a) Study Sites:

Sabarmati River

The Sabarmati is the name given to the combined streams the Sabar and Hathmati. The Sabarmati basin extends over states of Rajasthan and Gujarat having an area of 21,674 Sq km. The basin is bounded by Aravalli hills on the north and north-east, by Rann of Kutch on the west and by Gulf of Khambhat on the south. The basin is roughly triangular in shape with the Sabarmati River as the base and the source of the Vatrak River as the apex point. Sabarmati originates from Aravalli hills at an elevation of 762 m near village Tepur, in Udaipur district of Rajasthan. The total length of river from origin to outfall into the Arabian Sea is 371 km. Important industries are textiles, leather and leather goods, plastic, rubber goods, paper, newsprint, automobile, machine tools, drugs and pharmaceuticals etc. have been developed along the course of Sabarmati river whose effluence are the constant source of pollution affecting the biota present in it.

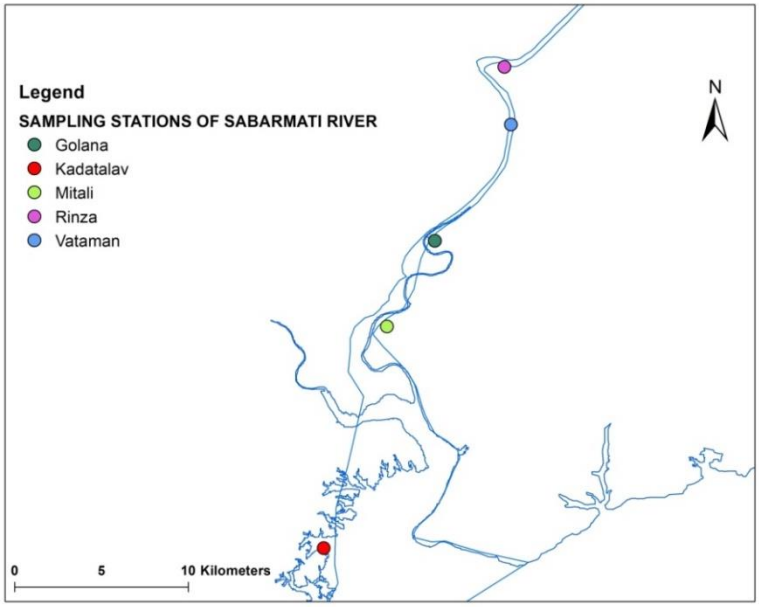

Fig.1. Landing centers of *Tenualosailisha* in Sabarmati River, Gujarat.

Mahi River

The Mahi basin extends over states of Madhya Pradesh, Rajasthan and Gujarat having total area of 34,842 Sq km. It is bounded by Aravalli hills on the north and the north-west, by Malwa Plateau on the east, by the Vindhyas on the south and by the Gulf of Khambhat on the west. Mahi is one of the major interstate west flowing rivers of India. It originates from the northern slopes of Vindhyas at an altitude of 500 m in Dhar district of Madhya Pradesh. The total length of Mahi is 583 km. It drains into the Arabian Sea through the Gulf of Khambhat. The major part of basin is covered with agricultural land accounting to 63.63% of the total area. Hydro Power stations are located in Mahi at Bajaj Sagar dam and at Kadana Dam. Vadodara is the only important urban center in the basin. There are not many industries in the basin.

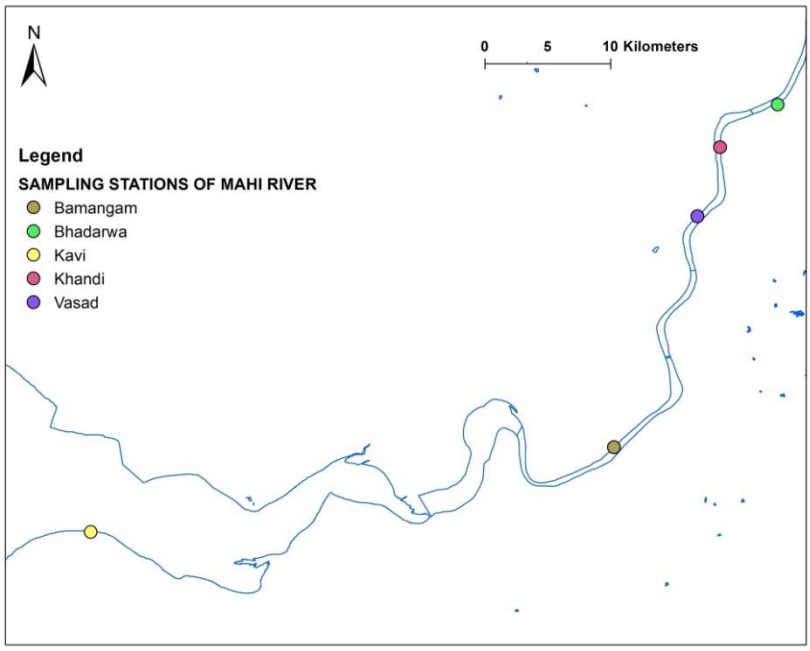

Fig.2. Landing centers of *Tenualosailisha* in Mahi River, Gujarat.

Narmada River

Narmada is the largest west flowing river of the peninsular India. Narmada flows westwards through a rift valley between the Vindhyan Range on the north and the Satpura Range on the south. It rises from Maikala range near Amarkantak in Madhya Pradesh, at an elevation of about 1057 m. Narmada basin extends over states of Madhya Pradesh, Gujarat, Maharashtra and Chhattisgarh having an area ~1 Lakh Sq.km. Its total length from its source in Amarkantak to its estuary in the Gulf of Khambhat is 1,310 km. There are several islands in the estuary of the Narmada of which Alia bet is the largest. The Narmada is navigable upto 112 km from its mouth. *Tenualoshailisha* forms an important fishery in the Narmada estuary and some distance upstream.

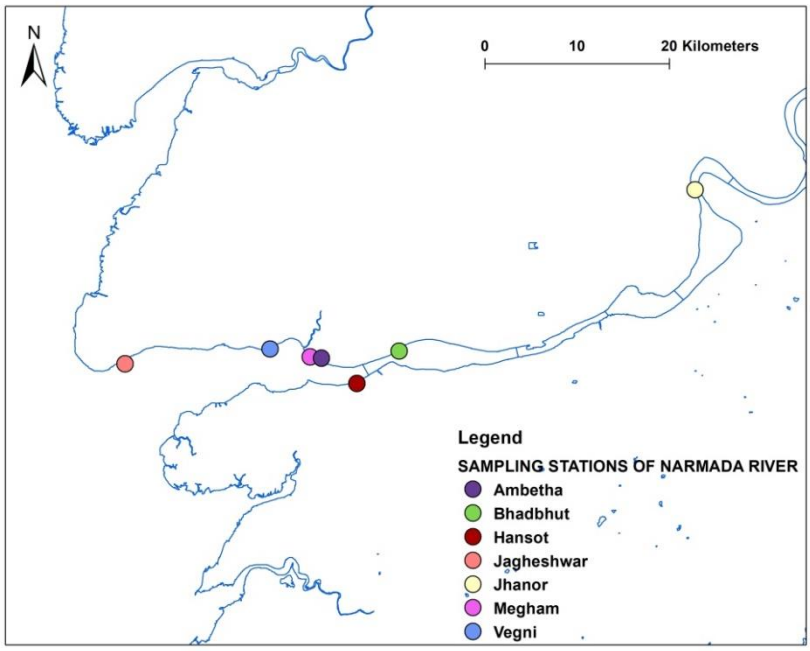

Fig.3. Landing centers of *Tenualosailisha* in Narmada River, Gujarat.

Tapi River

The Tapi is the second largest west flowing river of the Peninsular India and is known as 'the twin' or 'the handmaid' of the Narmada. It originates near Multai reserve forest in Madhya Pradesh at an elevation of 752 m and flows for about 724 km before outfalling into the Arabian Sea through the Gulf of Cambay. The Tapti River along with its tributaries flows over the plains of Vidharbha, Khandesh and Gujarat and over large areas in the state of Maharashtra and a small area in Madhya Pradesh and Gujarat. The basin extends over states of Madhya Pradesh, Maharashtra and Gujarat having an area of ~ 65,000 Sq.km.

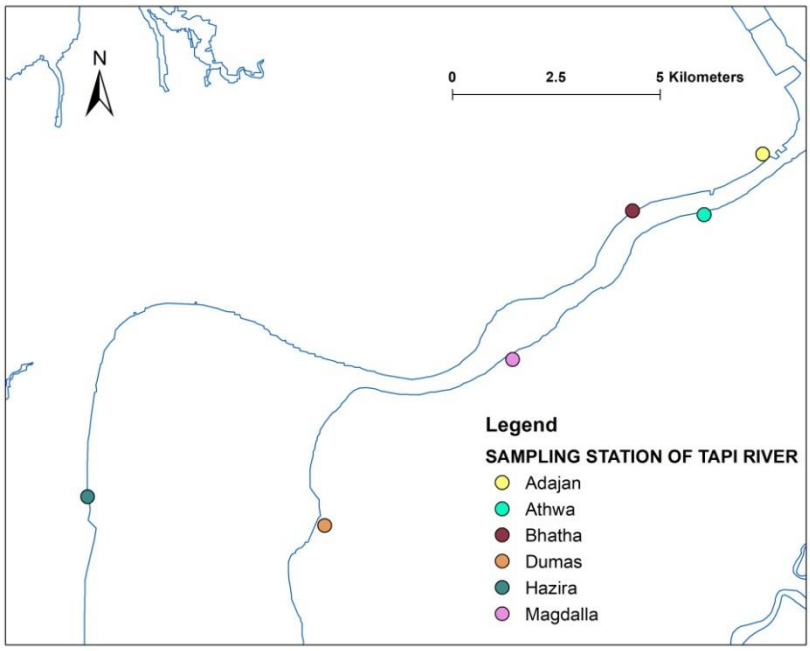

Fig.4. Landing centers of *Tenualosailisha*Tapi River, Gujarat.

b) Nomenclature and systematic position

There are three types of this fish, for example, *Tenualosailisha*, *H. keele* and *H. toli*. Of all these 3 species just *H. ilisha* goes to the upstream of the stream for rearing. Every single other species of Hilsa for example H. *toli* and H. *keele* are marine and furthermore breed in the marine condition. The particular name of this fish ilisha was first proposed by Hamilton- Buchanan in 1822. Hamilton-Buchanan (1822) named it as *Clupeailisha*. Regan (1917) made the new variety Hilsa to incorporate the Hilsa like clupeoids of the Indo-Pacific and gave a clear meaning of *Hilsailisha* and the related species H. *toli* and H. *kanagurta*. Regan's classification had remained a legitimate animal classification until Munro (1955) put out it to other genera. Fowler (1934) proposed the nonexclusive name for this fish as *Tenualosa*. Munro (1955) depicted the marine and freshwater hilsa fish of Ceylon as Tenualosailisha. Bhuiyan (1964) depicted this fish as Hilsailisha. He additionally referenced in his book that Munro named this fish as *Tenualosailisha* gathered from the marine and freshwater condition of Ceylon. Different creators of the sub mainland, for example, Jhingran (1975), Rahman (1989), Talwar and Jhingran (1991) depicted the fish as *Hilsailisha*. Day (1878) detailed that the non-attendance of the spots in the grown-up and just youthful ones have line of them.

Phylum – Chordata

Subphylum – Vertebrata

Superclass – Gnathostomata

Grade – Teleostomi

Class – Actinopterygii

Subclass – Neopterygii

Division –Teleostei

Order – Clupeiformes

Suborder – Clupeoidei

Family – Clupeidae

Sub family – Alocinae

Genus – Tenualosa

Species – *Tenualosailisha* (Hamilton, 1822)

Fig.5. *Tenualosailisha* of different age groups caught off the waters of Bhadbhut village, Narmada River, Gujarat

c) **Studying the spatio-temporal diversity and distribution of *Tenualosailisha***

The secondary data from different literary sources has been used to study the availability of *Tenualosailisha* in different parts of Persian Gulf and South Asian countries. Mapping software like ArcGIS and BHUVAN has been used to design the maps. The diversity of fishes and fishable organisms is very high and economical in Gujarat state owing to the longest coastline in the country. Fisheries in Gujarat constitute for about 70-80% export to China and many South Asian countries garnering a large amount of economic returns to the state. In this objective, the current distribution and spatio-temporal distribution of Hilsa in Gujarat has been discussed. The rivers of Gujarat has been surveyed throughout for the presence and absence of *Tenualosailisha* but the major focus during this work has been laid on Narmada River as it was found to having the highest landing river of Hilsa fisheries. It has a wide scope of dissemination and happens in marine, estuarine and riverine situations.

d) Identification of *Tenualosailisha* by taxonomical and molecular processes

The beginning of taxonomy can be probably traced back to John Ray (1628-1705). Ray introduced the complex grouping system and greatly improved the language description. He used the genus and species method of naming organisms. Prior to that the Greeks and Romans, notably Aristotle (384-322 B.C.) is credited with the introduction of taxonomy and had accurate knowledge of naming accurate organisms, mainly fishes and cetaceans.

Karl Linnaeus (1707-1778) is considered the father of taxonomy. He created a system of keys for recognizing and naming an organism. His first classification of nature (plants, animals and minerals) appeared in 1735 and was accepted by the taxonomical world.

Linnaeus further gave names to the groups naming them as class. The first was class and each was divided into orders, which then were broken down further to genera (genus singular) and species. By 1800 other workers introduced family as a category between order and the genus. Finally, the classes were grouped into higher categories called phyla.

The 10th edition of *SystemaNaturae* is the first publication to adhere strictly to binominal nomenclature, one of the International Rules states that no name published prior to this is valid. The International Rules of Zoological Nomenclature require that whenever a new species is discovered and described the name of the designated species must be called a Holotype on which the species is based. Other species used in describing the new species become paratypes, and the data collected from them is included in the description. It is customary for ichthyologists to give the designated type to (country of origin) museums for permanent preservation. This then can be reviewed and used for research by professional ichthyologists and students.

The work on freshwater systems of the Indian subcontinent started with the British officers of East India Company; they took great interest in natural history of the region; and Hamilton (1822) wrote an account on the aquatic fish diversity

of Ganga in 'The Fishes of the Ganges'. One of the most important contributions to the fisheries studies was made by Francis Day in his book 'Fishes of India' (1875-1878). Literature now available on fishes in Indian subcontinent include Hora's work 1937-1942 and most recent are by Talwar and Jhingran (1991), and Jayaram (1999). Day (1875-1878) was the first to give an account of the freshwater fishes of Western Ghats and suggested that the Indian fresh water fish fauna resembles closely to that of Eastern countries like Burma, China and Malaysia.

Diversity of life on earth is one of most striking aspects of our planet earth; hence knowing number of species on earth is most fundamental questions in science (Camilo *et al*., 2011). It is widely recognized that not only is the biotic diversity on planet Earth currently undergoing a mass extinction, but that the true extent of the extinction is unknown. Although prominent marker taxa such as endangered vertebrates are well studied, most taxa remain to be discovered (Blaxter, 2004).

Molecular taxonomy - DNA taxonomy

Molecular taxonomy or DNA 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.

International units for the identification for the commercial products, having electronic barcodes, are used by the tradesman to get some information about the concerned product. This barcode acts as an id for an article and it varies for each individual item that we purchase. This is actually the “universal product code” method (Savolainen et al. 2005), which is known as a barcode in the retail business. Building upon this idea Paul Hebert, from University of Guelph, in Canada developed the use of part of the mitochondrial gene as a universal ‘identification’ marker for living organisms (Savolainen et al. 2005). The whbole notion of product barcode is based on the arrangement of 10 alternate numbers in 11 positions to create 100 billion unique numbers which can then be used as an individual product id (Hebert *et al*. 2003). Genomic DNA can be used in the same manner but the problem here is we only have 4 bases to work with. This problem is solved when we look at the enormous size of the available DNA in the animal cell. It has been calculated by (Hebert *et al*. 2003) just taking 15 sites of nucleotide positions can create a possibility of 15 codes, which is huge compared to the artificial barcode system. Some of the 4 sequences are very highly conserved and other regions provide diversity to be checked for at least intraspecific levels.

The DNA barcoding workflow: Species identification through barcoding is usually achieved by the retrieval of a short DNA sequence – the barcode from a standard part of the genome (i.e. COI in animal case) from the specimen under

investigation. 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 (i.e. a new haplotype or geographical variant), or it can suggest the existence of a newly encountered species.

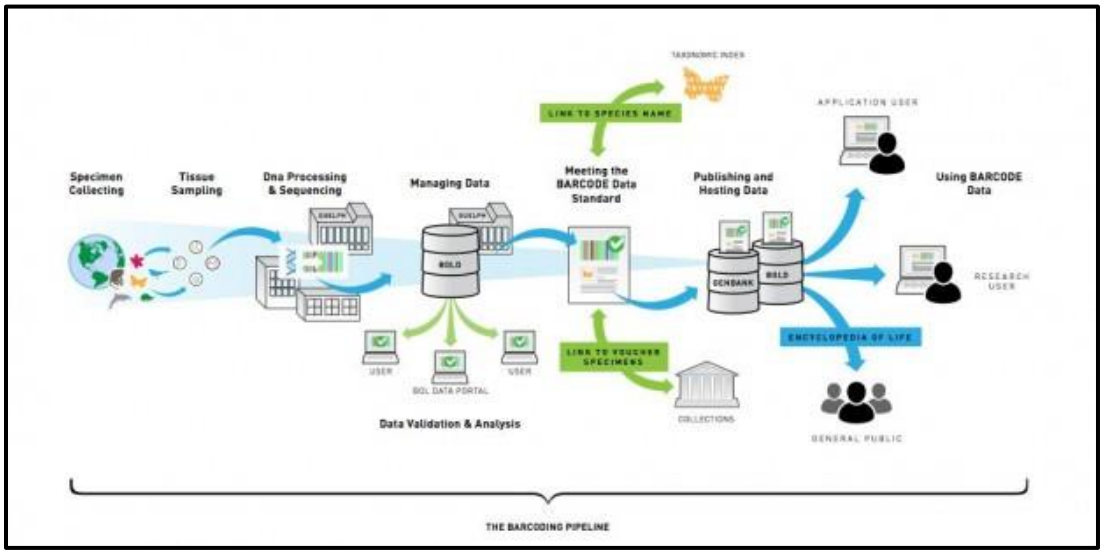

Fig.6. Steps of DNA Barcoding (Image: Barcode of life)

The two main ambitions of DNA barcoding are to

(i) Assign unknown specimens to species and

(ii) Enhance the discovery of new species and facilitate identification, particularly incryptic, microscopic and other organisms with complex or inaccessible morphology (Hebert *et al*., 2003).

Central to the efficacy of DNA barcoding is the selection of a suitable region of DNA. Its mutation rate must be slow enough so that intraspecific variation is minimized but sufficiently rapid to highlight interspecific variation. It must be

relatively easy to collect and should have as few insertions or deletions as possible to facilitate sequence alignment. The COI gene has proved to be suitable for the identification of a large range of animal taxa, including gastropods (Remigio*et al*., 2003), springtails (Hogg *et al*., 2004), butterflies (Hebert *et al*., 2004; Hajibabaei et al 2006). This region is rapidly gaining currency represents approximately the first half of the gene and is 648 base pairs, The target fragment is relatively easy to amplify with standard primers (Folmer*et al*., 1994) and thus cheap. It is frequently being used for phylogenetic and evolutionary inference and is considered by some researchers (Miya *et al*., 2000) as a very good gene for that purpose.

Mitochondrial DNA

Mitochondrial genome is one of the most frequently used loci in phylogenetic and phylogeographic analysis and is becoming increasingly possible to sequence and analyse this genome in its entirely from diverse taxa (Muller 2006). As the COI gene chosen for present study is the part of mitochondrial DNA the detailed structure of mt-DNA genome of the fish *Rivulusmarmoratus* is depicted below (Jae-Seong Lee et.al. 2001).

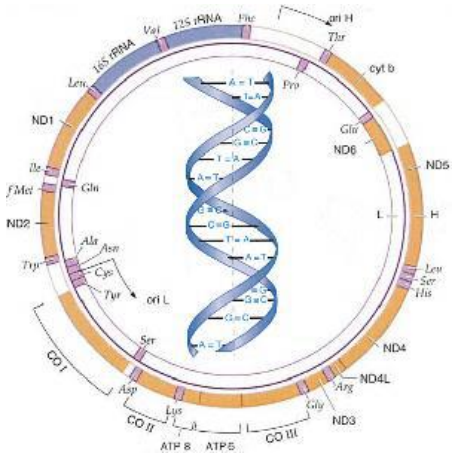

Fig 7: Various gene markers on associated with Mitochondrial DNA(Image: Barcode of Life)

This is the complete mitochondrial genome with two rRNAs, 22tRNAs and 13 protein-coding genes with two control regions. Interestingly the fishes' mitochondrial genome has two control regions (Lee et.al. 2001).

In the mitochondrial genome COI gene is an approximate 656bp region (site occupied shown in Fig. 3). This gene encodes part of the terminal enzyme of the respiratory chain of mitochondria. Within-species variation for this gene is low compared with between-species variation (Ward, 2009); with the use of this ~600basepair of sequence from the 5'terminus of the gene (Hebert et al. 2003). Even though shorter fragments of COI have also been found to be effective for the identification of specimens with degraded DNA, where a 650 base sequence is not easily obtainable (Hajibabaei*et al*. 2007).

The need for comprehensive and reliable species identification tools combined with early barcoding success with fishes (Savolainen*et al*., 2005; Ward *et al*., 2005) initiated the formation of the Fish Barcode of Life campaign (FISH-BOL) initiative. FISH-BOL is an international research collaboration that is assembling a standardized reference DNA sequence library for all fishes. This campaign has the primary goal of gathering DNA barcode records for all the world's fishes, some 30,000 species. It promises a powerful tool for extending understanding of the natural history and ecological interactions of fish species. Therefore, the data generated from FISH-BOL will tremendously add to the evolutionary history of the most diverse group of vertebrates on earth.

The need of barcoding fishes include species identification, area range of known species, finding previously overlooked species and enabling identifications where traditional methods like reference of the Day's Volume – I and II by Francis Day are not enough. The analysis is studying the mitochondrial cytochrome c oxidase I (COI) gene.

Fish species identification by molecular analysis has been utilized for many years. Initially, allozyme differences were used (Avise, 1975), followed by mtDNA examination (Avise, 1995). DNA-based methods have several advantages over their protein-based counterparts because DNA is less sensitive to degradation (Hanner*et al*., 2011) and can be accessed in all stages from egg to adult. Perhaps most importantly, DNA sequence data are easier to replicate and interpret across laboratories, so this technique has been very successful and widely used also. Bartlett & Davidson (1991) were among the first to use mtDNA sequencing for fish identification, showing that cytochrome b sequences could discriminate four species of tuna (*Thunnus* spp.). They subsequently proposed forensically important nucleotide sequences (FINS) as a means of identifying fishes.

The overarching goal of FISH-BOL is to barcode all the world's fishes. Initially, it aims at obtaining barcode records from a minimum of five specimens per species, increasing to at least five specimens per FAO area. It may be necessary to analyse more specimens for freshwater species because of their greater degree of spatial genetic differentiation (Ward *et al*., 1994). However, because collections depend on the interests and locations of FISH-BOL participants, some species will be widely barcoded, while others will receive little attention. As most of the

barcoding work has been concentrated on marine fish only, giving less importance to freshwater fishes.

Specimen Sampling & Preservation

In diverse disciplines of scientific study it is often necessary to preserve a whole specimen separate from a subsample of tissue. This specimen and its tissue will be used for different types of analysis

A specimen as a museum voucher will likely be preserved in formalin; the same individual(s) cannot be used in a molecular investigation for phylogenetic relationships, population genetics, or some other genetic study. In these cases differential preservation techniques are required. The method used for retaining viable tissue(s) to be used in future DNA extractions and molecular investigations (Ivanova, 2009).

Fixing of specimen is also required for diverse types of morphological studies.

1. Samples used for DNA studies were taken only from living, frozen or anesthetized specimens, DNA cannot be obtained from specimens or tissues that have been exposed or fixed in formalin.

2. Tissue for DNA studies can be taken from any part of the organism, but were most commonly taken from a piece of excised muscle (muscle plug), liver, gills or a piece of fin clipped from the specimen.

3. DNA cross-contamination (this is contamination of DNA from one sample with DNA of another sample) is always a serious issue that can compromise results from molecular studies.

To avoid cross-contamination of DNA; tissue plug were obtained using scalpel, knife and razor blade. Fin clip, scissors and forceps were thoroughly cleaned by soaking the instruments into high concentration Ethanol (i.e. 70% or greater Ethanol).

4. The sampling specimen needs to adopt the sterile laboratory techniques in the field. Wiping instruments with 70% or greater Ethanol and then ignited with a lighter (field sterilization technique).

5. Upon completion of collecting individual specimens from the sampling site, the individuals from which a fin clip, gills or tissue (muscle) plug removed were filled in sampling tubes.

6. Each tube contained only the tissue, Liver, or Gills of one specimen, as there can be confusion. Each tube had a unique label or number on the outside that exactly matches the information in field notes about the species.

Information includes location of specimen, latitude and longitude, date of collection and collectors. As the tubes were small, usually alphanumeric code were used to label the identity.

7. Fill the tube with 90% Ethanol (fill it till 3/4th of sampling tubes) and apply labels to all individuals sampling vials/tubes.

8. With sharp (sterile) scissors the Liver, Gills were successfully removed and were placed into the properly labelled tubes filled with 90% Ethanol. The same procedure was used for removing a tissue (muscle) plug, though instead of scissors a sharp (sterile) scalpel was used to remove muscle.

9. Sterilize the forceps, scissors, scalpel or knife and move on to the next fish specimen. Again, sterilize by plunging the instruments exposed to the previous sample of DNA (or tissue) into high concentration ethanol. (70% or greater ethanol) and ignited with a lighter.

10. Close each tube filled with specimen and label all required information on tubes as well as in field notes and place the tubes in icebox. This specimen tubes were kept cool in icebox till the isolation of DNA from specimen.

11. The specimen after tissues/Gills/Liver was collected was wrapped in an airtight zip bag with a label on it. (Such as name, date, location of sample)

12. Once required Tissue, Gills, or liver were obtained from the fish that was preserved for morphological studies.

Voucher preparation and Fixing Specimens in PBS with 30 % glycerol solution (Preservation):

The vouchers can be stored in various forms (Body parts, tissues, detailed photographs or whole specimen).

The Fish samples collected were washed thoroughly with tap water, and then the fish were wiped with tissue/blotting paper. After tissue samples were taken from the specimen it was important to maintain voucher specimen that could be used for either later verification of identification (voucher) or for morphological studies.

The specimen has a tag with the alphanumeric code used for the tissues. This tag/label was placed on an air-tight zip bags. These specimens were preserved in -80° Freezer.

DNA Extraction:

Genomic DNA was extracted using gills and muscles of Fish in an initial weight of approximately 20- 25 mg and extraction was carried out using QIAGEN DNeasyBlood and Tissue kit Protocol for the DNA isolation is as follows (Zetzsche*et al*., 2008):

- Fish was washed before proceeding for DNA isolation using 70% ethanol and again rinsed with sterile distilled water.
- Approximately 25 mg of tissue (preferably Gills/muscle) was dissected and chopped into fine pieces. Fish tissue was transferred into 1.5 ml sterile micro centrifuge tube and 180 µl ATL buffer from kit was added to the tube.
- Mechanically crushed fish tissue 20 µl of proteinase K was added and mixed thoroughly by vortexing. Sample was incubated at 56º C in water bath overnight.
- A treatment of 5 µl RNAase was added and was kept for 3 hours at a temperature of 37º C.
- To clear lysate 200 µl buffer AL was added and mixed thoroughly by vortexing.200 µl ethanol (96-100%) was added to the above solution and mixed again by vortexing.
- A mixture was transferred to the DNeasy mini spin column placed into a 2 ml collection tube. Columns centrifuged at 8000 rpm for 1 minute. Flow-through was discarded.

- DNeasy mini spin column was placed in new 2ml collection tube and 500 µl buffer AW1 was added. Colum was centrifuged at 8000 rpm for 1 minute. Flow- through was discarded.
- DNeasy mini spin column was placed in new 2ml collection tube and 500 µl buffer AW2 was added. Colum was centrifuged at 14,000 rpm for 3 minute to dry the DNeasy membrane. Flow- through and collection tube was discarded.
- Columns were placed in 1.5 ml microcentrifuge tubes. 50 µl buffer AE was added directly onto the DNeasy membrane and incubated at room temperature (25º C) for 10 minutes.
- DNA was eluted by spinning the DNeasy columns at 8000 rpm for 1 minute. DNA was stored at -20º C.

Reagents supplied with kit

- Lysis buffer – Buffer ATL and Buffer AL
- Wash Buffer – Buffer AW1 and Buffer AW2
- Elution Buffer- Buffer AE
- Proteinase K

Quantification of genomic DNA:

After extraction of genomic DNA, quantification was done according to Sambrook*et al*., 1982. 10 µL of extracted DNA was dissolved in 30 µl of Tris buffer (pH 8) and O.D. was taken at 260 and 280 nm (Powerwave HT Microplate Spectrophotometer, BioTek). Quantity of DNA was calculated by using following formula:

The quality was assessed by taking the (O.D. at 260nm)/ (O.D. at 280nm). Samples which showed the O.D. between 1.6-1.8 were taken for further work.

Polymerase Chain Reaction (PCR)

PCR was carried out using 1X final concentration of ReadyMix™ Taq PCR Reaction Mix (Sigma) and, template DNA (50 ng/µl). The reaction was carried out in Thermal cycler (Applied BiosystemsVeriti®)

Final concentration of PCR reagents in reaction mixture (20 µl)

- 1x ReadyMix™ Taq PCR Reaction Mix (10 µl)
- 10 pmole of each primer
- 50-100 ng template DNA

Cytochrome c Oxidase subunit I gene (COI) amplification

COI gene in Fish was amplified in a volume of 20 µl containing 10 µlTaq PCR reaction mix, 10pmol forward primer (FishF2_t1), 10 pmol reverse primer (FishR2_t1), 50 ng template DNA and sterile ion-free water(to make up the final desired volume). Amplification was carried out in Thermal cycler (Applied BiosystemsVeriti®).

Reactions were amplified through 35 cycles with the following conditions (Folmer et al., 1994):

Denaturation: Thirty seconds at 95°C

Annealing: Forty Seconds at 50°C

Extension: Three minutes at 72°C. This was followed by a final extension step at 72°C for ten minutes. Initial denaturation was carried out at 95°C for four minute.

Table 1: Primer list with sequence

SR NO.	PRIMERS USED	SEQUENCE	REFERENCE
1.	FishF2_t1 (Forward)	TGTAAAACGACGGCCAGTCGACTAATCATAAAGATATCGGCAC	**(Ivanova*et al.*,2007)**
	FishR2_t1 (Reverse)	CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA	**(Ivanova*et al.*,2007)**
2.	LCO1490 (Forward)	GGTCAACAAATCATAAAGATATTGG	(**Folmer*et al.*,1994**)
	HCO2198 (Reverse)	TAAACTTCAGGGTGACCAAAAAATCA	(**Folmer*et al.*,1994**)
3.	FISH-BCL (Forward)	TCAACYAATCAYAAAGATATYGGCAC	(**Baldwin *et al.*, 2009**)
	FISH-BCH (Reverse)	ACTTCYGGGTGRCCRAARAATCA	(**Baldwin *et al.*,2009)**

Agarose gel electrophoresis

Table 2: Composition of 10X TBE buffer

Tris base	108 g
Boric acid	55 g

0.5 M EDTA (pH 8)	40 ml

Above mentioned chemicals were mixed in a volumetric flask and solution was made up to 1 litre by adding distilled water. Buffer was subjected to autoclave treatment as mentioned above. 10X TBE were diluted to 1X prior to use.

TrisHCl EDTA buffer:

Two hundred millilitre (ml) buffer solution of pH 8.0 was prepared by dissolving chemicals as follows:

Table 3: Composition of Tris-HCl EDTA

Chemical	**Required Strength in buffer**	**Amount added in 200 ml**
Tris-HCl	10 mM	0.24g
EDTA	1 mM	0.06g

Ethidium bromide:

Stock solution of 10 mg/ml concentration was prepared using double distilled water and stored in falcon tubes.

DNA loading dye:

10 ml loading dye was prepared by mixing sucrose 5.0 g (50 %), bromophenol blue 0.025 g (0.25 %), xylene cyanol 0.025 g (0.25 %).

Plate preparation and casting the gels:

Cleaned agarose gel casting cassette and comb were wiped with methanol and open sides of the tray were sealed with gel sealing tape. The comb was placed in the given slits of the plate. Calculated amount of agarose in TBE buffer was mixed to prepare 2 % solution. The agarose was dissolved completely in the buffer by heating the mixture at 80-85oC in microwave oven and was cooled to 50oC. Ethidium bromide was added in a final concentration of 0.6 mg/ml and mixed well. Liquid was gently poured into the casting tray before it gets solidified. The combs and sealed tape were removed slowly after complete solidification of the agarose gel.

Preparation of samples and scanning of gels:

The amplified DNA samples having approximately 5 µl volume were mixed with 2 µl gel loading dye and were carefully loaded in the wells using gel-loading tips. 100 bp Marker Electrophoresis was carried out at 180V. The gel images were recorded in JPEG or TIF formats using gel documentation system (Biorad, USA). The gels were analyzed by using the software Image lab version 3.0 (Biorad, USA).

Purification of amplified PCR products

Purification of COI gene amplified products were done using GenElute™ PCR Clean-up kit (cat no. NA 1020-1kt) using following protocol

• Column preparation: GenElute plasmid mini spin column was assembled in 2.0 ml collection tube provided with kit. 0.5 ml of column preparation solution was added to the columns and centrifuged at 12,000 g for 30 seconds.

• Add 20 µl of PCR product to 100 µl of binding solution. After mixing properly the bfsolution was transferred to the binding column. Columns were centrifuged at 16000 g for 1 minute. Flow-through was discarded.

• Binding column was placed in a collection tube and 0.5 ml of wash solution was added to the binding column and centrifuged at maximum speed for 1 minute. This step was repeated in order to remove the impurities.

• To dry the binding membrane completely and to prevent the alcohol contamination in preceding reactions columns were centrifuged at maximum speed for 3 minutes.

• Columns were transferred to the fresh 2 ml collection tube and 50 µl of Elution solution was applied to the centre of each column.

• DNA elution was carried out at maximum speed for 1 minute.

• Eluted DNA (PCR product) was stored at -20º C.

Cycle Sequencing

Sequencing was carried out using BigDye® Terminator v 3.1 Cycle sequencing kit. The BigDye Terminator v3.1 Cycle Sequencing Kit provides the required reagent components for the sequencing reaction in a ready reaction, pre-mixed format.

Cycle sequencing was performed in 10 µl volume. Reaction mixture was prepared as follows. Two reaction tubes were prepared for forward sequencing primers and reverse sequencing primers. In this experiment COI amplification primers (FishF2_t1 and FishR2_t1) served as sequencing primers.

Table 4: Quantity of reagents used for Cycle Sequencing

Reagent	Quantity
Terminator Ready Reaction mix v 3.1	4 µl
Big dye Sequencing buffer	1 µl
Template	150 - 300 ng
Primer	10 pmole
Deionized water	To make the volume up to 10 µl

Before going to amplification reaction mixture was mixed well in an individual tube and spun down briefly.

Amplification was carried out in Thermal cycler (Applied BiosystemsVeriti®). Reactions were amplified through 35 cycles with the following conditions:

Initial denaturation at 96°C for 2 min, followed by 30 cycles of 96°C for 30 sec, annealing at 50°C for 15 sec, and extension at 60°C for 4 min, followed by indefinite hold at 4°C (Ivanova et al., 2005).

Purification of Cycle Sequencing Products

In order to achieve optimal results, complete removal of unincorporated dye terminators before performing capillary electrophoresis. Excess dye terminators in sequencing products can obscure data in early part of the sequence and can interfere with base calling. Purification was done using BigDyeXTerminator® Purification Kit. Kit contains SAM™ Solution and BigDye® XTerminator™ Solution. Following purification protocol was followed:

- Cycle sequencing reaction plate was spun at 100 g for 1 minute.
- Purification reaction premix was prepared by adding 10 µl of BigDyeXTerminator® to 45 µl of SAM™ solution. Total reaction had 55 µl for each well.

- After removing seal of the 96-well plate 55 µL of SAM™ Solution/XTerminator® Solution premix was added to each well.
- Plate was sealed using MicroAmp® Clear Adhesive Films and subjected to vortex for 30 minutes at 2000 rpm on IKA® Vortex 4 digital.
- After vortexing plate was centrifuged at 1000 rpm for 2 minutes.
- Plate kept at room temperature (25ºC) before going to capillary electrophoresis.

Capillary electrophoresis

- Capillary electrophoresis of cycle sequenced products was Performed on 3500 XL platform (Applied biosystems)
- Instrument software 3500 was used to give commands to instrument. Dye Set Z and the Sequencing Install Standard, BigDye® Terminator v3.1 Kit was used to create the BigDye® Direct spectral calibration information to apply to the data.
- BigDye® mobility and calibration files were used for optimal basecalling with the BigDye® Cycle Sequencing Kit v 3.1.
- Plate containing cycle sequenced products was loaded on Position A/ Position B.
- Sample information was loaded on plate preparation mode.
- Capillaries were filled with POP-7™ polymer.

The approximate run time for 24 samples was 2 hours and 30 minutes.

Analysis of Sequence and Submission of Barcodes to Barcode of Life Database (BOLD)

Sequence analysis was done using sequencing analysis version 5.4 (Applied Biosystems) and BioEdit, biological sequence alignment editor (Ibis Biosciences). Consensus sequences generated after aligning gene sequences from forward and reverse primers. These sequences were subjected to Sequence match analysis using Basic Local Alignment Search Tool (BLAST) on NCBI.

Consensus sequences which showed significant match with the earlier identified data on NCBI were submitted to BOLDSYSTEMS according to the guidelines provided onto BOLD website (http://www.boldsystems.org/). For few species where NCBI data was not available were subjected to detailed and thorough morphological analysis and submitted to BOLD.

e) Population dynamics and understanding Hilsa fishery status

Understanding the ecology and the population of any species is very important to ascertain the biodiversity status of any ecosystem and for the case of aquatic ecosystem in a particular species.

The length weight relationships are required in population dynamics and fisheries stock assessment (Gulland, 1983). Until the early 1960s, length weight relationships were calculated mostly using log transformed mean weights of fishes in different classes (Nomura, 1962). The following decade, scientific pocket calculators and computers made it easy to use data on individual fishes and to compare statistically linear predictive regressions through covariance analysis. As log transformations introduce a negative bias in the estimate of the weights of large specimens Ricker (1973, 1975) used functional regression. While accepted by many statisticians, it was widely used by fishery scientists in the 1970s.

Statistical packages for mainframes (1980) and powerful personal computers and programs (1990) made it easy to estimate no-linear relationships without transformations. While there is no doubt that the non-linear fitting approach combined with least squares or maximum likelihood statistics are a powerful tool to describe and compare length-weight relationships (Kimura 1980; Saila*et al* 1988; Cerrato 1990), each of these approaches has advantages and drawbacks in real life situations.

Length–weight relationships are important because they: (a)allow the conversion of growth-in-length equations to growth-in-weight, for use in stock assessment models; (b) allow the estimation of biomass from length observations; (c) allow an estimate of the condition of fish; and (d) are useful for between-region comparisons of life histories of a certain species (Wootton 1991;Pauly 1996; Petrakis and Stergiou, 1995; Goncalves*et al*., 1996).Relationships between different types of lengths (length–length relationships), for which little information seems to be available for Mediterranean species, are also very important for comparative growth studies (Froese and Binohlan, 1998; Pauly*et al*, 1998).

Length-Weight relationship & Condition Factor [Kn]

The weight (W) of fishes (and other organisms) is exponentially related to their length (L) according to the equation W = aLb, where a is the intercept and b is the slope of the log-transformed relation (Le Cren 1951, Froese 2006). Based on the slope (b) of the relation between weight and length, one can check whether the growth of a fish species is isometric (b = 3, all fish dimensions increase at the

same rate), hypoallometric (b < 3, a fish increases less in weight than predicted by its increase in length, i.e., it becomes more elongated as it grows; also termed negative allometric) or hyperallometric (b > 3, a fish increases more in weight than predicted by its increase in length, i.e., it becomes less elongated or more roundish as it grows; also termed positive allometric). Weight–length relations (WLRs) can be used for converting lengths into biomass, determining fish condition, comparing fish growth among areas, and as a complement to species-specific reproduction and feeding studies (Petrakis and Stergiou 1995, Koutrakis and Tsikliras 2003, Froese 2006). Thus, they are an important component of fisheries biology and when properly calculated they can be very useful to fisheries management.

Number of species, sample size, length range and preservation

1. At least 10 species should be included in a submission, including some for which no information on WLRs was previously available in FishBase (Froese and Pauly 2011); exceptions may apply when the work concerns very rare species or the biodiversity of a very restricted area, in which case less than 10 species could be included;

2. Adequate sample size of about 100 specimens, i.e., there is no need to kill thousands of specimens only for WLR estimates; for rare species fewer measurements are acceptable and sacrificed specimens should be deposited in a museum collection for further research; for endangered and protected species, nonlethal methods or specimens of opportunity (by-catch etc.) should be used;

3. Coverage of a full size range (from juveniles to adults close to their asymptotic sizes), ideally with the number of specimens being equally distributed among size classes (e.g., 10 small, 10 medium-size, and 10 large specimens), in order to avoid over- or underestimation of b. Thus, samples taken with selective gear (e.g., a single mesh size of gillnets or a single hook size in longlines) are not generally appropriate because of the narrow length ranges sampled;

4. Ideally, the sampling period should extend over a full year cycle, but, in any case, all seasons should be covered;

5. The preservation technique should be clearly stated (and the duration the samples were preserved) and kept the same for all samples per species.

Total length was taken from the tip of snout to the caudal fin end in cm and weight was taken in gram. A total of 250 individuals at each year in Bhadbhut, Narmada River were used for study.

The length-weight relationship expressed as

$$W = aL^b$$

Logarithmic transformation of the above formula gives a linear equation, that is:

$$\text{Ln } W = \ln a + b \times \ln L$$

Where

W = weight in gram;

L = total length in cm;

a and b are constants

A graph of log W against log L forms a straight line with a slope of b and a Y-axis (log W) intercept of log a and invariably, b is close to 3.0 for all species.

The relationship was established for both male and female by linear regression of the natural logarithms of the length and weight data following Pauly (1983). Conversion of the resultant transformed equation to the original equation was achieved by rewriting the equation as

$$W = ea \times Lb$$

Confidence limit for slope (b) estimated following King (1995) and using the formula:

$$95\% \text{ confidence limit} = b \pm t \times Sb$$

Where,

b = slope in length weight relationship

t = table value of t (t test at 95 % confidence

$$Sb^2 = (1/ (n -2)) (Sy^2 / Sx^2) b^2)$$

Where,

Sb^2 = variance of slope

Sy^2=sum of square Y

Sx^2=sum of square X

n = number of observations

The regression analysis, Analysis of co variance (ANOCOVA) on the regression equations, t test on b and r value were carried out following standard statistical procedures (Snedecor, 1961;Snedecor and Cochran,1967). Condition factor values were assessed for male, females and pooled data. Data analysed for various length

group for different months to understand the well-being of the species and also to infer information on reproductive behavior, feeding habits etc. (Froese, 2006). Condition factor/Ponderal index/Fulton's condition factor (Fulton, 1904) was estimated using formulae:

$$K = 100\ W/L^3$$

Where,

W = weight of fish in gram (gm)

L = length of fish in centimeter (cm).

Modified condition factor (Ricker, 1975) was estimated following formula:

Modified condition factor = 100 W/ Lb

Where,

W = weight of fish (gm)

L = length of fish (cm)

b is b value in length weight relationship.

Relative condition factor 'Kn' (Le Cren,1951) was estimated by using formulae

Kn = W / ^w

Where,

W = actual weight of fish in gram (gm)

^w = Expected weight

^w = Log W* = log a + b log L

Where, W* = Average of W

Stock assessment

Indirect stock assessment techniques have improved considerably through the development of population dynamics, and the use of computers and adequate statistics have increased their precision. Direct stock assessment methods such as fisheries acoustics are still improving, largely because of benefits from technological progress. Although fish behaviour is not directly represented through parameters in the stock assessment methods, there is an increasing awareness that behaviour is a major limiting factor in the accuracy of abundance estimates. To illustrate the reasons behind this awareness, we review how fish behaviour, grouped into habitat selection, aggregation patterns, avoidance reactions and learning, may influence the most common stock assessment methods.

Tenualosailisha is capable of withstanding a wide range of salinities and migrating long distances upstream (up to 1287 km). The majority of the population feed and grow mainly in the sea before migrating to fresh water for spawning (Haroon 1998). Juveniles develop and grow in fresh water, but soon migrate to the ocean, where they spend most of their life span. The fishery for hilsa contributes about 15% of the total annual fish production of the country. The fishery provides direct or indirect employment to about 2% (2.5 million) of the entire population of the fishermen(Bhaumik, 2013).Hilsa are harvested by different types of crafts and gears in different seasons and environments. In the recent past, the fishery in inland waters had been declining, but the total catch has remained stable (about 200,000 mt annually) due to an increase in catch from the marine sector. The inland sector contributed 32.7% to the total

Hilsa catch of 229,714 mt during 2000-2001. In the inland sector, most Hilsa are caught in the Narmada River (66.2% during 1998-99), from both fresh water and estuarine reaches. The decline in the inland sector of the fishery, particularly in the Padma River, has been a cause of concern (Haldar*et. al*. 1992; Hussain *et. al*. 1998; Islam 1998; Mazid 1998; Rahman et. al. 1998; 1999; Amin et. al. 2000 a,b; Rahman et. al. 2000; Amin et. al. 2001; Haldar et. al. 2001; Mazid 2001; Rahman et. al. 2001; Amin et. al. 2002; 2003). There are a large number of vessels fishing for Hilsa in all sectors and most are unregistered. This makes it difficult to estimate fishing effort. During the peak Hilsa-fishing season (July - September) the number of vessels and types of fishing gear used to catch Hilsa increases and many non-traditional fishermen, day labourers and unemployed people participate. These, plus the migratory habits of the fish, add to the difficulty in obtaining reliable estimates of annual fishing effort in each sector. In spite of all these difficulties, it is essential to monitor the catch and production trends and to develop a catch monitoring system. The continuous unregulated catch of Hilsa will adversely affect the productivity of the population. Therefore, the present study was undertaken to provide an estimate of the catch per unit effort, total annual production etc. of Hilsa. These aspects will help in formulating management and conservation policies for Hilsa in India.

f) Study of water quality parameters in the landing centres of Narmada River, Gujarat

The water samples were collected randomly from different landing sites in 500ml or 1litre plastic bottles. These samples were tested seasonally for

general physico-chemical parameter analysis. The various parameters were carried by methodology prescribed from American Public Health Association (APHA, 1960). All the parameters have been measured in departmental research laboratories as well as for confirmatory processes in private labs and are mentioned in accordance to universal units.

Table 5. Physico-chemical parameters with standard units of measurements

PARAMETER	UNITS OF MEASUREMENT
Water surface temperature	°C
Dissolved oxygen	mg/L
pH	---
Alkalinity	mg/L
Total Hardness	mg/L
Total Dissolved Solids	mg/L
Turbidity	NTU

g) Analysis of proximate composition to investigate the nutritional values of *Tenualosailisha*

The study of mineral elements present in living organisms is of biological importance; since many of such elements take part in some metabolic processes and are known to be indispensable to all living things (Shul'man, 1974). The body usually contains small amount of these minerals, some of which are essential nutrients, been components of many enzymes system and metabolic mechanisms, and as such contribute to the growth of the fish. The most important mineral salts are that of calcium, sodium, potassium, phosphorous, iron, chlorine while many others are also needed in trace amounts. The deficiency in these principal nutritional mineral elements induces a lot of malfunctioning; as it reduces productivity and causes diseases, such as inability of blood to clot, osteoporosis, anaemia etc. (Shul'man, 1974). Moreover, the measurement of some proximate profiles such as protein contents, carbohydrates, lipids, moisture contents and ash percentage is often necessary to ensure that they meet the requirements of food regulations and commercial specifications (Watermann, 2000). There are a couple of reports on the nutritive estimations of Hilsa. Hilsa is a transitory fish and it voyages a large number of kilometers from upstream of stream to mid sea. Various locales of the Narmada River and the mouth of the River contain various kinds of nourishment (phyto- and zooplankton). Different types of food make proximate composition a bit different and also different part of the body makes the composition different as well. Biochemical composition of fish flesh may vary within the same species of fish depending upon the fishing season, age, sex and habitat. The variation is also found within the different region of the body. In fishes, proximate composition means the composition of the fish flesh. Fish flesh

contains four basic ingredients in varying proportions major nutrients such as water (70 - 80%), protein (18 - 20%), fat (5%) and minerals (5%) and minor nutrients such as vitamin, carbohydrate. It has high nutritional value in terms of fats and proteins that are not commonly available in other foods. The present study details with the proximate analysis of protein, fat, moisture and ash content in Hilsa.

The proximate composition of fish was determined by conventional methods of Association of Official Analytical Chemicals on weight basis. At the beginning, initial weight of the sample (W1) was taken. The collected samples were dried in an oven at about 75°C for 24 hours and were transferred to desiccator to cool down. Thereafter the weight of the sample (W2) was taken again. The moisture content was planned after keeping a known and weighed amount of body muscle in an oven at 65°C for 48 hours. The same muscle sample is then weighed again and the amount of moisture found in the tissue was calculated by the following formula: Moisture (%) = (W1 – W2) x 100/W1.

The total protein was estimated following the method of Lowry *et al.* (1951) using FolinCiocalteau's solution. Known amount tissue was homogenized in distilled water. After homogenization the sample was sonicated during 30 seconds and centrifuged at 2500 rpm for 15 minutes in centrifuge. The supernatant was collected and was used for determination of protein. The protein values were obtained from a linear standard curve which was prepared previously using Bovine Serum Albumin (BSA sigma) as standard and expressed in mg protein per mg of sample tissue. The optical density (O.D.) was measured using a standard

spectrophotometer at 620 nm. For the estimation of fat content, the dried samples left after moisture determination were finely grinded and the fat was extracted with a nonpolar solvent, ethyl ether using a soxhlet. After extraction, the solvent was evaporated and the extracted materials were weighed. The ash content of a sample is the residue left after ashing in a laboratory furnace at about 550 - 600°C till the residue became white.