprinidae	LRnt	Or	C
95	*Puntius stolickanus* (Day)	Cyprinidae	LRlc	Or	C
96	*Puntius terio* (Hamilton)	Cyprinidae	LRnt	Or	C
97	*Puntius ticto* (Hamilton)	Cyprinidae	LRnt	Or	C
98	*Esomus danricus*(Hamilton-Buchanan)	Cyprinidae	LRlc	Or	O
99	*Setipinna phasa* (Hamilton-Buchanan)	Engraulididae	LRnt	Fd	C
100	*Glossogobius giuris* (Hamilton-Buchanan)	Gobiidae	LRnt	Fd	C
101	*Heteropneustes fossilis* (Bloch)	Heteropneustidae	VU	Fd	O
102	*Rhinomugil corsula* (Hamilton)	Mugilidae	VU	Fd/Or	H
103	*Macrognathus aral* (Bloch and Schneider)	Mastacembelidae	LRnt	Fd/Or	C
104	*Macrognathus morehensis* (Arunkumar and Tombi)	Mastacembelidae	LRlc	Fd/Or	C
105	*Macrognathus pancalus* (Hamilton)	Mastacembelidae	LRnt	Fd/Or	C
106	*Mastacembelus armatus* (Lacepede)	Mastacembelidae	LRlc	Fd/Or	C
107	*Nandus nandus* (Hamilton-Buchanan)	Nandidae	LRnt	Or	C
108	*Notopterus notopterus* (Pallas)	Notopteridae	EN	Fd	O

109	*Notopterus chitala* (Hamilton-Buchanan)	Notopteridae	EN	Fd	C
110	*Olyra longicaudata* (McClelland)	Bargridae	LRnt	Or	C
111	*Pisodonophis chilkensis* (Chaudhuri)	Ophichthidae	LRnt	Fd	C
112	*Pangasius pangasius* (Hamilton-Buchanan)	Pangasiidae	CNE	Fd	C
113	*Bagarius bagarius* (Hamilton)	Sisoridae	VU	Fd	C
114	*Gagata cenia* (Hamilton)	Sisoridae	LRnt	Fd/Or	C
115	*Gagata dolichonema* (He)	Sisoridae	LRlc	Fd/Or	C
116	*Hara hara* (Hamilton)	Sisoridae	LRlc	Or	C
117	*Hara Jerdoni* (Day)	Sisoridae	LRlc	Or	C
118	*Hara horai* (Misra)	Sisoridae	NE	Or	C
119	*Conta conta* (Hamilton-Buchanan)	Sisoridae	NE	Or	C
120	*Conta pectinata* (Ng)	Sisoridae	LRlc	Or	C
121	*Sisor barakensis* (Vishwanath and Darshan)	Sisoridae	VU	Or	C
122	*Sisor rhabdophorus* (Hamilton)	Sisoridae	LRlc	Or	C
123	*Sisor chennuah* (Ng and Lahkar)	Sisoridae	DD	Or	C
124	*Glyptothorax indicus* (Talwar)	Sisoridae	LRlc	Or	C
125	*Glyptothorax cavia* (Hamilton)	Sisoridae	LRlc	Or	C
126	*Glyptothorax telchitta* (Hamilton)	Sisoridae	LRlc	Or	C
127	*Ompok pabda* (Hamilton)	Siluridae	VU	Fd	C
128	*Ompok pabo* (Hamilton)	Siluridae	EN	Fd	C
129	*Wallago attu* (Schneider)	Siluridae	VU	Fd	C
130	*Neotropius atherinoides* (Bloach)	Schilbeidae	LRlc	Fd	C
131	*Ailia coila* (Hamilton)	Schilbeidae	VU	Fd	C
132	*Clupisoma garua* (Hamilton)	Schilbeidae	VU	Fd	C
133	*Clupisoma Montana* (Hora)	Schilbeidae	VU	Fd	C
134	*Eutropiichthys murius* (Hamilton)	Schilbeidae	LRnt	Fd	C
135	*Eutropiichthys vacha* (Hamilton)	Schilbeidae	VU	Fd	C

136	*Amphipnous cuchia* (Hamilton-Buchanan)	Synbranchidae	VU	Fd	C
137	*Microphis deocata* (Hamilton-Buchanan)	Syngnathidae	LRnt	Or	O
138	*Tetradon cutcutia* (Hamilton-Buchanan)	Tetradontidae	LRnt	Or	O

Note: Feeding habit: O= Omnivorous, C= Carnivorous, H=Herbivorous, **Economic importance:** Fd=Food fish, Or= Ornamental fish. **Conservation status:** According to IUCN (2010) and CAMP (1998), DD= Data deficient, NE= Not evaluated, VU= Vulnerable, EN= Endangered, CNE= Critically endangered, LRnt=Lower risk near threatened, LRlc=lower risk least concern.

DISCUSSION

5. DISCUSSION

5.1. WATER QUALITY

5.1.1 Temperature

Temperature controls fish growth and has profound effects on the chemistry, physical and biochemical reactions of aquatic organisms. No other factor has much influence as temperature as far as solubility of gas and salts in water is concerned (**Welch, 1952**). In the present study, the mean air temperature of the River Kaljani had 30.5^0C and in captivity at room temperature was 28^0C. This result was supported by similar finding of **Acharjee (2013**), where that highest air temperature recorded in hill stream river Relli was 32.3^0C and 32.4^0C for river Teesta.

Kaljani river water temperature ranged from 19^0 C to 36^0C with a mean value of 28.45^0C in the present study. Whereas, in captivity it was 27^0C to 32^0C with a mean value of 29.54^0C. These findings were supported by **Dubey (1978),** who reported 8.5^0C to 26^0C from rivers of Bhutan Himalaya and 29.6^0C from Trishuli river in Nepal (**Thapa *et al.*, 2010**). **Barat and Jha (2002) and Bhadra *et al.*, (2003)** reported water temperature to range from 17.5^0C to 34 ^{0}C in the rivers Mahananda and Torsa; **Saha (2014)** 16.9^0C to 29.5^0C in Shutunga river at Cooch Behar district and 26.5^0C to 26.9^0C in Lotchka river in Darjeeling **Roy (2014)**. The optimum temperature required for growth of carp was reported to be 27^0C to 32^0C (**ICAR, 2006**). In the present study, the range of temperature was appropriate for the good growth of fishes. Good growth of fish was observed in summer season than winter season in the present study. For this reason in captivity, the water temperature was

maintained from 27^0C to 32^0C in the winter season for broodstock development. This report was supported by **Weldermeriam (2013)** who reported that standard temperature for sustaining aquatic life varies between 28^0C to 30^0C. From the present investigation it was observed that water temperature increased when air temperature was high. This means that in summer season the water temperature increases and corroborates with the findings of **Wetzel (2001).**

5.1.2. pH (Hydrogen Ion Concentration)

pH measures the basic or acidic nature of water. Most natural waters are generally alkaline due to presence of sufficient quantity of carbonates. Average Hydrogen ion concentration (pH) of the Kaljani river water was 7.82. The highest value found during the rainy season from July to August 2014 was due to rainfall and low decomposition. (**Pandit *et al.*,2001).** They reported that pH is dependent on the amount of CO_2 and inversely proportional to the activity of photosynthesis**.** The mean value of pH in the captive condition water was 7.88. The pH value in captivity was constant due to strong buffering capacity of water and was found in both natural water system and captive water to be very approximate to the normal limit of 6.5 to 8.5 as suggested by the **BIS (1991**) and 7 to 8.5 by **WHO (1992).**

From the present investigation, it was also observed that Kaljani river water and laboratory water (Captive condition) were alkaline in nature. The higher value of pH (8.2) during rainy season may be due to the leaching of dolomite because, Kaljani river is connected with small streams arising from Buxa Reserve Forest. In captive condition, water pH was also high indicating that ground water also probably contained calcium carbonate. The present investigation agreed with the findings of **Renold *et al.* (1998),** who found 6.5 to 8.7 in the surface water of upper Khumbu region of Nepal; **Mondal *et al.*, (2011a** and **2011b**) and **Mozumder *et al.*, (2015)** observed alkaline pH in Torsa

river, Kaljani river and Mahananda river water. In the present study, the pH showed positive correlation with hardness (r = 0.776, P<0.01) and negatively correlation with free CO_2 (r = -0.292,) and total alkalinity (r = -0.180) in Kaljani river, respectively. Similar relations have been observed by **Acharjee (2013)** in river Teesta**.**

5.1.3. Specific Conductivity

The range (110 µS cm^{-1} to 180 µS $cm^{-1)}$, mean value (151.27 ± 22.30) of specific conductivity of River Kaljani was lower than the range (240 µS cm^{-1} to 250 µS cm^{-1}), mean value (246.14± 3.18) of the captive condition water. The optimum level (110 to 250 µS cm^{-1}) of Specific Conductivity water bodies studied was found within the prescribed range (300 µS cm^{-1}) of **(BIS, 1991).** The Specific Conductivity, as observed was higher in captivity than river water. This could be due to higher value of dissolved solids, than ions in the water **(Bhatt *et al.*, 1999)**. The specific conductivity between captive and Kaljani river water had negatively correlation (r = -0.100, P<0.01). In the present study, specific conductivity was found to be higher during winter season which may be due to low flow rate of water and increased in dissolved solids. Low values of specific conductivity during rainy season may be due to high water level for rainfall. Similar observations were also recorded by **Thapa *et al.*, (2010)** from Nepal**; Mandal *et al.*, (2012); Acharjee (2013) Saha (2014)** and **Mozumder *et al.*, (2015)** from different rivers of northern region of West Bengal.

5.1.4. Total Dissolved Solids (TDS)

In natural waters, dissolved solids are composed mainly of carbonates, bicarbonates, chlorides, sulphates, phosphates, and nitrates of calcium, magnesium, sodium, potassium, iron and manganese. The present study showed that the average value

of total dissolved solids in river Kaljani varied from 80 to 120 mg L^{-1} with mean value of 135 (± 31.96) mg L^{-1}. Total dissolved solids of breeding tank water were 250 to 260 mg L^{-1} with mean value 255 mg L^{-1} (± 3.97). The present study showed that the TDS of the sampling water ranged from 80 mg L^{-1} to 260 mg L^{-1} in the both the system of culture which was as per the **BSI (1991)** standard of 500 mg L^{-1}. Further, the TDS level was higher than the earlier workers like **Mondal *et al.*, (2011a** and **2011b**) and **Mozumder *et al.*, (2015)** who also worked on Torsa river, Kaljani river and Mahananda river water. Present investigation also corroborated with the observations of other workers like **Kumar *et al.*, (2014)** where TDS was 126.33 to 170.33 mg L^{-1} in Kali river; **Patela and Vaghanib (2015)** on Par River, Gujarat TDS (227 to 250.6 mg L^{-}) and **Baitule *et al.*, (2015)** (240 to 430 mg L^{-}) in Nag River, Nagpur city.

5.1.5. Dissolved Oxygen

Dissolved oxygen (DO) plays a vital role for aquatic life, and oxygen content depends on many factors such as temperature, photosynthesis, presence of algae, decomposition activities, pollution and the level of aeration. In the present investigation, the average concentration of dissolved oxygen found in the Kaljani river water was 10.98 (± 0.914) which was quite adequate and characteristic of hill streams. During the study period, dissolved oxygen in captivity was maintained at 6.3 mg L^{-1} to 7.6 mg L^{-1} with the help of aeration. This level of dissolved oxygen seemed significant for the survival and activity of *Botia* species. From the present study, dissolved oxygen was higher than the standard limit of DO (5.0 mg L^{-1})**.** According to **Swingle (1967)** in dissolved oxygen content ranging between 1.0 - 5.0 mg l^{-1} fishes can survive, but their reproduction is poor and growth is slow. **Mondal and Barat (2004)** also reported that dissolved oxygen concentration less than 5.0 mg l^{-1}, is not considered conducive for fish growth. The maximum DO (12.4 mg l^{-1}) found during winter in the present study, may be due to low

temperature. The decreased water temperature during winter season has a greater capacity to hold DO than warm water and probably led to a lower rate of respiration thereby allowing maximum DO in winter **(Welch, 1952).** In the present study, DO showed negative correlation with water temperature ($r = -0.765$, $P<0.01$). Present investigation also corroborated with the observations of other works like **McColl (1972)** and **Acharjee (2013**) in their studies on New Zealand Lakes, Teesta and Relli respectively.

5.1.6. Free Carbon Dioxide

Carbon dioxide present in surface water in the form of carbonic acid is called Free Carbon dioxide. Carbon dioxide dissolveds in water more readily and the dissolution depends on temperature, pressure and mineral content of the water. The variation of CO_2 was due to the absorption by aquatic plants for photosynthesis and the activity of other living organisms. In the present study, the mean of Free Carbon dioxide concentration of river water was 3.76 mg L^{-1} (± 0.801) and breeding tank water was 6.67 mg L^{-1}(± 0.519). Lowest value of Free Carbon dioxide (2.3 mgL^{-1}) was found in July was probably due to high rain fall and highest value recorded in December may be due to high decomposition load. In the present study Free CO_2 showed negative correlation with pH ($r = -0.292$, $P<0.01$) in Kaljani river. **Dhanze *et al.* (1998)** observed negative correlation of Free CO2 with alkalinity; and **Acharjee (2013)** also observed negative correlation between Free CO_2 and pH in their study on Teesta and Relli.

5.1.7. Total Alkalinity

Alkalinity measures the buffering capacity of water and is caused by calcium carbonate and bicarbonate and also to some extent to phosphates and organic matter. Kaljani river water and aquarium water were medium productive as, total alkalinity

ranged from 40 to 90 mg L^{-1}. **Jhingran (1991)** considered total alkalinity of 40 to 90 mg L^{-1} as medium productive. Kaljani river water showed bicarbonate alkalinity mostly whereas, Captive water showed carbonate alkalinity some timed as pH values were greater than 8.2 (**Trivedy and Goel, 1984). Barat and Jha, (2002**), observed bicarbonate alkalinity of river Mahananda and **Acharjee (2013)** observed it is river Teesta and Relli. In the present study, total alkalinity was positively correlation with pH ($r = 0.834$, $P<0.01$) in Kaljani river. **Dobriyal and Singh (1988), Trivedi (1988)** reported similar positive significant correlation of total alkalinity with pH.

5.1.8. Total Hardness

Total hardness of water is an important component to determine the suitability of water for domestic and industrial uses. Hardness is caused by multivalent metallic cations and certain anions present in the water. The principla hardness-causing cations are the divalent calcium, magnesium, strontium, ferrous iron and mangnous ions. Average value of total hardness of the river water was 24.0 mg L^{-1}(± 4.03) and the captive water was 27.29 mg L^{-1}(± 1.29). The results of total hardness indicated that water of both condition was soft (**Swingle, 1967).** According to **Boyd (1982),** normal fish culture needs at least 20 mg L^{-1} total hardness. The values of total hardness were soft in the present study and it agreed with the observations **Barat and Jha, (2002); Roy and Barat, (2011); Mandal *et al.*, (2011); Mandal *et al.*, (2012)** and **Acharjee, (2013).** The total hardness of water was positively correlation with pH ($r = 0.021$) and this result was supported by **Acharjee (2013)** in river Teesta and Relli.

5.1.9. Ammonium-N

The results of the ammonium-nitrogen (NH_4-N) revealed that in both the systems there was no toxic effect due to ammonia and suggested that the low concentrations may be due to the fact, that aquatic autotrophs rapidly utilize ammonium ions preferring these to nitrates; as such NH_4-N does not reach harmful concentrations. Sewage has large quantities of nitrogen matter, thus its disposal tends to increase the ammonia content of the water (**Trivedy and Goel, 1984**). Distribution of NH_4-N as found in the present study corroborates with the distribution of NH_4-N in unpolluted rivers as mentioned by **Wetzel (2001). Jha and Barat (2003)** recorded, NH_4-N concentration to vary from 0.006 to 0.072 mg L^{-1} in Mirik lake, **Thapa *et al.* (2010)** observed to haveNH_4-N ranged from nil to 0.013 mg L^{-1} in the Trishuli River of Nepal. The NH_4-N of water was negatively correlated with dissolved oxygen (r = -0.302) in the Kaljani river. **Jana and Barat (1984)** and **Acharjee (2013)** observed similar relations between NH_4-N and dissolved oxygen.

5.1.10. Nitrite-N.

Nitrite-N is a very unstable ion and gets converted into either ammonia or nitrate depending upon the conditions prevailing in the water (**Trivedy and Goel, 1984**). The range (0.001 mg L^{-1} to 0.027 mg L^{-1}), mean value (0.009 mg L^{-1} ± 0.110) of NO_2-N of river Kaljani was found slightly higher than the range (nil to 0.008 mg L^{-1}), mean value (0.003 mg L^{-1} ± 0.003) of the Captive water NO_2-N. In the present study toxicity due to Nitrite-nitrogen (NO_2-N) was negligible. **Barat and Jha, (2002)** reported NO_2-N level 0.002 to 0.030 mg L^{-1} in Mahananda river and again, **Jha and Barat (2003)** reported similar low values of NO_2-N ranged from 0.002 to 0.032 mg L^{-1}in Mirik lake. Range of NO_2-N in the

two rivers (Torsha and Teesta) of northern part of West Bengal were 0.001 to 0.008 mg L^{-1} and 0.006 to 0.014 mg L^{-1} respectively (**Bhadra *et al.*, 2003; Acharjee, 2013).**

5.1.11. Nitrate–N

The nitrate-N is one of the most oxidisable forms of nitrogen and is essential for plant nutrient and also associated with sewage and sullage discharge (**Barat and Jha, 2002**). Mean nitrate-nitrogen concentration of the river water was 0.312 mg L^{-1} (± 0.220) and the Captive 0.215 mg L^{-1}(± 0.086). According to **Lester (1969)** Kaljani river water and captive water was very clean <0.5. The quantities of NO_3-N as found in the present investigation were slightly higher than the distribution of NO_3-N as mentioned by **Wetzel (2001)** in unpolluted rivers. In the present study nitrate- nitrogen was greater than ammonium-nitrogen and nitrite- nitrogen. So, water quality was good because nitrate-nitrogen is a good form of nitrogen as it can be returned to the nitrogen cycle quickly. Similar type of observation was done by **Bhadra *et al.*, (2003)** who reported NO_3-N level in river Torsha 0.090 to 2.200 mg L^{-1}. **Acharjee (2013)** reported NO_3-N level in Teesta to range from 0.032 to 0.062 mg L^{-1}. NO_2-N studied in the water of Lukha River, Meghalaya to vary from 2.14 to 12.81 mg L^{-1} (**Lamare and Singh, 2016).**

5.1.12. Phosphate-P

Phosphorus is an essential nutrient for the growth of organisms and helps in the primary productivity of a water body. The presence of phosphate in large quantities in fresh waters indicates pollution through sewage and industrial wastes. Phosphate-phosphorous (PO_4-P) concentration of the river water ranged (0.012 mg L^{-1} to 0.197 mg L^{-1}) with mean value 0.101 mg L^{-1}(± 0.060) and ranged (0.110 mg L^{-1} to 0.318 mg L^{-1})with mean value 0.172 mg L^{-1} (± 0.078) of captive water. PO_4-P concentration in the Kaljani river and Captive condition were normal level. According to **CPAB (2008),** the

maximum permissible limit of phosphate is 5.0 mg l^{-1} for inland surface water. Similar observation had been made by different workers in different rivers of North Bengal like **Barat and Jha, (2002); Roy and Barat, (2011); Achajee,(2013); Saha, (2014)** and **Mozumder *et al.*, (2015).**

5.2. FISH BIOLOGY

5.2.1. Fish growth parameters

5.2. 1.1. Gonado-Somatic Index (GSI)

Gonado Somatic Index (GSI) is a reliable indicator of gonadal maturity; as the weight of the gonad increases with maturity when it spawns; there is a reduction in the weight of the gonad on account of the release of gametes (**Vlaming *et al.*, 1982**). Gonado-somatic index is particularly helpful in identifying days or seasons of spawning as the ovaries of gravid females rapidly increase in size just prior to spawning. The present study revealed that Gonado-somatic index increased from April and declined from September. The Index was higher in female than male of *Botia* species. The maximum value of GSI was observed during the spawning time of *Botia* species and this observation has been supported by **Bouain and Sian (1983)** who reported GSI was indicative of fish spawning in temperate and tropical regions. The average Gonado-somatic Index of *Botia* species were, *Botia almorhae* (11.96±10.29), *Botia dario* (8.34±5.4), *Botia lohachata* (13.86±11.50) and *Botia rostrata* (10.29±9.01). Among the *Botia* species *Botia lohachata* had the highest GSI and *Botia rostrata* had the lowest. GSI reduced after spawning of *Botia* species and this study was supported by **Vlaming *et al.* (1982).**

The Coefficient of Correlation between Gonado-somatic Index of male and female of *Botia* species were *Botia almorhae* (0.87), *Botia dario* (0.99), *Botia lohachata* (0.949) and *Botia rostrata* (0.817). The Coefficient of Correlation (r) among all

relationship showed significance at $p \leq 0.01$ and positively correlated. Similar observations were made by **Dey *et al.*, (2015d** and **2015c)** on *Botia dario* and *Botia lohachata*.

From the present investigation, it was found that GSI increased with the increase of gonad size of *Botia* species. GSI values of both males and females followed more or less the same trend. The peak GSI value was found only in the breeding season and so it confirms that *Botia* species breeds only once a year. *Botia almorhae*, *Botia lohachata* and *Botia rostrata* spawned from June to August and *Botia dario* from May to July. Similar types of observations were made on ornamental fish by many authors like **Joshi and Pathani (2009**) on *Botia almorhae*; **Oliveira *et al.*, (2015)** on *Hemiramphus brasiliensis* from Brazil; **Dey *et al.*, (2015d, 2015c and 2015d**) on *Botia dario, Botia lohachata* and *Botia rostrata*; **Pal and Mahapatra (2016)** on *Amblypharyngodon mola* and **Dey *et al.*, (2016)** on *Barilius barila.*

5.2. 1.2. Condition Factor

Condition factor or K- factor or Ponderal index of fish is an Index used to monitor feeding intensity and growth rate (**Oni *et al.*, 1983**). From the present investigation, Condition Factor was for *Botia almorhae* (1.390), *Botia dario* (1.788.), *Botia lohachata* (1.538) and *Botia rostrata* (1.399). According to **Le Cren (1951)**, "K" > 1 indicates good general condition of fish. Therefore, in Captivity Condition Factor of *Botia* species was good. Among the four *Botia* loaches, *Botia dario* had the highest Condition Factor because, *Botia dario* was healthy or robust than the others fish. Fish with high value of 'K' are heavy for its length, while with low K are lighter **(Bagenal and Tesch, 1978)**. Similar type of work on Condition Factor was made by **Arockiaraj *et al.*, (2004**) on *Mystus montanus* (4 to 9); **Bindu (2006)** on *Etroplus suratensis* (2.29 to

3.2) and *Horabagrus brachysoma* (1.13 to 1.38) respectively; **Rahman *et al.*, (2016)** on *Mystus vittatus* (0.95 to 1.32) and **Lal *et al.*, (2016)** on *Mystus armatus* (0.22 to 2.84).

5.2. 1.3. Length-Weight Relationship

Length- Weight relationship gives us history and morphological comparisons between different fish species or between different fish by the Least-Square Method from logarithmic data. The association of degree between weight-length variables can be calculated by the determination of Coefficient of Correlation (r). The Coefficient of Correlation of *Botia almorhae* was 0.811; *Botia dario* 0.802; *Botia lohachata* 0.753 and *Botia rostrata* 0.936. The Coefficient of Correlation (r) of *Botia rostrata* was more significant than other species. The Coefficient of Correlation (r) showed significance at $p \leq 0.01$. The theoretical value of "b" (regression coefficient) in length-weight relationship is reported to be 3 when the body form of fish remains constant at different length, that is, the growth is isometric (**Allen, 1938**). If this value is less than or more than 3 it indicates that growth is allometric (**Bagenal and Tesch, 1978**). When 'b' value was greater than 3, then the growth was a positive allometric growth. However, $b<3$ showed a negative allometric growth, or isometric growth when equal to 3.0.

During the present investigation, the value of 'b' was greater than 3. This indicated that growth pattern of fish population was allometric but, *Botia lohachata* growth pattern was isometric (b=3.006). *Botia lohachata* growth was slightly higher than isometric growth but present value showed, positive allometric growth pattern in captivity. *Botia almorhae* (b=4.027), *Botia dario* (b=4.005) and *Botia rostrata* (b=3.138) indicated positive allometric growth because all values were higher than 3.0. These results suggested, that all species showed positive allometric growth and that the fish grew in proportion to the length in captive condition. **Beverton and Holt (1957)** stated, that serious departures from isometric growth (n= 3.0) are rare. In the present study, 'b'

value of *Botia lohachata* was almost equal to 3. According to **Tesch (1971)** and **Wootton (1990)** 'b' value depends on many factors like habitat, degree of stomach fullness, gonadal maturity; sex and so on. Similar observations were made by authors like **Islam *et al.*, (2012)** on *Sillaginopsis panijus* Bangladesh; **Jan *et al.*, (2014)** on *Schizothorax plagiostomus* from Kashmir; **Rejitha and Pillai (2015)** on six coral reef fishes and **Dey *et al.*, (2016)** on *Barilius barila***.**

5.2.2. Fecundity and fertilization rate

Absolute fecundity refers to the number of eggs produced per female per year (**Wootton, 1979**), and can also be defined as the number of mature Oocytes present in the ovary immediately before spawning (**Bagenal, 1963**). Fecundity is an important tool to understand the reproductive capacity of a fish species and in regulating the rate of reproduction to changing environmental condition**.** Population size of fish was also dependent on fertilization rate. The percentage of fertilization depended on the quality of brood stock.

Average fecundity of *Botia* species was *Botia almorhae* (18539 ± 3828), *Botia dario* (22573 ± 4949), *Botia lohachata* (18053 ± 7331) and *Botia rostrata* (18698 ± 2772). The average fertilization rate had *Botia almorhae* (90.03%), *Botia dario* (82.09 %.), *Botia lohachata* (95.98%) and *Botia rostrata* (67.60%).

In the present study it was observed, that among the four *Botia* loaches *Botia dario* had high fecundity and *Botia rostrata* the lowest fecundity **Dey *et al.*, (2015b, 2015c** and **2015d)**. It was also found that individual fecundity increased with body weight and length **Dey and Barat (2015)**. This finding was supported by workers like **Nikolsky(1969); Guar and Pathani (1996); Islam *et al.*, (2012)** and **Dey *et al.*, (2016).**

The Coefficient of Correlation of *Botia* species was between gonad weight and body weight, gonad length and body length and fecundity and body weight; *Botia almorhae* (0.819, 0.611 and 0.848); *Botia dario* (0.948, 0.410 and 0.676); *Botia lohachata* (0.70, 0.865 and 0.832) and *Botia rostrata* (0.889, 0.949 and 0.956) respectively.

All the Coefficient of Correlation (r) among all relationships of *Botia* species showed significance at $p \leq 0.01$. All correlation of *Botia* species showed that fecundity increased with the gonad weight, gonad length, body weight and body length. These findings were supported by **Simpson (1951); Bagenal (1957); Sarkar *et al*.,(2004)** and **Mahapatra *et.al*, (2004).**

The fertilized eggs were transparent and unfertilized ones were opaque and white. Fertilization rate of *Botia* loaches in captivity was high. Highest fertilization was observed in *Botia lohachata*.Similar type of captive breeding and fertilization rate were reported by **Kimmel *et al.(*1995)** on *Danio rerio*, **Udit *et al*. (2014)** on *Puntius sarana*, **Dey *et al*. (2014)** on *Devario aequipinnatus* and **Dey *et al*., (2016)** on *Barilius barila.*

5.2. 3. Captive breeding standardization

The loaches of *Botia* species are good candidate species for ornamental fish. They are near to the door of extinction due to indiscriminate fishing. Loaches do not breed spontaneously in captivity and as such breeding technique was developed with the help of synthetic hormone in captivity. Artificial breeding of wild spawners under controlled conditions is often implemented as a conservation method for endangered freshwater fish species, to produce fry for the enhancement or restoration stocking (**Philippart, 1995; Poncin and Philippart, 2002).**

Four different doses of WOVA-FH hormone (0.5 ml/kg as1st dose, 0.25 ml/kg as 2nd dose, 0.025ml/Fish as 3rd dose and 0.0125 ml/fish as 4th dose) were used, with the

best response to reproduction obtained from the dosage of WOVA-FH of 0.025 ml/ fish. Among the four different doses 3rd dose had appropriate for the captive breeding of *Botia* loaches. The higher fertilization, hatching and survival rates were found in fish injected with 0.025 ml/fish. Same dose of WOVA-FH (0.025 ml/fish) hormone was injected to both male and female.

Spawning was observed after 4-5 hours in Set-up-3, 7, 11 and 15 which fishes were injected 0.025 ml/ fish of WOVA-FH. The present study demonstrated the successful breeding of four species of genus *Botia* in captive condition with little dose 0.025ml/fish WOVA-FH (**Dey and Barat, 2015a).**

The present study revealed that flowing water was essential for induced spawning of *Botia species*. The spawning behaviour of *Botia species* was similar to the Indian Major Carps like flowing water systems. *Botia* species easily matured and bred successfully under captive condition **Dey and Barat (2015)** on *Botia almorhae*; **Dey *et al.*, (2015d, 2015c and 2015b)** on *Botia dario, Botia lohachata* and *Botia rostrata.*

The latency period is described as the time interval between injection of hormone on a female fish and starting the spawning (egg release by the female) of fish. In the present investigation, latency period of *Botia almorhae* was between 05.00 to 05.30 hours; for *Botia dario* it was 5 to 6 hours; *Botia lohachata* was 4 to 5 hours and in *Botia rostrata* was between 4.30 and 05.00 hours in fish injected with a dosage of 0.025ml WOVA-FH per fish respectively. It was observed that latency period of *Botia* species was short than other species. *Botia dario* was highest latency period than other three species. Similar types of finding was reported by **Udit *et al.*, (2014**) on *Puntius sarana;* (**Purkayastha *et al.*, 2012**) on *Ompok pabda* and **Dey *et al.*,(2016)** on *Barilius barila*.

According to **Kiyzhanovsky (1949),** spawning type of *Botia* species was Pelagophils (fishes which spawn freely in column of water and the eggs float). *Botia* loaches spawn once in a year (**Dey and Barat, 2015b; Dey *et al.*, 2015d, 2015c and 2015b**). Similar type of captive breeding of ornamental fishes was reported by many authors *Danio rerio* **(Kimmel *et al.*,1995);** *Macrognathus aculeatus* (**Das and Kalita, 2003**); *Etroplus suratensis* **(Bindu, 2006);** *Puntius gelius* **(Sarma,2008**); *Devario aequipinnatus (***Dey *et al.*, 2014**) and *Sahyadria denisonii* (**Sajeevan *and* Anna Mercy, 2016**).

5.2.4. Breeding behaviour study of fish

Spawning behaviour was observed during the night or afternoon in absence of light. Male fishes were more actively involved in spawning. At the time of spawning, they made a loud cracking sound repeatedly. Six types of breeding behaviour were observed during spawning time like a) male hitting the female on snout, b) male hitting the female fish in the vent region more frequently, c) fighting between the males, d) male chasing the female, e) male and female fish were embraced together and swimming and f) Cannibalism behaviour. In the present investigation, no jumping activity on the surface, no nest building activity and did not showed any types of parental care of *Botia* species. Both male and female exhibited swimming movements in pairs, circling and pushing the female on the abdomen during courtship and similar type of study was done by **Guthric and Mutz (1993)** on *Brachydanio rerio*; **Kharbuli *et.al,* (2004)** on *Danio aequipinnatus;* **Angami (2012)** on *Danio dangila* and *Puntius chola*

The male initiates the motivation for courtship and in the process nudges the female with the snout and pushes the female upwards and then bends down and brings its genital pore in proximity with the female's genital pore enticing and interlocking the

female with the pelvic and anal fin. This study was supported by **Angami (2012).** Similar type of breeding behaviour was observed in other fishes by many workers like, **Hutchings *et al.*, (1999)** on *Gadus morhua*; **Anna Mercy *et al.*, (2003)** on *Pristolepis marginata;* **Chandran *et al.*, (2013)** studied on breeding behaviour of *Pseudosphromenus cupanus;* **Paray *et al.*, (2013)** on breeding behavior of the *Channa striatus* and **Behera *et al.*, (2016)** observed pairing and chasing courtship behaviour of climbing perch, *Anabas testudineus.*

5.2.5. Embryonic development

The colour of fertilized eggs was whitish and transparent initially and then changed to creamy as the embryonic development proceeded. The embryonic development of *Botia* species were divided into eight stages-Zygote, Cleavage, Blastula, Gastrula, Segmentation, Pharyngula, Hatching and Early larval period **(Dey and Barat, 2015; Dey *et al.*, 2015b; 2015c and 2015d).**

In the present study, egg incubation period ranged between *Botia almorhae* (15.30 and 16.00 hours), *Botia dario* (14.30 to 14.40 hours), *Botia lohachata* (14.00 to 14.30 hours) and *Botia rostrata* (15 to 15.30 hours). The incubation period was also lower than other species **(Dey and Barat, 2015**; **Dey *et al.*, (2015b, 2015c and 2015d).**

The first cleavage occurred at 28 minutes (*Botia almorhae*), 25 minutes (*Botia dario*), 24 minutes (*Botia lohachata)* and 26 minutes (*Botia rostrata*) after the eggs were fertilized of *Botia* species. First cleavage formed in *Botia* species within 28 minutes and this development took less time than other ornamental fish. similar type of observation was made by **Kimmel *et al.* (1995)** after 40 minutes in *Danio rerio;* **Udit *et al.* (2014)** reported first cleavage occurred after 30 minutes in *Puntius sarana*, **Dey *et al.* (2014)**

after 45 min. in *Devario aequipinnatus* and Dey *et al.,* (2016) after 34 minutes in *Barilius barila*.

5.2.6. Supplementary feed for larval rearing of fish

According to **Nikolsky (1963),** juveniles of *Botia* species are "euryphagic" because they consume different types of food. At first larvae, *Paramecium* sp. were fed and then *Artemia* after 3days, the larvae consumed small sized zooplanktons of *Botia* species in captivity **Dey *et al.*, (2015b, 2015c and 2015d)** and **Dey and Barat (2015).**

In the present study, good growth was observed in Tank-D (only minced snail or bivalve flesh fed) than other experimental tanks where Tank-A was fed only commercial fish feed, Tank-B: live zooplanktons and Tank-C: boiled minced meat. The growth rates were similar in Tank B and Tank C. Lowest growth rate was observed in Tank-A. Similar type of observation was reported on ornamental fish culture by **Karim and Hossain (1972)** on *Mastacembelus pancalus;* **Serajuddin and Mustafa (1994)** on *Mastacembelus armatus*; **Das and Kalita (2003)** on *Macrognathus aculeatus*; **Williot *et al.*, (2009)** on *Acipenser sturio;* **Rahman and Awal (2016)** on *Channa striatus*; **Ghosh *et al.*, (2016)** on Clownfish, *Amphiprion Clarkii*.

5.3. HISTOLOGICAL STUDY OF THE FISH GONADS

Immature ovary of *Botia* species are small, elongated, thread like and translucent without visible oocytes. Immature stage of ovary of *Botia* species was seen from February to March.

The pre-spawning phase or developing phase of ovary was found to be during March to May. Developing stage of testis of *Botia* species were found from April to May. Spawning phase of ovary of *Botia almorhae, Botia lohachata* and *Botia rostrata* were found to be during June to August and *Botia dario* was found to be during May to July.

The testes were milky whitish, long and flat, narrower behind, ribbon-like and increased in size. Spawning phase of testis of *Botia* species were found during May to September.

Post ovulated ovary of *Botia* species were elongated, thread like and transparent. Post-spawning phase of ovary of *Botia almorhae, Botia lohachata* and *Botia rostrata* were found to be during September to October. Post ovulated ovary of *Botia dario* was found to be during August to October. The Post spawning stage of testis of *Botia* species were found from October to February. Histological features of post spawning stage of testis were presence of spermatogonia (SG). Spermatogonia(SG) and Residual spermatozoa(RSP). This finding was similar to (**Sanwal and Khanna 1972**).

In the present investigation it was found that gonad developmental stages were similar Indian major carp or minor carps.Present study was similar with other works done by **Roy and Manda (2015)** on *Labeo bata*; **Chakraborty and Choudhury (2015)** on *Notopterous notopterous;* **El-Nasr (2016)** on *Gerres filamentosus* from Hurghada Red Sea, Egypt; **Mahmud *et al.*, (2016)** on *Channa striata* from Bangladesh; **Silva *et al.*, (2016)** on *Cynoscion leiarchus;* and **Sales *et al.*, (2016)** on *Hypostomus francisci* from Brazil.

5.4. DNA BARCODING OF FISH

The blast search analyses of sequences were carried out for strengthening of the sequenced data. The phenotypical identification of the present studied species of *Botia* showed 100% similarity with same species sequence in Genbank. **Hebert *et al*., (2004)** proposed a concept; a short nucleotide sequence of mitochondrial genome will act as a DNA barcode of species identification of eukaryotic in particular animals. The technology has proven to be a rapid tool for precise identification of biological specimens. DNA barcoding works under the principle that interspecies variations are

greater than the intraspecies variations allowing one to distinguish the species using nucleotide sequences. Six hundred fifty (650) nucleotide bases of 5 Cytochrome C oxidase sub – unit I gene (COI) have been accepted as universal barcode to delineate animal life in this planet. Identification of juveniles and immature stages of loach are very difficult using traditional taxonomic approach. Therefore, molecular phylogenies help resolve the taxonomic confusion of species.

In the present investigation, evolutionary distances among *Botia* genus ranged from 0.004 to 0.200. The interspecies Kimura's 2- parameter pair-wise distance was highest (0.200) between *B. modesta* and *B. lohachata* and lowest (0.004) for *B.almorhae* and *B. lohachata*. Best fit models for COI dataset was Hasegawa-Kishino-Yano (HKY+ I) model for different population of *Botia* and closely related species such as *B. lohachata* and *B. almorhae* .

The nucleotide sequences of COI gene were aligned in order to determine the phylogenetic relationship among 6 species of *Botia*. The phylogenetic tree showed that *B. almorhae* and *B. lohachata* formed a monophyletic group (supported by 100% bootstrap value) and then constituted one clade with *B. kubotai*. Other Asian species, *B. rostrata, B. striata, B. dario B. modesta and B. macrocanthus* also contributed to this clade but are distant to native *Botia* species**.**

The Barcode ID number of four *Botia* species was **SDP657007-17** (*Botia almorhae*), **SDP657005-17** (*Botia dario*), **SDP657002-17** (*Botia lohachata*) and **SDP657006-17** (*Botia rostrata*). The present study thus highlighted the validity of DNA barcoding to differentiate the loaches at the species level and helped to understand the loaches in different reaches of rivers of Terai region of West Bengal.

DNA barcoding of fishes in different parts of the globe has gained momentum and is well established in Australia **(Ward *et al.*, 2005).** In Indian waters, similar types of findings were reported on barcoding by **Lakra *et al.*, (2009, 2011); Chandra *et al.*, (2012); Ambili *et al.*, (2014); Persis *et al.*, (2009); Ajmal *et al.*, (2010); Ajmal et al., (2011); Akbar *et al.*,(2010); Prasanna *et al.*,(2011) and Kannan *et al.*, (2014).**

5.5. ICHTHYOFAUNA DIVERSITY OF RIVER KALJANI

Kaljani river originating from Eastern Himalaya has the richest fish diversity among all other rivers of Cooch Behar district. The present study revealed, 138 fish species belonging to 31 families in the river **(Dey *et al.*, 2015a)**.

The most dominant fish families contributing to the study were Cyprinidae (50 species) and Sisoridae(14 species). The less dominant family than Cyprinidae were Bagridae (11 species) and Cobitidae (8 species). The families Belontiidae, Channidae, and Schilbeidae contributed to 6 species. Mastacembelidae represented 4 species and Balitoridae, Badidae and Siluridae represented 3 species each. Ambassidae, Amblycipitidae,Clupeidae and Notopteridae contributed 2 species whereas, other families Anabantidae, Anguillidae, Aplocheilidae. Belonidae, Chacidae, Clariidae, Engraulididae, Gobiidae, Heteropneustidae, Mugilidae, Nandidae, Ophichthidae, Pangasiidae, Synbranchidae, Syngnathidae and Tetradontidae all contributed 1 species each. Among the 138 species, 55 species had food value, 58 species ornamental value and 25 species both ornamental and food value.

The evaluation of conservation status of the fishes and the results of the present study also revealed that 25.36% of the fishes belonged to the lower risk near threatened (LRnt), 29.71% to vulnerable (VU), 29.71% lower risk least concern (LRlc) 2.17% not evaluated (NE), 9.42% endangered (EN), 0.72% critically endangered (CEN) and 2.89%

data deficient (DD) category). Month wise availability of fish species were high in the months of November (2012) to April (2013) and September (2013). Chhat Bhelakopa (Site -4) had the richest diversity than the other sites. *Pangasius pangasius*, a critically endangered species was found in this region. *Tenualosa toil*, the Chinese herring, was also found at Chhat Bhelakopa (Site-4) during monsoon only.

In the present study an attempt had been made to explore the available indigenous ornamental fish fauna of West Bengal. Ornamental fishes were dominant over the food fishes. All the three types of feeding habits of fishes namely carnivorous, omnivorous and herbivorous were available in this region. About 97 species of fishes are carnivorous, 28 species are omnivorous and 13 species are herbivorous fish.

Similar findings had been reported by **Ghosh and Lipton (1982)** where, 172 species were noted from North East India; **Choudhury (2005)** reported 297 fish species; **Goswami *et al.*, (2012)** recorded 422 species from North East India; **Mahapatra *et al.*, (2015)** reported 190 fish species from West Bengal; **Acharjee *et al*. (2013, 2014a and 2014b)** reported 65 species from Teesta river, 25 species from river Relli and 20 species of loaches from Darjeeling Himalaya; **Dey *et al.*, (2015f)** reported 141 species from the three districts of Eastern Himalayan region; **Dey and Sarkar (2015)** recorded 107 species from Torsa river; **Das (2015)** recorded 105 species from Torsha and **Debnath (2015)** recorded 73 species from Gadadhar river, Cooch Behar, respectively.

SUMMARY AND CONCLUSION

6. SUMMARY AND CONCLUSION

The present thesis entitled "**Breeding behaviour, embryonic development and barcoding of the ornamental loaches (Cobitidae: Cypriniformes) of Terai region of West Bengal, India"** comprises of 7 chapters (Introduction, Review of Literature, Materials and Methods, Results, Discussion, Summary and Conclusion and Bibliography) was executed for a period of three years (August 2012 to July 2015). With the following objectives of the study, 1) to estimate the limnochemistry of River Kaljani, 2) standardize breeding behaviour, 3) embryonic development, 4) captive breeding techniques and 5) barcoding of Endangered and Vulnerable *Botia* loaches. The following important findings of the thesis are presented:

- Four sampling area in the river Kaljani namely Amlaguri, Chhatoa, Jaigir Chilakhana and Chhat Bhelakopa were selected for collection of water samples and fishes. Physico-chemical parameters were studied in river Kaljani and Captivity (in aquariums) or Laboratory for rearing of *Botia* loaches.
- The average Physio-Chemical parameters of River Kaljani recorded were air temperature (30.5^0C), water temperature (28.45^0C), pH(7.82), Specific Conductivity (151.27 μS cm^1), Total Dissolved Solids (135 mg L^{-1}), Dissolved Oxygen (10.98 mg L^{-1}), Free Carbon Dioxide (3.76 mg L^{-1}), Total Alkalinity (69.78 mg L^{-1}), Total Hardness (24.0 mg L^{-1}), Ammonium-nitrogen (NH_4-N) (0.017 mg L^{-1}), Nitrite-nitrogen (NO_2-N) (0.009 mg L^{-1}), Nitrate-nitrogen (NO_3-N) (0.312 mg L^{-1}) and Phosphate-phosphorous (PO_4-P) (0.101 mg L^{-1}).
- The mean of Laboratory (captive) Physico- Chemical parameters were air temperature (29.54^0C), pH(7.88), Specific Conductivity (246.14 μS cm^1), Total Dissolved Solids (255 mg L^{-1}), Dissolved Oxygen (6.3 to 7.6 mg L^{-1}), Free

carbon dioxide (6.67 mg L^{-1}), Total Alkalinity (54.68 mg L^{-1}), Total Hardness (27.29 mg L^{-1}), Ammonium-nitrogen (NH_4-N) (0.002 mg L^{-1}), Nitrite-nitrogen (NO_2-N) (0.003 mg L^{-1}), Nitrate-nitrogen (NO_3-N) (0.215 mg L^{-1}) and Phosphate-phosphorous (PO_4-P) (0.172 mg L^{-1}) .

- In the present study, for both river Kaljani and Captive study water was soft, alkaline in nature with high Dissolved Oxygen and medium productive condition. The temperature was good for growth of the fishes of Kaljani river water and Laboratory water (Captive condition). TDS of the sampling water ranged from 80 mg $^{L-1}$ to 260 mg $^{L-1}$ which was within limits **(BSI, 1991).** Lowest value of Free Carbon Dioxide was found in July due to high rainfall and highest in December probably due to high decomposition load in river Kaljani. The average concentrations of ammonium-nitrogen (0.017 mg L^{-1}), nitrite-nitrogen (0.009 mg L^{-1}) and nitrate-nitrogen (0.312 mg L^{-1}) were negligible. Phosphate-phosphorou**s** concentration in the Kaljani river and captive condition were also within normal range (0.101 mg L^{-1}).
- The average Gonado-somatic Index (GSI) data of *Botia* species revealed to be *Botia almorhae* (11.96±10.29), *Botia dario* (8.34±5.4), *Botia lohachata* (13.86±11.50) and *Botia rostrata* (10.29±9.01). Among the *Botia* species, *Botia lohachata* had the highest GSI and *Botia rostrata* the lowest. The Coefficient of Correlation (r) between Gonado-somatic Index of female and male of *Botia* species were, *Botia almorhae* (0.87), *Botia dario* (0.99), *Botia lohachata* (0.949) and *Botia rostrata* (0.817). The results showed significance at $p \leq 0.01$ and were positively correlated. It was further revealed that GSI increased with increase of the gonad size of *Botia* species. The peak GSI values were found only during breeding season. This confirmed that *Botia* species breeds only once a year. *Botia*

almorhae, Botia lohachata, Botia rostrata spawned from June to August whereas; in *Botia dario it* was from May to July.

- Condition Factor or K- factor in Captive condition for *Botia almorhae* was 1.390, *Botia dario* was 1.788., *Botia lohachata* was 1.538 and *Botia rostrata* was 1.399. The values indicated good general condition of fish as 'K' was greater than 1.0.Among the four *Botia* loaches *Botia dario* had highest Condition Factor and this was also revealed by its health condition (robustness).
- Length- Weight relationship gives us history and morphological comparisons between different fish species or between different fish by the Least-Square Method from logarithmic data, and the association of degree between weight-length variables can be calculated by the determination of Coefficient of Correlation (r). The Coefficient of Correlation of *Botia almorhae* was 0.811; *Botia dario*: 0.802; *Botia lohachata*: 0.753 and *Botia rostrata*: 0.936. The coefficient of correlation (r) of *Botia rostrata* was more significant than other species. The Coefficient of Correlation (r) showed significance at $p \leq 0.01$. *Botia almorhae* (b=4.027), *Botia dario* (b=4.005) and *Botia rostrata* (b=3.138) indicated positive allometric growth because all values were higher than 3.0. These results suggested that all species show positive allometric growth and that the fish grows in proportion to the length in Captive condition.
- Average fecundity of *Botia almorhae, Botia dario, Botia lohachata* and *Botia rostrata* were 18539, 22573, 18053 and18698 respectively and fertilization rate 90.03%, 82.09 %, 95.98 % and 67.60 % respectively
- In the present study it was observed that among the four *Botia* loaches *Botia dario* had high fecundity and *Botia rostrata* the lowest fecundity. It was found that individual fecundity increased with body weight and length.

- *Botia* loaches are near to the door of extinction due to indiscriminate fishing for its high ornamental value. These loaches do not breed spontaneously in captivity. Breeding technique was developed with the help of synthetic hormone in captivity. Four different doses of WOVA-FH hormone (0.5 ml/kg as1st dose, 0.25 ml/kg as 2nd dose, 0.025ml/Fish as 3rd dose and 0.0125 ml/fish as 4th dose) were used, and the best response to reproduction was obtained from the dosage of WOVA-FH of 0.025 ml/ fish. The higher fertilization, hatching and survival rates were found in fish injected with 0.025 ml/fish in Set-ups- 3, 7, 11 and 15. Same dose of WOVA-FH hormone was injected to both male and female
- The latency period of *Botia* species were; *Botia almorhae* (05.00 to 05.30 hours), *Botia dario* (5 to 6 hours), *Botia lohachata* (4 to 5 hours) and *Botia rostrata* (4.30 and 05.00 hours) for fish injected with a dosage of 0.025ml WOVA-FH per fish. It was observed that latency period of *Botia* species was shorter than other species. *Botia dario* had the highest latency period than the other three species.
- Spawning behaviour was observed during the night or afternoon in absence of light. Male fishes were more actively involved in spawning. At the time of spawning, they made loud cracking sound repeatedly. Six types of breeding behaviour were observed during spawning time like a) male hitting the female on snout, b) male hitting the female fish in vent the region more frequently, c) fighting between the males, d) male chasing the female, e) male and female fish were embraced together and swam and f) Cannibalism behaviour.
- The colour of fertilized eggs was whitish and transparent initially and then changed to creamy as the embryonic development proceeded. The embryonic development of *Botia* species was divided into eight stages-Zygote, Cleavage, Blastula, Gastrula, Segmentation, Pharyngula, Hatching and Early larval period.

In the present study, egg incubation period ranged as follows: *Botia almorhae* (15.30 to 16.00 hours), *Botia dario* (14.30 to 14.40 hours), *Botia lohachata* (14.00 to 14.30 hours) and *Botia rostrata* (15 to 15.30 hours). The incubation period was also lower than the other species. The first cleavage occurred at 28 minutes for *Botia almorhae*, 25 minutes for *Botia dario*, 24 minutes for *Botia lohachata* and 26 minutes for *Botia rostrata* after the eggs of *Botia* species were fertilized. First cleavage formed in *Botia* species within 28 minutes and this development took less time than other ornamental fishes reported.

- At first, larvae were fed with *Paramecium* sp. then *Artemia* after 3 days. The larvae consumed small sized zooplanktons of *Botia* species in captivity. In the present study, good growth was observed in Tank-D (only minced snail or bivalve flesh fed) than other experimental tanks where Tank-A was fed only commercial fish feed. Tank-B with live zooplanktons and Tank-C with boiled minced meat. The growth rates were similar in Tank B and Tank C. Lowest growth rate was observed in Tank-A.
- After captive breeding of wild *Botia* species, F_1 generation of *Botia* loaches were aqua-ranched into the natural environment of the river system.
- The pre-spawning phase or developing phase of ovary was found to be during March to May. Developing stage of testis of *Botia* species was found from April to May. Spawning phase of ovary of *Botia almorhae, Botia lohachata* and *Botia rostrata* were found to be during June to August and *Botia dario* was found to be during May to July. The testes was milky whitish, long and flat, narrower behind, ribbon-like and increased in size. Spawning phase of testis of *Botia* species was found during May to September. Post-spawning phase of ovary of *Botia almorhae, Botia lohachata* and *Botia rostrata* was found to be during September

to October. Post ovulated ovary of *Botia dario* was found to be during August to October. The Post spawning stage of testis of *Botia* species was found to be from October to February.

- In the present investigation, evolutionary distances among *Botia* genus ranged from 0.004 to 0.200. The interspecies Kimura's 2- parameter pair-wise distance was highest (0.200) between *B. modesta* and *B. lohachata* and lowest (0.004) for *B. almorhae* and *B. lohachata.*
- The phylogenetic tree showed that *B. almorhae* and *B. lohachata* formed a monophyletic group (supported by 100% bootstrap value) and then constituted one clade with *B. kubotai*. Other Asian species *B. rostrata, B. striata, B. dario B. modesta and B. macrocanthus* also contributed to this clade but are distant to native *Botia* species.
- The Barcode ID number of four *Botia* species was **SDP657007-17** (*Botia almorhae*), **SDP657005-17** (*Botia dario*), **SDP657002-17** (*Botia lohachata*) and **SDP657006-17** (*Botia rostrata*). The present study thus highlighted the validity of DNA barcoding to differentiate the loaches at the species level and helped to understand the loaches in different reaches of rivers of Terai region of West Bengal.
- The present study permitted to study the Ichthyodiversity of river Kaljani, Cooch Behar district, West Bengal, 138 fish species were recorded which belonged to 31 families. The most dominant of the fish families contributing to the study was Cyprinidae: 50 species and Sisoridae: 14 species. The less dominant family than Cyprinidae was Bagridae contributing 11 species and Cobitidae: 8 species. Among the 138 species, 55 species had food value, 58 species ornamental value and 25 species both ornamental and food value.

- The evaluation of conservation status of the fishes and the results of the present study revealed that 25.36% of the fishes belonged to lower risk near threatened (LRnt), 29.71% vulnerable (VU), 29.71% lower risk least concern (LRlc), 2.17% not evaluated (NE), 9.42% endangered (EN), 0.72% critically endangered (CEN) and 2.89% data deficient (DD) category, respectively. Month wise availability of fish species were high in the months of November (2012) to April (2013) and also September (2013). Chhat Bhelakopa (Site -4) had the richest diversity than the other sites. *Pangasius pangasius* is a critically endangered species, found in this region. *Tenualosa toil, a* Chinese herring, was also found at Chhat Bhelakopa (Site-4) only during monsoon. About 97 species of fishes are carnivorous, 28 species are omnivorous and 13 species are herbivorous.

It may be concluded from the present study, that Physico-Chemical parameters of River Kaljani in Cooch Behar district was not polluted and good for propagation of *Botia* loaches which are highly sensitive to temperature and water pollution. *Botia* species can be easily matured and bred successfully under captive conditions with the help of synthetic hormone WOVA-FH. The present study also highlighted the validity of DNA barcoding to differentiate the loaches at the species level and helped to understand the loaches in different reaches of rivers of Terai region of West Bengal. *Botia* species are near to the door of extinction due to indiscriminate fishing for its high ornamental value. So, establishment of proper sanctuaries in the selected areas of Terai and Dooars rivers, floodplain and reservoirs is recommended for conservation of this species. This thesis is useful for fish breeders, aquarium keepers and those involved in or interested in the study of indigenous fish.

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LIST OF PUBLICATIONS

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2. **Dey A., Sarkar D. and Barat S. (2015).** Spawning biology and proper dose of hormone for captive breeding of Vulnerable fish, *Botia rostrata* (Gunther), in Cooch Behar, West Bengal, India. *International Journal of Applied Research*, 1(11):767-768.
3. **Dey A., Sarkar D. and Barat S. (2015).** Spawning biology, embryonic development and rearing of Endangered loach *Botia lohachata* (Chaudhuri) in captivity. *International Journal of Current Research*. 2015b; 7(11):22208-22215.
4. **Dey A., Sarkar D. and Barat S. (2015).** Spawning biology, embryonic development and captive breeding of Vulnerable loach *Botia dario* (Hamilton). *Journal of Entomology and Zoology Studies*, 3(6):183-188.
5. **Dey A., Sarkar D., Singh M. and Barat S. (2017).** DNA barcoding of four ornamental fishes of genus *Botia* from Eastern Himalaya. *International Journal of Science and Research*, 6(6): 608-611.

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Research Article

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Ichthyofauna Diversity of River Kaljani in Cooch Behar District of West Bengal, India

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ABSTRACT

The present study was conducted to generate a primary database on ichthyofauna diversity of river Kaljani flowing through Cooch Behar district of West Bengal, India. 138 indigenous fish species belonging to 31 families were identified. The family Cyprinidae represented the largest diversity accommodating 20 genera and 50 species. Amongst all the fishes 58 species have ornamental value and 55 species the food value. Ornamental fishes are dominant over the food fishes and carnivorous fishes are dominant over the omnivorous and herbivorous fishes. According to IUCN (International Union for Conservation of Nature) and CAMP (Conservation Assessment and Management Plan) the conservation status of the fishes are listed as, 1(0.72%) species as Critically Endangered,13(9.42%) species as Endangered 41(29.71%) species as Vulnerable, 35 (25.36%) species as at Lower Risk Near Threatened, 41(29.71%) species as Lower Risk Least Concerned,4 (2.89%)species as Data Deficient and 3(2.17%) species as Not Evaluated. It is concluded, that anthropogenic pressure arising out of agriculture run offs, indiscriminatory use of fishing with new fishing technologies and widespread habitation of people have contributed to the vulnerability of the fish diversity.

Keywords:*Ichthyofauna diversity, Kaljani river, Cooch Behar, Ornamental fish, Conservation status.*

INTRODUCTION

Cooch Behar district being situated near the state of Assam, and lying between 25^0 57′47″ to 26^0 36′2″ North latitude and between 89^0 54′35″ to $88^0$47′44″ East longitude, is unique in its topography and climatic characteristics. It has a total water stretch of approximately 6121 ha including hill stream rivers, beels and others aquaculture resources and fishes are invariable living components of these water bodies. These organisms are important food resources and good indicators of the ecological health of the waters they inhabit. The diversity within the fresh water ecosystem has a great importance in terms of the livelihood and the economic importance of the people living around it. Accordingly the relation between the biodiversity and human well-being is inter-related and is being promoted increasingly through the concept of ecosystem services provided by the species. Biodiversity is essential for stabilization of ecosystem, protection of overall environmental quality for understanding intrinsic worth of all species on the Earth [1]. The Cooch Behar district shows close similarities with the North Eastern States of India, particularly Assam, in terms of its richness and magnificent biodiversity. The North Eastern region of India is considered to be one of the major hotspots of freshwater fish biodiversity in the world[2]. Earlier studies report 230 fish species from the North Eastern India by Sinha[3] and 422 species reported from North East India by Goswami *et al*.,[4] Kar *et al*.,[5] also reported 69 species from North Eastern India.

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RESEARCH ARTICLE

SPAWNING BIOLOGY, EMBRYONIC DEVELOPMENT AND REARING OF ENDANGERED LOACH, *BOTIA LOHACHATA* (CHAUDHURI) IN CAPTIVITY

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ABSTRACT

Botia lohachata or "Y- loach", an endangered and vulnerable fish, has both ornamental and edible value. Schistosomiasis or snail fever, a serious disease affecting human, domestic animals and wild animals is naturally controlled by the "Y-loach" in eating the freshwater snail and therefore plays a significant role in controlling the disease. The aim of the present study was, therefore, to breed this ornamental fish in captivity, study the embryonic development and conserve the fish in its natural habitat. The fecundity of females ranged from 3,731 to 23,120. The fish spawns in flowing water system at night. Embryonic and post embryonic development was recorded for 45 days. Each fish was given a dose of 0.025ml of WOVA-FH, a synthetic hormone, for induced breeding. The fertilized eggs measuring 0.9-1mm in diameter were observed to be demersal, nonadhesive and optically transparent. The average fertilization rate was found to be 95.98 %.The average Gonado-Somatic Index of female *Botia lohachata* was 24.46 and male 3.2. The embryos hatched after 14.30 h of fertilization from the chorion and measured 2.5 mm in total length. Correlation studies between total length, body weight, gonad weight, gonad length, fecundity and GSI were found to be significant ($p \leq 0.01$). The present work thus contributed to cover the deficient information for embryonic development of *Botia lohachata.* The embryonic development and captive breeding of this fish can play a great role in the conservation and its habitat protection.

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INTRODUCTION

Botia (loaches) is a genus of freshwater fish of the family Botiidae. *Botia lohachata* is an endangered species (CAMP, 1998) and vulnerable (IUCN, 2010) and has both ornamental and economical fish food value. The fishes are very colourful having bright bands, peaceful nature, lesser scales and barbles. The distribution of this tropical loach is native to India, Nepal, Bangladesh and Pakistan. *Botia lohachata* can reach an average length of 11cms and exist up to 10years. They lead a nocturnal life but adapt quickly in captive condition. They feed during the day time in captive condition, and prefer animal feeds like *Daphnia*, worms and Brine shrimp. They are benthic feeder but are also capable of feeding in mid and surface water. The "Y-loach", like many of its relatives, consumes some of the common aquarium type of snails. The fish is sometimes purchased mainly to get rid of infestation of snails in an aquarium.

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The "Y-loach" is a scavenger but does not eat fish wastes. Schistosomiasis, also known as "bilharzias", "bilharziosis" or "snail fever" is a serious disease affecting human and domestic animals as well as wild animals. The intermediate host of the parasite which causes the disease is a species of freshwater snails. The snail eating loach is one of the many natural controls of the freshwater snail population and plays its part in controlling the disease. "Y- loach" makes a cracking sound. This is either produced by forcing air through the gills and may be connected with feeding on the surface of the water or alternately produced by specialized teeth in the throat of the fish that appear to aid in the extraction of snails muscle from their shells.

Literature available on loaches are discrete .Information available show results on spawning biology and fecundity of *Cobitis taenia* (Juchno and Boron, 2006), fecundity of *Botia dario* (Hossain *et al*., 2007), spawning behaviour of *Sabanejewia vallachica* (Bohlen, 2008) and spawning biology of *Botia almorhae* (Joshi and Pathani, 2009) and diversity of loaches in Darjeeling, West Bengal (Acharjee, and Barat,

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Spawning biology, embryonic development and captive breeding of vulnerable loach *Botia dario* (Hamilton)

Dey A, Sarkar D, Barat S

Abstract
Botia dario is an vulnerable species (CAMP, 1998) [4] having both ornamental as well as edible value. Breeding experiments in captivity were conducted successfully for the ornamental fish *B. dario* in May 2013-2015 using synthetic hormone. This fish spawned only in running water system. The fecundity of the females ranged from 13,880 to 27,510. The average fertilization rate was found to be 82.09 %. The average Gonado-Somatic Index of B. *dario* for female was 13.21 and for male 3.4. About 14.30-14.40 hours after fertilization the embryo hatched out from the chorion of the egg. The present work contributed to the deficient information on embryonic development, induced breeding and breeding behaviour of *B. dario*.

Keywords: *Botia dario*, Captive breeding, Embryonic development and Conservation.

1. Introduction
Botia (Indian loach) is a genus of freshwater fish of the loach family Botiidae. It is a large genus with about 20 species. *Botia dario* is one of the most active loach being distributed in Bangladesh, Bhutan and North-East India. *B. dario* known as "Queen loach", with yellow golden stripes on a black background look very attractive. *B. dario* is an vulnerable species (CAMP, 1998) [4] having both ornamental and edible value. Nine species of genus *Botia* were recorded from India (Kottelat, 2004) [13]. One special characteristic of this loach group is the ability to produce a loud "cracking" sound which is commonly heard during feeding time. This sound stems from a special type of pharyngeal teeth that are used to extract snails from their shells. The fish of this genus possess a pair of razor-like spines under their eye sockets. These spines normally lie flat, but may be extended when the loach feels threatened. The snail eating loach is one of the many natural controls of the freshwater snail population and plays a great role in controlling the disease. The proposed study was therefore undertaken in embryonic development, standardizing artificial breeding and fry and fingerling rearing of *B. dario*. There is scanty information available on loaches. Literature available show results on spawning biology and fecundity of *Cobitis taenia* by Juchno and Boron (2006) [11], fecundity of Botia *dario* by Hossain *et al*. (2007) [7], spawning behaviour of *Sabanejewia vallachica* by Bohlen (2008) [3], spawning biology of Botia *almorhae* by Joshi and Pathani (2009) [10] and diversity of loaches in Darjeeling, West Bengal by Acharjee and Barat (2014) [1]. Literature is on behaviour, breeding or conservation aspects of loaches is lacking. These lacunae inspired us investigate the behaviour, embryonic development and conservation of the *Botia dario* loach species which may contribute to some extent to the information database and conservation approach of the natural resource.

2. Materials and methods
Collection and Experimental site
B. dario weighing on an average from 3 gm to 5 gm were collected(May 2013-2015) from sampling sites located at Bhelakopa, Dwitia Khanda of Cooch Behar district, West Bengal, India lying at $26^{0}18$'North latitude and $89^{0}34$'East longitude. After collection the fishes were oxygen packed in sterile polythene bags and kept in cartons for transport to the Fishery Wet Laboratory of Uttar Banga Krishi Viswavidyalaya, Cooch Behar. In the laboratory the fishes were transferred to suitable aquariums for regular rearing and maturation.

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Spawning biology and proper dose of hormone for captive breeding of vulnerable fish, *Botia rostrata* (Gunther), in Cooch Behar, West Bengal, India

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Abstract
Botia rostrata is an vulnerable species (CAMP, 1998) having high ornamental value. Breeding experiments in captivity were conducted successfully for the ornamental fish, *Botia rostrata* using synthetic hormones. Induced breeding of *B. rostrata* with WOVA-FH at 0.025 ml per fish was achieved. Sex ratio 1:1 (male: female) were maintained in two trials. These fish spawned only in running water system. The fecundity of females ranged from 14,103 to 21, 352. The average fertilization rate was found to be 67.60%. The present work contributed to the information lacking on induced breeding and breeding behaviour of *Botia rostrata.*

Keywords: *Botia rostrata,* Fecundity, Captive breeding and Conservation.

1. Introduction
Botia rostrata [1], commonly known as "Ladder Loach", is a vulnerable fish [2] and very rare in the Terai region of West Bengal. *Botia rostrata* belongs to the family Cobitidae and is very beautifully coloured indigenous ornamental fish and has a great value in the ornamental fish market. It is distributed in West Bengal, Assam and Bangladesh. Many ornamental fishes like *D. aequipinnatus*, *D. dangila*, *D. rerio*, *B. bendelisis*, *B. rostrata*, *E.danricus*, *P. shalynius*, *L. guntea*and so on are exported out of the State as an aquarium fish and also consumed by the local populace [3].

2. Materials and Methods
2.1 Collection and experimental site
The sampling sites located at Bhelakopa, Dwitiya Khanda of Cooch Behar district of West Bengal, India which lies at $26^0$18' North latitude and $89^0$34'East longitude. Live fishes were sampled from different sampling sites of Kaljani River. The fishes were packed in polythene bags filled with 1/3 water and 2/3 pure oxygen and then packed in cartons transport to site. The experiments were conducted in the Wet Laboratory in the Fishery laboratory in Uttar Banga Krishi Viswavidyalaya, Cooch Behar, West Bengal, India. The fishes were regularly observed for their maturation.

Fig 1: Matured female fish

Fig 2: Matured male fish

Fig 3: One month old fish

2.2 Fecundity and Fertilization rate
The fecundity of a fish is defined as the number of ova found in the ovary of a female fish prior to spawning was determined. Absolute fecundity was also calculated according to the method of Hartman and Conkle [4] using the expression F=nG/g where, 'F' is Fecundity; 'n' is mean numbers of eggs in all samples, 'G' is weight of ovaries and g is weight of samples. After 1 hour of spawning 2 litre of water and eggs were collected from the hatchery.

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DNA Barcoding of Four Ornamental Fishes of Genus *Botia* from Eastern Himalaya

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Abstract: ***Botia loaches are high demanding species having both ornamental and economical important food value and contribute to a major share of the world market for beautiful coloured indigenous ornamental fish. Their identification is difficult due to morphological variation. Genetic variation amongst the four species namely Botia alomorhae, Botia dario, Botia lohachata and Botia rostrata using mtDNA were studied. Cytochrome Oxidase I (COI) gene (655 bp) was amplified using PCR and sequenced. The pairwise genetic distances among Botia species ranged from 0.002 to 0.112. The interspecies Kimura's 2- parameter pair-wise distance was highest (0.112) between Botia dario and B. lohachata and lowest (0.002) for B.almorhae and B. lohachata. The phylogenetic tree showed that B. almorhae and B. lohachata formed a monophyletic group (supported by 100% bootstrap value). The present study is helpful for identification of the Endangered and Vulnerable Botia species from Eastern Himalayan region and population studies, management and conservation programs.***

Keywords: Genus *Botia*, Cytochrome Oxidase subunit I gene, DNA Barcoding

1. Introduction

Ornamental fishes are called 'living jewels' for their beautiful colour and playful behaviour and are typically small sized; attractive and bizarre shaped in appearance [4]. The tropical ornamental fishes from North Eastern and Southern provinces of India are in great demand in the hobbyists market like loaches, barbs, badis, zebra fishes, catfishes and glass fishes. Terai and Dooars regions of Eastern Himalaya are considered as "hot spot" for fresh water fish biodiversity [3]. A great number of species have been reported from Cooch Behar district on fish biodiversity and 10 species of loaches are available in Cooch Behar district [3]. The fishes of the family Cobitidae are popularly known as 'Loach'. The loaches are high demanding species having both ornamental and economical important food value and contribute to a major share of the world market for beautiful coloured indigenous ornamental fish. Among the loaches, *Botia dario* (Hamilton-Buchanan) commonly known as "Queen loach*"* or "Rani Mach*"*, *Botia rostrata* (Gunther), commonly known as "Ladder loach", are vulnerable fishes [9] whereas, *Botia almorhae* (Grey), commonly known as "Almorha loach*"* and *Botia lohachata* (Chaudhuri), popularly known as "Y-loach*"* or "Tiger loach*"* or "Lohachata*"*, are endangered species [9] are distributed widely in North-East India and Bangladesh. These loaches are considered as Endangered. The Endangered status of the loaches are mainly because of the deterioration of the environment particularly, water quality which may be due to agricultural run-offs or pesticidal effect of tea gardens in the Terai and Dooars regions, and big-water bodies being fragmented into small water bodies; thus drying up the water. Their identification is difficult due to morphological variation especially amongst *Botia almorhae*, *Botia lohachata* and *Botia rostrata.*

DNA barcode is a new tool for taxon recognition and classification of biological organisms based on sequence of a fragment of mitochondrial gene, Cytochrome Oxidase I (COI). In view of the growing importance of the fish, DNA barcoding for species identification, molecular taxonomy and fish diversity conservation is essential [14]. The DNA barcoding is based on a small sequence of about 655bp of mitochondrial gene Cytochrome oxidase subunit I (COI) with universal primers [7]. The present study was, therefore, focussed to establish the genetic variation amongst the four species namely *Botia alomorhae*, *Botia dario*, *Botia lohachata* and *Botia rostrata* using mtDNA and to show the genetic distance between them. No other literature is available on DNA barcoding of the four *Botia* species. These lacunae instigated the present investigation on molecular identification of the above four mentioned *Botia* loach species.

2. Materials and Methods

Sampling site

River Kaljani situated in Cooch Behar district covers a stretch of about 9 Km upto the lower reaches of the river, that is, from Amlaguri in the north to Chhat Bhelakopa in the south. The sampling areas which were divided into four sites and having a distance of 3 km between them included Amlaguri (26^0 34' N latitude and 89^0 58' E longitude), Chhatoa (26^0 32' N latitude and 89^0 58' E longitude), Jaigir Chilakhana (26^0 31' N latitude and 89^0 58' E longitude) and Chhat Bhelakopa (26^0 29' N latitude and 89^0 58' E longitude). Live fishes were sampled from different sampling sites of Kaljani River. The fishes were identified following their general body form, morphometric and meristic characteristics according to Talwar and Jhingran [19] and Jayaram [10].

Paper Presentation in Conferences

1. Participated and presented a paper entitled "Fish Diversity of River Kaljani in Cooch Behar District of West Bengal, India" in the 'Animal Science' section sponsored by DST, Govt. Of West Bengal, at the **22nd West Bengal State Science and Technology Congress – 2015** held during 28th February and 1st March, 2015.

2. Participated and presented a paper entitled "Spawning Biology, Embryonic Development and Rearing of Endangered Loach, Botia lohachata (Chaudhuri) in captivity" in the **International Conference on Aquatic Resources and Sustainable Management** held from 17 to 19 February, 2016 at Science City, Kolkata, organised by Central Calcutta Science and Culture Organisation for Youth.