# ANALYSIS OF MORPHOLOGY AND MITOCHONDRIAL GENE SEQUENCES OF *Labeo calbasu* (CYPRINIFORMES: CYPRINIDAE) FROM BANGLADESH

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**November-2018**

## DECLARATION

I hereby declare that the dissertation entitled "**ANALYSIS OF MORPHOLOGY AND MITOCHONDRIAL GENE SEQUENCES OF *Labeo calbasu* (CYPRINIFORMES: CYPRINIDAE) FROM BANGLADESH** " is an authentic record of the work done by me and that no part there of has been presented for the award of any degree, diploma, associateship, fellowship or any othersimilar title.

8th November, 2018 **(Liton Chondra ShutraDhar)**

**Bangladesh**

Dated: 06/11/2018

## CERTIFICATE

Certified that the dissertation entitled "**ANALYSIS OF MORPHOLOGY AND MITOCHONDRIAL GENE SEQUENCES OF *Labeo calbasu* (CYPRINIFORMES: CYPRINIDAE) FROM BANGLADESH** "is a record of independent research work carried out by **Liton Chondra Shutra Dhar** during the period of study from July 2014 to November 2018 under our supervision and guidance for the degree of **Master of Philosophy (**Genetics and Molecular Biology, Zoology**)** and that the thesis has not previously submitted anywhere for the award of any degree, diploma, associateship, fellowship or any other similar title.

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## ABSTRACT

Resolving any taxonomic unceratainty before taking appropriate conservation strategy of a species is a priority task. Status of *Labeo calbasu* in Bangladesh is marked as endangered. Since there exist other *Labeo* species in the country, unambiguous identification of *Labeo calbasu* is important. In this perspective, both morphology and mitochondrial gene sequences were utilized to identify the species, compare it with other related species and to study phylogeny of the group. The species were collected from Kaptai Lake, Rangamati, Chittagong. Total Genomic DNA was isolated and certain regions of mitochondrial genome were amplified through PCR technique. The PCR products were sequenced. The sequenced regions covered parts of mitochondrial *16SrRNA*, *ND1* and *ND2*, and complete sequences of three *tRNA* genes, i.e., $tRNA^{Ile}$, $tRNA^{Gln}$, $tRNA^{Met}$. Multiple sequence alignment of sequenced *16SrRNA* gene with two other sequences of *L. calbasu* from Indian subcontinent revealed 4.8% polymorphic sites. On the other hand, 19.96% polymorphic sites were detected when the sequenced region of *16SrRNA* gene was aligned with nine other *Labeo* species, one *Catla* species, two *Cirrhinus* species and one *Tor* species. Similarly, sequenced regions of other genes were aligned to find intra-species and inter-species polymorphic sites. Phylogenetic trees were also constructed to undertand relationship among the *Labeo* species. Overall, present work could be contributive in sustainable management of this endangered *Labeo* species from a conservation point of view.

# Contents

# List of Abbreviations

µg : Micro grams

µl : Micro litre

µM : Micro molar

AFLP : Amplified Fragment Length Polymorphism

AFS : Soft rays of anal fin

AFR : Branched rays of anal fin

bp : Base pair

BLAST : Basic Local Alignment Search Tool

EDTA : Ethylenediaminetetraacetic Acid

CTAB : Cety Trimethylammonium Bromide

gDNA : Genomic DNA

C : Caudal fin

Cca :*Cyprinus carpio*

CC : *Catla catla*

Cm :*Cirrhinus mrigala.*

CV : Coefficient of variation

D : Dorsal fin

DFS : Soft rays of Dorsal fin

DFR : Branced rays of dorsal fin

GRC : Gill rakers on the outer gill arch

IN : India

IUCN :International Union for Conservation of Nature

IOW : Inter-orbital width

LH : Head length

La : *Labeo angra*

Lb : *Labeo bata*

Lc : *Labeo calbasu*

Lbo : *Labeo boggut*

Ld : *Labeo dussameri*

Ldd : *Labeo dycellius dycollus*

Lf : *Labeo frimbriatus*

Lg : *Labeo gonius*

Lr : *Labeo rhota*

LCPD : Length of caudal pedancle

LSS : Later line scale count

*mt genome* : Mitochondrial genome

N : Number

*NCBI* : National Center for Biotechnology Information

*ND1* : NADH dehydrogenase subunit 1

*ND1* : NADH dehydrogenase subunit 2

$O_L$ : Origin of L-strand replication

P :Pectoral fin

PFR :Pectoral fin ray

PS : Present study

SD : Standard deviation

SL : Standared length

Tt : *Tor tor*

*tRNA* : Transfer RNA

V : ventral fin

VFR : Number of ventral rays

*16S rRNA* : 16S ribosomal RNA

UPGMA : Unweighted Pair Group Method with Arithmetic Mean

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# Chapter 1

# INTRODUCTION

Chapter 1

# INTRODUCTION

## 1.1. Background and scope of the study

Major portion of Bangladesh lies in the Ganges-Brahmaputra-Meghna Delta (GBM Delta). It has a lot of natural and artificial water reservoirs. Among them, Kaptai Lake is the largest man-made fresh water lake in South East Asia. The Lake was constructed for hydro-electric power generation by damming in Karnaphuli River near Kaptai Upzilla under the Rangamati district. Physically the Lake is H shaped, surface area cover 58,300 hactartes ("Kaptai Lake - Banglapedia,"). It has drowned almost the whole of the middle-Karnafuli valley and the lower reaches of the Chengi, the Kasalong and the Rinkhyong Rivers. The Lake has made a substantial contribution to the national economy through freshwater fish production, navigation, irrigation and flood control.

The basin is confined within Rangamati and embraces upzilla of Rangamati Sadar, Kaptai, Nannerchar, Langadu, Baghaichhari, Barkal, Juraichhari and Belaichhari. A significant number of flora and fauna have recorded from the lake and surrounding hilly forest areas. Haldar et al.,( 2007) recorded 66 species of indigenous fish; Basak et al., (2016) recorded 84 fish species which consist of 75 finfish and 9 shell fish. Among the finfish, 52 are indigenous freshwater fish species, 10 exotic species and 13 marine species. In shellfish group seven species are prawn and shrimp, 1 crab and 1 pond tortoise species. The most abundant freshwater fish species are *Catla catla, Labeo rohita, Clarias batrachus, Cirrhinus cirrhosus,* and *Channa punctatus.* Few numbers of marine fishes have been recorded including *Latescal carifer, Euthynnus affinis* and *Mugil cephalus*. *Macrobrachium rosenbergii* and *Penaeus monodon* abundantly recorded crustacean species. According to IUCN Bangladesh, a total 20 species are recorded threatened for Bangladesh of them 7 are vulnerable, 10 endangered and 3 critically endangered species. Among the fishes most of them are available in the lake.

Ten exotic fish species (*Hypophthalmicthys molitrix, Ctenopharyngo donidella, Cyprinus carpio, Puntius gonionotus, Oreochromis niloticus, Oreochromis mossambicus, Pangasius*

*hypopthalmus*, *Mylopharyngodon piceus*, *Aristichthys nobilis* and *Clarias gariepinus*) recorded from the Kaptai Lake. In 2016, Basak et al., published present current record of *Labeo calbasu* from Kaptai Lake. This speciesis the most important carp species next to the three major carps *i.e. Labeo rohita*, *Catla catla* and *Cirrhinus mrigala* (Chondar, 1999). It is a popular fish in Bangladesh having for good taste, less intramuscular bones and high protein content (Chondar, 1999; Rahman, 2005.; Talwar, 1991). This fish species supports an important commercial fishery in different countries mainly in Bangladesh and India (Pathak and Jhingran 1977, Gupta and Tyagi 1992, Singh *et al.* 1998, Chondar 1999, Dwivedi *et al.* 2004, Nautiyal *etal.* 2004). Recently it has made its entry in ornamental fish markets of India (Gupta *et al.* 2012) and also has been reported to be exported from India as indigenous ornamental fish (Gupta and Banerjee 2014).

### 1.2. Reasons for decline of *L. calbasu* from the Kaptai Lake

Khan et al., (2000) documented *L. calbasu* as a endangered species for Bangldesh while Molur and Walker, (1998) reported the species is near threatened for India. But the wild populations sotck of Kalibasu at Kaptai Lake has sharply declined due to overharveating, fragmentation of habitat and breeding ground, aquatic pollution, and several other man made reasons which are affecting its feeding, migration and spawning (Hossain et al., 2010).

### 1.3. Objectives of the Study

The major objectives of the present study were to:

- To analyze the partial mitogene of *L.calbasu* species
- Molecular characterization of the mitochondrial gene of *L. cabasu*
- Use sequence information from mitochondrial genes to find the phylogenetic position of *L. calbasu*

### 1.4. Characterization of the study area

Kaptai Lake of the southern region of Bangladesh is selected as the study area based on the distribution of candidate species. *L. calbasu* is exhibiting a distribution of fragmented

populations restricted to Karnafuli valley and Chengi, Kasalong and Rinkhyong rivers (Basak et al., 2016). Karnafuli valley and Chengi, Kasalong and Rinkhyong basins in Chittagong Hill tract are originating from the Lushai hills near the Indo-Bangladesh border. A dam was constructed on Karnafuli River at Kaptai Upzilla. Due to the construction of dam, a vast lake of water has formed the reservoir known as Kaptai Lake. The watershed of Karnafuli reservoir lies within tropics. Main watersheds are elongated in the North-South direction, which lies within narrow valleys between parallel ridges of hills, through which the main portion of the river has cut its channel. The topography of Karnafuli river basin is fairly flat and low at downstream but the upstream portion is quite rugged. The river travels a long distance to reach the sea. The natural habitat of fishfauna is mainly found in Karnafuli estuary and it's upstream.

The present study, Kaptai Lake systems which include the habitats of *L. calbasu*, *L. gonia*, *L. bata* and *L. rohita* were short listed for based on the available literature and the survey during the course of collection (detail of sampling and sampling sites are included in section 3.1., Table 3.1, Fig.3.1 and Fig. 3.2).

**1.5. Description of the species**

**1.6. *Labeo calbasu*** (Hamilton-Buchanan)

*Cyprinus calbasu* Hamilton-Buchanan, 1822, *Fishes of Ganges*: 297, 387, pl.2

*Labeo calbasu*: Day, 1877, *Fishes of India*: 536, pl.126; Day,1889, Fauna Br. India, Fishes,1:259; Hora,1921, Rec. Indian Mus., 22(3): 182; Macdonald, 1984, 1984, J. Bombay nat. Hist. Soc., 44: 529

**Taxonomic status**

Phylum: Vertebrata

Subphylum: Craniata

Superclass: Gnathostomata

Series: Pisces

Class : Teleosstei

Subclass: Actinopterygii

Suborder: Actinopterygii

Order : Cypriniformes

Family: Cyprinidae

Genus: *Labeo*

Species: *calbasu*

It is a freshwater fish species; mainly inhabits in rivers but also well established in natural lakes, reservoirs, streams, ponds, beels, baors, haors and canals (Bhuiyan, 1964). Kalibaus (*Labeo calbasu*) (Hamilton 1822) is a teleost fish species distributed in Bangladesh, India, Pakistan, Myanmar, Thailand and also South China. Once this species was abundant in all

natural water bodies: rivers, beels (relatively large water bodies with static water in the Ganga-Brahmaputra floodplains of Bangladesh), haors (wetlands in the northeastern part of Bangladesh which physically are bowl- or saucer-shaped shallow depressions), baors (oxbow lakes, found mostly in the moribund delta as in northeastern Bangladesh) and Lakes of Bangladesh

**Distinguishing characters**

**D** iii-iv 13-16; **A** ii-iii 5; **P** i 16-18; V I 8

Body stout and rather deep, head fairly large and conical, its length less than body depth, snout depressed and fairly pointed, devoid of lateral lobe, studded with pores, eyes moderate, not visible from underside of head, the diameter about 3.3 times in head, mouth inferior, lips thick and conspicuously fringed, both lips with a distinct inner fold, barbells two pairs (rostral amd maxillary). Doral fin with a fairly long base, inserted midway between snout tip and base of caudal fin. Caudal fin deeply forked, scales moderate, lateral line with 40-44 scales, lateral transverse scale-rows 5 to 6 between lateral line and pelvic fin base, predorsal scales 15 to 18.

Colour in life, blackish-green, lighter below, flanks buff pink or with scarlet spots with dark edges which may form stripes, fins black, upper lobe of caudal fin usually tipped with white (Fig. 1.1)

**Fig. 1.1.** *Labeo calbsu* - Specimen collected form Kaptai Lake

**1.7. *Labeo rohita*** (Hamilton-Buchanan)

*Cyprinus rohita* Hamilton-Buchanan, 1822, *Fishes of Ganges*: 301, 388, pl.36, fig)

*Labeorohita*: Day, 1877, *Fishes of India*: 538, pl.127 fig 4; Day,1889, Fauna Br. India, Fishes,1:262; Macdonald,1948,J. Bombay nat. Hist.Soc, fig.9.

*Labeohorai* Fowler, 1924, Proc, Acad. nat. Sci. Philad., 76:95, fig.8 (type-locality: Sutej river. Ludhiana, Punjab)

**Taxonomic status**

Phylum: Vertebrata

Subphylum: Craniata

Superclass: Gnathostomata

Series: Pisces

Class : Teleosstei

Subclass: Actinopterygii

Suborder: Actinopterygii

Order : Cypriniformes

Family: Cyprinidae

Genus: *Labeo*

Species: *rohita*

Rhu is the natural inhabitant of freshwater sections of the rivers of Bangladesh, India, Pakistan, Burma and Nepal (Jhingran and Pullin, 1985). Rhu has also been introduced in Sri Lanka. In Bangladesh, this species is mostly available in the Padma Brahmaputtra system and Halda river system in Chittagong (Alam et al., 2013).

**Distinguishing characters**

**D** iii-iv 13-16; **A** ii-iii 5; **P** i 16-18; V I 8

Body moderately elongated, snout fairly depressed projects beyond mouth, devoid of lateral lobe, eyes large, not visible from underside of head, the diameter 4 to 6 times in head, mouth small and inferior, lips thick and fringed with a distinct inner fold to each lip, barbells a pairs of maxillary barbels concealed in lateral groove. Dorsal fin inserted midway between snout tip and base of caudal fin. Caudal fin deeply forked, scales moderate, lateral line with 40-44 scales, lateral transverse scale-rows 6 to 6.5 between lateral line and pelvic fin base, predorsal scales 12 to 16.

Colour in life, bluish along back, become silvery on the flanks and beneath, with a reddish mark on each scale during breeding seasons (Fig. 1.2).

**Fig.1.2.** *Labeo rohita*- specimen collected from local fish markets of Dhaka city

**1.8. *Labeo bata*** (Hamilton-Buchanan)

*Cyprinus bata* Hamilton-Buchanan, 1822, *Fishes of Ganges*: 283, 386

*Labeobata*: Day, 1877, *Fishes of India*: 542, pl, 129, fig 5; Day,1889, *Fauna Br. India*, Fishes,1:268, Murthy,1977, Proc. Indian Acad.Sci, 85 B(3): 134(Identity discussed)

**Taxonomic status**

Phylum: Vertebrata

Subphylum: Craniata

Superclass: Gnathostomata

Series: Pisces

Class: Teleosstei

Subclass: Actinopterygii

Suborder: Actinopterygii

Order: Cypriniformes

Family: Cyprinidae

Genus: *Labeo*

Species: *bata*

The minor carp *L. bata* is a freshwater benthopelagic, potamodromous cyprinid in Bangladesh. This species can also be found throughout the Indian sub-continent, including Bangladesh, India, Myanmar, Nepal and Pakistan, inhabiting rivers, canals, *haors*, ponds and ditches (Talwar, 1991). Nowadays, this fish is being cultured commercially.

**Distinguishing characters**

**D** iii-iv 13-16; **A** ii-iii 5; **P** i 16-18; V I 8

Body elongate, eyes large, not visible from underside of head, the diameter about 4 to 4.3 times in head, mouth inferior, lips thin lower lip slightly fringed and folded back, barbells a pair of minute maxillary only, not easily perceptible. Dorsal fin inserted nearer snout-tip than base of caudal fin.

Colour in life, golden-yellow above and on dorsal half of flanks, silvery on lower half of flanks and belly, an irregular black blotch present on anterior scale of lateral line (Fig. 1.3).


**Fig.1.3.** *Labeo bata* specimen collected from local fish markets of Dhaka city

**1.9. *Labeo gonius*** (Hamilton-Buchanan)

*Cyprinus gonius* Hamilton-Buchanan, 1822, *Fishes of Ganges*: 292, 387, pl.4

*Labeo gonius*: Day, 1877, *Fishes of India*: 537, pl.127, 1; Day, 1889, Fauna Br. India, Fishes,1:261; Shaw and Shebbeare,1937,J Asiat, Soc Beng., 3:53, pl.2, fig.2 and text-fig.51

**Taxonomic status**

Phylum: Vertebrata

Subphylum: Craniata

Superclass: Gnathostomata

Series: Pisces

Class: Teleosstei

Subclass: Actinopterygii

Suborder: Actinopterygii

Order: Cypriniformes

Family: Cyprinidae

Genus: *Labeo*

Species: *gonius*

Gonia is a common species of minor carps distributed in freshwaters bodies of Pakistan, India, Nepal and Myanmar (Talwar, 1991). In Bangladesh, this species is recorded from geographically isolated different water bodies (haors, baors, beels and rivers) theose are belong to the districts of Kishoreganj, Narsingdi and Noakhali and today is being cultured for commercial purpose(K.M et al., 2016).

**Distinguishing characters**

**D** iii-iv 13-16; **A** ii-iii 5; **P** i 16-18; V I 8

Gonia has shiny color in his life with small scales, Body elongate, head small, snout blunt, rostal flods overlap the upper lips with no lateral lobe, mouth narrow, lips thick and fringed, with a distinct inner fold in their entire circumference, barbells two pairs maxillary shorter than the rostal. Dorsal fin inserted much near to the tip of snout than base of caudal fin; it upper edge concave. Pelvic fin inserted below the middle of dorsal, does not reach the anal fin. Caudal fin is forked (Fig.1.4).

**Fig.1.4.** *Labeo gonius*- Specimens collected from local fish market from Dhaka city

# Chapter 2

# REVIEW OF LITERATURE

Chapter 2

# REVIEW OF LITERATURE

## 2.1. Morphometrics and meristics

Ichthyologists around the world have recorded 32,500 fish species. All these belong to freshwater and marine fish species (Coad and Murray, 2006). To know the species systematics and population genetic structure, fish taxonomist has a strong interest on morphological characters. All fishes have common measureable and countable traits.Such trait considered as powerful tool for measuring discreteness and relationships among various taxonomic categories (Hubbs and Lagler, 1964).

Generally, genetic marker is used in molecular biology for research purpose is too sensitive and have low level of gene flow and have a relatively low of exchange between stocks (Ward and Grewe, 1995). Therefore, molecular markers and partial sequence of conserve regions may not be sufficient to analyse intragenric variation among populations. However, phenotypic traits may detect morphological differention that due to environmental change in habitat of partially-isolated populations (Carvalho and Hauser, 1998). Well documented studies on fish taxonomy and population stock structure in fishes by morphological traits have conducted by Jayasankar et al., (2004). Choudhury and K Dutta, (2012) have findout interrelationships of five species of the genus *Labeo* by morphometric analysis. Mahfuj et al., (2017) observed morphological variations of *Labeo bata* populations (Teleostei: Cyprinidae) in six rivers of Bangladesh by landmark-morphometric method. Begum et al., (2013) has shown morphological and genetic variations in wild and hatchery populations of Gonia (*Labeo gonius*, Hamilton) using truss measurement and allozyme markers. Hossen et al., (2018) findoutpopulation parameters of the minor carp *Labeo bata* (Hamilton, 1822) in the Ganges River of northwestern Bangladesh by morphometric traits. Hossain et al., (2010) have shown morphometric and meristic variations of the endangered carp, Kalibaus *Labeo calbasu*, from stocks of two isolated Rivers, the Jamuna and Halda, and a Hatchery.

Sometimes environmental parameters may induce morphological variations among the isolated fish population and phenotypic plasticity in fish morphology. Such type of phenotypic plasticity of fish allows them to respond environmental changes by the modification in their

physiology and behavior which leads to changes in their morphology, reproduction or survival that mitigate the effects of environmental variation. Early investigators used descriptive statistics and univeriate analyses separately on each meristic and morphometric character. But these did not always yield effiective results (Surre et al., 2011). The multivariate technique such as principal component analysis, factor analysis, cluster analysis, and discrinant analysis have been adopted in the fish identification and population structure of fishes which give always positive results (Nowak et al., 2008; Quilang et al., 2007; Trapani, 2003).

The study of shape variation has advanced from measuring simple linear distances to deriving geometric variables. The shift form traditional morphometrics to more complex geometric functions was facilitated by the development of image processing tools. Recently developed geometric methods to morphometric analysis are generally categoried as either landmark methods or out methods. The landmark method analyses data derived form discrete, morphometric points, linear distances between point and geometric relationships among points, where as outline methods quatify boundary shapes of structure such as scales so that patterns of shape variation within and among groups can be evaluated.

Meristic characters generally reffered to the characters that corresponded to body segments such as vertebrae numbers and fin rays counts. Now, any countable structure is used in meristic study, including number of scales, gill rakers and so on. These characters are usefull because they are clearly distinguisible. Also meristic characters are easier to treat statistically, so comparisons can be made between populations and among the species, whereas, morphometric studies are based on a set of measurements which represent size and shape variation and are continuous data. Therefore, the predictive abilities of morphometrics and meristic characters differs statiscally. Thus morphometric data should be analysed separately from meristic data in multivariate analyses (Ihssen et al., 1981).

Fishes are considered to morphologically more variable than most other vertebrates having relatively higher within- population coefficient of variation of phenotypic characters (Carvalho, 2005). The differentiable variation in morphology among fish populations has been suggested as indicative of the presence of stock structuring and restricted movement among geographically isolated populations. Highly dispersible morphological characters have found on marine and

estuarine species that revealed spatial separation of populations on morphometric characters and gill rackers counts.

Even though, well documented morphometric and meristic studies have found, which provide evidence for identification of species and stock discrimination in fishes, the application of morphometrics in stock identification is complicated by the fact that phenotypic variations in these characters are often influenced by environmental factors and are not directly related to particular differences in the genome always (Clayton, 1981). Therefore, several new techniques using molecular markers came into practice to indentification of species and to detected stock structure and genetic variations of the organism.

## 2.2. Mitochondrial DNA

Eukaryotic cell has one copy of the entire nuclear genome in its nucleus. In contrast, every cell contains several thousand mitochondria depending on energetic requirements. Mitochondria contain a trace amount of genetic materials that is essential component for eukaryotes. It is well known that mitochondria prossess their own genome and the machinery for replication, transcription and protein synthesis (Ankel-Simons and Cummins, 1996).This organelle has been found to play a central role in metabolism (oxidative phosphorylation), apoptosis, thermogenesis, and aging.

Animal mtDNA is a circular in structure and a high copy number has observed in the mitochondrial cells (Fig. 2.1).

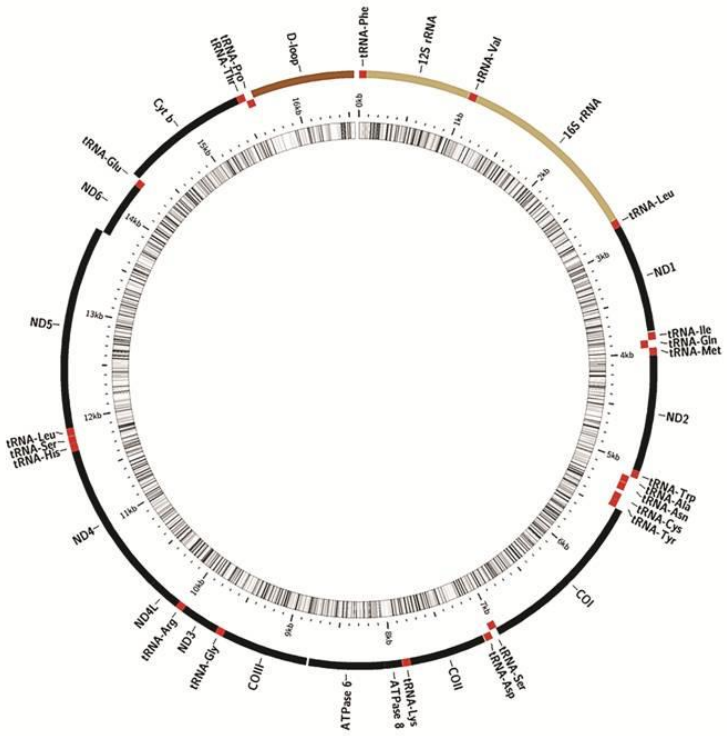


**Fig. 2.1**. Cyprinids mitochondrial genome, lebels outside ring indicate the loctions of the 2 *rRNAs*, the 13 mitochondrial-encoded polypeptides of OXPHOS and the light strand origin and *tRNAs*.

Researchers compare animal mtDNA with fungi, plants and protists. But animal mtDNA show little genomic variation in size it range from 16 to 20 kb in length, coding for 40 genes; 2 of them encoded of the ribosomal RNAs, 22 transfer RNAs and 13 encode polypeptides of the oxidative phosphorylation system (OXPHOS) (Hartl et al., 1997(30). The mitochondria have 4 regions: D-Loop, *rRNA*, *tRNA* and gene that code for protein. The regions that code for *rRNA*, tRNA and protein regon are called the "coding region". The Displacement-Loop (D-Loop) has a non-coding D-loop region often reffered to as the "hypervariable region" which containsessential transcription and replication elements (Garesse and Vallejo, 2001). In vertebrates, transcriptions are initiated bi-directionally at two promoters, PH and PL for heavy (H) and light strands (L) repectively within the D-Loop regulatory region (Shadel and Clayton, 1997). The evolution rate of this region is much faster than the whole mDNA molecules. The study shows it evolves 5 to 10 times faster than the nuclear DNA (Brown et al., 1979) because of the absence of repair enzymes for errors in the replication nor for the damages of the DNA(Clayton, 1982). Teleosts fish shows high mutation rate among the vertebrates that leads a high variable sites in their intraspecies level (Huey et al., 2006). It also has a capacity to accumulate mutations that give it a faster rate of evolution than nuclear DNA in higher vertebrates (Brown et al., 1979).

Mitochondrial DNA process several favorable characteristic, requirement of only a small amount of fresh, frozen or alcohol-stored tissue, haploid, materal inheritance and extremely low probability fo paternal leakage (Cummins et al., 1998) change maily mutation through mutation recombination. All the features make mDNA a useful and one of the most frequently used markers in molecular systematic and has been widly employed to address questions of genetic diversity, population structure, phylogegraphy and population evolution of animals (Anderson et al., 1981; Ankel-Simons and Cummins, 1996; Domínguez-Domínguez et al., 2008; Stamatis et al., 2004). There are some disadvantages with this marker like as maternal inheritance which does not provide information about males in populations but display different dispersal behavior to females

It is almost ten years since the polymerage chain reaction(PCR) has been used for amplifying and sequencing of microbial, plant and animal nuclear and mitochondrial genes (Kocher et al., 1989). From 1990's an increasing number of studies have been published making use of selected parts of mitochondrial and nuclear genome. The mitochondrial DNA control

region, which includes the D-loop in vertebrates, also known as AT rich region in invertebrates, does not code for a functional gene (Saccone et al., 1987) and therefore more sensitive to protein loci as a maker to score intraspecific variation of many organisms (Caccone et al., 1996). Such kind of genetic variability is pivotal to maintain the capability of restocked fish to adapt to a changing environment(Nguyen et al., 2006).

The mitochondrial *16S rRNA* is structural, non coding gene. Its transcript is the large subunit ribosomal RNA that in a conserved secondary structure and in association with proteins froms the large subunits of mitochondrial ribosomes. This gene frequently used for molecular systematics of several animal taxa. *16S rRNA* gene sequencing provide accurate species identification of various groups of fish (Ziyadi, 2012). However, the use of *16S rRNA* gene sequencing in Fish taxonomy is still often reserved for the Ichthylogist. Fish that are difficult to identify by morphoetric and meristic characters and where mistakes in identification often occur, *16S rRNA* gene sequencing is most useful tool. For rarely encountered fish, their morphomertic profiles are often poorly defined or not included in existing identification system databases. Fish taxonomist also sometimes encounters rare fish species with typical phenotypes which can lead to mis-identification. *16S rRNA* gene sequencing can provide genus identification in >100% and species identification up to 99.99% of these circumstances. *16S rRNA* gene have also found effective in bivalves (Bendezu et al., 2004), crustaceans (Grandjean et al., 2002) and fishes (Craig, 2007; Doiuchi and Nakabo, 2006; Ilves and Taylor, 2009; Sparks and Smith, 2004; Vinson et al., 2004; Wiley et al., 1998)

Therefore, sequencing of *16S rRNA* gene is much more crediable and is now considered the standard for identification of most Fish species. Recently a large amount of fish 16 rRNA gene sequence data have been generated and deposited in various databases like as NCBI GeneBank database, which has BLAST search matching of query sequences from any fishes. In the genomics era, the availability of fish genome sequences has also confirmed the representativeness of *16S rRNA* gene in comparison to complete genome for fish taxonomic studies.

ND genes have found effective for identification of a variety of organisms including mammals (Anderson et al., 1982, 1981; Bibb et al., 1981; Chomyn et al., 1985), amphibians

(Roe et al., 1985), insects (Clary et al., 1982; Garesse, 1988), protozoa (Koslowsky et al., 1990; Pritchard et al., 1990; Souza et al., 1993), plants ((Oda et al., 1992), and algae (Michaelis et al., 1990), as well as filamentous fungi (Brown et al., 1989; De Vries et al., 1986). In the plants liverwort (Ohyama et al., 1986)), tobacco (Shinozaki et al., 1986), and rice (Hiratsuka et al., 1989), ND genes not only identify the species but various multiple disorders of humans are associated with mutations of the mitochondrial ND genes (Wallace, 1992) have been identified.

The *mtND1* gene is one of the 13 protein-coding genes in mitochondrial DNA involved in respiration (Gerber et al., 2001). The complete length of *ND1* is 957. Variant of *ND1* is associates with the cancer (Akouchekian et al., 2011),type 2 diabetes mellitus (Yu et al., 2004) and other human diseases. The NADH dehydrogenase subunit 2 (*ND2*) enzyme is encoded by the MT-*ND2* gene and its size in *L. calbasu* is 1044 bp (Yang et al., 2012). As MT-*ND1*, MT-*ND2* is one of the 13 mitochondrial genes and it also takes part in the transfer of electrons and hydrogen to produce ATP through the progress of phosphorylation. Previous researcher found nucleotide variation in *ND2* gene of bird which is effective for genetic variation and diversity with species(Astuti and Prijono, 2016).

The animal mitochondrial genome geneally encoded 22 tRNA genes. Although their arrangement is variable among invertebrates mitochondrial genome, a typical gene arrangement highly conserved among vertebrates mitochondrial genome from jawless fish to mammals with some eceptions. The typical secondary structure for nuclear tRNA genes was estimated, and each nucleotide position of the structure was numbered 1–73 from the 5'- to 3'-ends(Sprinzl and Vassilenko, 2005). These mitochondrial tRNA genes are interspersed between ribosomalRNA and protein-coding genes. Each tRNA sequence can be folded into a cloverleaf structure except $tRNA^{Ser}$ , contain an amino acid stem, an anticodon stem, adihydrouracil loop (DHU) stem, and a TψC stem.

On the otherhand, mitochondrial DNA has been the most popular marker in Phylogenetic analysis of fish for its maternal mode of inheritance and high mutation rate. In recent years, to solve the ambiguity that's related to the phylogenetic relationship. Besides 16S rRNA, other commonly used mitochondrial genes are the ctychrome oxidies I (*COI),* ctychrome b (*cyt b*) and the small subunit ribosomal RNA (*12S rRNA*). While *COI* and *Cyt b* are mitochondrial coding

genes, the *12S rRNA* and *16S rRNAs* are structural, non coding genes. Their *rRNA* transcription from part of mitochondrial ribosomes where they play important catalysic roles during translation of *mRNA*s to proteins, the *rRNA*s have secondary structure with stem and loops and some regions of the molecules are active in biochemical process and thus more conserve in their sequence. The combination of variable and conserve regions within the same gene is probally one of the reasons why *16S rRNA* has become one of the most popular genes for reconstructing animal phylognetics.

To study fish phylogeny, the *16S rRNA* gene is very useful due to the gene presence in almost all fishes, often existing as a multigene family, or operons, the function of the *16S rRNA* gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time and the *16S rRNA* gene (1500 bp) is large enough for informatics purposes (Patel, 2001).Using *16S rRNA* sequences, numerous fish genera and species have been reclassified and renamed, phylogenetic relationships have been determined(Basudha, 2016; K. K et al., 2016; Lakra et al., 2013; Sarkar, 2012; Singh et al., 2015, 2018).

However, there has been no report on the phylognetic analysis of *L. calbasu* from Bangladesh based on the mitochondrial NADH dehydogenase subunit 1 and 2 gene (*ND1*) and (*ND2*). *ND1*gene have been found to be a useful marker for inferring intraspecific variations, biogeography and identification of digenean trematodes(van Herwerden et al., 2000), solving controversy over subgenric taxonomic classification bassed on morphological characters of a small group of monkey from South America and Panama (Tagliaro et al., 2005).

### 2.3. Bangladesh scenario

Now genetics has become an important tool in Bangladesh for taxonomical, phylogenetical, biogeographical and population genetics. In recent years, researchers when working on taxonomy, population structure, gene flow study, they are adding morphometric, meristics and hydrological data for the accuracy of the species or the population. These types of research have been conducted on marine and freshwater fish species those have economic value. Over the last quarter of century numerous genetic studies have been undertaken to study

molecular taxonomy, determine variation and population structure of fresh water fishes using a variety of molecular techniques. Many authors had used allozymes electrophoresis technique for identification of major Carps (*Catla catla, Labeo rohita* and *Cirrhinus cirrhosus*) and their hybrids (M.r.i et al., 2011;Hoshan et al., 2012; Hossain et al., 2013). With the progression of molecular techniques like as RFLP'S, RAPD'S and mtDNA sequencing have quickly adopted by many authors (Mollah et al., 2009). Begum et al., (2013) have taken *16S rRNA* for molecular characterization of *Clarius batracus* species other author have taken COI gene for indentification of Hilsha and cat fish (Ahmed and Reza, 2018; Boidya et al., 2015; Smriti et al., 2017; Tabassum et al., 2017), *16S rRNA* gene for *Clarius garieus* (Miah et al., 2013; Sultana et al., 2015)(Alam et al., 2015). Munira et al., (2016) have used cyt b gene for molecular detection of cattle and buffalo species meat. Mazumder and Alam, (2009) have used PCR-RFLP and the mitochondrial DNA D-loop region for the genetic variability and differentiation in hilsa shad, *Tenualosa ilisha* (Clupeidae, Clupeiformes) populations,Shifat et al.,(2003) use of RAPD fingerprinting for discriminating two Populations of Hilsa shad (*Tenualosa ilisha* Ham.) from Inland Rivers of Bangladesh.

# Chapter 3

# MATERIALS AND METHODS

Chapter 3

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## 3.1. Sampling

Nine samples of Kalbasu were collected from Kaptai Lake in Rangamati, Nanner Char, Langadu Upzillas and the waterfall near the Barkall boat station which is located at Barkal Upzilla (Fig. 3.1, 3.2 and Table 3.1). Specimens of *L. calbasu* were morphologically identified in the lab following the taxonomic key by Talwar, (1991). A small portion of muscle tissue from trunk of right side of each specimen was excised and stored in deep refrigerator. For further study, the specimens were fixed and preserved in 70% alcohols (Voucher specimen was deposited at Molecular Biology and Genetics research Lab, Department of Zoology, University of Dhaka).

## 3.2. Ecological observations

The sampling sites fall within the 'IUCN Bangladesh'designated' 'Bio-Ecological zone: 5C Kaptai Lake' which is rich in biodiversity (Bio-ecological Zones of Bangladesh, 2002). This is an artificial lake and has since flooded over 68,800 ha of forest valley and the Chittagong hill tracts districts (Bio-ecological Zones of Bangladesh, 2002). This wetland is surrounded by evergreen forest. Fig 3.1and 3.2 show the sampling sites, which is part of Kaptai Lake, the land use map shows that maximum land is hilly area, agriculture land and human establishment and rest of the areas are water bodies. In November 2016, Lake's water was observed in flowing condition and transperant state in the sampling period. A FGD (Focous Group Discussion) conducted with the local fishermen. The species abundance in the Lake has been reduced drastically due to over fishing.

**Table 3.1. Details of sampling sites**

| River basin | Geographical location | | Date of capture | N |
|---|---|---|---|---|
| Kaptai Lake | 22°37'38.28"N | 92°12'44.48"E | December 2017 | 2 |
| Waterfall nearest Barkal upzilla | 22°43'21.03"N | 92°22'3.57"E | December 2017 | 2 |
| Kaptai Lake | 22°50'58.37"N | 92°14'56.76"E | December 2017 | 1 |

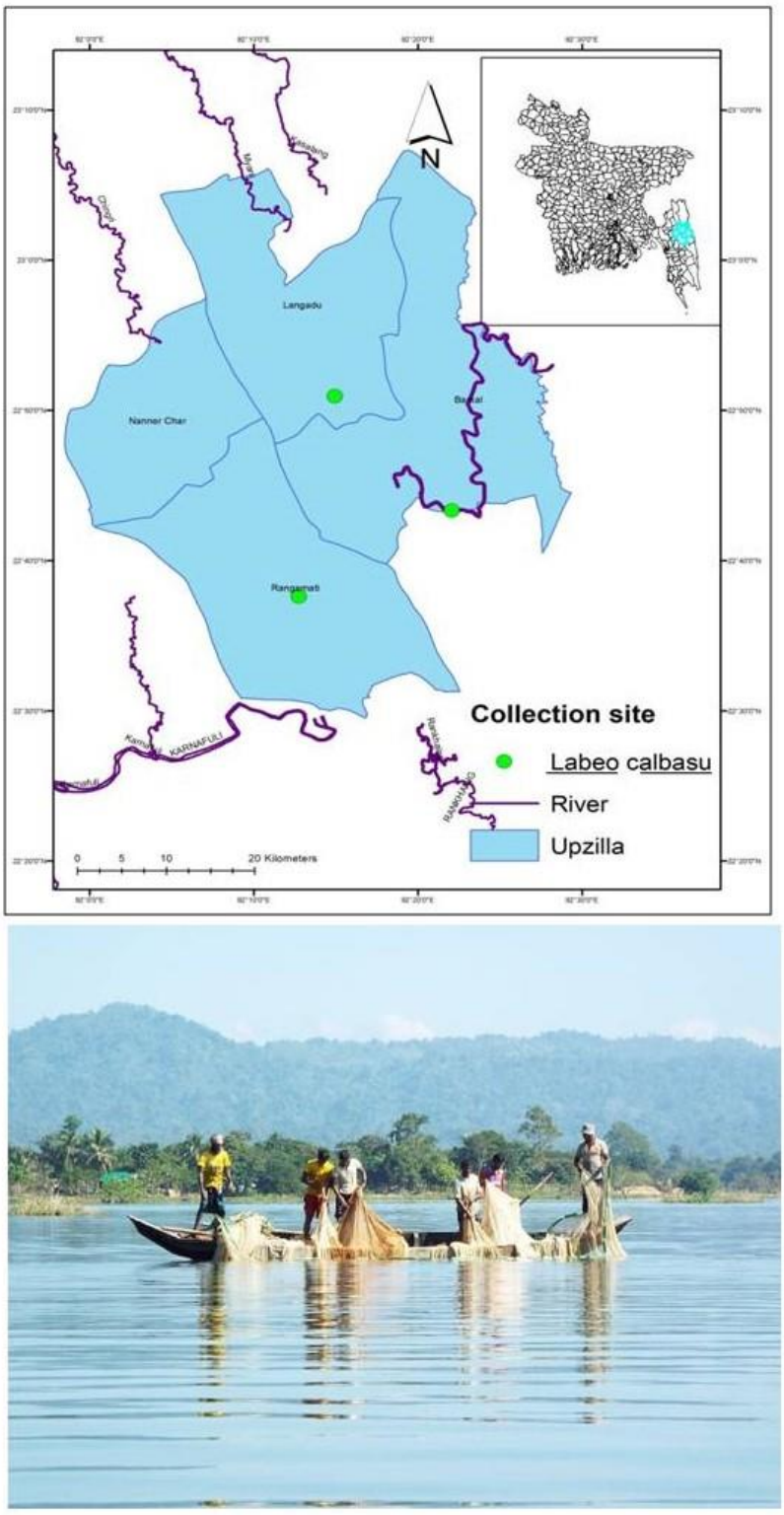



**Fig.3.1.** Map of study area showing the sampling sites of *L. calbasu*, river and lake connectivity

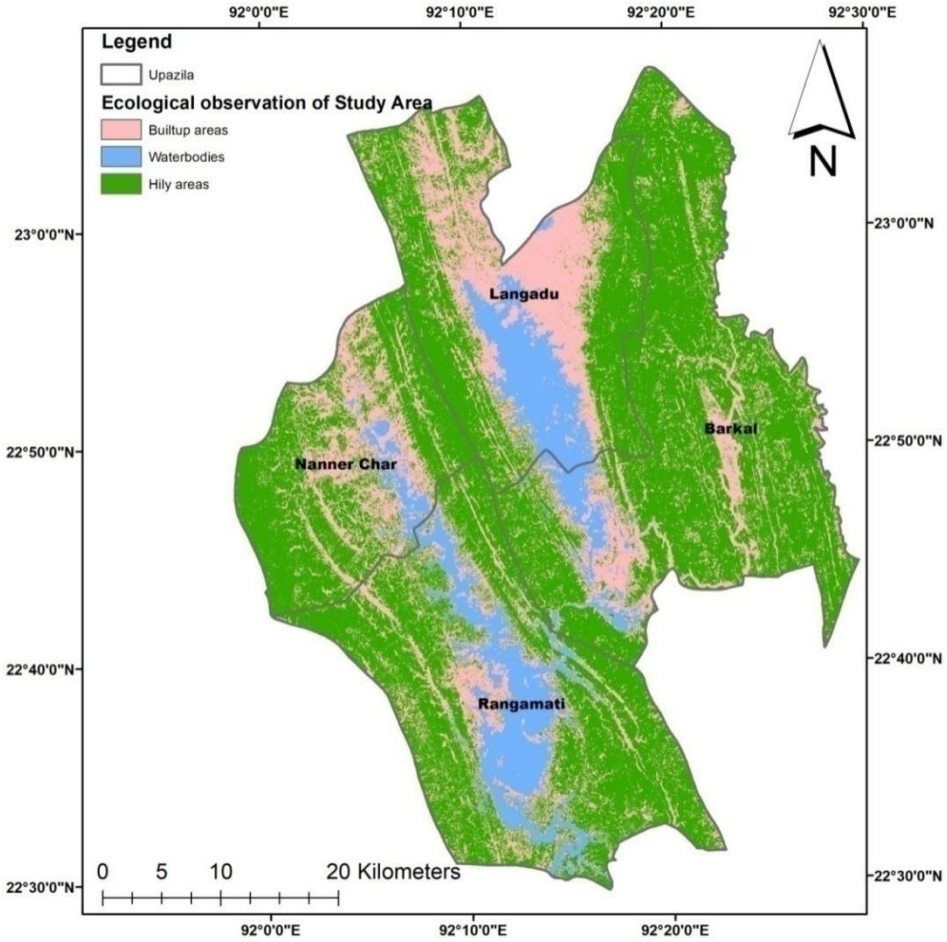



**Fig.3.2.** Map of present study areas showing the water bodies from where *L. calbasu* was collected

### 3.3. Morphometrics and meristics measurements

#### 3.3.1. Laboratory Work

Collected sample from different sites were tansfered to lab and used for morphogical study and molecular study.

#### 3.3.2. Morphometric Measurements

Fourteen morphometric characters were measured along the body of fish to the nearest 0.05 mm with digital callipers and metallic ruler, following the conventional method described byHubbs and Lagler, (1964). Thirteen measurable characters were selected for the morphometric study (Table 3.2 and Figure 3.3). All the measurements and counts were taken followingKottelat, (1990). All length measurements (L) were taken parallel to antero-posterior body axis. Wide of mouth was measured between the points of lateral edges of jaws of the mouth.

#### 3.3.3. Meristic Counts

A number of 16 meristic variables based on Holcik et al.(1989), were count in each specimen by direct observation. Abbreviations used for meristic characteristics are: D1, Dorsal fin spines; D2, Dorsal fin branched rays; A1, Anal fin spines; A2, Anal fin branched rays; P1, Pectoral fin spines; P2, Pectoral fin branched rays; Pel1, Pelvic fin spines; Pel2, Pelvic fin branched rays; Ll, Lateral line scales; Squ.Sup,Scales above lateral line; Squ.Inf, Scales below lateral line; CP, Caudal peduncle scales; Ac, Around Caudal peduncle scales; Gr1, Outer gill rakers; Gr2, Inner gill rakers;Vn, Vertebrate number. To avoid human error all morphological measurement were performed by the same investigator.

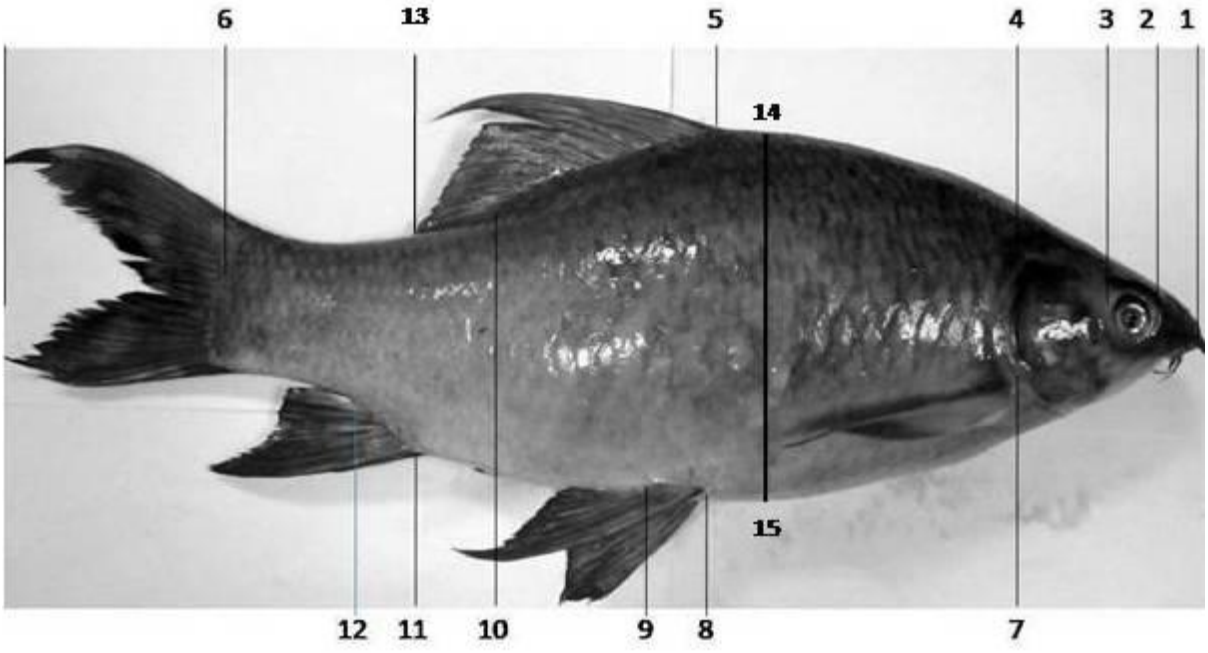



**Fig. 3.3.** Image of a *L. calbasu* depicting marks used to infer morphometric character measurements

**Table 3.2. Description of morphometric characteristics**

| Character | Code | Description | Number |
|---|---|---|---|
| Standard Length | SL | Snout tip to the midpoint of caudal fin origin | 1-6 |
| Head length | HL | Snout tip to the posterior edge of operculum | 1-4 |
| Pre dorsal length | | Snout tip to the origin of dorsal fin | 1-5 |
| Pre pelvic length | | Snout tip to the origin of pelvic fin | 1-8 |
| Pre anal length | | Snout tip to the origin of anal fin | 1-11 |
| Width of mouth | | Length between the points of lateral edges of jaws of the mouth | Not shown |
| Eye length | | Length (along axis) of the orbit | 2-3 |
| Snout length | | Distance from the tip of the snout to the anterior margin of the eye | 1-2 |
| Inter-orbital width | IOW | Measured as the shortest distance between the fleshy margins of the orbits | Not shown |
| Head width | | The distance between the two points of the head | Not shown |
| Length of Caudal Peduncle | LCPD | The distance between the base of the anal fin and the posterior edge of the hyural plate | 6-12 |
| Dorsal fin base length | | Length of dorsal-fin base (from base of 1st to base of 5th rays) | 5-13 |
| Body depth | | The vertical distance between the dorsal and ventral margins of the body | 14-15 |

### 3.4. Data Analysis

To eliminate any size effect in the morphometric data setbody depth, predorsal, preanal, prepelvic length were divided by standared length; snout, Iow, head length were divided by the eye diameter; head length was divided by the snout length, head width, wide of mouth, LCPD, dorsal finbase length and finally standared length was divided by head length to remove size-dependent variation in morphometric characters.

#### 3.4.1. Univariate coefficients of variation

The coeffeiceient of variation (CV) is widely used measure in external quality assessment, laboratory medicine as well compute for morphometric character. The coefficient of variation (CV) was computed for each character using the Van Valen, (2005)

CV = (100 x SD) $X_m$

Where, SD is the standad deviation and $X_m$ is the mean of transformed measuremed measurements of characters in each species.

#### 3.4.2. PCA (Principal Component Analysis) analysis:

For PCA analysis 13 morphometric variables were selected. Each variable was transformed into same unit for PCA which was performed by trial version XLSTAT software. All descriptive statistics were performed by IBM SPPS statistics software.

#### 3.4.3. Principal component analysis (PCA) step by step

A principal component analysis is concern with explaining the variance-covariance structure of a set of variables through a few linear combinations of these variables.

Its general objective are (I) data reduction technique and (2) interpretation.

**Data preprocessing**

**Compute "covariance matrix"**

Covariance is a measure of the extent to which corresponding elements from two sets of ordered data move in the same direction. We use the following formula to compute covariance.

Cov($X$, $Y$) = Σ ( $X$i - $X$m) ( $Y$i - $Ym$ ) / $N$

*Cov (X) = E (E(X - Xm)' * E(X - Xm))*

Here X is the data set, Xm is the mean of corresponding columns or variables, and similarly for y. E is the mean.

**Compute "eigenvetors" of matrix**

**Principal Component Analysis (PCA) algorithm**

Algebracally, principal components are particulary linear combination of the p random variables $X_1$, $X_2$---------- Xp. Geomatrically, these linear combinations represent the selection of a new coordinates system obtainted by rotaing the orginal system with $X_1$, $X_2$---------- Xp as the coordinates axes.The new axecs represent the directions with maximum variability and provide a simpler and more parcimous description of the covariance structure.

Principal component soley depended on covariance matrix ∑(or the coorelation matrix ***p***) of $X_1$ $X_2$---------- X***p***

Let the random vector **Ẋ** =[$X_1$, $X_2$---------- Xp] have the covariance matrix ∑ with eigenvalues $\lambda_1 \geq \lambda_2 \geq$---------- $\geq \lambda_p \geq 0$

Considered the linear combinations

$Y_1$ = **á**$_1$**X** = **á**$_{11}$**X** + **á**$_{12}$**X**+----------+ **á**$_{1p}$**X*****p***

$Y_2$ = **á**$_2$**X** = **á**$_{21}$**X** + **á**$_{22}$**X**+----------+ **á**$_{2p}$**X*****p***

Y***p*** = **á*****p***$_1$**X** = **á*****p***$_1$**X** + **á*****p***$_2$**X**+----------+ **á**$_{pp}$**X*****p***

Then var ($Y_1$) = **á**$_i$∑**Xa**$_1$ I = 1,2,----------***p***

cov ($Y_i$,$Y_k$) = **á**$_i$∑**Xa**$_k$ *I, k* = 1,2,----------***p***

The first principal component is the linear combinations with maximum variance. That is it maxizes Var ($Y_1$) = **á**$_i$∑**Xa**$_1$. It is clear thatVar ($Y_1$) = **á**$_i$∑**Xa**$_1$ can be increased by multiplying

any $\mathbf{a_1}$ by some constant. To eliminate this indeterminacy, it is determinted to restrict attention to cofficeient vactor of unit length. We therefore define

First principle component = linear combination $\mathbf{á_1X}$ that maxizes

Var $\mathbf{á_1X}$ subject to $\mathbf{á_1}$ $\mathbf{a_{1=1}}$

Second principle component = linear combination $\mathbf{á_2X}$ that maxizes

Var $\mathbf{á_2X}$ subject to $\mathbf{á_2}$ $\mathbf{a_{2=}}1$

Cov ($\mathbf{á_1X}$ $\mathbf{á_2X}$)

At this ith steps

Second principle component = linear combination $\mathbf{á_2X}$ that maxizes

Var $\mathbf{á_2X}$ subject to $\mathbf{á_2}$ $\mathbf{a_{2=}}1$

### 3.5.1. Genomic DNA Isolation (gDNA)

Muscle tissue of *L. calbasu* was processed for genomic DNA (gDNA) following the phenol-chloroform procedure with minor modification (Lu et al., 1997).

**Required apparatus**

| **Apparatus name** | **Details** |
|---|---|
| Auto vortex, 51W | Heidolph ,Germany |
| Camera | Samsung, 12MP |
| Centrifuge machine | S4150,Eppendorf, Germany |
| Distilled water machine | 11123904J, GFL, Germany |
| Electronic balance | AR1140, Ohaus Coro USA |
| AGE Electrophoresis Unit | Fisons, FEC-370 |
| Incubator | Memmert, Germany |
| Latex Examination Gloves | Xtend$^{TM}$, Malaysia |
| Magnetic Stirrer ,CB162 | Struart |
| Microwave Oven | Whiripool |
| Microcentrifuge Tubes 1.5 | StarLab S.r.l, Italy |
| Micropipette | K-83-16966, Gilson, France |
| Power packs | PP3000, Biometra |
| PCR machine | Applied Biosystems,Thermo fisher scientific (Veriti 96 Well thermal cycle) |
| Refrigerator | -20$^{0}$C Platinum 500, Angelantony, Italy |

| | |
|---|---|
| UV transilluminator | WealTec, USA |
| Water bath | GTR214, Memmert, Germany |

**Required Glassware and others materials**

| | |
|---|---|
| Beakers | Aluminium foils |
| Eppendorf tubes 10 µl,20 µl,100,200 µl, 500 µl | Blotting papers |
| Measuring cylinders | Tray |
| Petridishes | Test tubes |
| Masking tape | Cotton |
| Forceps | Glass rod |
| Pipette tips | Surgical gloves |

**Required Chemicals**

| | |
|---|---|
| EDTA | Tris base(Merck,Germany; 669.96**)** |
| Glacial Acetic Acid | Ethidium bromide solution 1% (10mg/ml, ROTH, German). |
| Hydrochloric Acid | Sodium Chloride |
| Phenol Chloroform | Agarose powder |
| Distilled water | Tris HCL |
| NaOH | RNAase |
| 100 ml 50X TAE | 100 1X TAE |
| Distilled water | Proteinase K |
| 70% Ethanol | Isopropanol |
| GeneRuler 1kb DNA Ladder (Thermo Scientific, USA) | GeneJET PCR Purification kit (#K0701, #KO702) |

**Stock solution**

**Tris HCl (pH-8.0)**

Molecular weight 121.14 gm/1000ml

| | |
|---|---|
| Tris base | 12.11gm(1M) |
| Distilled water | 70ml |
| HCL | 4.5ml |

Adjust pH to 8.0 using added additional HCl or NaOH. Make up the volume to 100ml added additional $dH_20$ and stored at room temperature.

**0.5M EDTA (pH-8.0)**

EDTA (molecular weight) 372.24 g/mol

| | |
|---|---|
| 1 M | 37.224 gm/100 ml |
| 0.5 M | 18.612 gm/100 ml |

Adjust pH to 8.0 using NaOH and stored at room temperaute

**10N NaOH**

| | |
|---|---|
| Na pellet | 40gm |
| Distilled water | 100ml |

**CTAB**

Ingredients for 100ml

| | |
|---|---|
| 1M Tris HCl (PH:8) | 10ml |
| 0.5M Na2 EDTA (PH:8) | 4ml |
| Nacl | 8.18g |
| CTAB | 2g |

SDW(/d$H_2O$) PH:8

Before use, added 2µl of 2 mercapto ethanol to make 1ml CTAB buffer

**TE buffer**

Ingredients for 100ml

| | |
|---|---|
| 1M Tris Cl (pH-8.0) | 1ml |
| 0.5M EDTA (pH-8.0) | 0.2ml |

**RNAase**

RNAase - 10 mg/ml of RNAase buffer

**Proteinase K**

20mg/ml Proteinase K powder dissolve in 50mM Tris-HCl, 2mM calcium acetate, PH:8

**3.5.2. Genomic DNA Isolation Protocol:**

Protocol for gDNA isolation from muscle tissue of *L. calbasu*

0.05 gm of muscle tissue was taken in 1.5 ml eppendorf tube then added 400µl of CTABbuffer after that tissue was homogenized with the help of sterilized glass rod.

⇩

Next grinded sample was transferred into a sterilized 1.5 ml microcentrifuge tube.

⇩

In addition, 10µl Proteinase K (11mg/ml) was added as well as the sample was mixed thoroughly by the vortex machine.

⇩

After vortex. the sample was incubated at 55 degree celsius in shaking water bath for 120 minutes

⇩

Incubated samples were taken out from the bath and 10µl of RNAase solution was added. Then mixed sample was vortexed thoroughly also incubated it at room temperature for 30 minutes.

⇩

Carefully, total sample was centrifuged at 13,000 RPM using the centrifuged machine.

⇩

Sample was collected from the centrifuged machine then transferred to another 1.5µl eppendorf tube.

⇩

Equal volume of Phenol chloroform was added in the same volume of colected sample also inverted for 1minute.Whole sample was centrifuged for 5 minutes at 13,000 RPM. As a result two distinct layers were formed in the centrifuged tube.

The upper supernatant was cautiously collected from centrifuged tube and transferred into an eppendorf tube with the help of micropipettes

⇩

.

Same volume of Isopronol solution was added in collected supernatant solution and inverted the sample carefully for several times.

⇩

Then sample was centrifuged for 5 minutes at 13,000 RPM as well as Isopropanol was totally removed by drainage technique.

⇩

After drainage, 500µl of 70% ethanol was added in the tube furthermore it was centrifuged for 1 min at 13.000 RPM.

⇩

Total ethanol was discarded from the tube applying the drainage technique

⇩

Tube was dried in air for the suspended DNA

⇩

In addition, 20µl nuclease free water was added in the DNA pellets as well as incubated for 10 min at 37 degree Celsius.

⇩

Finally isolated gDNA sample was stored at $-20^0$C for further reactions.

### 3.5.3. Agarose electrophoresis and visualization of gDNA bands

The gDNA sample was examined through 1% agarose gel electrophoresis (10x7cm) with ethidium bromide incorporated in 1X TAE buffer and 1KB DNA ladder.

**Stock solution preparation:**

| **TAE Buffer 50X (PH-8.2-8.5)** | | **Preparation of 1X TAE buffer from 50X** | |
|---|---|---|---|
| Tris base | 24.2g | TAE buffer 50X | 1ml |
| Glacial acetic acid | 5.71ml | | |
| 0.5M EDTA | 1 0.0ml | Distill water | 49ml |
| Make up the solution to 100ml add distilled water and stored at room temperature | | prepared 1X buffer store at room temperature for further use | |

**Ethidium bromide preparation**

| | |
|---|---|
| Ethidium bromide | 10mg |
| Distilled water | 1ml |

**Agarose gel 1% preparation**

| | |
|---|---|
| Agarose M (Gel strength1%) Bio Basic Canada INC. | 0.50gm |
| 1X TAE | 50 ml |

### 3.5.4. Gel preparation, sample loading and Gel run:

According to manufacturer procedure, gel casting unit was arranged

**1% agarose gel preparation and sample loading**

Firstly, 0.5gm of agarose powder and 50ml 1X TAE buffer was poured in a beaker

⇩

Secondly, agarose solution was heated at 60 seconds on an oven

⇩

Thirdly, when powder was completely melted then solution was taken out from oven as well as the solution was kept at room temperature for slightly warm condition.

⇩

Fourthly, 0.2µl of ethidium bromide was added in the solution and mixed up thoroughly.

⇩

Fifthly, the solution was poured in a already adjusted gel comb gel casting plate

⇩

Sixthly, the solution was allowed to solidify at room temperature for 30 minutes

⇩

After that, carefully the comb was removed from the gel. The gel was placed in electrophoresis unit.

⇩

Next 1X TAE buffer was poured into the electrophoresis unit as electrolyte.

⇩

4µl gDNA + 1µl loading dye = total 5µl volume of samples and 3µl 1kb gene ruler were loaded in each wells of gel

⇩

Electrophoresis was done at 80V constant voltage and 44miliampere for 18 minutes

⇩

After completing electrophoresis, the gel was taken out from the unit. Then it was placed on UV chamber for observation of band using UV transilluminator.

### 3.6. Primer designing

Primers were designed, using the alignments of complete mitochondrial genome sequences of nine *Labeo* species as well as other closely related to the Kalibasu species. The aligned sequences were Angra (*L. angra*), Bata (*L. bata*), Boggut (*L. boggut*), Malabar (*Labeo*h*edussumieri*), Heel-gorya (*L. d. dyocheilus*), (Fringed-lipped) *L. fimbriatus*, Indian medium carp (*L. gonius*), Pangusia (*L. pangusia*), Rhou (*L. rohita*). Three sets of primers designed are as follows –

| Forward | Sequence(5́ -3́) | Reverse | Sequence (5́ - 3́) |
|---|---|---|---|
| LCA3-F | CCC CGT TAA ACC TCA CCA CT | LCA3-R | TCA CCA GGT TCG GTA GGT CT |
| LCA4-F | ACA GCC TAT TAA GGG CCA ACC | LCA4-R | AAG CTC CAA AGG GTC TTC TCG |
| LCA7-F | GCACCATTCGACTTAACAGAAGG | LCA7-R | TCAGGGGTTTGTTGTGCTTG |

#### 3.6.1. Primer dilution:

**Stock solution**

Supplied primers were in powder form. It was needed to be diluted. Dilution was done adding 200µl of deionized water poured into the supplied primers tubes. From each tube 10 µl of forward and reverse primer solutions were taken into another eppendrof tube then 90 µl of nuclease freewater was added. The forward and reverse primer's tubes were centrifuge for 60 seconds. Prepared stock primer's solutions were store at deep refrigerator for PCR reactions.

**2.5 µl working primers solution preparation**

2.5µl solution was taken from each forward and reverse 100µl stock primer solutions then added 97.5 µl deionized water in each eppendrof tube. Prepared solutions were stored at deep refrigerator for further PCR reactions.

**5X gDNA dilution**

5µl of gDNA was taken from extracted gDNA solution and then added 95µl deionized water. Prepared template DNA was stored at deep refrigerator for further use.

### 3.6.2. Polymerase Chain Reaction (PCR)

PCR amplification was performed in 15µl volume for test reaction and final reaction was performed at 45µl volume following the standard procedure of Lu et al., 1997 (Table 3.4 and Table 3.5). To check DNA contamination, a negative control was set up omitting template DNA from the reaction mixture. All PCR reactions were carried out in Gradient Applied Biosystem PCR machine.

**Table.3.4. Master mix preparation**

| Chemical name | 15µl PCR reaction for test |
|---|---|
| Go Tag® G2 Green Master Mix,REF# M7822, system lot#0000236523, Size: 100 reaction, storage temp-10℃ Promega, USA | 7.5 µl |
| Nuclease free water (REF#P119A, Lot#0000226975,1.25ml, Promega, Madison,WI USA) | 4.6µl |
| Forward primer | 1.2µl |
| Reverse primer | 1.2µl |
| Template DNA | 0.5µl |
| Total volume = | 15µl |

Table.3.5. **Thermocycler conditions**

| | Degree | Time |
|---|---|---|
| Initial denaturation | 95ºC | 3m |
| Denaturation | 95ºC | 30s |
| Annealing | 53º C | 30 s, |
| Elongation | 72ºC | 30 s, |
| Final elongation | 72ºC | 5m |
| Hold | 23ºC | ∞ |

### 3.6.3. PCR product test Run in gel

5µl of PCR products from each samples and 3µl of GeneRuler were loaded in each wells. Then ectrophoresed was performed in 1% agarose gel for 18 minutes at constant voltage 80V and 44 milliampere .The gel was visualized under UV transilluminator and documented.

### 3.6.4. PCR product purification

The PCR products were purified using Thermo Scientific GeneJET PCR Purification kit following the manufacturer's instruction

400µl volume of binding buffer was added with 40µl PCR amplified products then the whole sample mixed up thoroughly.

⇩

The total volume was transferred to the Genejet purification column then the column was centrifuged for 30-60 s at 13,000RPM and after completion of the process flow through was discarded from the Genejet purification column.

⇩

In addition, 700 µl of Wash Buffer was added to the Genejet purification column as well as column was centrifuged for 30-60s at 13,000 RPM and flow-through was discarded from the column.

⇩

Empty Genejet purification column was additionally centrifuged for 1 min to completely remove any residual substances.

⇩

Genejet purification column was transferred to a clear 1.5 microcentrifuge tube. 50µl of elution buffer was poured in the centre of the GeneJET purification column membrane after that the column was centrifuged for 60s.

⇩

The GeneJET purification column was discarded and purified DNA was stored at deep refrigerator for sequencing purpose

### 3.7. Gene sequencing

The purified PCR products were sended to Macrogen, Co.Ltd, Korea for sequencing there same primers were used for next generation sequencing.

### 3.8. DNA chromatograph and BLAST search

The ab DNA sequences files were checked using FinchTV version 1.4.0. After checking the sequences, BLAST search was performed on the NCBI database (https: //blast .ncbi .nlm. nih. gov/Blast.cgi).

### 3.9. Sequence assembly

Sequences were edit and assembling using and Bioedit version 7.0.4 (Hall, 1998). Pairwise and multiple alignment of sequences was performed using EMBL-EBI website (https://www.ebi.ac.uk/Tools/psa/) and Unipro Ugene version 1.2.9. Alignment was than manually check and corrected.

### 3.10. A, T, G, C, GC and AT count

Python programming was used for counting A, T, G, C, GC and AT content

### 3.11. tRNA gene indentification and Structure

The tRNA scan-SE search server (http://lowelab.ucsc.edu/tRNAscan-SE/) and the ViennaRNA Web Services (http://rna.tbi.univie.ac.at) with default parameters were used for indentification tRNA gene and construction of stem-loop secondary structures.

### 3.12. Phylogenetic analysis

Morphological phylogenetic tree was constructed using the Mesquite version 3.1bassed on 13 characters that was make character matrix data, Molecular phylogenetic analysis was performed in MEGA version 7.1(Kumar et al., 2016) using the Maximum likelihood (ML) method. The trees were analysis using bootstrap test 1000 replicates.

### 3.13. DNA sequence translation

*ND1* and *ND2* nucleotide sequences were translated into amino acid using Expasy-translation tools (https://web.expasy.org/translate/). Among the *ND1* translated amino acid results 5′3′ frame 3 was chosen for comparative study. For *ND2* 5′3′ frame 2 was chosen for the comparative analysis.

# Chapter 4

# RESULTS

Chapter 4

# RESULTS

## 4.1. Morphometric analysis

### 4.1.1. Morphometry of *L. calbasu*

The total length of the studied fish sample was 45.47 cm, standared length 36.83cm, head length 7.62cm, body depth 14.47 cm, fork length 9.39 cm and the body weight 1kg and 800 gm. Morphpological characteristics of fish presented in Fig 4.1. Taxonomic formulaof *L. calbasu*,D III/15; P 15; Lt 40, conformed to the sample.

### 4.1.2. Morphometric traits

The species-wise descriptive statistics of different criteria*viz.*, minimum, maximum, mean, standared deviation; the coefficient variantion (CV) of all morphometric traits and the Principal component analysis (PCA)were carried out. The results are as follows.

### 4.1.3. Descriptive statistics of Morphometric traits

Descriptive statistics for each of the mophometric variables of *L. calbasu*, *L. bata*, *L. gogius*, and *L. rohita* are presented in Table 4.1 and 4.2 repectively. Generally low coeffeicients of variation were obtained for the morphometric characters of *L. gonius* (0.52-16.66%), *L. bata* (1.53-15.83%), *L. rohita* (2.30-15.83%) and *L. calbasu* (5-16.25%).

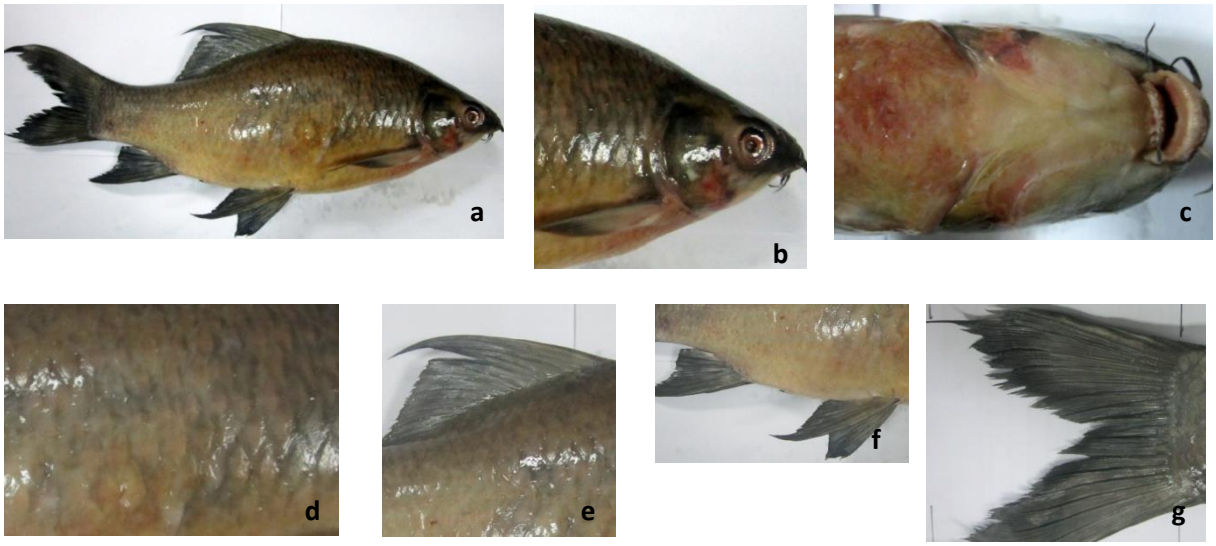



Fig. 4.1. Morphological characteristics of the Kalbasu fish (*Labeo calbasu*), a. Exteranal features, b. Lateral view of mouth, c. Frontal view of mouth, d. Scales of dorsal side, e. Dorsal fin, f. Pelvic and pectoral fin, g. Cadual fin

**Table 4.1:** Descriptive statistics of transformed morphometric variables, the coefficient of variation (*CV*) of each measurement of *L. calbasu* and *L. rohita*

| | ***L. calbasu* (n = 9) PS** | | ***L. calbasu* (n = 37) IN** | |
|---|---|---|---|---|
| | **Mean ± SD (min-max)** | **CV** | **Mean ± SD (min-max)** | **CV** |
| **SL/Body depth** | 3 ± 0.34 (2.6-3.6) | 11.33 | 3.3 ± 0.28 (2.9-3.9) | 8.48 |
| **SL/LH** | 4.4 ± 0.44 (3.6-5.2) | 10 | 3.9 ± 0.22 (3.4-4.4) | 5.64 |
| **SL/Predorsal length** | 2 ± 0.12 (1.8-2.2) | 6 | 2.3 ± 0.19 (1.8-2.7) | 8.26 |
| **SL/ Preanal length** | 1.2 ± 0.07(1.1-1.4) | 5.83 | 1.3 ± 0.03 (1.2-1.4) | 2.30 |
| **SL/Prepelvic length** | 1.8 ± 0.09 (1.6-1.9) | 5 | 1.9 ± 0.06 (1.8-2.1) | 3.15 |
| **Snout/Eye** | 1.1 ± 0.09 (1-1.2) | 8.18 | 1.9 ± 0.23 (1.3-2.3) | 12.10 |
| **Iow/Eye** | 2.5 ± 0.5 (2-3.5) | 20 | 1.9 ± 0.43 (1.3-3.0) | 22.63 |
| **LH/Eye** | 4 ± 0.6 (3.3-5.5) | 15 | 4.1 ± 0.49 (3.3-5.5) | 11.95 |
| **LH/Snout** | 3.6 ± 0.53 (3-4.5) | 14.72 | 2.4 ± 0.18 (2.2-2.9) | 7.5 |
| **LH/Head width** | 1.3 ± 0.14 (1-1.5) | 10.76 | 1.6 ± 0.13 (1.3-2) | 8.12 |
| **LH/Wide of mouth** | 3.2±0.34 (3-4) | 10.62 | 3.6 ± 0.54 (2.5-5.5) | 15 |
| **LH/LCPD** | 1.2 ± 0.15 (1-1.4) | 12.5 | 1.6 ± 0.17 (1.3-2) | 10.62 |
| **LH/Dorsal finbase** | 0.8 ± 0.13 (0.6-1) | 16.25 | 0.9 ± 0.19 (0.8-2) | 21.11 |

**Table 4.2:** Descriptive statistics of transformed morphometric variables, the coefficient of variation (*CV*) of each measurement, of *L. bata* and *L. gonius*

| | ***L. rohita* (n = 15)** | | ***L. bata*** (n=8) | | ***L. gonius*** (n=6) | |
|---|---|---|---|---|---|---|
| | **Mean ± SD (min-max)** | **CV** | **Mean ± SD (min-max)** | ***CV*** | **Mean ± SD (min-max)** | ***CV*** |
| **SL/Body depth** | 3.8 ± 0.18 (3.5-4.1) | 4.73 | 3.9 ± 0.31 (3.5-4.4) | 7.94 | 3.7 ± 0.19 (3.4-4) | 5.13 |
| **SL/LH** | 4.1 ± 0.23 (3.8-4.7) | 5.6 | 4.6 ± 0.32 (4.1-5) | 6.95 | 4.4 ± 0.39 (4-5.1) | 8.86 |
| **SL/Predorsal length** | 2.1 ± 0.06 (2-2.2) | 2.85 | 2.1 ± 0.16 (1.7-2.2) | 7.61 | 2.1 ± 0.3 (1.5-2.2) | 14.28 |
| **SL/ Preanal length** | 1.3±0.03 (1.2-1.3) | 2.3 | 1.3 ± 0.02 (1.2-1.3) | 1.53 | 1.2 ± 0.04 (1.2-1.3) | 3.33 |
| **SL/Prepelvic length** | 1.8±0.19 (1.2-2) | 10.55 | 2 ± 0.06 (1.9-2.1) | 3 | 1.9 ± 0.01 (1.94-1.98) | 0.52 |
| **Snout/Eye** | 1 ± 0.14 (0.9-1.4) | 14 | 0.9 ± 0.09 (0.7-1) | 10 | 0.84 ± 0.04(0.8-0.9) | 4.76 |
| **Iow/Eye** | 2.4 ± 0.38 (2-3.5) | 15.83 | 1.5 ± 0.07 (1.4-1.6) | 4.66 | 2.1 ± 0.35 (1.8-2.7) | 16.66 |
| **LH/Eye** | 4.4 ± 0.59(3.5-5.9) | 13.4 | 3.8 ± 0.34 (3.3-4.4) | 8.94 | 2.1 ± 0.2 (1.7-2.4) | 9.52 |
| **LH/Snout** | 4.2 ± 0.51(3.2-4.9) | 12.14 | 4.1 ± 0.47 (3.6-4.8) | 11.46 | 4.8 ± 0.17 (4.5-5) | 3.54 |
| **LH/Head width** | 1.4 ± 0.13 (1.1-1.6) | 9.28 | 1.6 ± 0.13 (1.4-1.8) | 8.12 | 1.5 ± 0.13(1.2-1.6 | 8.66 |
| **LH/Wide of mouth** | 3.7 ± 0.36 (3.2-4.7) | 9.72 | 2.9 ± 0.19 (2.7-3.3) | 6.55 | 3.3± 0.33 (3-4) | 10 |
| **LH/LCPD** | 1.5 ± 0.17 (1.3-1.8) | 11.33 | 1.2 ± 0.19 (1-1.5) | 15.83 | 1.2 ± 0.11 (1.1-1.4) | 9.16 |
| **LH/Dorsal finbase** | 1.1 ± 0.1 (0.8-1.3) | 9.09 | 0.9 ± 0.06 (0.9-1.1) | 6.66 | 1 ± 0.14 (0.8-1.2) | 14 |

**Principal Component Analysis (PCA) among *L. calbasu, L. bata. L. gonius* and *L. rohita* species**

Principal component analysis was carried out factoring the correlation matrix of the morphometric data among the four *Labeo* species.

**PCA among *L. calbasu, L. bata. L. gonius* and *L. rohita* species**

PCA of the 13 significant variables (Table 4.1 and 4.2) among *L. calbasu, L. bata, L. gonius* and *L. rohita* yielded two principal components (PCA1 and PCA2) accounting for 82.13% of the total variation in the original variables (Table 4.3). The factor loading of the first three components are shown in (Table 4.4). The first component was mainly defined by measurements of SL/Pre- dorsal length, SL/Pre-anal length, SL/Body depth, LH/Snout length, LH/Head width, whereas, the second component was mainly correlated with length of Head (LH)/Dorsal finbase, LH/Width of mouth, LH/LCPD and Iow/Eye. These indicated that the above morphometric characters contribiuted the maximum to differentiate *L. calbasu*, *L. rohita*, *L. bata*, and *L. gonius*. The scatter diagram based on PCA clearly distinguishes four *Labeo* species which are evidently distinct as separate species (Fig. 4.2 and 4.3).

**Table 4.3.** Summary of principal component analysis (PCA) for the morphometric variables of *Labeo* species

| Component | Eigenvalue | Variability % | Cumulative % |
|---|---|---|---|
| 1 | 6.143 | 47.254 | 47.25 |
| 2 | 4.534 | 34.879 | 82.13 |
| 3 | 2.323 | 17.867 | 100.00 |

**Table 4.4.** Factor loadings for the first three principal components formed from the morphometric variables of four *Labeo* species

| Morphometric variables | PC1 | PCA2 | PCA3 |
|---|---|---|---|
| SL/Body depth | 0.790 | 0.573 | 0.218 |
| SL/LH | 0.559 | -0.819 | 0.131 |
| SL/Pre-dorsal length | 0.775 | 0.631 | 0.026 |
| SL/Pre-anal length | 0.305 | 0.508 | 0.806 |
| SL/Pelvic length | 0.923 | -0.315 | 0.223 |
| Snout/Eye | -0.888 | -0.154 | 0.433 |
| IOW/Eye | -0.899 | 0.239 | -0.368 |
| LH/Eye | -0.526 | 0.169 | 0.834 |
| LH/Snout | 0.662 | 0.445 | -0.603 |
| LH/Head width | 0.984 | 0.011 | 0.176 |
| LH/ Width of Mouth | -0.483 | 0.842 | -0.239 |
| LH/LCPD | -0.320 | 0.910 | 0.264 |
| LH/Dorsal finbase | 0.216 | 0.967 | -0.136 |

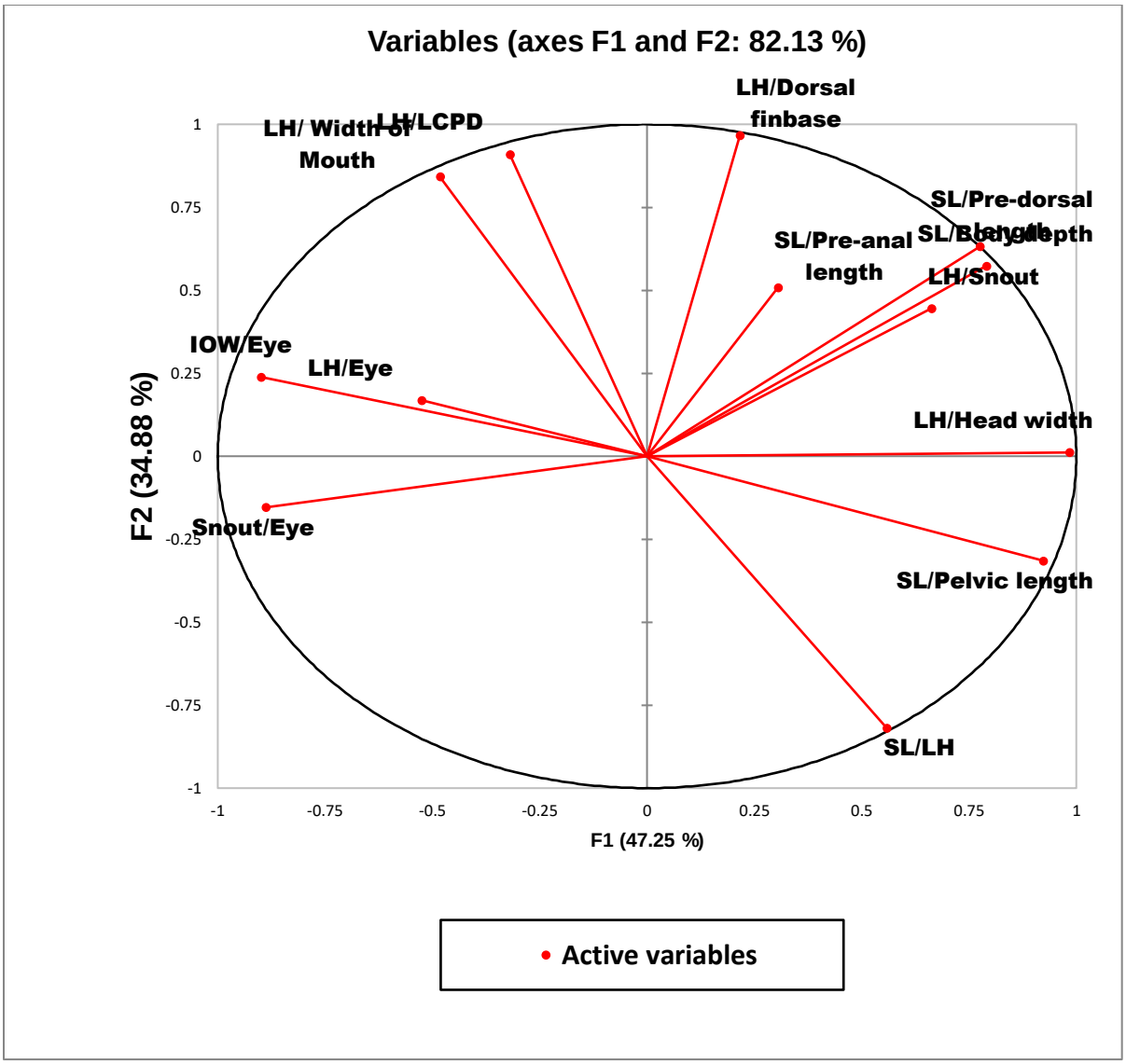



**Fig.4.2.** Scatter diagram based on PCA of significant morphometric variables among *L. calbasu*, *L. rohita*, *L. bata* and *L. gonius* specimens

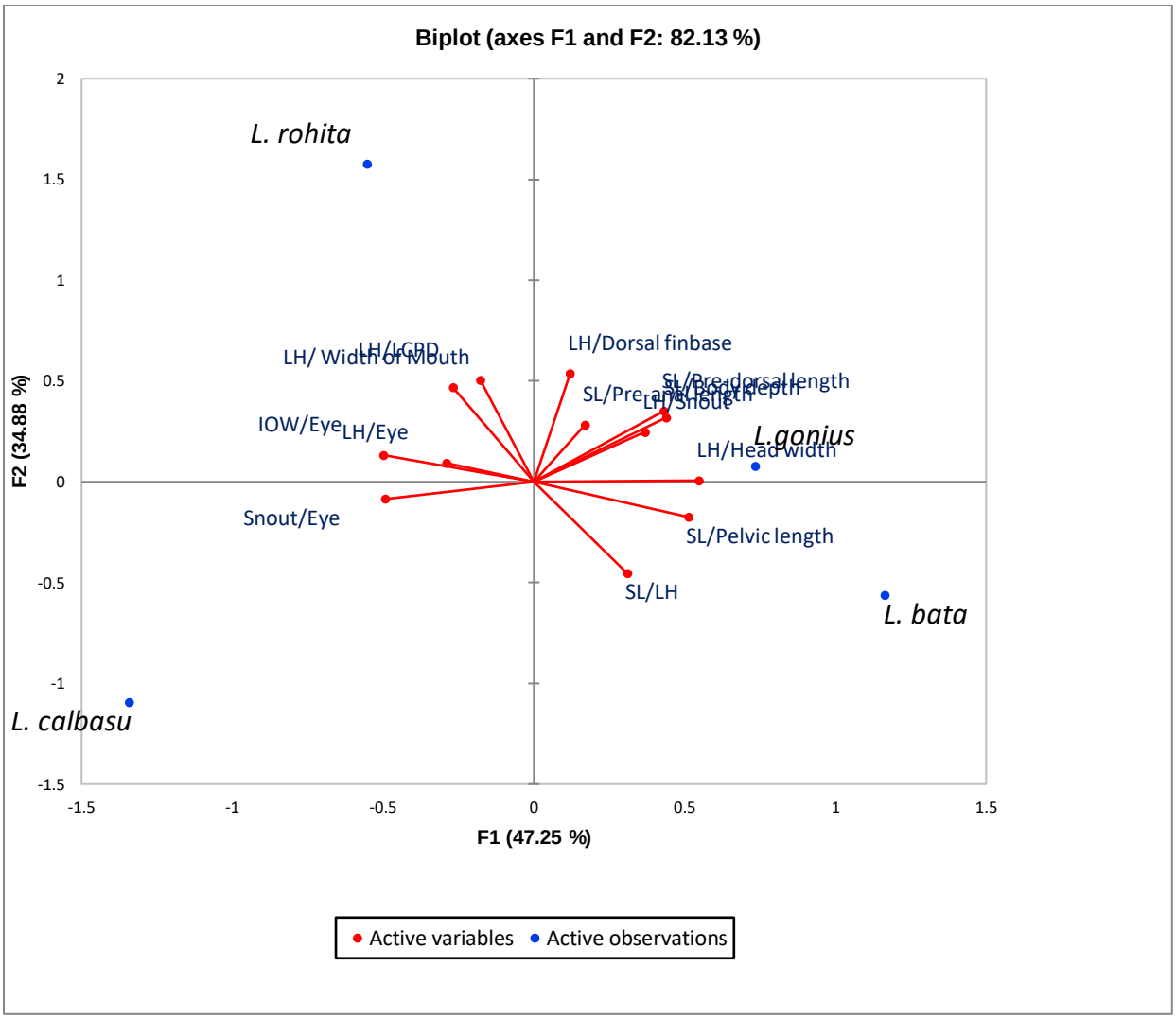



**Fig. 4.3.** Biplot diagram based on PCA of significant morphometric variables among *L. calbasu*, *L. rohita*, *L. gonius* and *L. bata* specimens

### 4.3. Meristic traits

Six meristic characters were conunted; structure and arrangement of gill rakers were examined to find out any variation among the 4 species viz., *L. calbasu*, *L.rohita*, *L. gonius* and *L. bata*.

Gill rackers of *L. clabasu* were slender and villiform structure (Fig. 4.3a) and that of *L.rohita*, *L. gonius and L. bata* were comparatively stout and blut tip (Fig. 4.3b, 4.3c and 4.3d)

#### 4.3.1. Descriptive statistics of meristic traits

Descriptive statistic for each of the mereistic variables of *L. calbasu*, *L. bata*, *L. gonius* and *L. rohita* are denoted in Table 4.4 and 4.5 respectively. Generaly low coefficients of variations (CVs) were obtained for the meristic characters of *L. calbasu* (0.0-15.7%), *L. rohita* (0.0-6.2%), *L. bata* (0.0-6.3%) and *L. gonius* (0.0-7.4%).

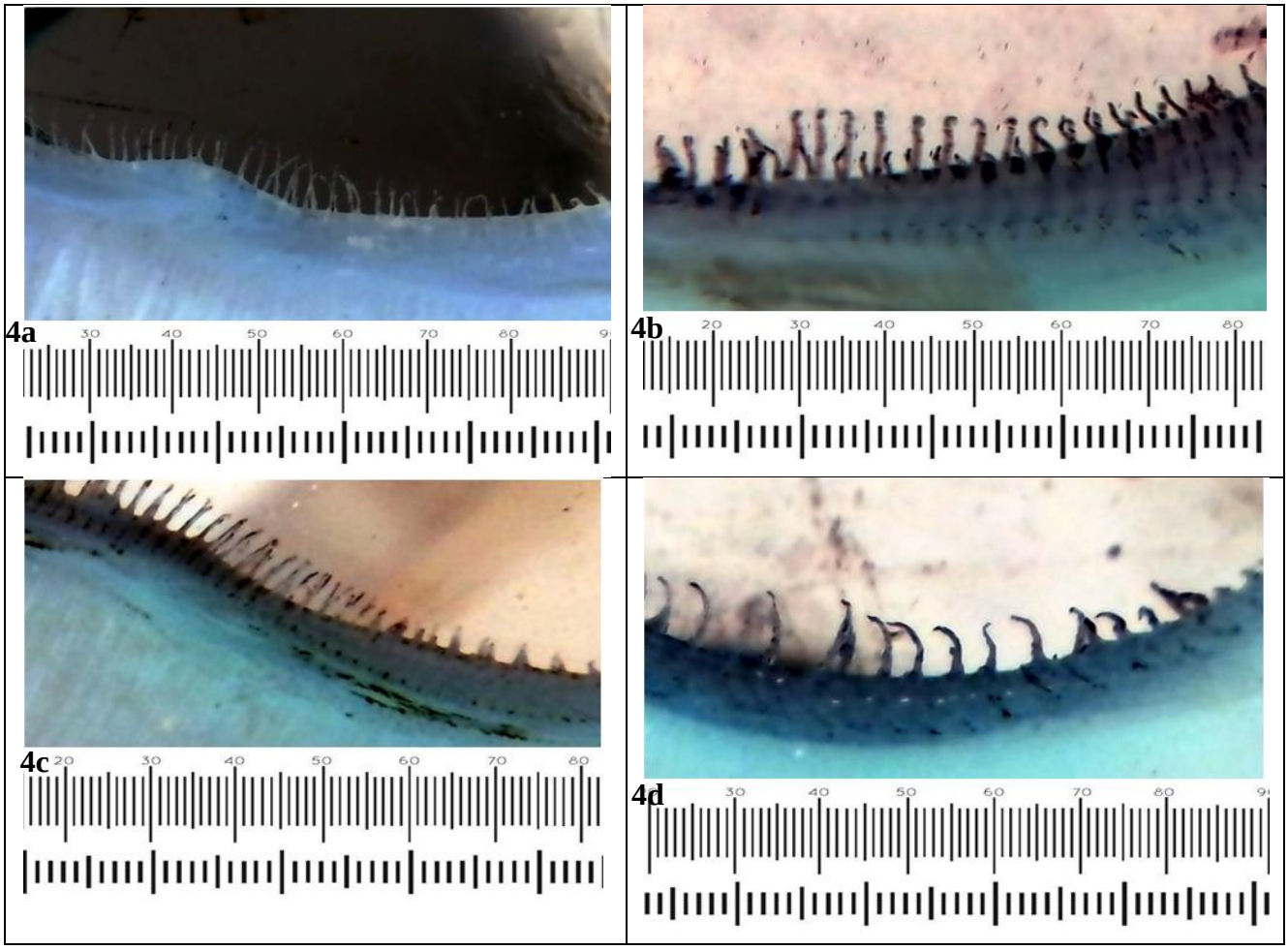



**Fig. 4.4.** Microscopic image of gill rackers of outer gill arch of *L. calbasu, L. rohita, L. gonius* and *L. bata*

**Table. 4.5.** Descriptive statistic of meristic values, the eocfficent variation (CV) of each measurement of *L. calbasu* and *L. rohita*.

| | ***L. calbasu*** | | | ***L. rohita*** | | |
|---|---|---|---|---|---|---|
| | **Mean ± SD (min-max)** | **CV** | **n** | **Mean ± SD (min-max)** | **CV** | **n** |
| **DFR** | 14.1 ± 0.9 (13-15) | 6.5 | 9 | 12.2 ± 0.5 (12-14) | 4 | 17 |
| **PFR** | 16.1 ± 0.3 (16-17) | 2 | 9 | 16.1 ± 0.4 (16-17) | 2.7 | 17 |
| **VFR** | 8 ± 0 (8-8) | 0 | 9 | 8 ± 0 (8-8) | 0 | 17 |
| **AFR** | 6.3 ± 1(5-8) | 15.7 | 9 | 6 ± 0(6-6) | 0 | 17 |
| **GRC** | 53 ± 4.35 (48-56) | 8.2 | 3 | 54.5 ± 3.4 (50-58) | 6.2 | 4 |
| **LLS** | 41.1± 1.1 (40-43) | 2.8 | 9 | 40.8± 1.1 (40-42) | 1.8 | 17 |

**Table. 4.6**. Descriptive statistic of meristic values, the eocfficent variation (CV) of each measurement of *L. gonius* and *L. bata*.

| | ***L. gonius*** | | | ***L. bata*** | | |
|---|---|---|---|---|---|---|
| | **Mean ± SD (min-max)** | **CV** | **n** | **Mean ± SD (min-max)** | **CV** | **n** |
| **DFR** | 13.5 ± 1 (13-15) | 7.4 | 4 | 9 ± 0 (9-9) | 0 | 8 |
| **PFR** | 15.75 ± 0.5 (15-16) | 3.17 | 4 | 15.2 ± 0.4 (15-16) | 3 | 8 |
| **VFR** | 8 ± 0 (8-8) | 0 | 4 | 8 ± 0 (8-8) | 0 | 8 |
| **AFR** | 6 ± 6(6-6) | 0 | 4 | 5 ± 0(5-5) | 0 | 8 |
| **GRC** | 57 ± 0 (57-57) | 0 | 2 | 34 ± 2.16 (31-36) | 6.3 | 4 |
| **LLS** | 77.5± 4.2 (73-82) | 2.8 | 4 | 37.7± 0.7 (37-39) | 1.8 | 8 |

### 4.4. Morphologicalphylogeny analysis

Phylogenetics were constructed using Mesquites heuristic search, single linkage cluster analysis (Distance: Distance from characters matrix) and UPGMA cluster analysis (Distance: Distances from characters matrix) methods. All the trees showed almost the same topology (Fig. 4.5). They consistently indicated that *L. bata*, *L. rohita* and *L. calbasu*were in the same group. The monophyletic group divided into a clade 1 and a single species *L. bata*. Clade 2 consists of *L. calbasu* and *L. rohita.*

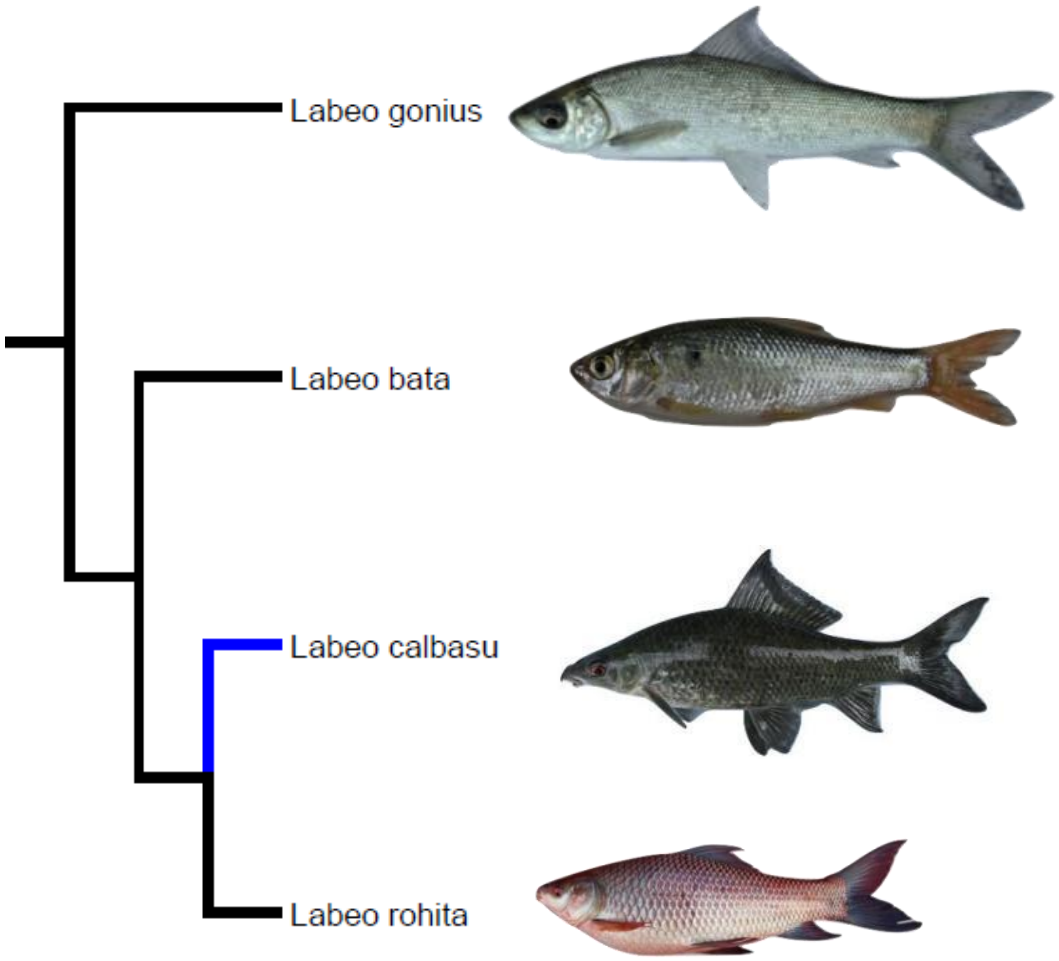



**Fig. 4.5.** Phylogenetic tree of *L. calbasu* from UPGMA cluster analysis (Distance: Distances from characters matrix)

### 4.5. Mitochondrial DNA analysis

Gel image of extracted DNA has been presented in the Fig.4.6.a. Total 14 set of primers have used in PCR reactions (3.6.1 section), only three worked primarily. The three PCR products of mitochondrial DNA have been visualized in the gel Fig.4.6.b and4.6.c .Sequences of the three amplified regions (named as LCA3, LCA4 and LCA7) are presented in the Fig. 13 b and 13 c (respectively). After checking the chromatogram of first sequence (LCA3), only 281 nucleotides could be determined unambiguously that correspond to positions 1134 to 1415 in the reference complete mitochondrial geome sequence of *L. calbasu* (AP012143.1). This portion covered a region of *16S rRNA* gene. Target sequence of LCA4 region was 867 bp long, however, after editing 794 bp was taken unambiguously which was placed from 1445bp to 2239 bp in the reference mt genome. The region covered 48.70% of the *16S rRNA* gene. LCA7 amplified sequence was 790 bp after checking the chromatograph, total 754 bp nucleotide sequence was found which was in the reference sequence from 3514 to 4278bp that covered partial ending regions of *ND1* gene, complete sequence of $tRNA^{Ile}$, $tRNA^{Gln}$, $tRNA^{Met}$ and starting regions of *ND2* gene.

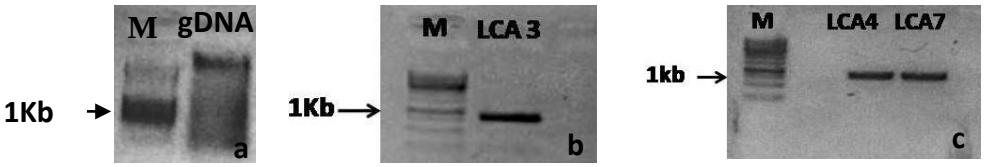



**Fig.4.6.** a) Gel showing gDNA band of *L. calbasu*; b) Agarose gel showing PCR band of LCA3 regions c) Gel showing PCR band of LCA4 and LCA7 band of *L. calbasu* M indicates the molecur weight marker

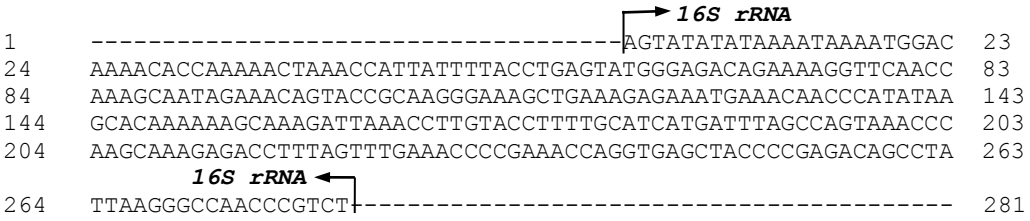



**Fig.4.7. LCA3 amplified regions**


**Fig.4.8. LCA4 amplified regions**

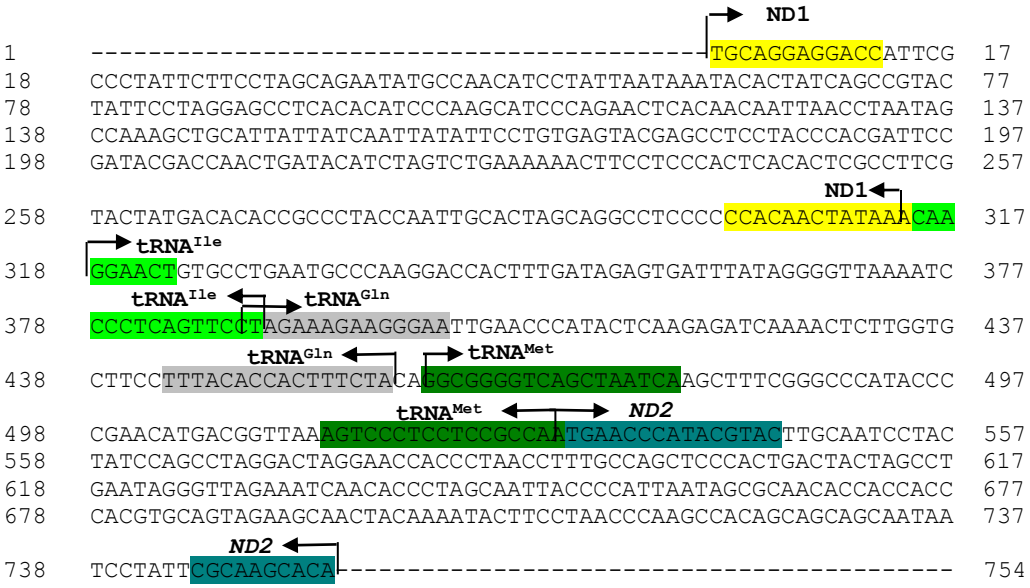



**Fig.4.9. LCA7 amplified regions**

### 4.5.1. BLAST search analysis

For confirmation of species, the LCA3, LCA4 and LCA7 sequences were aligned using BLAST at NCBI database. BLAST result of LCA3 showed 99% sequence similarity with the mitochondrial sequences of *L. calbasu* (AP012143.1), *L. rohita* (KR185963.1), *L. stolizkae* (JX042130.1), *C. catla* (JQ087872.1), *L. yunnanensis*(DQ845881.1), *L. chrysophekadion* (AP011199.1) and *L. angra* (AP011329.1) available in the NCBI database. In addition, it had 98% similarily with the mitochondrial sequences of *L. chrysophekadion* (EU136620.1), *L. pierrei* (AP011200.1), *C. catla* (KY419138.1), *L. dussumieri* (AP011384.1), *C. microlepis* (AP011357.1), *L. gonius* (KT001152.1), *L, fimbriatus* (KP025676.1) and *L. calbasu* (JQ231113.1) (Appendix I- Table. 4.7). The LCA4 search result showed that the sequence has 100% similarity with *L. calbasu* (AP012143.1) as well as 98% similarily with *L. dussuimieri* (AP011384.1), *L. rohita* (KR185963.1), *L. yunnanensis* (DQ845881.1), *L.* g*onious* (KT001152.1), *L. frimbriatus* (KP025676.1), *L. chrysophekadion* (AP011199.1), *L. rohita* (JN412817.1), *C. catla* (JQ087872.1)(Appendix I- Table 4.8). LCA7 search result showed that the sequence has 99% similarity with *L. calbasu* (AP012143.1), 97% similarily with *L. chrysophekadion* (AP011199.1), 96% similarity with among the species *Ldussumieri* (AP011384.1), *Catlacatla* (AP011355.1), *L. gonius* (KT001152.1), *L. fimbriatus* (KP025676.1), and 95% similarity found with *L. rohita* species(KR185963.1)(Appendix I- Table 4.9).

### 4.5.2. Gene structure, organization and composition

The *16S rRNA*, *ND1*, $tRNA^{Ile}$, $tRNA^{Met}$ and *ND2* gene were located on the H-strand. On the other hand, $tRNA^{Gln}$ gene was located on the L-strand. The overall base composition of the *L. calbasu* of *16S rRNA*region sequenced during the present work was 37.4% A, 20.4 % T, 23.2 % C and 18.% G, and the GC content was 42.4 %. The percentage of nucleotide composition of *ND1* (based on H strand) of Kalbasu was found to be A: 31 %, C: 32 %, G: 12 % and T: 23 %.

Nucleotide composition patterns for *ND1* and *ND2* were A>C>T>G and C>A>T>G, respectively (Appendix –III, Table 4.12). The LCA7 sequence of *L. calbasu* encodes three*tRNAs* ($tRNA^{Ile}$, $tRNA^{Gln}$ and $tRNA^{Met}$) which were identified using their anticonds and secondary structures (Appendix –III, Fig. 4.10). In *L. calbasu*, sequence overlapping between two genes, $tRNA^{Ile}$-$tRNA^{Gln}$ was observed (Fig.4.9).

### 4.6. Sequence alignment

#### 4.6.1. Intraspecific variation of sequence

For intraspecific comparisions, all three sequenced regions of *L. calbasu* were aligned with that same regions obtained fro two other samples available online (AP012143.1 and JQ231113.1). In the LCA3 region, out of 284 nucleotides, 6 (2.1%) were polymorphic sites (variable sites) and 278 (98.8%) monomorphic sites (conservere sites)(Table 4.11, Table 4.1, and Appendix II: Table 4.15). In the LCA4 region, 46 out of 813 (5.7%) sites were polymorphic (Table 4.11, Table 4.13, and Appendix II: Table 4.16). Similarly, in the LCA7 region, 50 out of 755 nucleotide positions showed variability, i.e.6.5% polymorphic sites (Table 4.11, Table 4.14, and Appendix II: Table 4.17).

#### 4.6.2. Interspecific variation of sequence

Interspecific comparisons were conducted among the selected 15 nucleotides sequences of Cyprinidae fish species (Table 3). All the sequences were taken from NCBI database. Multiple alignments of LCA3 region, here 41(14, 4%) were polymorphic sites (Table 4.11, Table 4.1, and Appendix II: Table 4.15) and 240 (84.5%) were conserves sites, for LCA4 region, 178(21.8%) were variable sites and 635(78.1%) were conserve sites (Table 4.11, Table 4.13, and Appendix II: Table 4.16) and LCA7 region, 184(24.3%) were polymorphic sites and 571 (75.62%) (Table 4.11, Table 4.14, and Appendix II: Table 4.17).

**Table 4.11.** The percentage of polymorphic sites found in the intraspecific and interspecific comparison between different Cyprinidae fish from Bangladesh and it adjacent countries

| Amplified regions | Polymorphic sites | |
|---|---|---|
| | Intraspecific | Interspecific |
| LCA3 (1134 to 1415 = 281 in the reference sequence of *L. calbasu* (AP012143.1) covers *16S rRNA* | 7(2.4)% | 41(14.5%) |
| LCA4 (from 1445bp to 2239 bp =794bp in the reference genome) cover 48% of *16S rRNA* | 46 (5.7%) | 178 (22.4%) |
| LCA7(from 3524 to 4278bp =754bp in the referenc genome) covers *ND1*,$tRNA^{Ile}$, $tRNA^{Gln}$,$tRNA^{Met}$ and *ND2* genes | 52 (6.8%) [*ND1*:42(13.3%); *ND2*: 5 (2.6%)] | 184(24.4%) [*ND1*:101(32.6%); *ND2*: 55 (24.6%)] |

**Table 4.12.** Polymorphic sites observed in LCA3 regions among the cyprinidae nucleotides sequences. The "-"sign indicates deletion of a nucleotide base pair

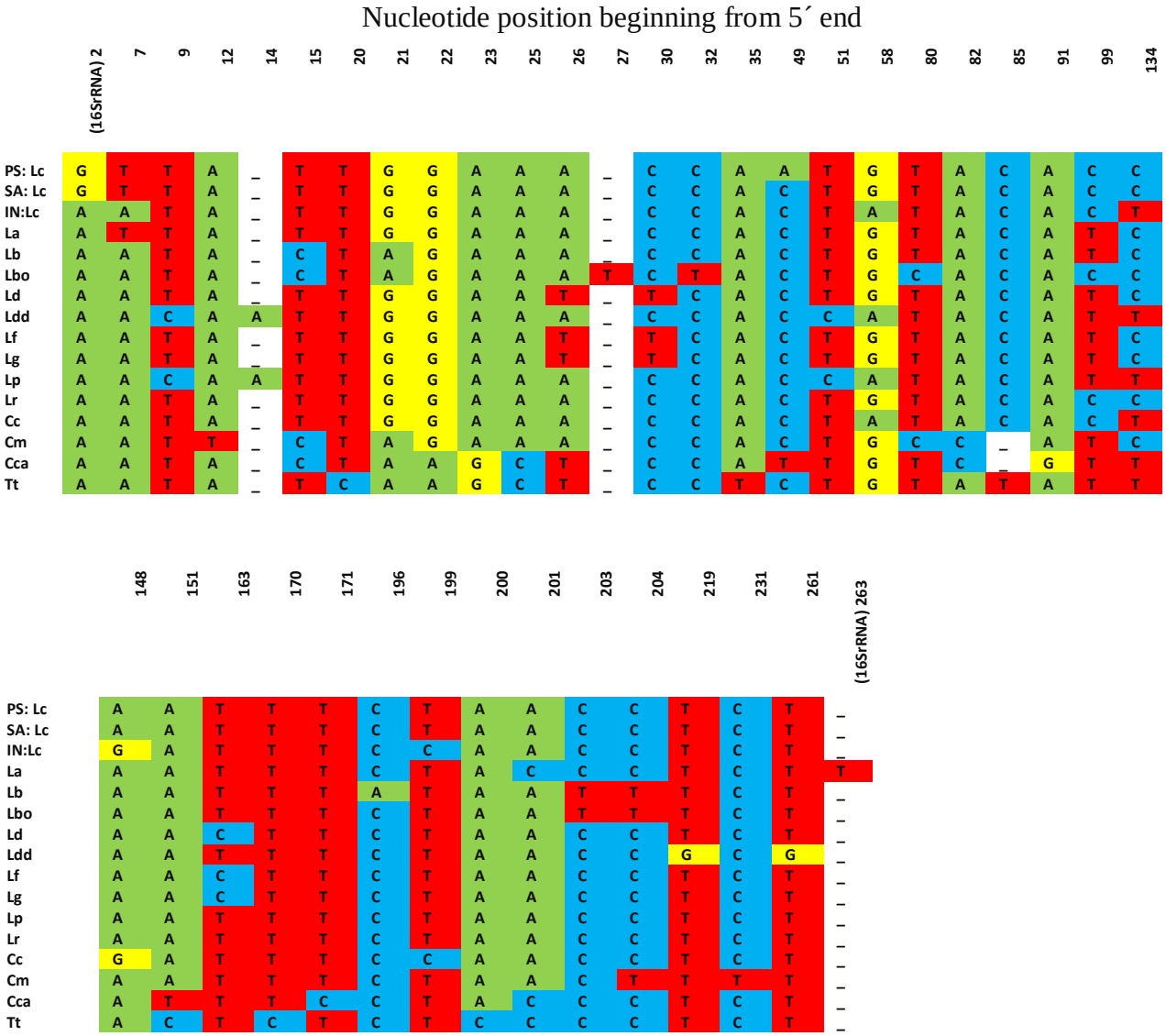
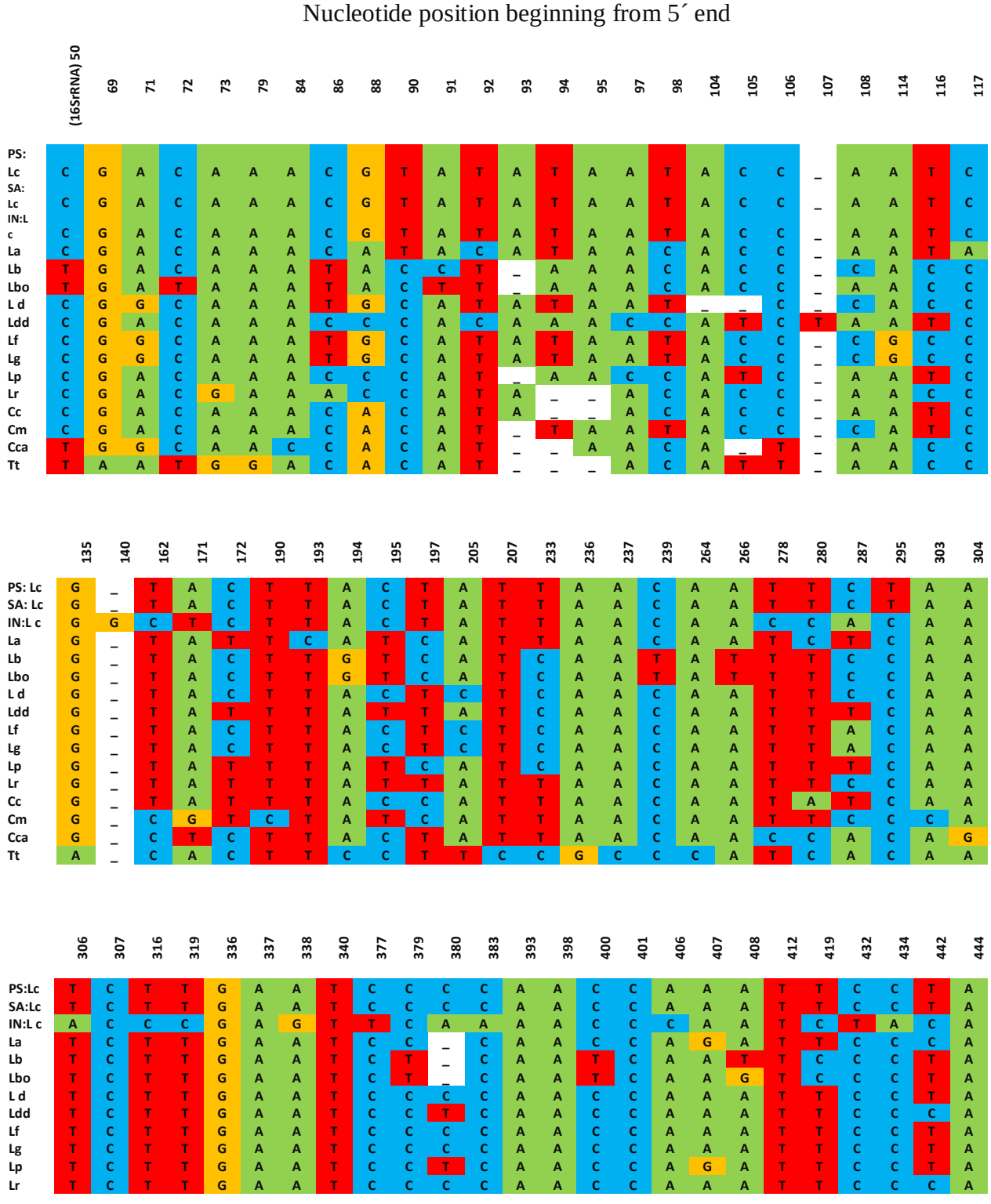
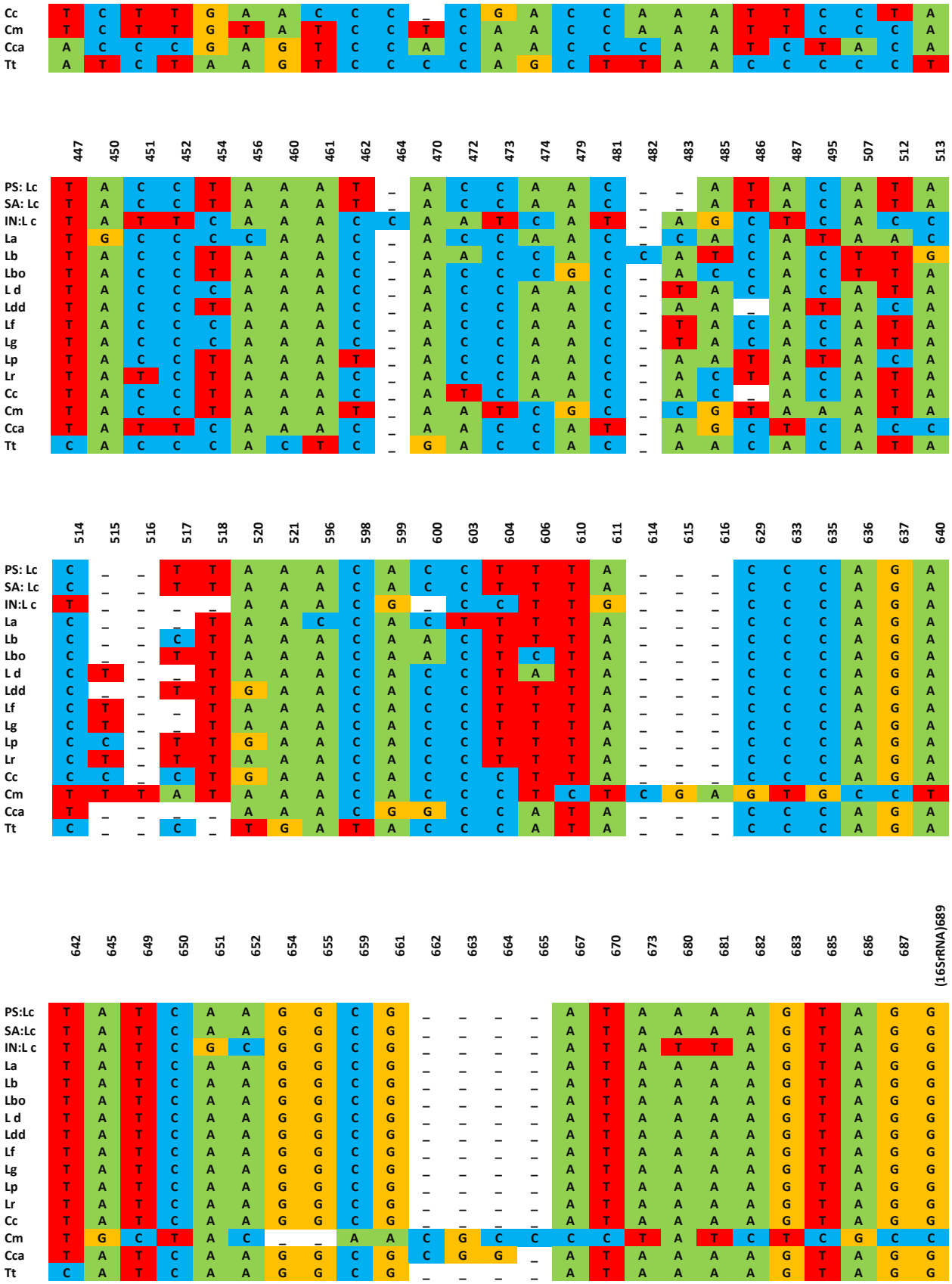


**Table 4.13** Polymorphic sites observed in LCA4 regions among the cyprinidae nucleotides sequences. The "-"sign indicates deletion of a nucleotide base pair

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Cm T C T T G T A T C C T C A A C C A A A T T C C C A
Cca A C C C G A G T C C A C A A C C C A A T C T A C A
Tt A T C T A A G T C C C C A G C T T A A C C C C C T
447 450 451 452 454 456 460 461 462 464 470 472 473 474 479 481 482 483 485 486 487 495 507 512 513
PS: Lc T A C C T A A A T _ A C C A A C _ _ A T A C A T A
SA: Lc T A C C T A A A T _ A C C A A C _ _ A T A C A T A
IN:L c T A T T C A A A C C A A T C A T _ A G C T C A C C
La T G C C C C A A C _ A C C A A C _ C A C A T A A C
Lb T A C C T A A A C _ A A C C A C C A T C A C T T G
Lbo T A C C T A A A C _ A C C C G C _ A C C A C T T A
L d T A C C C A A A C _ A C C A A C _ T A C A C A T A
Ldd T A C C T A A A C _ A C C A A C _ A A _ A T A C A
Lf T A C C C A A A C _ A C C A A C _ T A C A C A T A
Lg T A C C C A A A C _ A C C A A C _ T A C A C A T A
Lp T A C C T A A A T _ A C C A A C _ A A T A T A C A
Lr T A T C T A A A C _ A C C A A C _ A C T A C A T A
Cc T A C C T A A A C _ A T C A A C _ A C _ A C A T A
Cm T A C C T A A A T _ A A T C G C _ C G T A A A T A
Cca T A T T C A A A C _ A A C C A T _ A G C T C A C C
Tt C A C C C A C T C _ G A C C A C _ A A C A C A T A
514 515 516 517 518 520 521 596 598 599 600 603 604 606 610 611 614 615 616 629 633 635 636 637 640
PS: Lc C _ _ T T A A A C A C C T T T A _ _ _ C C C A G A
SA: Lc C _ _ T T A A A C A C C T T T A _ _ _ C C C A G A
IN:L c T _ _ _ _ A A C G _ C C T T G _ _ _ C C C A G A
La C _ _ _ T A A C C A C T T T T A _ _ _ C C C A G A
Lb C _ _ C T A A A C A A C T T T A _ _ _ C C C A G A
Lbo C _ _ T T A A A C A A C T C T A _ _ _ C C C A G A
L d C T _ _ T A A A C A C C T A T A _ _ _ C C C A G A
Ldd C _ _ T T G A A C A C C T T T A _ _ _ C C C A G A
Lf C T _ _ T A A A C A C C T T T A _ _ _ C C C A G A
Lg C T _ _ T A A A C A C C T T T A _ _ _ C C C A G A
Lp C C _ T T G A A C A C C T T T A _ _ _ C C C A G A
Lr C T _ T T A A A C A C C T T T A _ _ _ C C C A G A
Cc C C _ C T G A A C A C C C T T A _ _ _ C C C A G A
Cm T T T A T A A A C A C C C T C T C G A G T G C C T
Cca T _ _ _ _ A A A C G G C C A T A _ _ _ C C C A G A
Tt C _ _ C _ T G A T A C C C A T A _ _ _ C C C A G A
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La T A T C A A G G C G _ _ _ _ A T A A A A G T A G G
Lb T A T C A A G G C G _ _ _ _ A T A A A A G T A G G
Lbo T A T C A A G G C G _ _ _ _ A T A A A A G T A G G
L d T A T C A A G G C G _ _ _ _ A T A A A A G T A G G
Ldd T A T C A A G G C G _ _ _ _ A T A A A A G T A G G
Lf T A T C A A G G C G _ _ _ _ A T A A A A G T A G G
Lg T A T C A A G G C G _ _ _ _ A T A A A A G T A G G
Lp T A T C A A G G C G _ _ _ _ A T A A A A G T A G G
Lr T A T C A A G G C G _ _ _ _ A T A A A A G T A G G
Cc T A T C A A G G C G _ _ _ _ A T A A A A G T A G G
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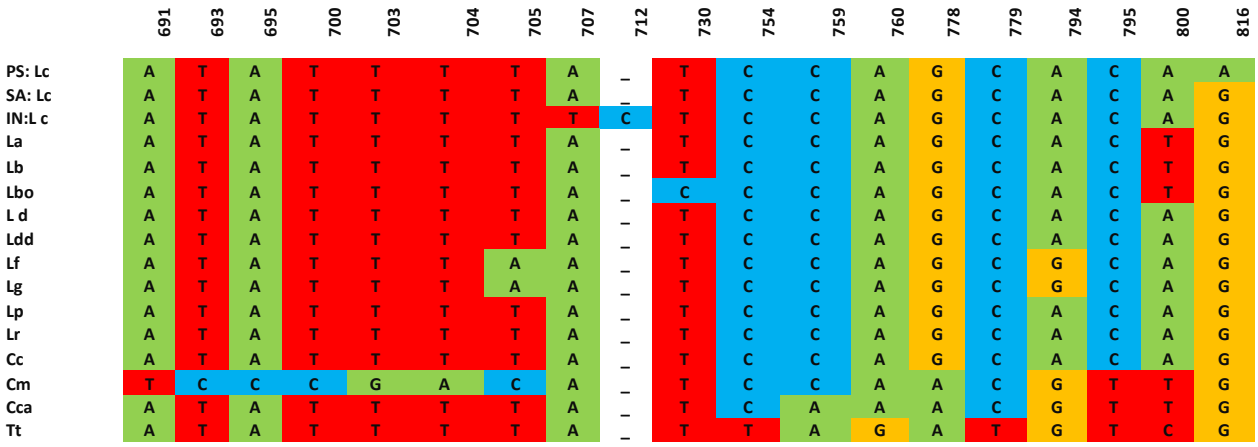
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Lp A T A T T T T A _ T C C A G C A C A G
Lr A T A T T T T A _ T C C A G C A C A G
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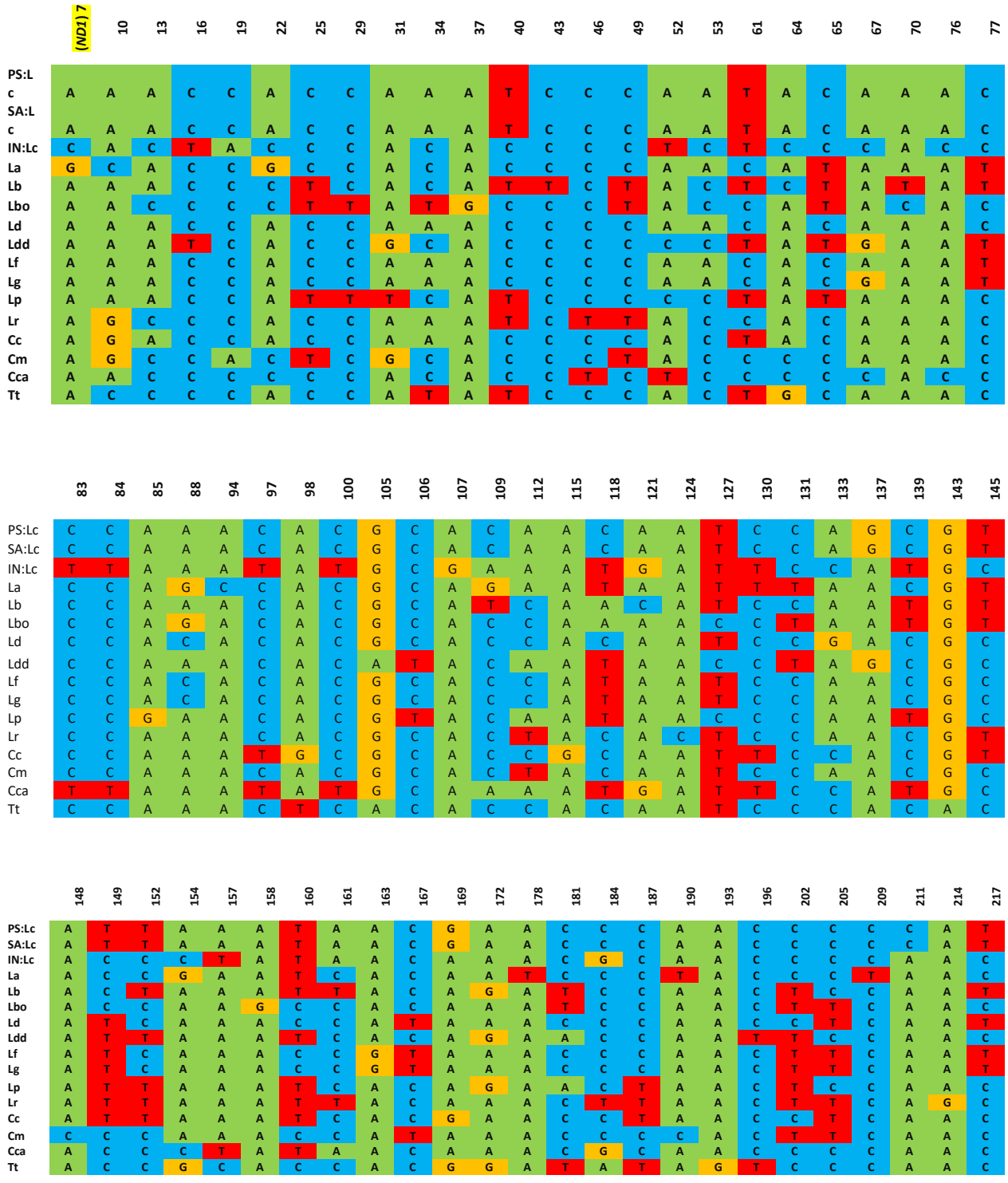
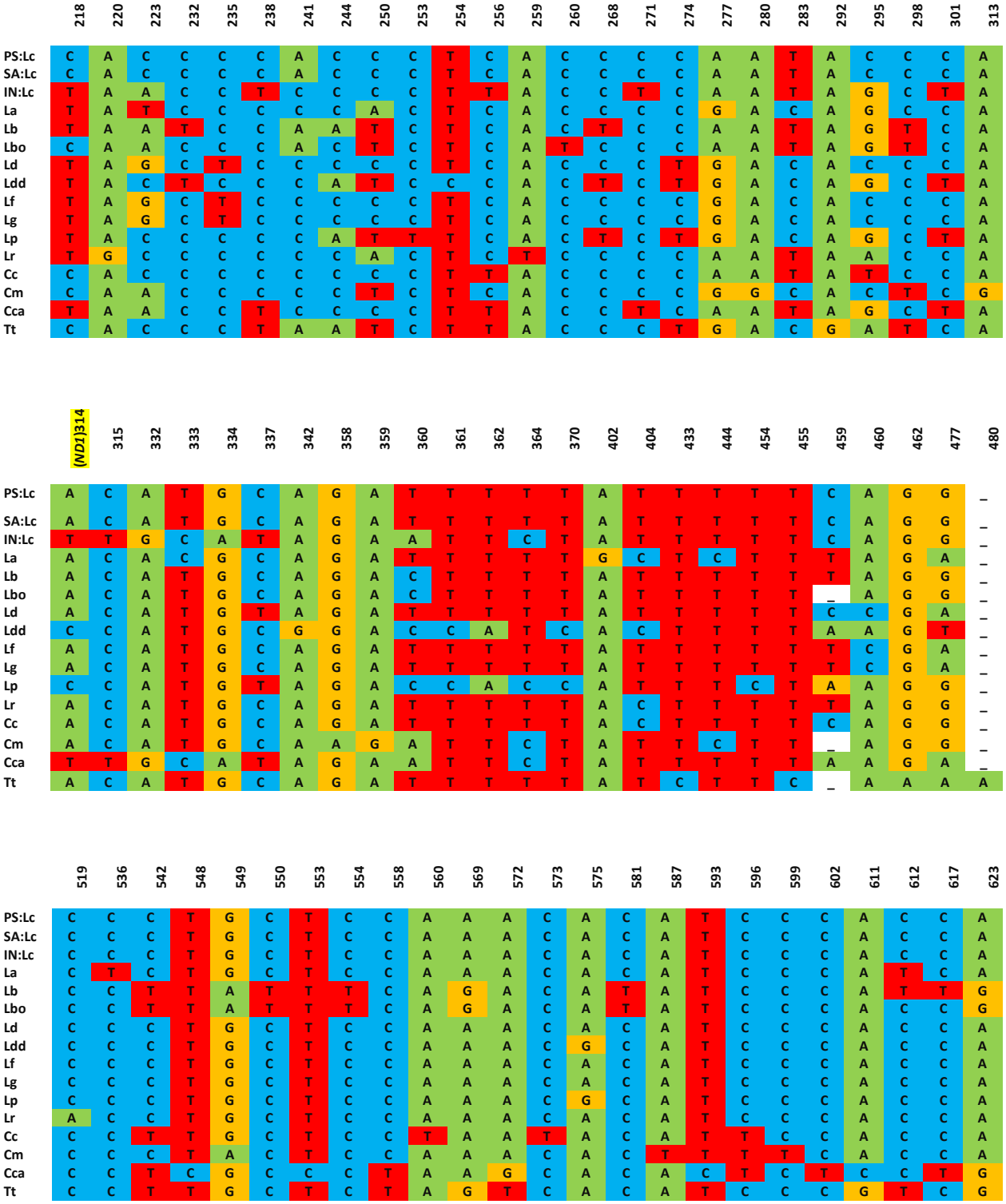
Cm T C C C G A C A _ T C C A A C G T T G
Cca A T A T T T T A _ T C A A A C G T T G
Tt A T A T T T T A _ T T A G A T G T C G

**Table 4.14.** Polymorphic sites observed in LCA7 regions among the cyprinidae nucleotides sequences. The "-"sign indicates deletion of a nucleotide base pair

Nucleotide position beginning from 5´ end

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IN:Lc T A A C C T C C C C T T A C C T C A A T A G C T A
La T A T C C C C C A C T C A C C C C G A C A G C C A
Lb T A A T C C A A T C T C A C T C C A A T A G T C A
Lbo C A A C C C A C T C T C A T C C C A A T A G T C A
Ld T A G C T C C C C C T C A C C C T G A C A C C C A
Ldd T A C T C C C A T C C C A C T C T G A C A G C T A
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Lg A C A T G C A G A T T T T T A T T T T T T C G A _ _
Lp C C A T G T A G A C C A C C A T T T C T A A G G _ _
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Cm A C A T G C A A G A T T C T A T T C T T _ A G G _ _
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IN:Lc C C C T G C T C C A A A C A C A T C C C A C C A
La C T C T G C T C C A A A C A C A T C C C A T C A
Lb C C T T A T T T C A G A C A T A T C C C A T T G
Lbo C C T T A T T T C A G A C A T A T C C C A C C G
Ld C C C T G C T C C A A A C A C A T C C C A C C A
Ldd C C C T G C T C C A A A C G C A T C C C A C C A
Lf C C C T G C T C C A A A C A C A T C C C A C C A
Lg C C C T G C T C C A A A C A C A T C C C A C C A
Lp C C C T G C T C C A A A C G C A T C C C A C C A
Lr A C C T G C T C C A A A C A C A T C C C A C C A
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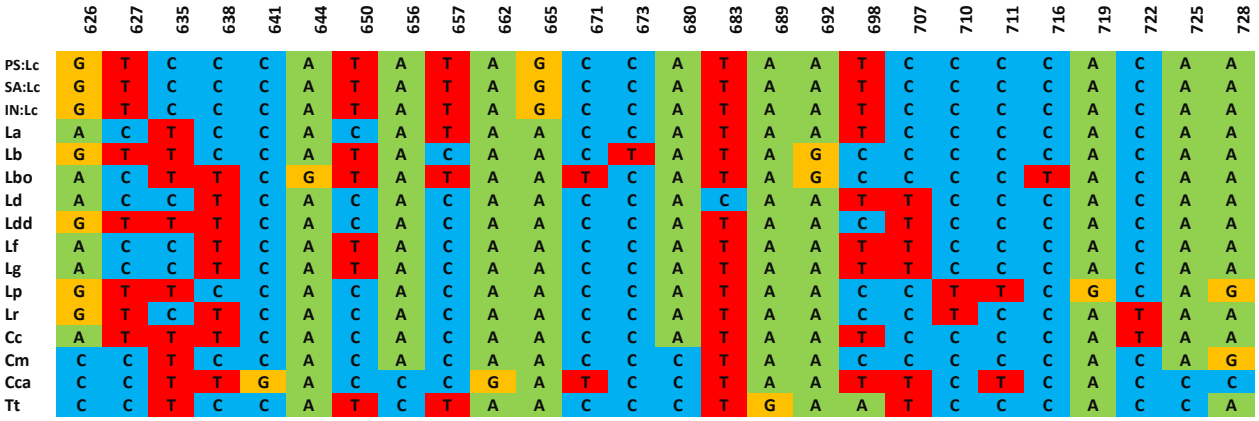
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La A C T C C A C A T A A C C A T A A T C C C C A C A A
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Lg A C C T C A T A C A A C C A T A A T T C C C A C A A
Lp G T T C C A C A C A A C C A T A A C C T T C G C A G
Lr G T C T C A C A C A A C C A T A A C C T C C A T A A
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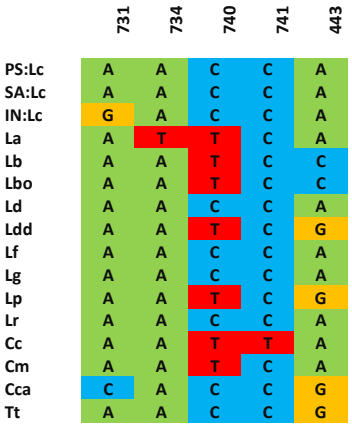
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Ld A A C C A
Ldd A A T C G
Lf A A C C A
Lg A A C C A
Lp A A T C G
Lr A A C C A
Cc A A T T A
Cm A A T C A
Cca C A C C G
Tt A A C C G

### 4.7. Phylogenetic analysis of *L. calbasu*

In order to assess molecular phylogenetic relationship of *L. calbasu*, LCA4 and LCA7 sequences were used. The length of the LCA4 was 794 bp and that of LCA7 was 754 bp. The multiple alignments of LCA4 and LCA7 sequences with respective regions of 18 complete mitochondrial genomes of cyprinids were used to construct phylogenetic tree. In this study, we have constructed phylogeny of *L. calbasu* using LCA4 and LCA7 sequences by ML, PS and NJ methods.The best phylogenetic relatedness inferred from ML and NJ method.

The yielded LCA4 phylogenetic tree by NJ method (Fig. 4.11) had a nearly same topology as that of LCA7 usingthe same method (Fig 4.12). In both trees all *Labeo* species was formed a separated monopylatic group where as other cyprinids fisheswere formed different clade.

In the present LCA4 phylogenetic tree *L. calbasu, L. rohita, L. boggut, L. bata, L. pangusia, L. d. dyocheilus L. dussumierie, L. gonius, L. angra* and *L. fimbriatus* fishes was fromed an independened group. Here this group was divided into three major clades according to statistical support. The fish of *L. calbasu* was observed a sister with *L. rohita* by 60% bootstrap support.*L. pangusia, L. d. dyocheilus* and *L. angra* were also formed a single clade with a bootstrap value of 81(Clade b in Fig.4.11)and this tree showed closest relation to *L. boggut* and *L. bata* having a bootstrap value of 100 (Clade A in Fig.4.11). Apart from these mentioned clades *L. dussumierie*, *L. gonius* and *L. fimbriatus* were formed a clade with a bootstrap value of 99 (Clade C in Fig.4.11).

Therefore, the LCA7 sequence based phylogenetic tree was showed a more conserved result amongst the closely related individuals. Here, *Labeo* monophyletic group was divided into four major clades.The *L. calbasu* and *L.rohita*were formed a sister clade with a bootstrap value of 63(Clade D in Fig.4.12).*L. dussumierie, L. gonius* and *L. fimbriatus* were also formed a clade with a bootstrap value of 100(Clade C in Fig.4.12) and the treewas showeda closest relation to *L. pangusia* and *L. d. dyocheilus* having a bootstrap value of 100 (Clade B in Fig.4.12). Apart from these *L. boggut, L. bata* were observed a sister clade with a bootstrap value of 99 (Clade A in Fig.4.12).

In the both topologies, cypriniformes fishes were clearly form a monophylic group when *Mystus cavasius*was used as outgroup.

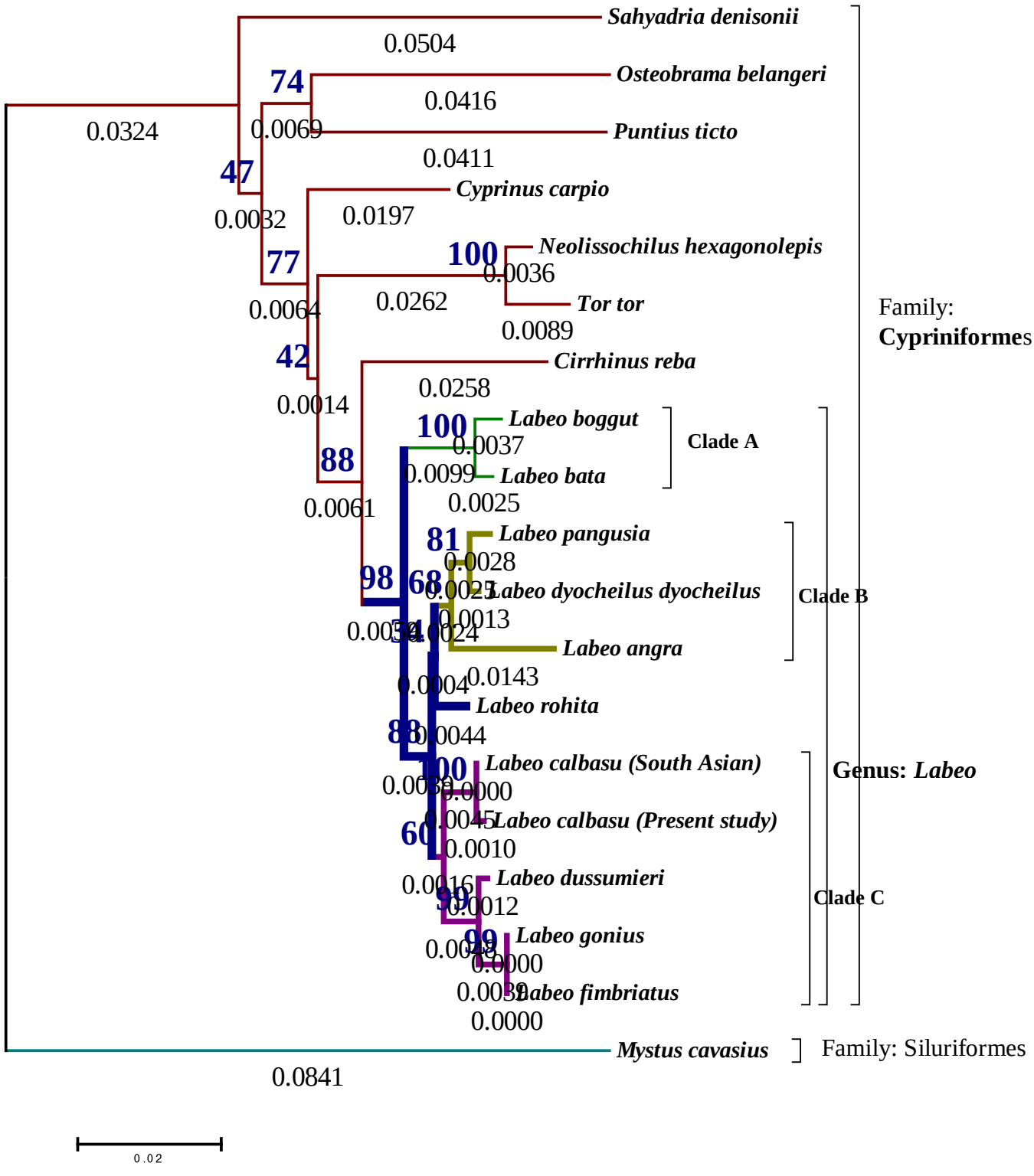


**Fig.4.11.** Neighbor-joining phylogenetic tree inferred from LCA4 sequences (794bp of *16S rRNA*) for all species and *Mystus cavasius* is as outgroup

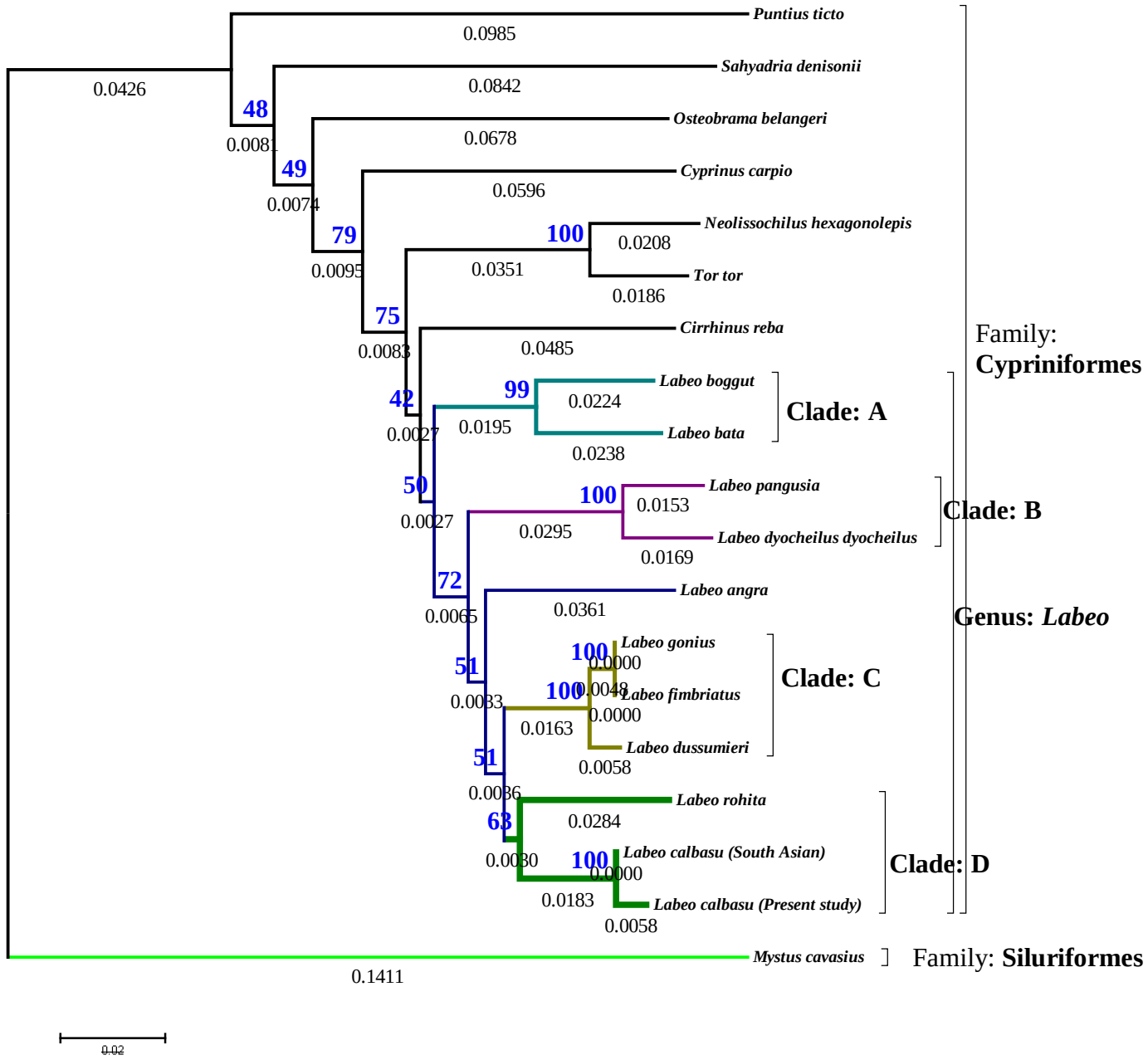


**Fig.4.12**. Neighbor-joining phylogenetic tree inferred from LCA7 sequences (754bp of *ND1*, $tRNA^{Ile}$, $tRNA^{Gln}$, $tRNA^{Met}$ and *ND2*) for all species and *Mystus cavasius* as outgroup

# Chapter 5

# DISCUSSION

Chapter 5

# DISCUSSION

## 5.1. Morphological identification

The kalbasu (*L. calbasu*) is an economically important fish species in Bangladesh and endemic to Asia. Due to change of aquatic environment and various human activities the wild population trend of this species is declining sharply and now this species is considered as threatened species in Bangladesh. Effective conservation plans for restoring this wild populations is hindered by unstable taxonomy. Statistical analysis and molecular marker have been successfully used to resolve the taxonomic conflict. The conventional techiniques like morphometric measurements, meristic counts, pigmentation pattern and colouration of the skin have been using to identify the species of *Labeo* genus. Sometimes morphological approach cannot be used in interspecies relation and population structure study because this approach is beset with problems including wide variation in the color pattern between mating and non-mating seasons of the same individuals of the same species. To solve the problems, we have used some statistical analysis which is very important from fisheries and aquaculture points of views.

*L. calbasu* can be distinguished from *Labeo* species by its two pair barbels on the mouth and distinct number of dorsal fin rays from *L. rohita, L. bata* and *L. gonius* (Talwar, 1991). In the present study, a series of phenotypic characters [13 morphometric characters (Table 4); and 4 out of 6 meristic characters (Table 11 and Table 12)] showed differences revealed by the Univariate co-efficients of variation to differentiate *L.calbasu, L. rohita, L. bata* and *L.*gonius. The multivariate analysis (Principal Component Analysis) using significant morphometric variables leading to the formation of bivariate scatter plot of principal component 1 against component 2 indicated clear separation of the two species without any inter-mixing individuals (Fig. 9 and 10) Structural variation observed on examination of gill rakers on the first gill arch (Fig. 13) of the four species also supported the distinct variation among the four species. The colour pattern (section 4.1. paragraph 1) observed in the adult specimens of *L.calbasu, L. rohita, L.gonius* (Fig. 1) and *L. bata* (Fig. 2) were also different(Choudhury and K Dutta, 2012). Moreover, the clearly distinguishable morphologic differences observed in the early juveniles of among the species

also emphasized the validity of *L. calbasu* as a different species from *L. rohita*, *L. gonius*, and *L. bata*.

The present study provides the pioneering report on the application of morphometric analysis of the selected species from Kaptai Lake. Morphometric studies have been widely used not only identify differences between fish taxa(Choudhury and K Dutta, 2012) but also examine phenotypic changes occur in their population due to envirmental change, anthropogenic factors.

**5.2. Molecular taxonomy based on mtDNA**

Mitochondrial gene sequences, esp., *16SrRNA, COI, 12SrRNA, ND1* and *ND2* are widely used in taxonomic identification of different species. Here, some selectecdregions of the mitochondrial genome that cover *16SrRNA* (partial), *ND1, ND2* and threetRNA genes have been sequenced to identify variations in the nucleotides. Available sequences of *L. calbasu* obtain from two other studies have been compared. In addition, sequences of *L. calbasu* have been compared with other *Labeo* species to study their relationship.

The *16S rRNA* nucleotides base composition of Kalbasu which is approximately similar to base compositions of orthologues found in other vertebrates and teleosts. The GC content of *L. calbasu* mitochondrial *16S rRNA, ND1* and *ND2* genes were always lower than AT content and this is also evident in other teleost species and similar result was observed in other cyprinids (Wang et al., 2008; Yue et al., 2006). The relative order of nucleotide composition in ND1 and *ND2* genes matches with the nucleotide pattern of *Catla catla* in the order: C>A>T>G and A>C>T>G (Bej et al., 2012). The organization of *tRNA* genes in this species was similar to other mitochondrial cyprinids mitochondrial genomes. In *L. calbasu*, the overlapping of nucleotides were between the $tRNA^{Gln}$- $tRNA^{Met}$ is similar to *Catlacatla* species.

Multiple sequence alignment of the *16SrRNA* gene sequence has revealed 53 variable sites out of 1097 bp long gene region (4.8%) when compared to that of two other studies of the same species (Table 13 and 14; Appendix-II, Table 19 and 20). Such variability could be

attributed to geographical variability of other two individuals, one from India and one from a South Asian country (though the country is mentioned).

On the other hand, multiple sequence alignment of Cyprinid fishes (10 *Labeo* species, one *Catla* species, two *Cirrhinus* species, one *Tor* species) has revealed 219 polymorphic sites out of 1097bp (19.96%) of 16SrRNA gene. Jahan *et al.*, (2017) performed a research at inter-species level using 16SrRNA gene of three individuals of *Labeo* (*L. rohita, L. bata and L.gonius*) species observed 23 variable sites out of 400 bp sequence (5.75%). Apparently, the difference in the percentage of polymorphic sites is due to variation in the number of species included in the study. Similar results were reported previously in other fish groups as well. Chanmthabam et al.,( 2018) observed 347 variable sites (42.79%) from 811bp from *Bangana dero* with Labeonin fishesof North East India, Basudha, (2016) 121/595 (20.34%) from Barbin fishes of North - East India, Lakra et al., (2009)170/597 (28.48%) from Indian Sciaenids, Lakra et al., (2010)119/493 (24.14%)in *Channa*species.Thus, the polymorphic site numbers varied depending on the included number of species and length of the sequence studied.

After analysis of LCA7 sequence we observed the sequence it was the combination of end portion of *ND1* gene sequence, full sequence of 3 tRNA genes (*tRNA$^{ILe}$*, *tRNA$^{Gln}$ and tRNA$^{Met}$*) and the beginning region of *ND2* gene sequence. *ND1* gene sequence was used in a very little cases of molecular identification, intra and interspecific research of fishes. However, mt*ND1*gene was used in several molecular researches on parasites and birds and mammals.Kirczuk et al., (2016) identified *Coregonus albula* species using *ND1* gene. They recorded 70 polymorphic sites out of 975 nucleotides from a protected and commercially exploited European Cisco (*Coregonus albula* L.) fish populations. The tRNA gene arrangements of *L. calbasu* (*tRNA$^{Ile}$*, *tRNA$^{Gln}$* and tRNA$^{Met}$) and the predicted tRNA structure were matched the previous records regarding other bony fishes (Appendix-III fig. 19). Castilho et al., (2007) reported 5 variable sites within *Galeus melastomus* species and 51 polymorphic sites among blackmouth (*Galeus melastomus*) and Atlantic sawtail catsharks, (*Galeus atlanticus*) species out of 1047 bp nucleotides sequences using the mt*ND2* marker. The present study recorded 6 polymorphic sites within *L. calbau* species and the 55 polymorphic sites among the *Labeo*

species out of 223 bp nucleotide sequences this recored has similariry with previous report (Castilho et al., 2007).

Our results indicated higher levels of intra and interspecies polymorphic sites variation in the amplified region of *ND1*compared to that of 16SrRNA and *ND2*.That is why *16S rRNA* is better choice for identification of species. In our study, for 16SrRNA, *ND1* and *ND2*, the intraspecific variation was much lower than the interspecific variation, which demonstrated the efficacy of *16S rRNA*, *ND1* and *ND2* for identifying *Labeo calbasu*. Hebert et al., (2004) proposed that interspecific divergence should be about ten times higher than intraspecific divergence. In our data, the level of variable sites of *16S rRNA* or *ND1* or *ND2* among congeneric species was higher than intraspecific variable sites (Table 12). Hence, these results showed that genes fragment sequences diagnose the *Labeo calbasu* species efficiently and accurately.

### 5.3. Phylogenetic relationship

*L. calbasu* is an economically important fish species and endanger to Bangladesh(Gupta and Banerjee, 2015). Wild populations have unstable taxonomy due to some phenotypic plasticity. Molecular markers have been successfully used to resolve the phylogenetic relation conflict which is better than the morphological traits based phylogenetic relation.For molecular evolutionary studies with more number of genes would give accurate phylogenetic information. In this present study, wild *L. calbasu* sample was collected from wild populations of Kaptai Lake and LCA4 (*16S rRNA*) and LCA7(*ND1*+*tRNA*$^{Ile}$+ *tRNA*$^{Gln}$+ tRNA$^{Met}$+*ND2*) mitochondrial regions were amplified, sequences and analyzed along with other Genebank nucleotide sequences of cyprinidae fishes. Total of 3 phylogenetic trees, 1 morphology based and other 2 molecular phylogenetic trees generated using LCA4 and LCA7 regions by Neighbor-Joining, Maximum Parsimony, Maximum Likelihood and Bayesian methods. All the topology was concordant. On the basis of their morphological traits, 4 *Labeo* fishes of the cyprinidae family were for morphological phylogenetic relationship study. In this topology, *L. calbasu* formed a sister group with *L. rohita*, this observation was consistent with the present molecular phylogenetic topologies.

Generated LCA4 and LCA7 phylogenetic tropolgy were congruent although they differ in details. In LCA4 topology, *L. calbasu* formgroup with the species *L. gonius*, *L.* f*rimbriatus, L. dussumieri* and *L. rohita*, in this group *L. calbasu* showed sister realtion with L. rohita by 60 bootstraps values on the other hand in LCA7 phylogenetic tree revealed sister relationship between *L. calbasu* - *L. rohita* with 63 boorstrap values and *L. gonius* - *L.* f*rimbriatus*. Several previous studies ((Behera et al., 2017; Yang et al., 2012; Yang and Mayden, 2010; Zheng et al., 2012) based on mitochondrial and nuclear genes has shown the similar tree tropology results. The present study confirmed the realationship between *Labeo calbasu- Labeo rohita*, and *Labeo gonius – Labeo frimbriatus* with significant bootstrap value but slight variation was observed in between the LCA4 and LCA7 topology because the different genes have different evolutionary rates and the same gene in different organisms may have different evolutionary rates.

# Chapter 6

# SUMMARY AND CONCLUSION

# SUMMARY AND CONCLUSION

Fish of the genera *Labeo* (family: Cyprinidae; subfamily: Cyprininae), commonly known as Kalbasu, is one of the important carp fish after the *L. rohita* and have considerable contribution in capture fisheries. This carp is endemic to Asia. However, due to anthropogenic factors such as overfishing and habitat degradation the populations of *L. calbasu* species are declining at an alarming rate. To improve the natural population distribution of Kalbasu, comprehensive conservation strategies, including habitat management, protection is needed (Khare *et al.,* 2014). Taxonomic stability of an organism is the essential platform before developing conservation action plans (Morrison *et al.,* 2009) which unfortunately is likely to be large constrain for Kalbasu conservation. Kalbasu systematics has been a subject of great interest to taxonomists. Several studies have attempted to resolve the taxonomic conflict within this group with norphologicla characters (Laskar *et al.,* 2013; Khare *et al.,* 2014). However, it was well known fact that nucleotide characters (genes) would resolve the taxonomic conflict better than the morphometreic characters. With this background, the present study was carried out to analyze the partial mitochondrial genes of *L. calbasu*and to investigate the evolutionary relationship of these species within Cyprinidae family. The species of *L. calbasu*was collected from wild population at Kaptai water reservoirs and the partial mitochondrial genes were amplified using three pairs of primers. These sequence reads were mapped against reported *L. calbasu* species. The partial mitochondrial genes, content and arrangement were similar as that of other fishes compared though polymorphic sites were detected. The data of present work might help in future population genetics study of *Labeo calbasu* targeted to conserve the species.

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# Appendix
# BLAST search results

# Appendix-I: BLAST search results

### Table 4.7. BLAST Results of LCA3 nucleotides sequence (281)

Sequences producing significant alignments:

| Description | Accession | Identities |
|---|---|---|
| *Labeo calbasu* mitochondrial DNA, complete genome | AP012143.1 | 99% |
| *Labeo rohita* mitochondrion, complete genome | KR185963.1 | 99% |
| *Labeo rohita* mitochondrial DNA, complete genome | AP011201.1 | 99% |
| *Labeo stolizkae* isolate N3 16S ribosomal RNA gene, partial sequence; mitochondrial | JX042130.1 | 99% |
| *Labeo rohita* mitochondrion, complete genome | JQ231111.1 | 99% |
| *Catla catla* mitochondrion, complete genome | JQ087872.1 | 99% |
| *Labeo stolizkae* KIZCXY20060059 16S ribosomal RNA gene, partial sequence; mitochondrial | GU168735.1 | 99% |
| *Labeo yunnanensis* 16S ribosomal RNA gene, partial sequence; mitochondrial | DQ845881.1 | 99% |
| *Labeo rohita* mitochondrion, complete genome | JN412817.1 | 98% |
| *Labeo chrysophekadion* mitochondrial DNA, complete genome | AP011199.1 | 98% |
| *Labeo chrysophekadion* clone 16 16S ribosomal RNA gene, partial sequence; mitochondrial | EU136621.1 | 98% |
| *Labeo angra* mitochondrial DNA, complete genome | AP011329.1 | 98% |
| *Labeo chrysophekadion* clone 14 16S ribosomal RNA gene, partial sequence; mitochondrial | EU136620.1 | 98% |
| *Labeo pierrei* mitochondrial DNA, complete genome | AP011200.1 | 98% |
| *Catla catla* mitochondrion, complete genome | KY419138.1 | 97% |
| *Labeo dussumieri* mitochondrial DNA, complete genome | AP011384.1 | 97% |
| *Cirrhinus microlepis* mitochondrial DNA, complete genome | AP011357.1 | 97% |
| *Catla catla* mitochondrial DNA, complete genome | AP011355.1 | 97% |
| *Labeo gonius* mitochondrion, complete genome | KT001152.1 | 97% |
| *Labeo fimbriatus* mitochondrion, complete genome | AP013340.1 | 98% |

**Table 4.8. BLAST Results of LCA4 nucleotides sequence (797)**

Sequences producing significant alignments:

| Description | Accession | Identities |
|---|---|---|
| *Labeo calbasu* mitochondrial DNA, complete genome | AP012143.1 | 100% |
| *Labeo dussumieri* mitochondrial DNA, complete genome | AP011384.1 | 98% |
| *Labeo rohita* mitochondrion, complete genome | KR185963.1 | 98% |
| *Labeo rohita* mitochondrial DNA, complete genome | AP011201.1 | 98% |
| *Labeo yunnanensis* 16S ribosomal RNA gene, partial sequence; mitochondrial | DQ845881.1 | 98% |
| *Labeo gonius* mitochondrion, complete genome | KT001152.1 | 97% |
| *Labeo fimbriatus* mitochondrion, complete genome | KP025676.1 | 97% |
| *Labeo chrysophekadion* mitochondrial DNA, complete genome | AP011199.1 | 97% |
| *Labeo rohita* mitochondrion, complete genome | JN412817.1 | 97% |
| *Labeo rohita* mitochondrion, complete genome | JQ231111.1 | 97% |
| *Latla catla* mitochondrion, complete genome | JQ087872.1 | 97% |
| *Catla catla* mitochondrion, complete genome | JQ838172.1 | 97% |
| *Labeo dyocheilus* dyocheilus mitochondrial DNA, complete genome | AP011328.1 | 97% |
| *Labeo pangusia* mitochondrial DNA, complete genome, specimen_voucher: CBM:ZF:11832 | AP013340.1 | 97% |
| *Catla catla* mitochondrial DNA, complete genome | AP011355.1 | 97% |
| *Labeo stolizkae* isolate N3 16S ribosomal RNA gene, partial sequence; mitochondrial | JX042130.1 | 98% |
| *Labeo stolizkae* isolate KIZCXY20060059 16S ribosomal RNA gene, partial sequence; mitochondrial | GU168735.1 | 98% |
| *Labeo parvus* mitochondrial DNA, almost complete genome, except for D-loop, V:CBM:ZF:12695 | AP013339.1 | 96% |
| *Labeo altivelis* mitochondrial DNA, complete genome | AP013322.1 | 96% |
| *Incisilabeo behri* mitochondrial DNA, complete genome | AP011356.1 | 96% |

**Table 4.9. BLAST Results of LCA7 nucleotides sequence (764)**

Sequences producing significant alignments:

| Description | Accession | Identities |
|---|---|---|
| *Labeo calbasu* mitochondrial DNA, complete genome | AP012143.1 | 99% |
| *Labeo chrysophekadion* mitochondrial DNA, complete genome | AP011199.1 | 96% |
| *Labeo dussumieri* mitochondrial DNA, complete genome | AP011384.1 | 95% |
| *Catla catla* mitochondrial DNA, complete genome | AP011355.1 | 95% |
| *Catla catla* mitochondrion, complete genome | KY419138.1 | 95% |
| *Labeo gonius* mitochondrion, complete genome | KT001152.1 | 95% |
| *Labeo fimbriatus* mitochondrion, complete genome | KP025676.1 | 95% |
| *Catla catla* mitochondrion, complete genome | JQ838172.1 | 95% |
| *Labeo rohita* mitochondrion, complete genome | KR185963.1 | 95% |
| *Labeo rohita* mitochondrial DNA, complete genome | AP011201.1 | 95% |
| *Labeo rohita* mitochondrion, complete genome | JN412817.1 | 95% |
| *Labeo rohita* mitochondrion, complete genome | JQ231111.1 | 95% |
| *Catla catla* mitochondrion, complete genome | JQ087872.1 | 95% |
| *Labeo parvus* mitochondrial DNA, almost complete genome, except for D-loop,voucher: CBM:ZF:12695 | AP013339.1 | 94% |
| *Labeo coubie* mitochondrial DNA, complete genome | AP012149.1 | 94% |
| *Bangana tungting* mitochondrion, complete genome | KJ737371.1 | 93% |
| *Labeo* sp. CBM-ZF-11756 mitochondrial DNA, complete genome, specimen_voucher: CBM:ZF:11756 | AP013326.1 | 93% |
| *Labeo angra* mitochondrial DNA, complete genome | AP011329.1 | 93% |
| *Incisilabeo behri* mitochondrial DNA, complete genome | AP011356.1 | 93% |
| *Labiobarbus leptocheilus* mitochondrial DNA, complete genome, specimen_voucher: UAIC:14301.20 | AP013315.1 | 93% |

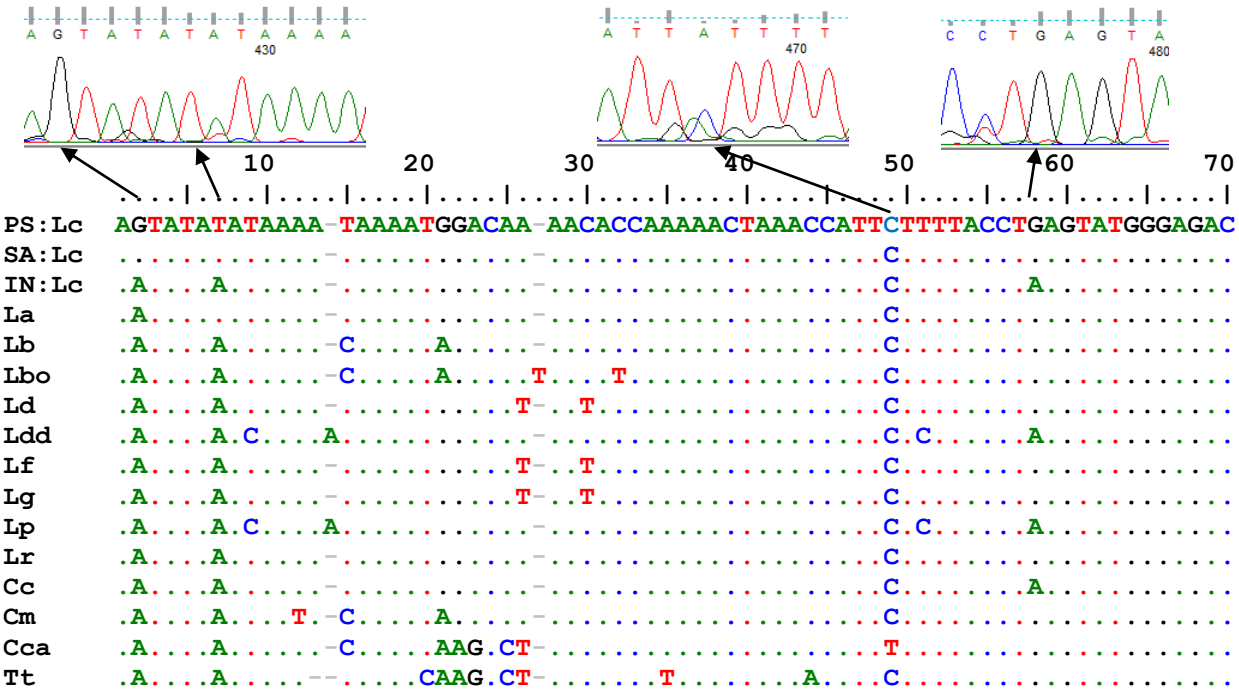
# Appendix –II

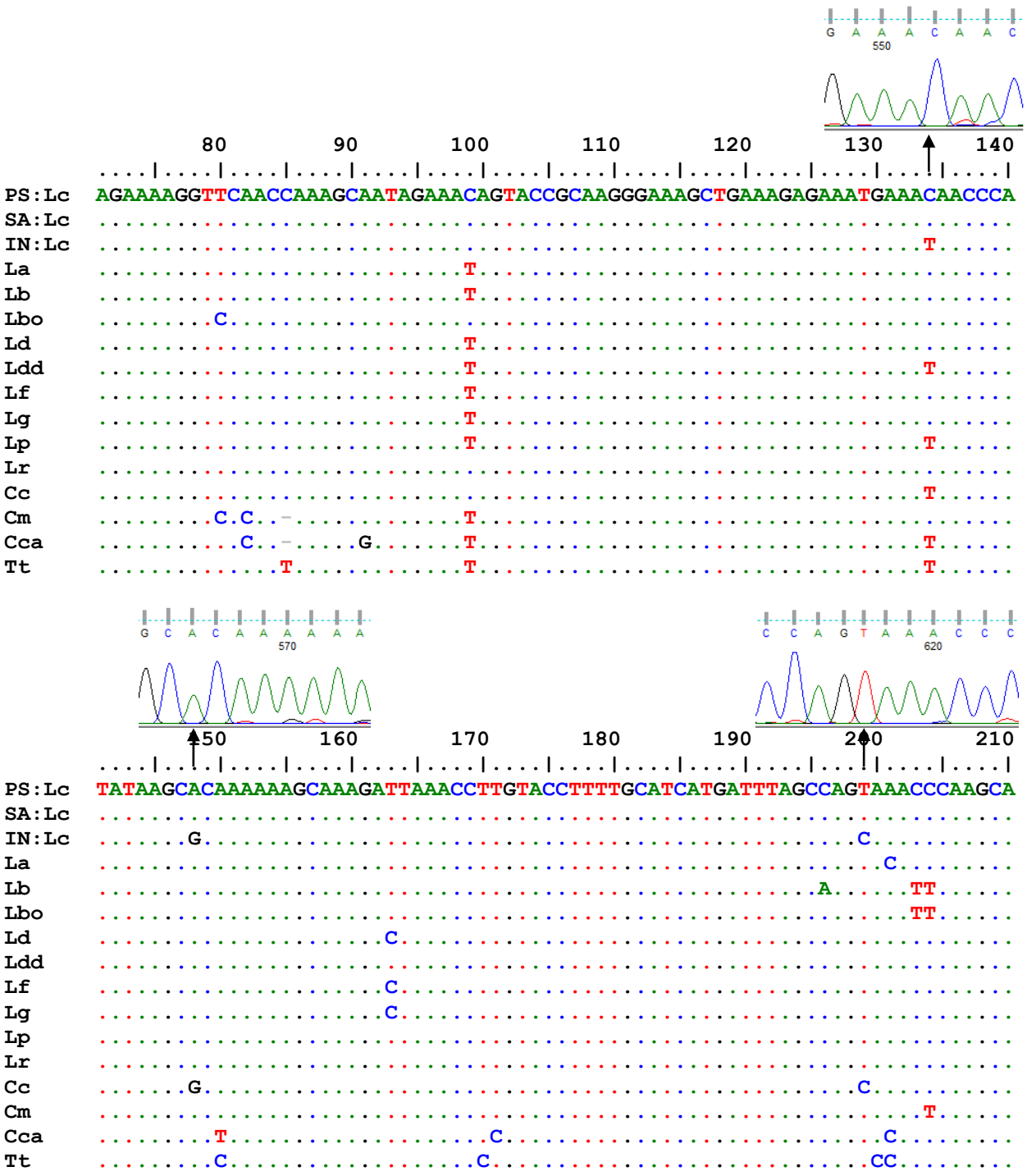
# Sequence alignments

# Appendix –II

## Sequence alignments

**Table 4.15.** Multiple alignments of the nucleotide sequences of the LCA3 regions of *L. calbasu* from present study. AP012143.1( *L. calbasu*), JQ231113.1 (*L. calbasu*), AP011329.1 (*L. angra*), AP011198.1 (*L.bata*), AP013338.1( *L. boggut*), AP011384.1 (*L. dussumieri*), AP011328.1 (*L. d. dyocheilus*), KP025676.1 (*L. fimbriatus*), KT001152.1 (*L.gonius*),NC_029451.1( *L.pangusia*), KR185963.1 (*L. rohita*), JQ838172.1 (*C. catla*), NC_017611.1 (C. mrigala), KU050703.1 (*C. carpio*), NC_027498.1 (*T. tor*) are from gene bank which are included with original sequence for easy comparison**.**

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Lf
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Lp
Lr
Cc
Cm
Cca
Tt
G C A C A A A A A A A
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620
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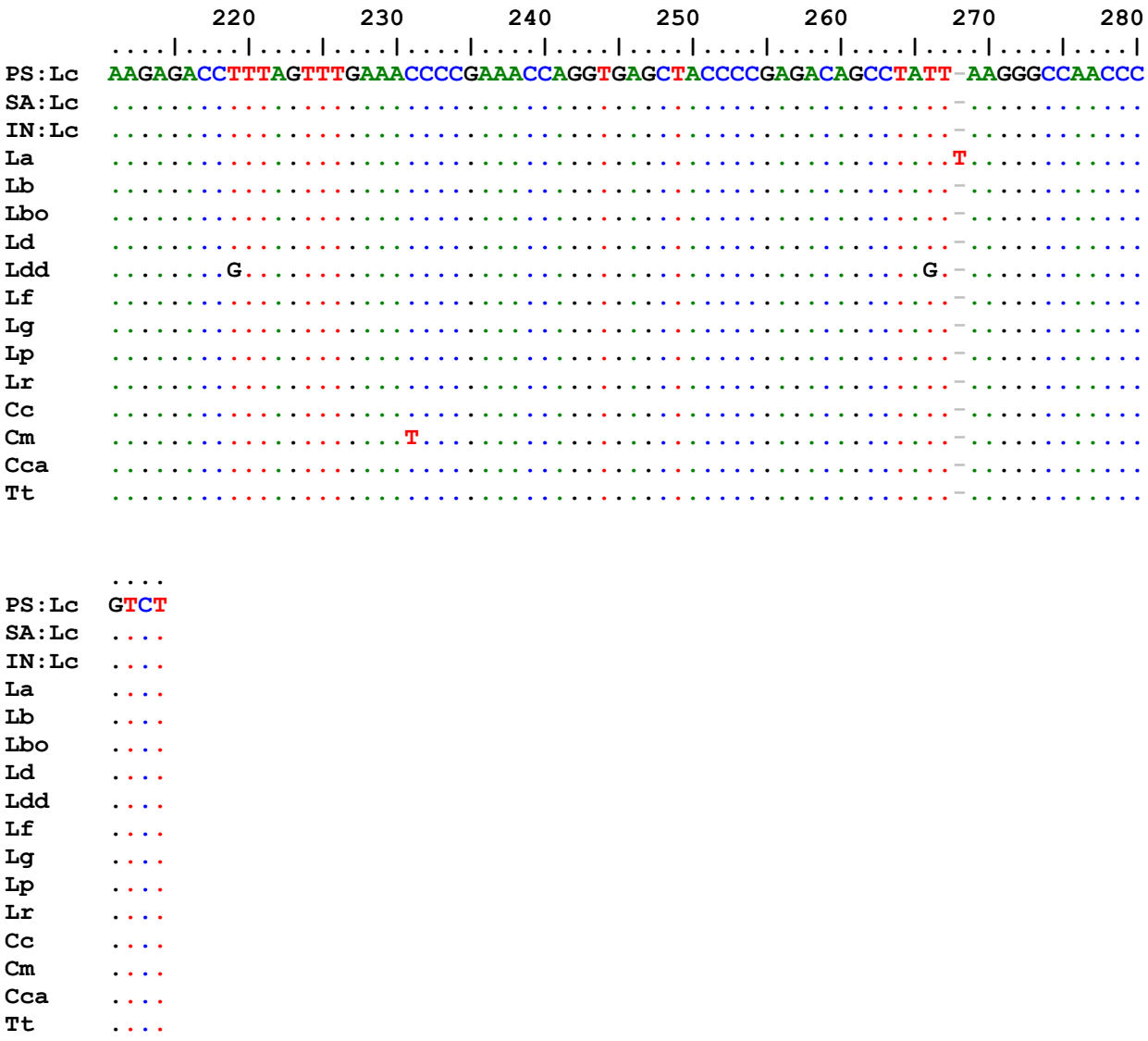
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Lf
Lg
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Lr
Cc
Cm
Cca
Tt
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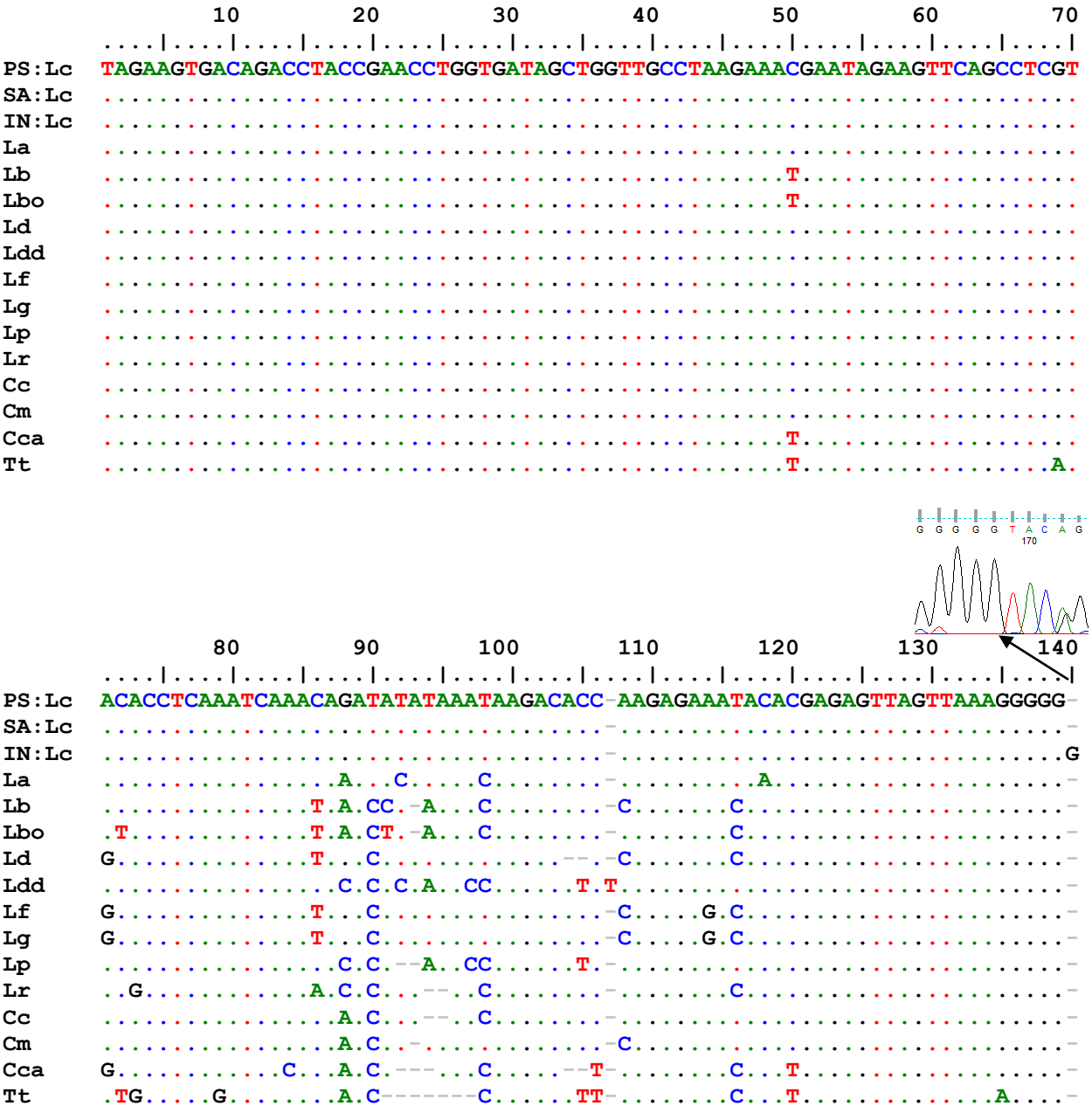
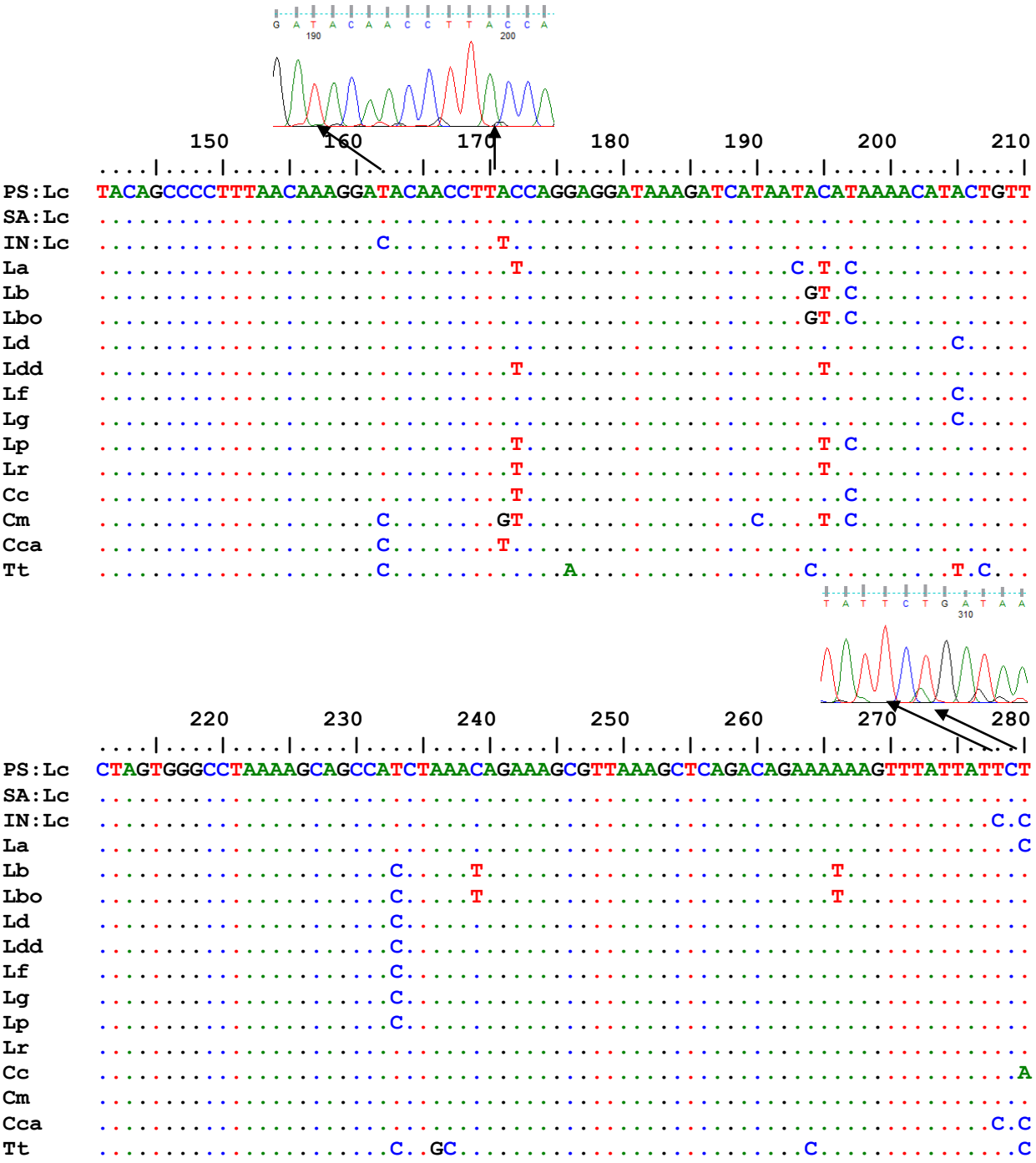
Lf
Lg
Lp
Lr
Cc
Cm
Cca
Tt

**Table 4.16.** Multiple alignments of the nucleotide sequences of the LCA4 regions of *L. calbasu* from present study. AP012143.1( *L. calbasu*), JQ231113.1 (*L. calbasu*), AP011329.1 (*L. angra*), AP011198.1 (*L.bata*), AP013338.1( *L. boggut*), AP011384.1 (*L. dussumieri*), AP011328.1 (*L. d. dyocheilus*), KP025676.1 (*L. fimbriatus*), KT001152.1 (*L.gonius*),NC_029451.1( *L.pangusia*), KR185963.1 (*L. rohita*), JQ838172.1 (*C. catla*), NC_017611.1 (C. mrigala), KU050703.1 (*C. carpio*), NC_027498.1 (*T. tor*) are from gene bank which are included with original sequence for easy comparison**.**

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Cca
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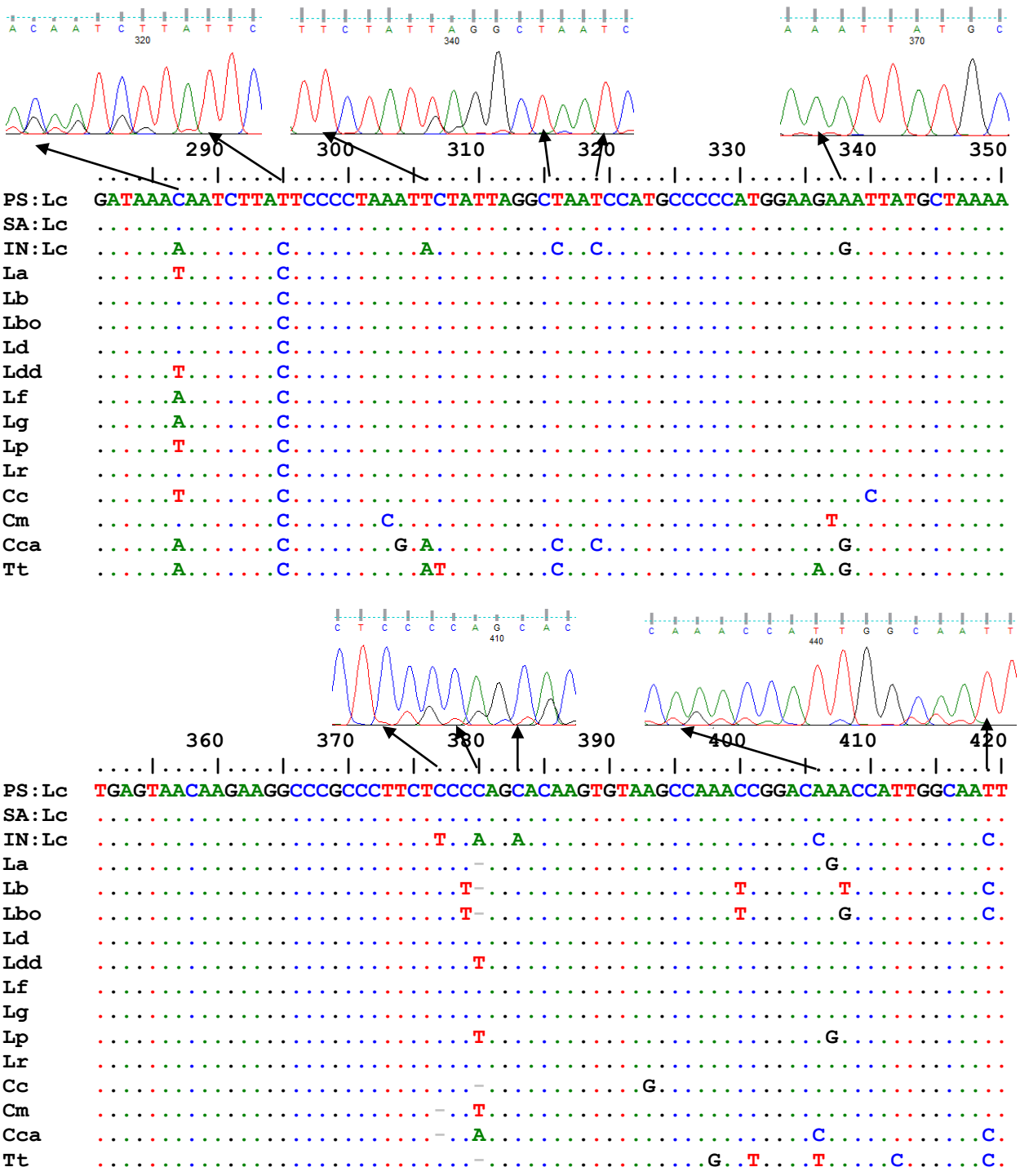
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Cc
Cm
Cca
Tt
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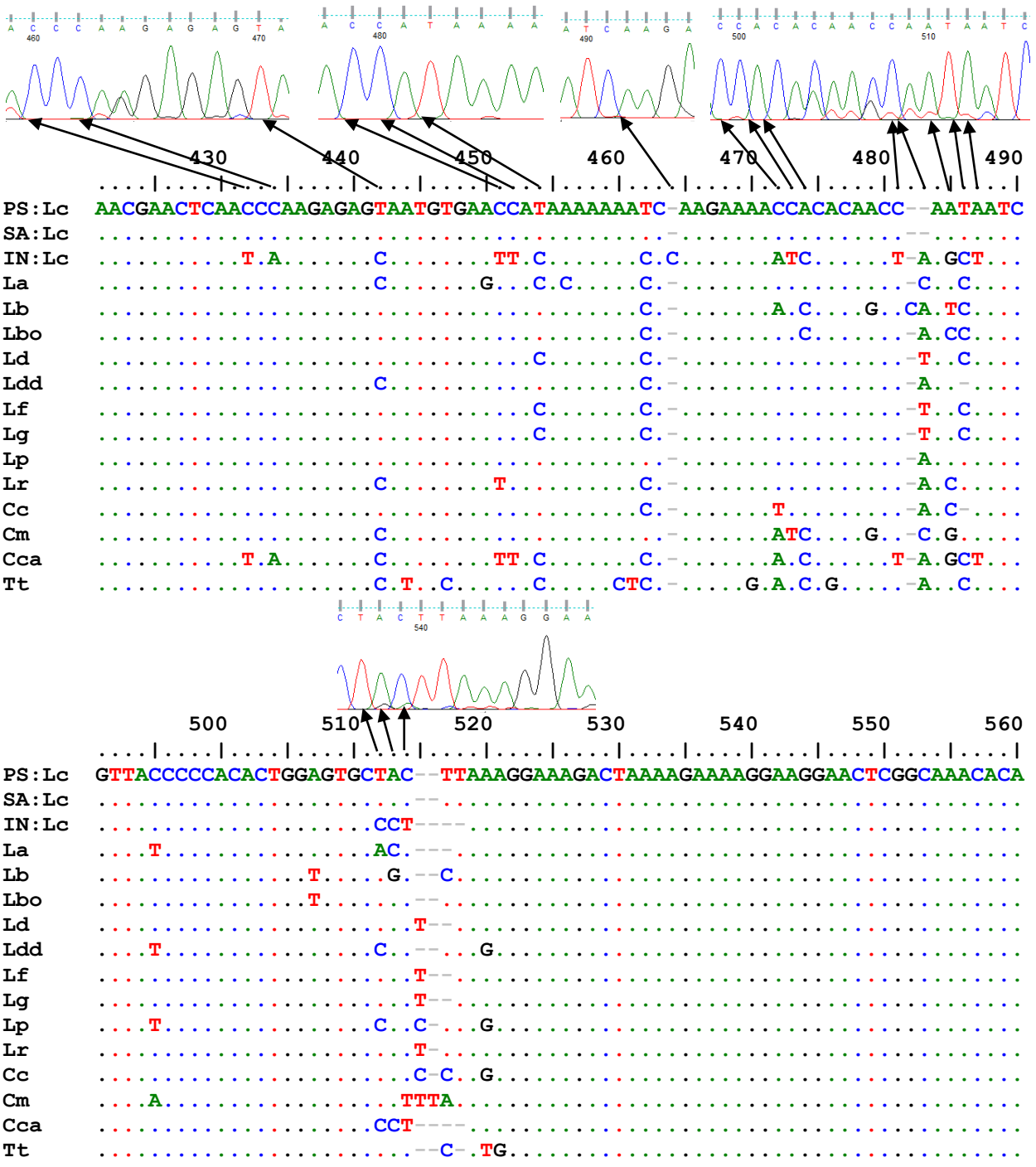
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510
520
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540
550
560
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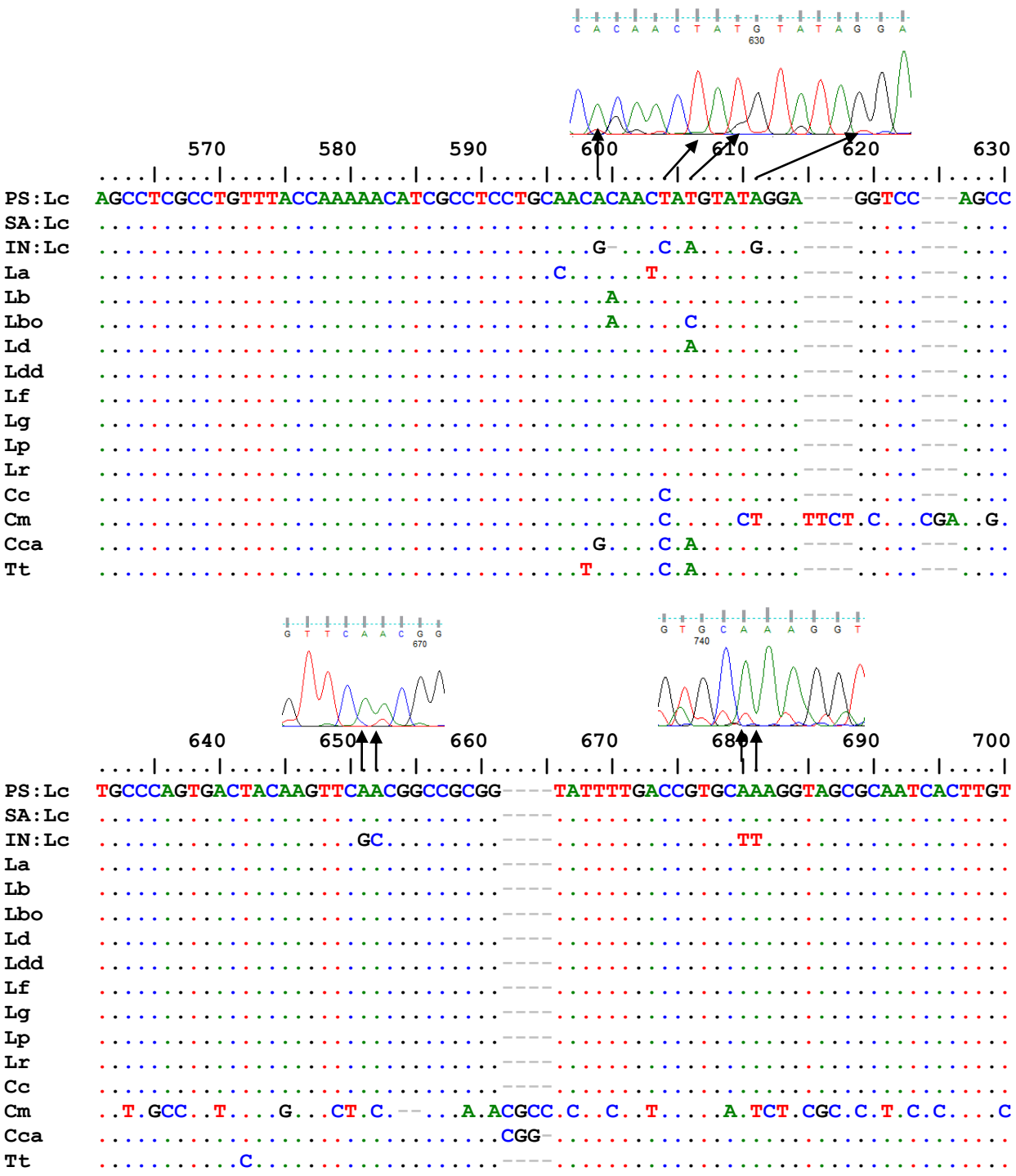
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Cca
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Tt

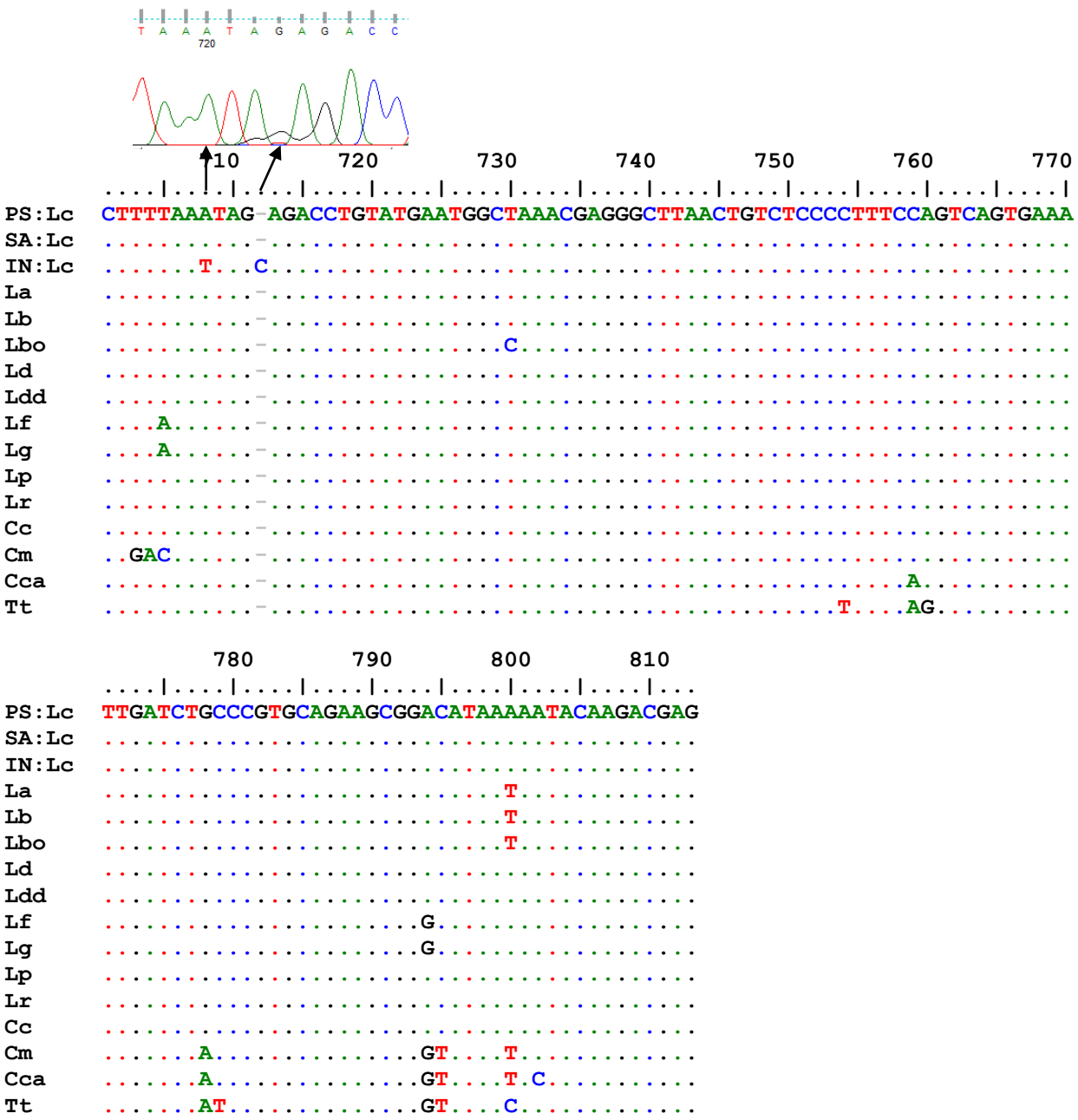
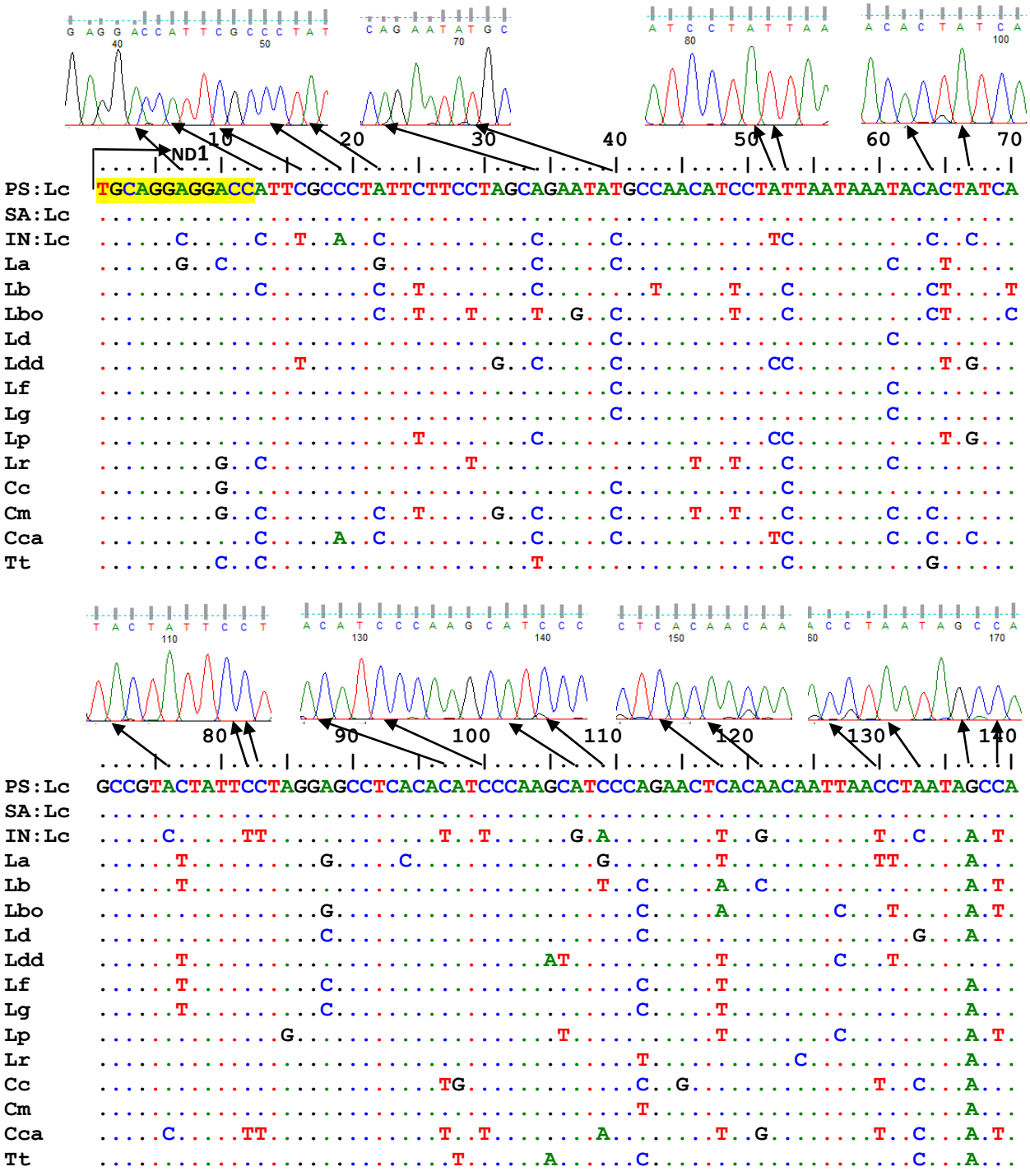
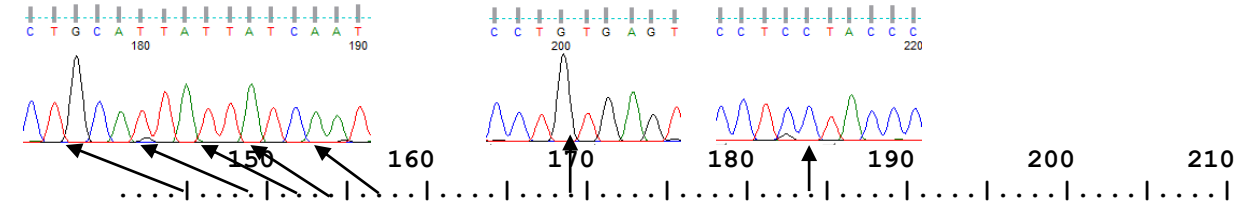
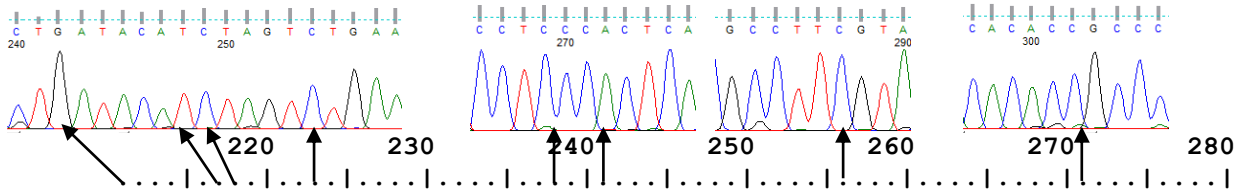

T A A A T A G A G A C C
720
10 720 730 740 750 760 770
PS:Lc CTTTTAAATAG-AGACCTGTATGAATGGCTAAACGAGGGCTTAACTGTCTCCCCTTTCCAGTCAGTGAAA
SA:Lc ..........-..............................................................
IN:Lc .......T...C..............................................................
La ..........-..............................................................
Lb ..........-..............................................................
Lbo ..........-................C.............................................
Ld ..........-..............................................................
Ldd ..........-..............................................................
Lf ....A.....-..............................................................
Lg ....A.....-..............................................................
Lp ..........-..............................................................
Lr ..........-..............................................................
Cc ..........-..............................................................
Cm ..GAC.....-..............................................................
Cca ..........-.....................................A....................
Tt ..........-.....................................T....AG.....
780 790 800 810
PS:Lc TTGATCTGCCCGTGCAGAAGCGGACATAAAAATACAAGACGAG
SA:Lc ..................................................
IN:Lc ..................................................
La ....................T.............
Lb ....................T.............
Lbo ....................T.............
Ld ..................................................
Ldd ..................................................
Lf ..................G...............
Lg ..................G...............
Lp ..................................................
Lr ..................................................
Cc ..................................................
Cm .......A..............GT....T.............
Cca .......A..............GT....T.C...........
Tt .......AT.............GT....C.............

**Table. 4.17.** Multiple alignments of the nucleotide sequences of the LCA7 regions of L. calbasu from present study. AP012143.1( *L. calbasu*), JQ231113.1 (*L. calbasu*), AP011329.1 (*L. angra*), AP011198.1 (*L.bata*), AP013338.1( *L. boggut*), AP011384.1 (*L. dussumieri*), AP011328.1 (*L.d. dyocheilus*),KP025676.1 (*L. fimbriatus*), KT001152.1 (*L.gonius*),NC_029451.1( *L.pangusia*), KR185963.1 (*L. rohita*), JQ838172.1 (*C. catla*), NC_017611.1 (C. mrigala), KU050703.1 (*C. carpio*), NC_027498.1 (*T. tor*) are from gene bank which are included with original sequence for easy comparison.

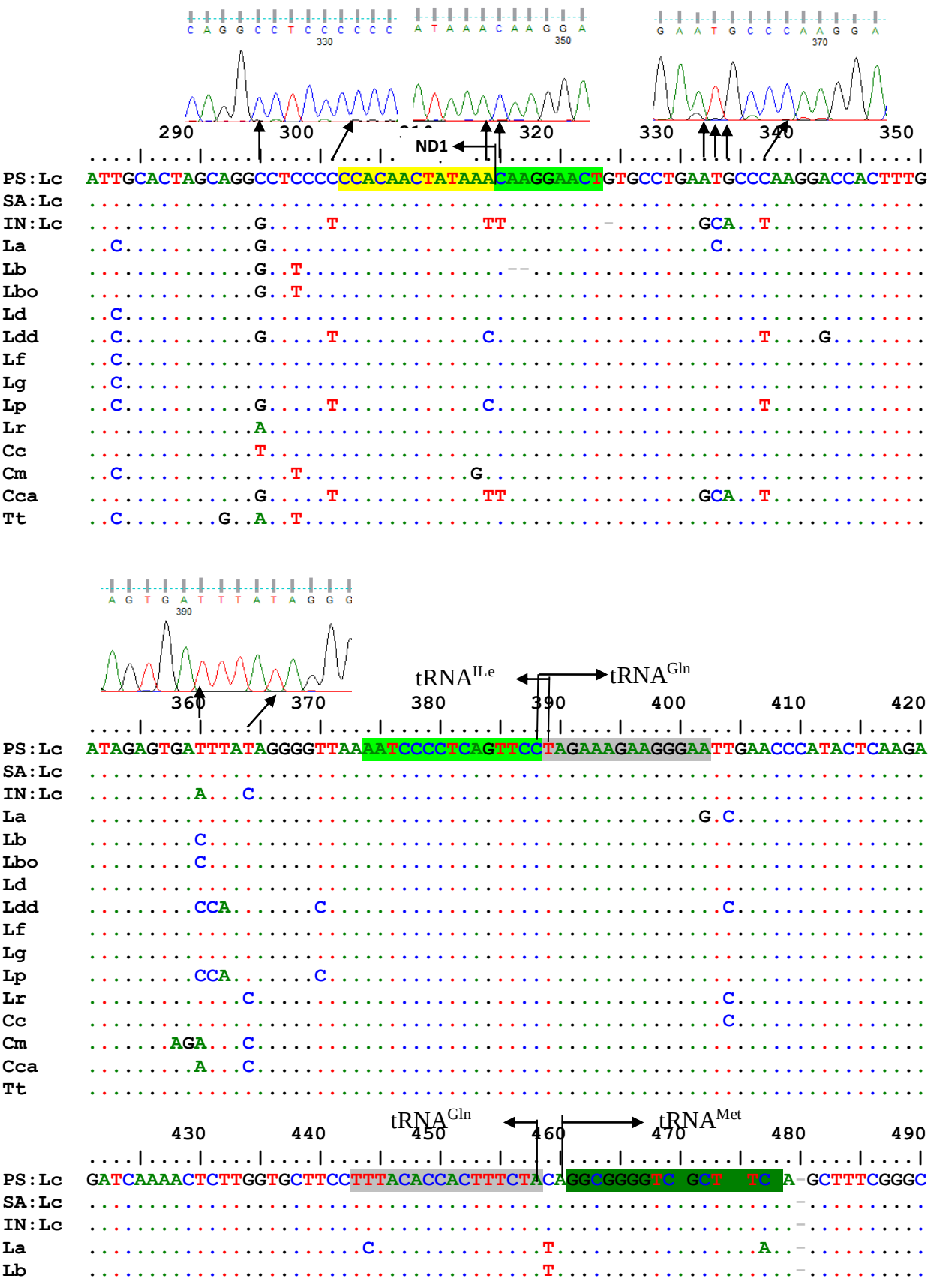


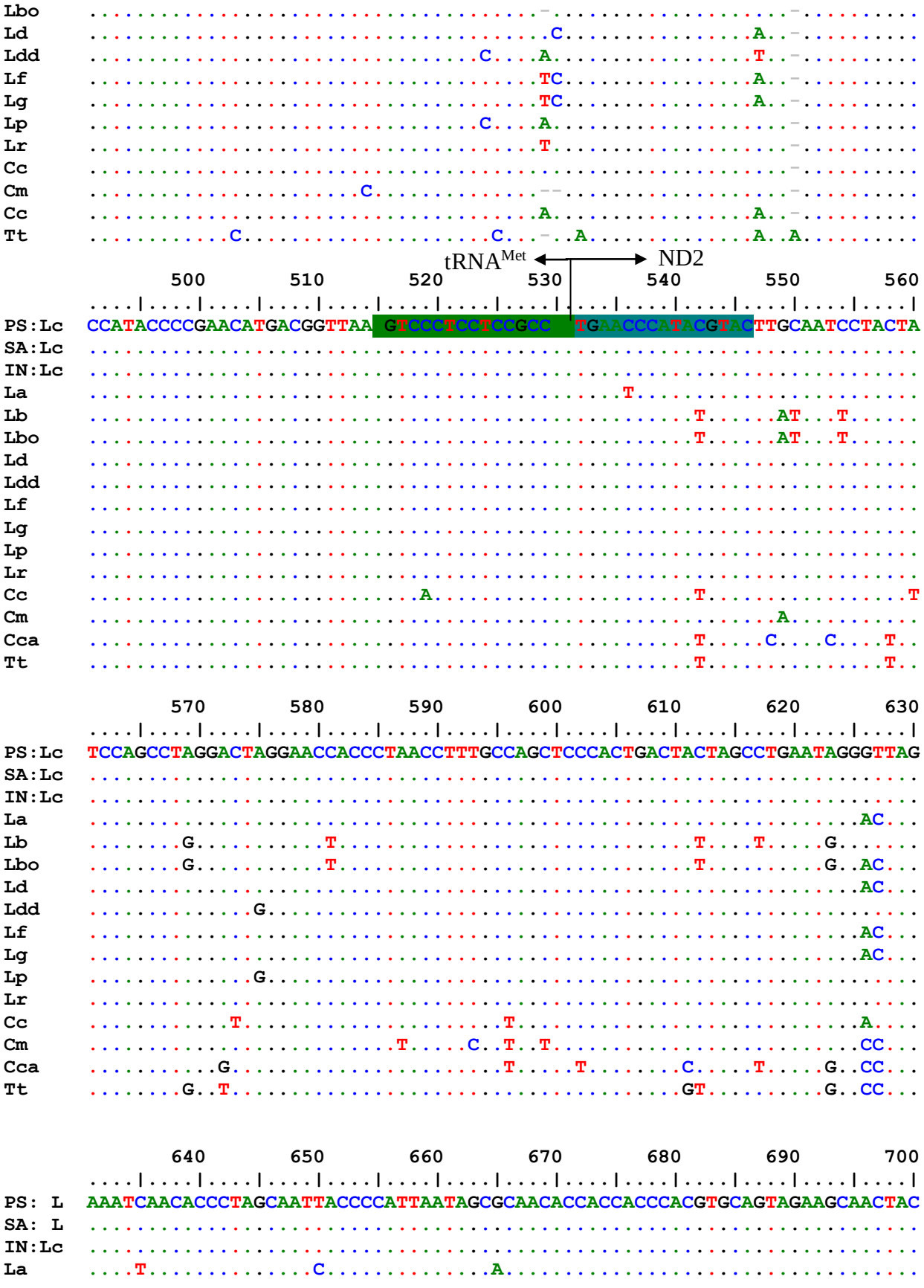



```
PS:Lc   AAGCTGCATTATTATCAATTATATTCCTGTGAGTACGAGCCTCCTACCCACGATTCCGATACGACCAACT
SA:Lc   ...............................................................................
IN:Lc   ....C...C..C.C..T..........A.............G.......................................
La      ........C..C.G......C.......A........T...........T.....................T.
Lb      ........C...........T.......A..G.......T.....................T.......
Lbo     ........C..C.....G.CC.......A...........T.....................T..T.....
Ld      ....C......C........CC.....T.A.....................................T.....
Ldd     ....C................C.......A..G........A..............T.....T........
Lf      ....C......C.......CC.G...T.A.....................................T..T.....
Lg      ....C......C.......CC.G...T.A.....................................T..T.....
Lp      ....C................C.......A..G........A.....T.............T........
Lr      ....................T.......A.............T..T.............T..T.....
Cc      ....................C.....................T.................T.....
Cm      ....C..CC..C........CC.....T.A.....................C...........T..T.....
Cca     ....C...C..C.C..T..........A.............G.......................................
Tt      ..A.C...C.TC.G..C..CC..........G.......T..A..T.....G..T.............
```

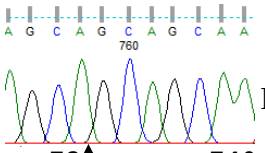



```
PS:Lc   GATACATCTAGTCTGAAAAAACTTCCTCCCACTCACACTCGCCTTCGTACTATGACACACCGCCCTACCA
SA:Lc   ...............................................................................
IN:Lc   A.....CT....A.............T..C.............T.............T.........
La      A.....CT....T.................C........A.....................G...
Lb      A......T....A........T...........A.....T.................T...........
Lbo     A.....C.....A.....................T.........T.....................
Ld      A......T....G...........T.....C.....................T..G...
Ldd     A.....CT............T........C..A.....T...C............T.....T..G...
Lf      A......T....G...........T.....C.....................G...
Lg      A......T....G...........T.....C.....................G...
Lp      A.....CT.....................C..A.....T..T.............T.....T..G...
Lr      A..G..CT.G...................C........A........T.....................
Cc      A.....C.....................C.............T.....................
Cm      A.....C.....A.................C........T.....................G..G
Cca     A.....CT....A.............T..C.............T.............T.........
Tt      A.....C.....................T.....A.....T.....T.................T..G...
```


ND1
tRNA^ILe
tRNA^Gln
tRNA^Gln
tRNA^Met
PS:Lc
SA:Lc
IN:Lc
La
Lb
Lbo
Ld
Ldd
Lf
Lg
Lp
Lr
Cc
Cm
Cca
Tt


tRNA^Met
ND2
500
510
520
530
540
550
560
570
580
590
600
610
620
630
640
650
660
670
680
690
700
PS:Lc
SA:Lc
IN:Lc
La
Lb
Lbo
Ld
Ldd
Lf
Lg
Lp
Lr
Cc
Cm
Cca
Tt
PS: L
SA: L
IN:Lc
La

```
Lb      ....T.....................C.......A........T.................G.....C..
Lbo     ....T..T.....G.....................A.....T.................G.....C..
Ld      .......T..........C......C.......A.................C.................
Ldd     ....T..T..........C......C.......A.....................................C..
Lf      .......T.....................C.......A.....................................
Lg      .......T.....................C.......A.....................................
Lp      ....T.............C......C.......A.....................................C..
Lr      .......T..........C......C.......A.....................................C..
Cc      ....T..T..........C......C.......A.....................................
Cm      ....T.............C......C.......A.................C.....................C..
Cca     ....T..T..G.......C.....CC....G..A.....T........C.....................
Tt      ....T.....................C........A..............C........G........A..
```




ND2

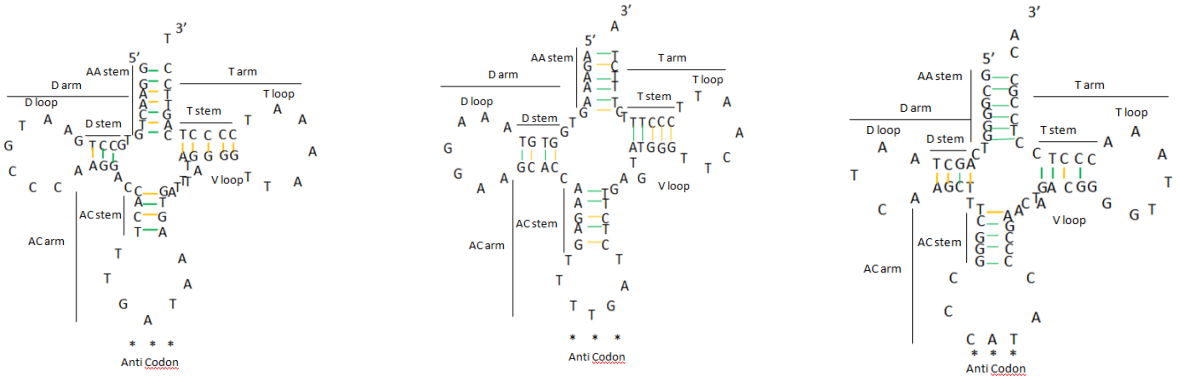
```
                710       720       730       740       750
                ....|....|....|....|....|....|....|....|....|....|....
PS:Lc           AAAATACTTCCTAACCCAAGCCACAGCAGCAGCAATAATCCTATTCGCAAGCACA
SA:Lc           ....................................................
IN:Lc           ..........................G.........................
La              ..............................T.....T................
Lb              ..............................T..C...................
Lbo             ..............T...............T..C...................
Ld              ......T.....................................................
Ldd             ......T.....................T..G...................
Lf              ......T.....................................................
Lg              ......T.....................................................
Lp              .........TT.......G........G..........T..G...................
Lr              .........T..........T.....................................
Cc              ....................T...............TT.....................
Cm              ..........................G..........T.....................
Cca             ......T...T............C..C..G..........G...................
Tt              ......T................C..............G........T...
```

# Appendix –III

# tRNA secondary structures

# Appendix –III

## tRNA secondary structures




*tRNA*$^{Ile}$ *tRNA*$^{Gln}$ tRNA$^{Met}$

**Fig. 4.12.** Sequences of *L. calbasu* mitochondrial tRNA genes, represented in theclover-form

**Table 4.10.** Nucleotide composition of *16S rRNA, ND1, tRNA$^{Ile}$, tRNA$^{Gln}$, tRNA$^{Met}$* and *ND2* genes of the *Labeo* species

| Genes | Species | Length | A% | T% | G% | C% | %C~G |
|---|---|---|---|---|---|---|---|
| *16S rRNA* | *L. calbasu(*PS) | 822 | 37 | 20 | 19 | 23 | 42.8 |
| *16S rRNA* | *L. calbasu*(SA) | 1688 | 37 | 19 | 19 | 23 | 43.3 |
| *16S rRNA* | *L. angra* | 1688 | 36 | 19 | 19 | 24 | 43.7 |
| *16S rRNA* | *L. bata* | 1687 | 36 | 19 | 19 | 17 | 43.1 |
| *16S rRNA* | *L. boggut* | 1688 | 37 | 19 | 19 | 23 | 43.1 |
| *16S rRNA* | *L. dussumieri* | 1687 | 36 | 19 | 19 | 23 | 43.5 |
| *16S rRNA* | *L. d.dycheilus* | 1690 | 37 | 19 | 19 | 23 | 43.5 |
| *16S rRNA* | *L. fibriatus* | 1691 | 36 | 19 | 19 | 23 | 43.5 |
| *16S rRNA* | *L. gonius* | 1691 | 36 | 19 | 19 | 23 | 43.5 |
| *16S rRNA* | *L. pangusia* | 1689 | 37 | 19 | 19 | 23 | 43.2 |
| *16S rRNA* | *L. rohita* | 1689 | 37 | 19 | 19 | 23 | 43.5 |
| ***16S rRNA*** | **Averge** | | **36.5** | **19.09** | **19** | **22.5** | **43.3** |
| *ND1* | *L. calbasu* (PS) | 323 | 31 | 23 | 12 | 32 | 44.9 |
| *ND1* | *L. calbasu*(SA) | 975 | 31 | 24 | 13 | 30 | 43.9 |
| *ND1* | *L.angra* | 975 | 30 | 24 | 14 | 31 | 44.2 |
| *ND1* | *L.bata* | 973 | 31 | 25 | 13 | 28 | 42.3 |
| *ND1* | *L.boggut* | 975 | 31 | 24 | 13 | 29 | 43.7 |
| *ND1* | *L.dussumieri* | 975 | 30 | 23 | 13 | 31 | 45.5 |
| *ND1* | *L. d. dyocheilus* | 975 | 31 | 23 | 14 | 30 | 44.6 |
| *ND1* | *L. fimbriatus* | 975 | 30 | 24 | 13 | 31 | 45.1 |
| *ND1* | *L. gonius* | 975 | 30 | 24 | 13 | 31 | 45 |
| *ND1* | *L. pangusia* | 975 | 31 | 24 | 14 | 29 | 44.2 |
| *ND1* | *L. rohita* | 975 | 30 | 24 | 14 | 31 | 45.4 |
| ***ND1*** | **Average** | | **30.5** | **23.8** | **13.2** | **30.2** | **44.4** |
| *tRNA$^{Ile}$* | *L. calbasu* (PS ) | 74 | 28 | 27 | 22 | 21 | 44.6 |
| *tRNA$^{Ile}$* | *L. calbasu* | 72 | 26 | 27 | 23 | 22 | 45.8 |
| *tRNA$^{Ile}$* | *L. angra* | 72 | 26 | 26 | 23 | 23 | 47.2 |
| *tRNA$^{Ile}$* | *L. bata* | 72 | 26 | 26 | 23 | 23 | 47.2 |
| *tRNA$^{Ile}$* | *L. boggut* | 72 | 26 | 27 | 23 | 22 | 45.8 |
| *tRNA$^{Ile}$* | *L. dussumieri* | 72 | 26 | 27 | 23 | 22 | 45.8 |
| *tRNA$^{Ile}$* | *L. d.dycheilus* | 72 | 26 | 23 | 25 | 25 | 50 |
| *tRNA$^{Ile}$* | *L. fibriatus* | 72 | 26 | 27 | 23 | 22 | 45.8 |
| *tRNA$^{Ile}$* | *L. gonius* | 72 | 26 | 27 | 23 | 22 | 45.8 |
| *tRNA$^{Ile}$* | *L. pangusia* | 72 | 27 | 23 | 23 | 25 | 48.6 |
| *tRNA$^{Ile}$* | *L. rohita* | 72 | 26 | 26 | 23 | 23 | 47.2 |
| ***tRNA$^{Ile}$*** | **Average** | | **26.2** | **26** | **23** | **22.7** | **46.7** |
| *tRNA$^{Gln}$* | *L. calbasu* (PS ) | 70 | 27 | 34 | 23 | 16 | 38.6 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *tRNA$^{Gln}$* | *L. calbasu* | 71 | 27 | 34 | 24 | 15 | 39.4 |
| *tRNA$^{Gln}$* | *L. angra* | 71 | 24 | 32 | 27 | 17 | 43.7 |
| *tRNA$^{Gln}$* | *L. bata* | 71 | 27 | 33 | 24 | 15 | 39.4 |
| *tRNA$^{Gln}$* | *L. boggut* | 71 | 27 | 34 | 24 | 15 | 39.4 |
| *tRNA$^{Gln}$* | *L. dussumieri* | 71 | 27 | 34 | 24 | 15 | 39.4 |
| *tRNA$^{Gln}$* | *L. d.dycheilus* | 71 | 24 | 34 | 27 | 15 | 42.3 |
| *tRNA$^{Gln}$* | *L. fibriatus* | 71 | 27 | 34 | 24 | 15 | 39.4 |
| *tRNA$^{Gln}$* | *L. gonius* | 71 | 27 | 34 | 24 | 15 | 39.4 |
| *tRNA$^{Gln}$* | *L. pangusia* | 71 | 25 | 34 | 25 | 15 | 40.8 |
| *tRNA$^{Gln}$* | *L. rohita* | 71 | 25 | 34 | 25 | 15 | 40.8 |
| ***tRNA$^{Gln}$*** | **Average** | | **26** | **33.7** | **24.6** | **15.2** | **40.2** |
| *tRNA$^{Met}$* | *L. calbasu* (PS) | 69 | 22 | 19 | 25 | 35 | 59.4 |
| *tRNA$^{Met}$* | *L. calbasu* (SA) | 56 | 27 | 24 | 30 | 43 | 59.4 |
| *tRNA$^{Met}$* | *L. angra* | 69 | 23 | 19 | 25 | 33 | 58 |
| *tRNA$^{Met}$* | *L. bata* | 69 | 22 | 19 | 25 | 34 | 59.4 |
| *tRNA$^{Met}$* | *L. boggut* | 69 | 22 | 19 | 25 | 35 | 59.4 |
| *tRNA$^{Met}$* | *L. dussumieri* | 69 | 22 | 19 | 25 | 35 | 59.4 |
| *tRNA$^{Met}$* | *L. d.dycheilus* | 69 | 22 | 20 | 25 | 33 | 58 |
| *tRNA$^{Met}$* | *L. fibriatus* | 69 | 22 | 20 | 25 | 33 | 58 |
| *tRNA$^{Met}$* | *L. gonius* | 69 | 23 | 19 | 25 | 33 | 58 |
| *tRNA$^{Met}$* | *L. pangusia* | 69 | 22 | 19 | 25 | 35 | 59.4 |
| *tRNA$^{Met}$* | *L. rohita* | 69 | 22 | 19 | 25 | 35 | 59.4 |
| ***tRNA$^{Met}$*** | **Average** | | **22.6** | **19.6** | **25.45** | **34.9** | **58.8** |
| *ND2* | *L. calbasu* (PS ) | 225 | 34 | 19 | 14 | 33 | 47.1 |
| *ND2* | *L calbasu* (SA) | 1045 | 35 | 21 | 11 | 32 | 44 |
| *ND2* | *L. angra* | 1045 | 35 | 20 | 11 | 30 | 43.7 |
| *ND2* | *L. bata* | 1045 | 35 | 21 | 11 | 32 | 43.8 |
| *ND2* | *L. boggut* | 1045 | 35 | 21 | 12 | 31 | 43.2 |
| *ND2* | *L. dussumieri* | 1045 | 35 | 20 | 12 | 32 | 44 |
| *ND2* | *L. d.dycheilus* | 1045 | 36 | 21 | 11 | 32 | 43.3 |
| *ND2* | *L. fibriatus* | 1045 | 35 | 21 | 11 | 32 | 43.5 |
| *ND2* | *L. gonius* | 1045 | 35 | 21 | 11 | 32 | 43.5 |
| *ND2* | *L. pangusia* | 1045 | 35 | 21 | 11 | 32 | 43.5 |
| *ND2* | *L. rohita* | 1045 | 35 | 20 | 11 | 33 | 45 |
| ***ND2*** | **Average** | | **35** | **20.5** | **11.4** | **31.9** | **44** |

# NCBI ACCESSIONS

**Table. 4.18.** List of NCBI accessions (relevant to the present study only are listed)

| S. No. | NCBI Accession No | Details |
|---|---|---|
| 1 | AP011329.1 | **Yang,L**., Arunachalam,M., Sado,T., Levin,B.A., Golubtsov, A.S., Freyhof,J., Friel,J.P., Chen,W.J., Hirt,M.V., Manickam, R., Agnew, M.K., Simons,A.M., Saitoh,K., Miya,M., Mayden,R.L. and He,S. Molecular phylogeny of the cyprinid tribe Labeonini (Teleostei: Cypriniformes) Mol. Phylogenet. Evol. 65 (2), 362-379 (2012) |
| 2 | AP011198.1 | **Saitoh,K**., Sado,T., Doosey,M.H., Bart,H.L. Jr., Inoue,J.G., Nishida,M., Mayden,R.L. and Miya,M. Evidence from mitochondrial genomics supports the lower Mesozoic of South Asia as the time and place of basal divergence of cypriniform fishes (Actinopterygii: Ostariophysi). Zool. J. Linn. Soc. 161, 633-662 (2011) |
| 3 | AP013338.1 | **Miya,M**. whole mitogenome of fishes. Unpublished |
| 4 | AP011384.1 | **Miya,M**. Whole mitochondrial genome sequences in Cypriniformes. Unpublished |
| 5 | AP011328.1 | **Miya,M**. Whole mitochondrial genome sequences in Cypriniformes. Unpublished |
| 6 | KP025676.1 | **Sahoo,L.,** Swain,S.K., Bej,D., Das,S.P., Das,P.C., Jayasankar,P. and Das,P. Complete mitochondrial DNA sequence of *Labeo fimbriatus* Unpublished |
| 7 | KT001152.1 | **Behera,B.K**., Kumari,K., Meena,D.K., Panda,D., Das,P., Bhakta,D., Pakrashi,S., Rout,A.K. and Sharma,A.P. Direct Submission. Submitted (30-MAY-2015) Central Inland Fisheries Research Institute, Barrackpore, Kolkata, West Bengal 700120, India |
| 8 | NC_029451 | **Miya,M**. and Sado,T. Direct Submission. Submitted (04-SEP-2013) Natural History Museum & Institute, Chiba, Department of Zoology; 955-2 Aoba-cho, Chuo-ku, Chiba, Chiba 260-8682, Japan |
| 9 | KR185963.1 | **Sahoo,L.,** Das,S.P., Patnaik,S., Bit,A., Meher, P.K., Jayasankar,P., Jena,J.K., Nagpure,N.S., Joshi,C.G., Kumar,D. and Das,P. NGS approach for resolving complete mitochondrial genome sequence of *Labeo rohita*. Unpublished |
| 10 | AP012143.1 | **Yang,L**., Arunachalam,M., Sado,T., Levin,B.A., Golubtsov,A.S., Freyhof,J., Friel,J.P., Chen,W.J., Hirt,M.V., Manickam,R., Agnew,M.K., Simons,A.M., Saitoh,K., Miya,M., Mayden,R.L. and He,S. Molecular phylogeny of the cyprinid tribe Labeonini (Teleostei: Cypriniformes) Mol. Phylogenet. Evol. 65 (2), 362-379 (2012) |
| 11 | JQ231113.1 | **Ravinder,K**. and Majumdar,K.C. The complete nucleotide sequence and gene organization of Labeo calbasu mitochondrial genome. Unpublished |

| | | |
|---|---|---|
| 12 | AB238969.1 | **Saitoh,K**.,Sado,T.,Mayden,R.L.,Hanzawa,N.,Nakamura,K.,Nis hida, M. and Miya, M. Mitogenomic evolution and interrelationships of the Cypriniformes (Actinopterygii: Ostariophysi): the first evidence towardresolution of higher-level relationships of the world's largest. J. Mol. Evol. 63 (6), 826-841 (2006)fresh water fish clade based on 59 whole mitogenome sequences |
| 13 | NC_008658.1 | **Saitoh,K**.,Sado,T.,Mayden,R.L.,Hanzawa,N.,Nakamura,K., Nishida, M. and Miya,M. Mitogenomic Evolution and Interrelationships of the Cypriniformes(Actinopterygii: Ostariophysi): The First Evidence Toward Resolution of Higher-Level Relationships of the World's LargestFreshwater Fish Clade Based on 59 Whole Mitogenome Sequences. J. Mol. Evol. 63 (6), 826-841 (2006) |
| 14 | NC_027498.1 | **Kumar,R.,** Goel,C., Kumari Sahoo,P., Singh,A.K. and Barat,A. Complete mitochondrial genome organization of *Tor tor* (Hamilton, 1822. Mitochondrial DNA, 1-2 (2015) In press |
| 15 | AME17836.1 | **Huang,Q.** and Wang,Z. The complete mitochondrial genome of Songpu mirror carp, Cyprinus carpio. Unpublished |
| 16 | YP_006073333.1 | **Ravinder,K.** and Majumdar,K.C. The complete nucleotide sequence and gene organization of Cirrhinus mrigala mitochondrial genome. Unpublished |
| 17 | AFJ21668.1 | **Bej,D.,**Sahoo,L.,Das,S.P.,Swain,S.,Jayasankar,P., Das,P.C.,Routray, P.,Swain,S.K., Jena,J.K. and Das,P. Complete mitochondrial genome sequence of Catla catla and its phylogenetic consideration. Mol. Biol. Rep. 39 (12), 10347-10354 (2012) |