CHAPTER-4:

RESULTS

4.1 Morphology based identification of the ornamental fishes

In this study a total of 27 specimens of ornamental fishes were collected from various locations of the state of Manipur, India. The morphological characters of these specimens were observed carefully and compared with described characters as mentioned in the leading taxonomic guide books, "The freshwater fishes of the Indian region" by KC Jayaram (1999) and "Inland fishes of India and adjacent countries" by PK Talwar and AG Jhingran (1991). Based on this study, following the standard family level taxonomic keys, the specimens were categorized under 9 families. The important aspects regarding identification of the studied specimens under each family is explained below.

4.1.1 Cyprinidae

A total of 11 different specimens (among our studied fishes) fall under this family. The specimens represented by sample code SGBK-FO2B and SGBK-OF4 were identified as *Puntius sophore*. *Puntius sophore* was first described as Cyprinus sophore by Hamilton in 1822. They are easily identifiable by the presence of 12 dorsal soft rays and 8 anal soft rays and the presence of distinct reddish line in the mid body length.

The specimen being coded by the sample ID SGBK-AUFO5 was identified as *Puntius chola*. Its morphological characters perfectly match with the one described by Talwar and Jhingran (Talwar and Jhingran, 1991). It can be noted that presence of deep body depth and no black dots or stripes are very helpful to diagnose this species from other congeners.

The specimen represented by the sample code SGBK-BF8 was identified as *Barbonymus gonionotus*. *Barbonymus gonionotus* is easily distinguishable from other congeners from the view that it is largest and its bright silver coloration in addition to body depth.

The specimens being represented by the sample codes SGBK-DF1, SGBK-DF2, SGBK-DF3 and SGBK-AUJF39 were identified as *Labeo bata*, *Labeo calbasu*, *Labeo gonius* and *Labeo boga* respectively. All these fishes have the identification key for the genus Labeo i.e., they have spindle shaped body, their mouths look very different, have a pronounced rostral cap, which covers the upper lip except when feeding. In case of *Labeo bata*, the body is elongate, its dorsal profile is more convex than the ventral. The snout slightly projects beyond the mouth, often studded with pores (Talwar and Jhingran, 1991). It is listed as thratened species by IUCN Bangladesh (2000). *Labeo calbasu* has dark blackish coloration and has a smaller inferior mouth surrounded by fleshy lips and the number of soft dorsal rays is 16 (Talwar and Jhingran, 1991). Presence of uniform and smaller scales can be used as a diagnostic character of *Labeo gonius* from its sister species. During fingerling stage, *Labeo bata* and *Labeo boga* have similar characteristics. A black spot is present at the caudal peduncle near the base of the caudal fin in case of *Labeo boga* .

The specimen represented by the sample code SGBK-AUFO35 was identified as *Cirrhinus mrigala*. The body is plain greyish in colour. Dorsal spines are absent. And the specimens being represented by the sample codes SGJB-AUECO1 and SGJB-AUECO2 were identified as *Rasbora daniconius* and *Esomus danricus*. *Rasbora daniconius* is identified easily by the presence of black stripe from eye to caudal fin, nearly complete lateral line with only last few scales lacking pores and slender body. There are 9 total dorsal softrays and 7 anal softrays. In case of *Esomus danricus,* the observed characteristics are all in agreement with those described by Talwar and Jhingran, 1991.

4.1.2 Siluridae

The specimens represented by the sample codes SGBK-AUFO37 and SGBK-AUFO7 were identified as *Wallago attu*. It is easily distinguishable from other congeners for having the characters like, broad head, snout depressed, body elongate and strongly compressed. The main distinguishing character is the presence of deeply cleft mouth with its corner reaching far behind eyes. Teeth in jaws are set in wide bands, Two pairs of barbels are present, maxillary barbels extending to anterior margin posterior of anal fin, mandibulary barbels to angle of mouth. Dorsal fin is small and anal fin is very long.

4.1.3 Sisoridae

The specimen represented by the sample code SGBK-AUFO27 was identified as *Sisor rabdophorus*. It can be easily distinguished by its long, thin body with numerous small bony plates and long filamentous extension on the caudal fin.

4.1.4 Bagridae

The specimen being represented by the sample code SGBK-FO3C was identified as *Mystus vittatus*. The observed characters like slightly compressed and elongate body, maxillary barbels extending beyond the pelvic fins and a narrow dusky spot often present on the shoulder etc. clearly matched with the described characters.

Labeo bata

Labeo gonius

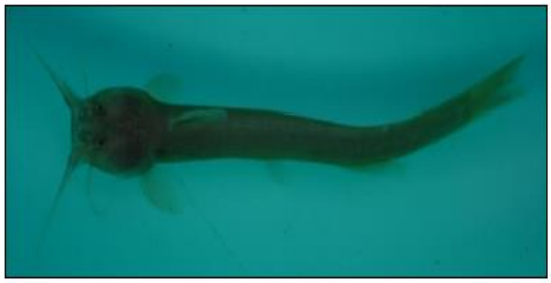
Labeo calbasu

Puntius sophore

Puntius chola

Cirrhinus mrigala

Plate 1. Specimens of the ornamental fish species- *Labeo bata, Labeo gonius, Labeo calbasu, Puntius sophore, Puntius chola, Cirrhinus mrigala.*



Amblyceps mangois

Rasbora daniconius

Channa orientalis

Channa punctatus

Badis badis

Esomus danricus

Plate 2. Specimens of the ornamental fish species- *Amblyceps mangois, Rasbora daniconius, Channa orientalis, Channa punctatus, Badis badis, Esomus danricus.*

4.1.5 Gobiidae

The fish being coded by the sample ID SGBK-OF16F was identified as *Glossogobius giuris*. Its characteristic features like flattened head, projecting lower jaw, pale body etc., help to diagnose this species.

4.1.6 Badidae

The specimen being represented by the code SGBK-OF29F was identified as *Badis badis*. This species can be distinguished easily from other groups of the genus only by the presence of cleithral spots and unique body pattern of light and dark striping.

4.1.7 Amblycipitidae

The specimen being represented by the sample code SGBK-OF31F was identified as *Amblyceps mangois*. It is easily distinguishable from its congeners in having a relatively short body and a caudal fin with upper and lower lobes of distinctly different shapes. Other observed characters also match with the described characters.

4.1.8 Nandidae

The specimen being represented by the sample code SGBK-BF6 was identified as *Nandus nandus* and the observed chracters match well with the described characters as mentioned by Talwar and Jhingran (1991).

4.1.9 Cobitidae

The specimens being represented by the sample codes SGBK-AUFO12, SGBK-AUFO17, SGBK-OF19F and SGBK-OF26F were identified as belonging to the genus *Lepidocephalus berdmorei*. It was thought previously as Lepidocephalus guntea but was finally identified as *Lepidocephalus berdmorei*. Further studies are detailed under discussion.

Barbonymus gonionotus

Mystus vittatus

Wallago attu

Sisor rabdophorus

Glossogobius giuris

Channa striata

Plate 3. Specimens of the ornamental fish species- *Barbonymus gonionotus, Mystus vittatus, Wallagu attu, Sisor rabdophorus, Glossogobius giuris, Channa striata.*

4.1.10 Channidae

The specimens being represented by the sample codes SGJB-FO1A and SGJB-BF2 were identified as *Channa punctatus*. The samples with sample code SGJB-BF1 was identified as *Channa striata* and the specimen being represented by the sample code SGJB-BF3 was identified as *Channa orientalis*. All the observed characters matched well with those described by Talwar and Jhingran, 1991.

The plates 1, 2 and 3 shows the whole specimen images of the ornamental fishes *Labeo bata, Labeo gonius, Labeo calbasu, Puntius sophore, Puntius chola, Cirrhinus mrigala, Amblyceps mangois, Rasbora daniconius, Channa orientalis, Channa punctatus, Badis badis, Esomus danricus, Barbonymus gonionotus, Mystus vittatus, Wallagu attu, Sisor rabdophorus, Glossogobisus giuris, Channa sriata respectively.*

Table 2. List of ornamental fish species collected with their local names in Manipuri and location (by GPS) from where they were collected. There are total 21 species which are being used in aquariums but only 17 species are classified as ornamental fishes but the remaining 4 species also have the potential to be used as ornamental fishes.

Order	Sl. No.	Species	Common Name in Manipuri	IUCN status	Ornamental relevance	Habitat	Location (generated through GPS)
Cypriniformes							
	1	*Cirhinus mrigala*	*Mirga*	LRnt	Classified	Jiri river	*24° 48′ 28.49″ N, 93° 06′ 38.99″ E*
	2	*Barbonymus gonionotus*	*Phabouachouba*	LC	Unclassified	Jiri river	*24° 48′ 0″ N, 93° 7′ 12″ E*
	3	*Labeo bata*	*Ngaton*	LRnt	Classified	Jiri river	*24° 48′ 0″ N, 93° 7′ 12″ E*
	4	*Glossogobius giuris*	*Ngamusengum*	VU	Classified	Jiri river	*24° 48′ 28.49″ N, 93° 06′ 38.99″ E*
	5	*Esomus danricus*	*Ngasang*	LRlc	Classified	Sana khong	*24° 48′ 09.64″ N, 93° 06′ 39.77″ E*
	6	*Rasbora daniconius*	*Ngasangmacha*	LC	Classified	Sana khong	*24° 48′ 09.64″ N, 93° 06′ 39.77″ E*
	7	*Labeo calbasu*	*Ngathi*	LRnt	Classified	Jiri river	*24° 48′ 0″ N, 93° 7′ 12″ E*
	8	*Labeo gonius*	*Khabak*	LC	Classified	Jiri river	*24° 48′ 26.18″ N, 93°07′ 20.70″ E*
	9	*Puntius chola*	*Phabou*	LC	Unclassified	Nambol	*24° 70′ 34.1″ N, 93° 82.9′ 88.6″ E*
	10	*Puntius sophore*	*Phabou*	LC	Unclassified	Nambol	*24° 70′ 34.1″ N, 93° 82.9′ 88.6″ E*
	11	*Lepidocephalus sp.*	*Ngakijou*	LC	Classified	Bishnupur	*24° 62′ 67.77″ N, 93° 76.4′ 98.8″ E*
	12	*Labeo boga*	*Ngaton*	LC	Unclassified	Jiri river	*24° 48′ 0″ N, 93° 7′ 12″ E*
Siluriformes							
	13	*Wallago attu*	*Sareng*	LRnt	Classified	Sanakhong	*24° 48′ 09.64″ N, 93° 06′ 39.77″ E*
	14	*Sisor rabdophorus*	*Hangoinga*	EN	Classified	Irang river	*24° 81′ 69.35″ N, 93° 50′ 77.91″ E*
	15	*Amblyceps mangois*	*Ngachikmacha*	LC	Classified	Jiri river	*24° 48′ 28.49″ N, 93° 06′ 38.99″ E*
	16	*Mystus vittatus*	*Ngasep*	LC	Classified	Jiri river	*24° 48′ 28.49″ N, 93° 06′ 38.99″ E*
Perciformes							
	17	*Channa striata*	*Porom*	LRnt	Classified	Sanakhong	*24° 48′ 0″ N, 93° 7′ 12″ E*
	18	*Channa punctata*	*Ngamu*	LRnt	Classified	Sanakhong	*24° 48′ 0″ N, 93° 7′ 12″ E*
	19	*Channa orientalis*	*Meitei ngamu*	VU	Classified	Sanakhong	*24° 48′ 0″ N, 93° 7′ 12″ E*
	20	*Badis badis*	*Napetnga*	VU	Classified	Jiri river	*24° 48′ 28.49″ N, 93° 06′ 38.99″ E*
	21	*Nandus nandus*	*Kharaobi*	LC	Classified	Jiri river	*24° 48′ 26.18″ N, 93°07′ 20.70″ E*

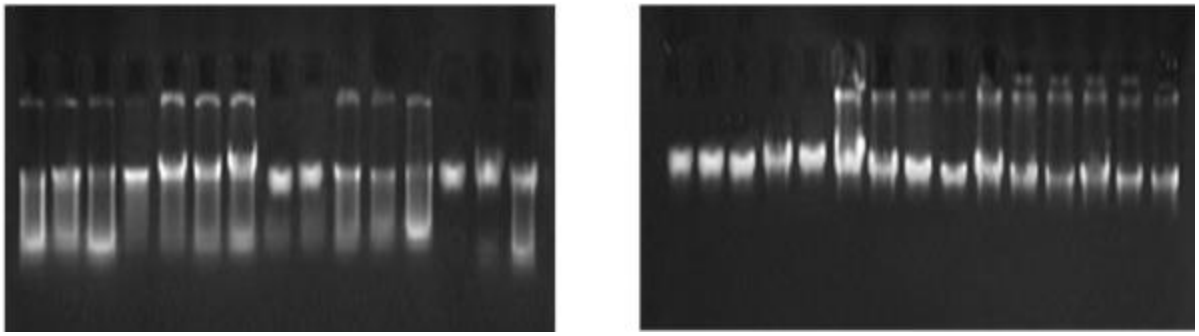
4.2 Molecular identification and characterization of the different species of ornamental fishes found in Manipur

4.2.1 Genomic DNA isolation from blood and tissue

The genomic DNA were extracted from all the 27 samples and the quality of the DNA was checked and visualized on agarose gel (figure 3.). The DNA extracted from most of the samples were greater than 10 kb which indicates that they were of high molecular weight. The purity checking in Spectrophotometer reveals that all the extracted genomic DNA were in the range of 50 – 250 ng/µl and revealed purity in the range of 1.35 – 1.60 in terms ratio of the absorbance at 260/280 nm.

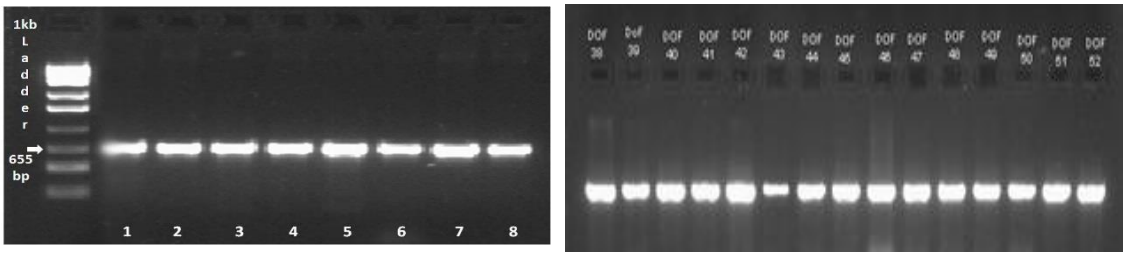
4.2.2 PCR amplification of COI DNA barcode and purification

The primer pair mentioned in methodology for amplification of full-length (655 bp) barcode led successful PCR amplification in all the cases. After purification, none of the PCR amplicons got degraded and therefore no smearing observed. Also there were no traces of primers on the purified PCR products that are essential for getting good sequencing result. The amplified PCR products and gel purified PCR products were shown in Figure 4.



(a) (b)

Figure 4. Few representatives of genomic DNA extracted pictures from blood and tissue samples. (a) tissue samples and (b) blood samples.

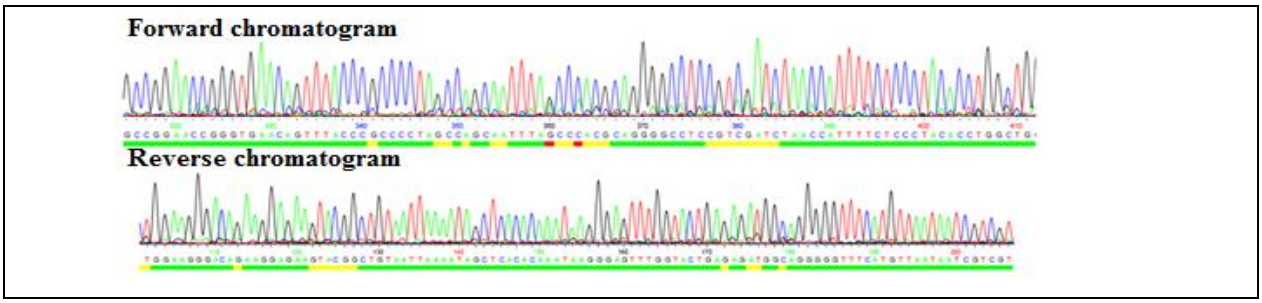


(a) **(b)**

Figure 5. COI DNA barcode PCR amplicons of ornamental fish samples (both **a** and **b**). The amplicons were of 655 bp length and was confirmed by comparision with standard 1 Kb marker by running on 1.5% agarose gel.

4.2.3 Final sequences

We received the sequencing results in the form of two chromatograms for each sample; one for the forward and other for the reverse. The software SeqScanner outputted the chromatograms in the form of original sequence (i.e. in ATGC format). As both the forward and reverse sequence represented the same gene location of the same sample, the reverse sequence being transformed in reverse complement form and match with the forward sequence revealed 100% alignment with any gap or indels. This 100% aligned sequence was considered the final sequence and its translation and protein BLAST result revealed 100% homology with partial amino acid array of mitochondrial COI gene. This result confirmed that the sequences are correct. Figure 5 was provided as an example of raw sequence annotation of the sample SGBK-AUFO35.



>AUFO35Forward(632nt)
TCGGCACAGCCCTTAGCTTACTTATTCGGGCAGAATTAGCACAACCTGGTGCTCTACTGGGTGATGACCAAATTTATAACG
TTATTGTTACTGCTCACGCATTCGTAATAATTTTCTTTATAGTAATACCAATTATGATTGGAGGCTTCGGAAACTGACTAGT
GCCCCTAATAATTGGAGCCCCTGACATAGCATTTCCACGTATGAATAACATAAGCTTCTGACTACTTCCACCATCCTTCCT
ACTACTGCTTGCATCTTCTGGAGTTGAAGCGGGAGCAGGAACAGGATGAACAGTATATCCGCCTCTTGCTGGGAATCTTG
CACATGCTGGAGCCTCAGTAGATTTAACCATTTTCTCCCTACACTTAGCAGGTGTCTCATCTATTCTAGCATCTATTAATTT
TATTACTACTATTATTAACATGAAACCCCCAGCCATCTCACAATATCAAACACCACTATTTGTTTGATCAGTGTTAATTAC
AGCCGTACTATTACTACTTTCCCTACCTGTACTAGCCGCTGGGATTACCATACTACTAACTGACCGAAATCTAAACACCAC
ATTCTTTGACCCCGCAGGGGGCGGAGACCCCATTCTCTACCAACATCTCTTCTGATTCTTTGGC

>AUFO35 reverse (658nt)
GGGTCTCCGCCCCCTGCGGGGTCAAAGAATGTGGTGTTTAGATTTCGGTCAGTTAGTAGTATGGTAATCCCAGCGGCTAG
TACAGGTAGGGAAAGTAGTAATAGTACGGCTGTAATTAACACTGATCAAACAAATAGTGGTGTTTGATATTGTGAGATGG
CTGGGGGTTTCATGTTAATAATAGTAGTAATAAAATTAATAGATGCTAGAATAGATGAGACACCTGCTAAGTGTAGGGAG
AAAATGGTTAAATCTACTGAGGCTCCAGCATGTGCAAGATTCCCAGCAAGAGGCGGATATACTGTTCATCCTGTTCCTGC
TCCCGCTTCAACTCCAGAAGATGCAAGCAGTAGTAGGAAGGATGGTGGAAGTAGTCAGAAGCTTATGTTATTCATACGTG
GAAATGCTATGTCAGGGGCTCCAATTATTAGGGGCACTAGTCAGTTTCCGAAGCCTCCAATCATAATTGGTATTACTATAA
AGAAAATTATTACGAATGCGTGAGCAGTAACAATAACGTTATAAATTTGGTCATCACCCAGTAGAGCACCAGGTTGTGCT
AATTCTGCCCGAATAAGTAAGCTAAGGGCTGTGCCGACTATTCCGGCTCAGGCACCAAATACCAGGTAGAGGGTGCCAAT
GTCTTTGTGGTTGGTTG

>AUFO35 (658nt) reverse complement
CAACCAACCACAAAGACATTGGCACCCTCTACCTGGTATTTGGTGCCTGAGCCGGAATAGTCGGCACAGCCCTTAGCTTA
CTTATTCGGGCAGAATTAGCACAACCTGGTGCTCTACTGGGTGATGACCAAATTTATAACGTTATTGTTACTGCTCACGCA
TTCGTAATAATTTTCTTTATAGTAATACCAATTATGATTGGAGGCTTCGGAAACTGACTAGTGCCCCTAATAATTGGAGCC
CCTGACATAGCATTTCCACGTATGAATAACATAAGCTTCTGACTACTTCCACCATCCTTCCTACTACTGCTTGCATCTTCTG
GAGTTGAAGCGGGAGCAGGAACAGGATGAACAGTATATCCGCCTCTTGCTGGGAATCTTGCACATGCTGGAGCCTCAGTA
GATTTAACCATTTTCTCCCTACACTTAGCAGGTGTCTCATCTATTCTAGCATCTATTAATTTTATTACTACTATTATTAACAT
GAAACCCCCAGCCATCTCACAATATCAAACACCACTATTTGTTTGATCAGTGTTAATTACAGCCGTACTATTACTACTTTC
CCTACCTGTACTAGCCGCTGGGATTACCATACTACTAACTGACCGAAATCTAAACACCACATTCTTTGACCCCGCAGGGG
GCGGAGACCC

The final annotated sequence
>AUFO35 (655nt)
CCTCTACCTGGTATTTGGTGCCTGAGCCGGAATAGTCGGCACAGCCCTTAGCTTACTTATTCGGGCAGAATTAGCACAACC
TGGTGCTCTACTGGGTGATGACCAAATTTATAACGTTATTGTTACTGCTCACGCATTCGTAATAATTTTCTTTATAGTAATA
CCAATTATGATTGGAGGCTTCGGAAACTGACTAGTGCCCCTAATAATTGGAGCCCCTGACATAGCATTTCCACGTATGAA
TAACATAAGCTTCTGACTACTTCCACCATCCTTCCTACTACTGCTTGCATCTTCTGGAGTTGAAGCGGGAGCAGGAACAGG
ATGAACAGTATATCCGCCTCTTGCTGGGAATCTTGCACATGCTGGAGCCTCAGTAGATTTAACCATTTTCTCCCTACACTT
AGCAGGTGTCTCATCTATTCTAGCATCTATTAATTTTATTACTACTATTATTAACATGAAACCCCCAGCCATCTCACAATAT
CAAACACCACTATTTGTTTGATCAGTGTTAATTACAGCCGTACTATTACTACTTTCCCTACCTGTACTAGCCGCTGGGATTA
CCATACTACTAACTGACCGAAATCTAAACACCACATTCTTTGACCCCGCAGGGGGCGGAGACCCCATTCTCTACCAACAT
CTCTTC

>lcl|Sequence 1 ORF:32..655 Frame +2
LYLVFGAWAGMVGTALSLLIRAELAQPGALLGDDQIYNVIVTAHAFVMIFFMVMPIMIGGFGNWLVPLMIGAPDMAFPRMNN
MSFWLLPPSFLLLLASSGVEAGAGTGWTVYPPLAGNLAHAGASVDLTIFSLHLAGVSSILASINFITTIINMKPPAISQYQTPLFV
WSVLITAVLLLLSLPVLAAGITMLLTDRNLNTTFFDPAGGGDPILYQHLF

Figure 6. Example of sequence editing. Sequences are shown as raw datas in the form of peaks. The red, blue, black and green peaks represents Thymine, Cytosine, Guanine and Adenine respectively.

4.2.4 Dataset characteristics

All the sequences were larger than 600 bp which assured that no NUMTs were amplified since the limit of NUMT hardly reaches 600 bp. After noisy ends of the sequences were trimmed, most of the sequences yielded 600 bp products except some sequences. All the sequences aligned with ClustalX showed no indels. The sequences when translated revealed coherent partial amino acid codes with fish mitochondrial COI gene frame without any stop codon. So, it was confirmed that the generated sequences were fragments of mitochondrial COI gene.

4.2.5 Submitted sequences in GenBank and BOLD

The final annotated sequences submitted in GenBank by the name as identified through morphological identification mentioned in chapter 4.1 of Result were found correct and assigned accession numbers (Table 4.). The sequences were also deposited in BOLD database under the project Genbank Fish with the code ANGBF and got the sequence IDs, ANGBF2206-12, ANGBF2207-12, ANGBF2208-12, ANGBF2209-12, ANGBF2210-12, ANGBF2211-12, ANGBF2212-12, ANGBF2213-12, ANGBF2214-12, ANGBF2215-12, ANGBF2216-12, ANGBF2218-12, ANGBF2219-12, ANGBF2220-12, ANGBF2221-12 and ANGBF2222-12.

4.2.6 DNA barcode based characterization of the ornamental fishes of Manipur

4.2.6.1 Species identification based on similarity match with database

Table 5 shows the comprehensive barcoding identification results based on the two databases, GenBank and BOLD. Both databases revealed definitive identity matches in the range of 98.37%-100% for consensus sequences of twelve species. These are *Puntius sophore, Barbonymus gonionotus, Labeo bata, Labeo calbasu, Labe ogonius, Wallago attu, Labeo boga, Sisor rabdophorus, Mystus vittatus, Amblyceps mangois, Cirrhinus mrigala and Channa punctata.* During the species identification search using the best 2 databases i.e., GenBank and BOLD, only 12 species were perfectly matched (98.37%-100%) in both GenBank and BOLD. AUFO5 (*Puntius chola*) has 99.38% homology with *Puntius chola* in

BOLD but has 95% homology with *Puntius brevis* in GenBank. So we consider our sample as *Puntius chola*. OF19F which matched 93% with *Lepidocephalus guntea* in GenBank was unable to match with any records in BOLD. Likewise another 2 samples, OF29F and AUECO2 which matched 98% with *Badis badis* and 100% with *Esomus danricus* in NCBI (GenBank) respectively were unable to match any records in BOLD. This signifies that a lot of data were still needed to be submitted in BOLD.

Table 3. Accession number of the sequences submitted in GenBank along with their sample codes and species name.

SL. No.	Sample code	Species	Accession no.
1	SGBK-FO2B	*Puntius sophore*	**JQ713844**
2	SGBK-BF6	*Nandus nandus*	**JQ713845**
3	SGBK-BF8	*Barbonymus gonionotus*	**JQ713846**
4	SGBK-DF1	*Labeo bata*	**JQ713847**
5	SGBK-DF2	*Labeo calbasu*	**JQ713848**
6	SGBK-DF3	*Labeo gonius*	**JQ713849**
7	SGBK-AUFO37	*Wallago attu*	**JQ713850**
8	SGBK-AUJF39	*Labeo boga*	**JQ713851**
9	SGBK-AUFO5	*Puntius chola*	**JQ713852**
10	SGBK-AUFO7	*Wallago attu*	**JQ713853**
11	SGBK-AUFO27	*Sisor rabdophorus*	**JQ713854**
12	SGBK-FO3C	*Mystus vittatus*	**JQ713855**
13	SGBK-OF4	*Puntius sophore*	**JQ713856**
14	SGBK-OF16F	*Glossogobius giuris*	**JQ713857**
15	SGBK-OF19F	*Lepidocephalus sp.*	*****
16	SGBK-OF26F	*Lepidocephalus sp.*	*****
17	SGBK-OF29F	*Badis badis*	**JQ713858**
18	SGBK-OF31F	*Amblyceps mangois*	**JQ713859**
19	SGBK-AUFO17	*Lepidocephalus sp.*	*****

20	SGBK-AUFO12	*Lepidocephalus sp.*	*
21	SGBK-AUFO35	*Cirrhinus mrigala*	**JQ713860**
22	SGJB-BF1	*Channa striata*	*
23	SGJB-BF2	*Channa punctata*	*
24	SGJB-BF3	*Channa orientalis*	*
25	SGJB-FO1A	*Channa punctata*	*
26	SGJB-AUECO1	*Rasbora daniconius*	*
27	SGJB-AUECO2	*Esomus danricus*	*

***-** to be submitted

Table 4. Sample identification results (homology comparison), based on GenBank and BOLD. Three samples (SGBK-OF19F, SGBK-OF29F and SGJB-AUECO2) were unable to match with any species in BOLD.

Sample code	**Accession no.**	**BOLD**		**GenBank**	
		%	**Species**	**%**	**Species**
SGBK-FO2B	**JQ713844**	100%	*Puntius sophore*	99%	*Puntius sophore*
SGBK-BF6	**JQ713845**	99.33%	*Nandus nandus*	87%	*Polycentropis abbreviate*
SGBK-BF8	**JQ713846**	100%	*Barbonymus gonionotus*	100%	*Barbonymus gonionotus*
SGBK-DF1	**JQ713847**	100%	*Labeo bata*	100%	*Labeo bata*
SGBK-DF2	**JQ713848**	100%	*Labeo calbasu*	100%	*Labeo calbasu*
SGBK-DF3	**JQ713849**	99.13%	*Labeo gonius*	99%	*Labeo gonius*
SGBK-AUFO37	**JQ713850**	100%	*Wallago attu*	100%	*Wallago attu*
SGBK-AUJF39	**JQ713851**	100%	*Labeo boga*	99%	*Labeo bata*
SGBK-AUFO5	**JQ713852**	99.38%	*Puntius chola*	95%	*Puntius brevis*
SGBK-AUFO7	**JQ713853**	100%	*Wallago attu*	100%	*Wallago attu*

SGBK-AUFO27	**JQ713854**	99.84%	*Sisor rabdophorus*	100%	*Sisor rabdophorus*
SGBK-FO3C	**JQ713855**	99.66%	*Mystus vittatus*	99%	*Mystus vittatus*
SGBK-OF4	**JQ713856**	100%	*Puntius sophore*	99%	*Puntius sophore*
SGBK-OF16F	**JQ713857**	99.17%	*Glossogobius giuris*	93%	*Parapercis ommatura*
SGBK-OF19F	-		No match	93%	*Lepidocephalus guntea*
SGBK-OF26F	-	83.87%	*Lepidocephalus guntea*	93%	*Lepidocephalus guntea*
SGBK-OF29F	**JQ713858**		No match	98%	*Badis badis*
SGBK-OF31F	**JQ713859**	98.37%	*Amblyceps mangois*	99%	*Amblyceps mangois*
SGBK-AUFO17	-	83.95%	*Lepidocephalus guntea*	92%	*Lepidocephalus guntea*
SGBK-AUFO12	-	84.04%	*Lepidocephalus guntea*	93%	*Lepidocephalus guntea*
SGBK-AUFO35	**JQ713860**	99.83%	*Cirrhinus cirrhosus*	99%	*Cirrhinus mrigala*
SGJB-BF1	-	96.39%	*Channa striata*	100%	*Channa striata*
SGJB-BF2	-	99.23%	*Channa punctata*	100%	*Channa punctata*
SGJB-B3	-	97.24%	*Channa harcourtbutleri*	100%	*Channa orientalis*
SGJB-FO1A	-	99.19%	*Channa punctata*	100%	*Channa punctata*
SGJB-AUECO1	-	97.54%	*Rasbora cf. dandia*	100%	*Rasbora daniconius*
SGJB-AUECO2	-		No match	100%	*Esomus danricus*

4.3 Sequence analysis of the DNA barcode region of the ornamental fishes

4.3.1 Disparity index test of pattern homogeneity

The probability of rejecting the null hypothesis that sequences have evolved with the same pattern of substitution can be judged from the extent of differences in base composition biases between sequences. A Monte Carlo test (500 replicates) was used to estimate the *P*-values. *P*-values smaller than 0.05 are considered significant and our analysis with the developed sequences generate a *P*-value of 0.00 which is perfectly significant.

4.3.2 Genetic divergence

The figure 6 clearly depicts the overall flow of congeneric and conspecific divergence calculated from the study. The mean value of congeneric and conspecific divergence were determined to be 0.217 and 0.006 respectively i.e., 21.7% and 0.6% respectively. The divergence gap between the species is clearly visible in comparison to the divergence within the species and thereby helps in easy discrimination of the species. The congeneric divergence were spectacularly higher than the conspecific divergences except in the case of *Lepidocephalus*. Therefore taking this point in consideration, we took two more sequences of *Lepidocephalus guntea* from Genbank and analyzed separately specifically for the genus *Lepidocephalus*. The detailed study is explained under discussion.

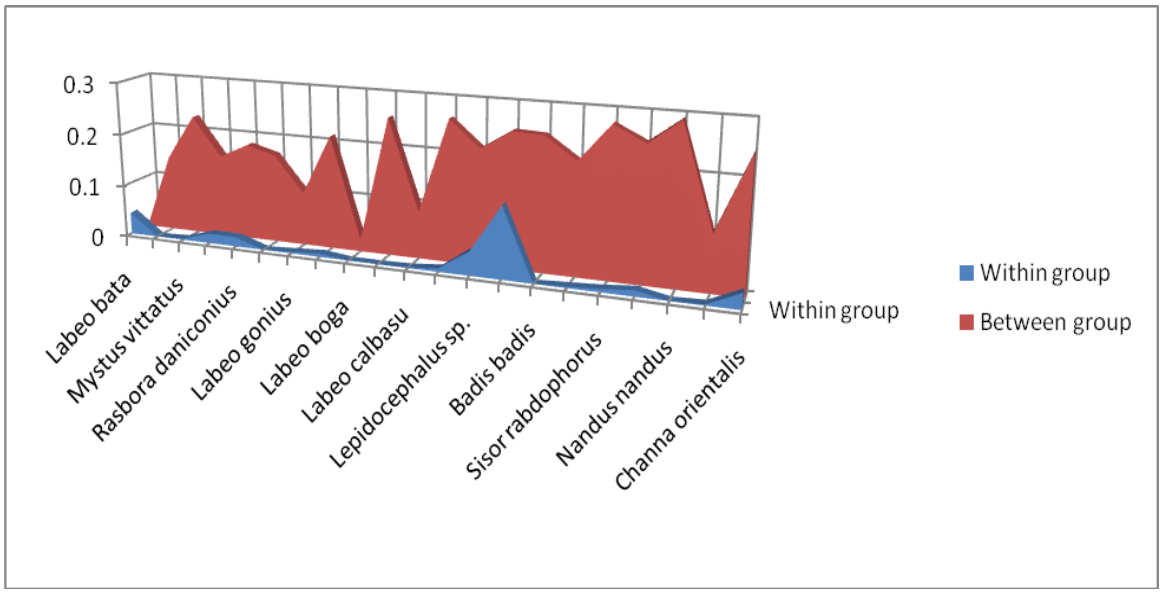


Figure 7. Chart showing genetic divergence within and between the species. It clearly shows that there is perfect gap between the divergence within species and between species which helps in easy delineation of the species.

4.3.3 Maximum Likelihood estimate of substitution matrix

For the estimation of substitution matrix by Maximum Likelihood, substitution patterns and rates were estimated under the General Time Reversible model. Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics. Each entry is the probability of substitution (r) from one base (row) to another base (column). The nucleotide frequencies are A = 25.81%, T/U = 29.78%, C = 27.29% and G = 17.12%. In our sequences the rate of transitional substitutions are far higher than that of transversional substitutions. It is highest in case of substitution of C to T/U (21.64%). Transversional substitution is highest in case of A to T/U (8.59%) and lowest in case of T/U to G with a rate of 0.86% only.

Table 5. Estimation of substitution matrix by Maximum Likelihood approach

	A	T/U	C	G
A	-	*8.59*	*7.80*	**8.77**
T/U	*7.45*	-	**19.84**	*0.86*
C	*7.38*	**21.64**	-	*1.13*
G	**13.22**	*1.50*	*1.80*	-

4.3.4 Maximum Likelihood estimate of Transition transversion bias

The estimated transition transversion bias was found to be 1.74. Substitution pattern and rates were estimated under Kimura-2-Parameter (K2P) model. The analysis involved 27 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$ +non-coding. All positions with less than 95% site coverage were eliminated.

4.3.5 Maximum Composite Likelihood estimate of the pattern of Nucleotide substitution

Each entry shows the probability of substitution (r) from one base (row) to another base (column). For simplicity, the sum of r values is made equal to 100. Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics. The nucleotide frequencies are 25.81% (A), 29.78% (T/U), 17.12% (C), and 27.29% (G). The rate of transitional substitution is highest in case of C-T (19.68%) while it is lowest in case of A-G with a rate of 7.77%. And the transversional substitution rate is highest in two cases one in case of A-T and the other in case of G-T both with a rate of 6.37%. Here also the rate of transitional substitutions are higher than that of transversional substitutions.

Table 6. Estimation of pattern of nucleotide substitution by MCL approach

	A	**T**	**C**	**G**
A	-	*6.37*	*5.84*	**7.77**
T	*5.52*	-	**18.03**	*3.66*
C	*5.52*	**19.68**	-	*3.66*
G	**11.72**	*6.37*	*5.84*	-

4.3.6 Nucleotide composition analysis

The frequencies of nucleotide composition were analyzed for the 27 sequences which were generated from 27 specimens of ornamental fishes found in Manipur, India, (figure 7, 8, 9 and 10 respectively). The overall nucleotide composition depicted in figure 4.3.2.clearly shows that in all the cases the frequency of G is lowest in comparison to the other three nucleotides i.e., T(U), C and A. In all the sequences, frequency of T(U) is high compared to the other three and highest (32.7%) in case of *Sisor rabdophorus* which is represented by the sample code SGBK-AUFO27. The frequency of T(U) is lowest (27.6) in case of SGBK-AUFO5 (*Puntius chola*). The frequency of C is highest (30.4%) in case of SGJB-FO1A (*Channa punctatus*) and lowest (24.9%) in case of SGJB-AUECO2 (*Esomus danricus*). The frequency of A is highest (28.4%) in case of SGBK-DF1 (*Labeo bata*) and lowest (22.8%) in case of SGBK-OF29F (*Badis badis*). And the frequency of G is highest (19.1%) in case of SGBK-OF16F (*Glossogobius giuris*) and lowest (15.1%) in case of SGBK-AUFO12 (*Lepidocephalus sp.).*

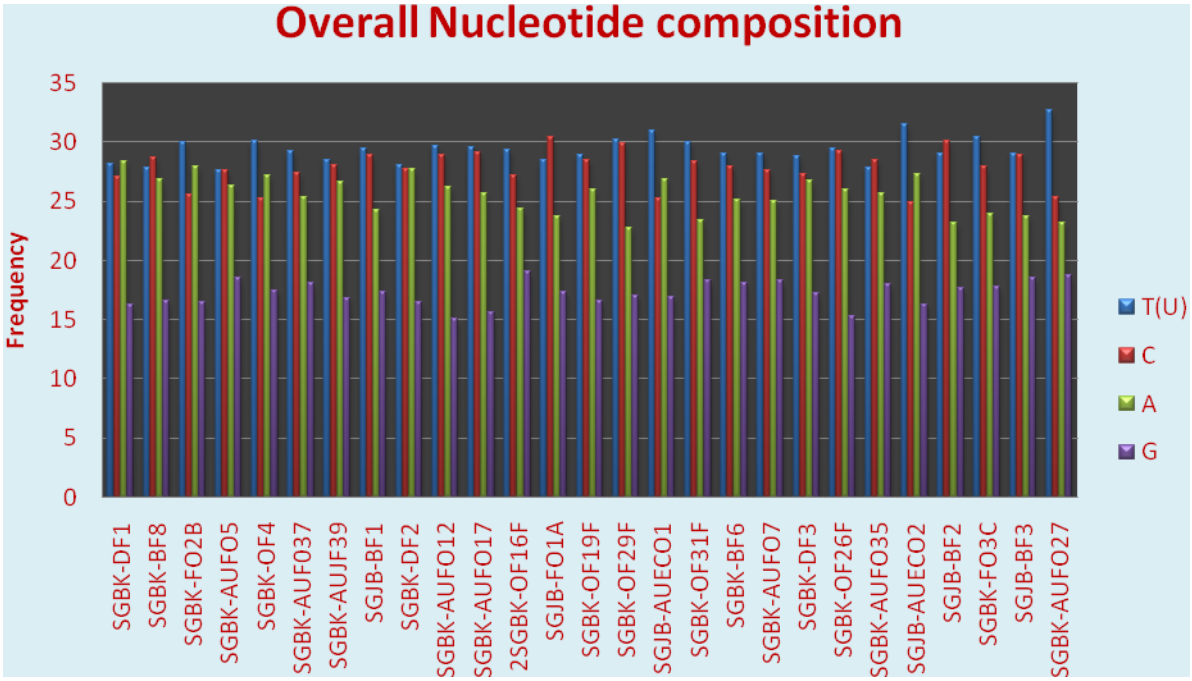


Figure 8. Overall nucleotide composition of the 27 sequences under study. SGBK-AUFO27 representing the specimen *Sisor rabdophorus* has highest frequency of T(U) than any other sequences.

Further it was observed that in 1st codon position the frequencies of the different nucleotides almost goes in uniform way (figure 8.) showing conserved nature but in 2nd and 3rd codon position, the nucleotide frequencies are variable, more variable in 2nd codon position.

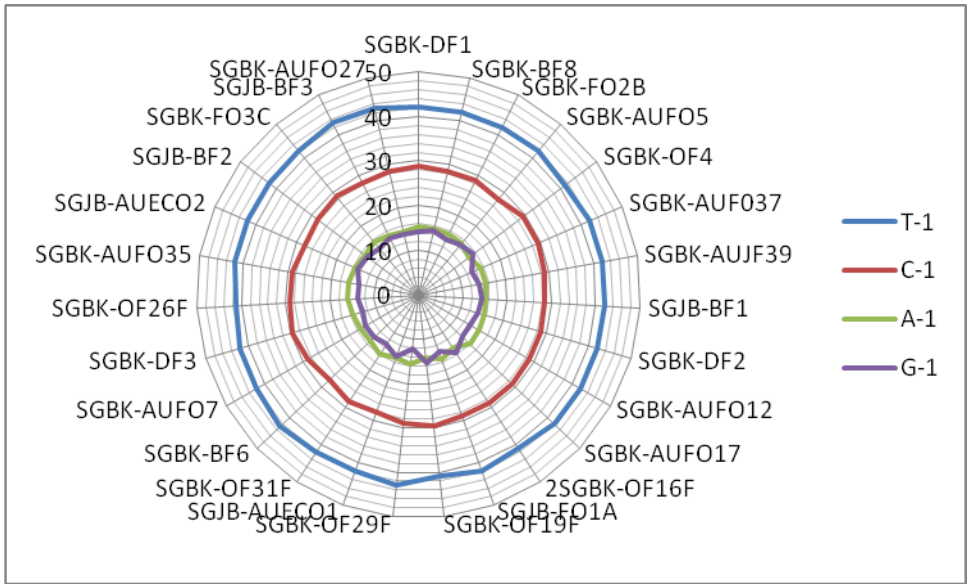


Figure 9. Nucleotide composition in 1st codon position of the 27 sequences generated

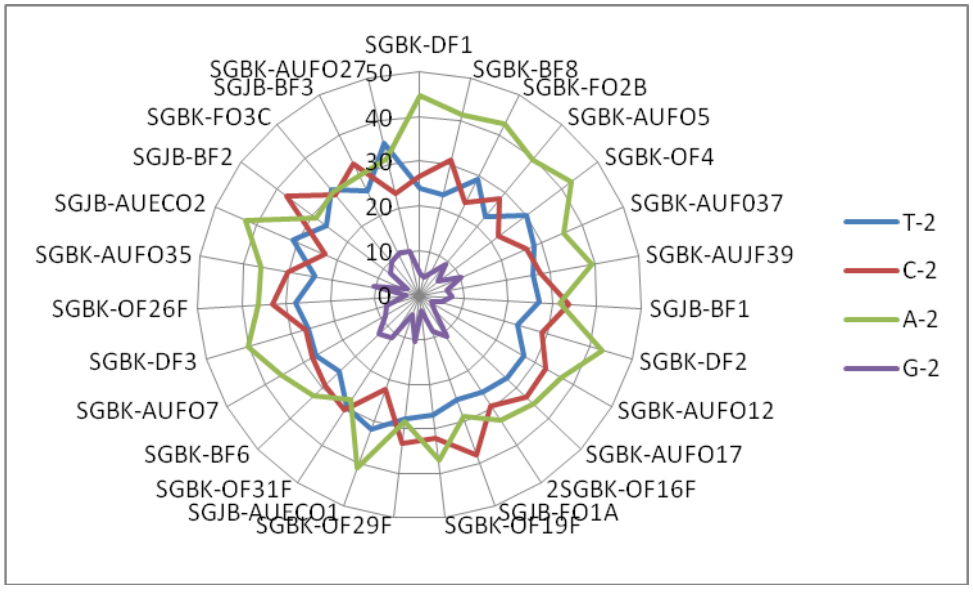


Figure 10. Nucleotide composition in 2nd codon position of the 27 sequences generated

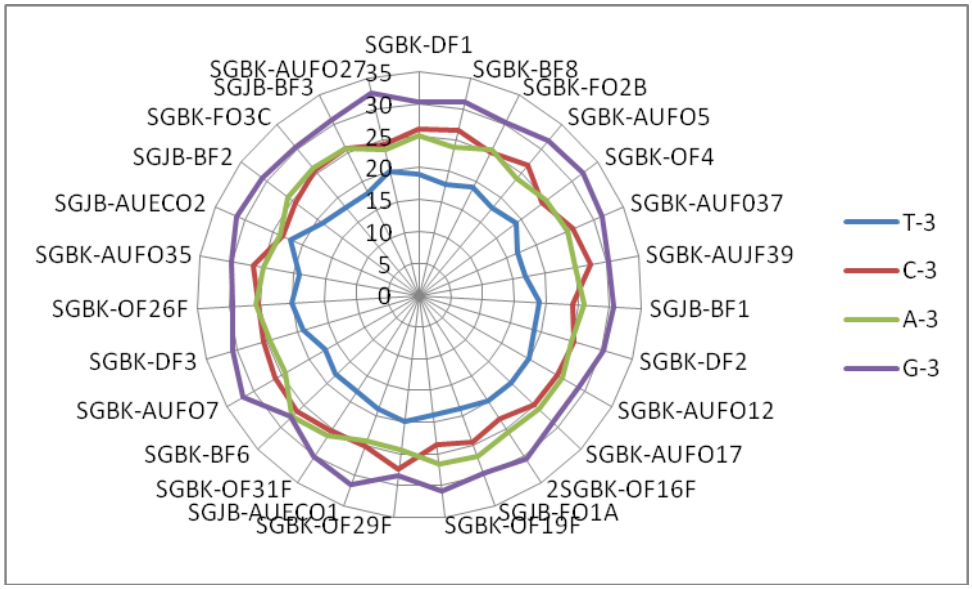


Figure 11. Nucleotide composition in 3rd codon position of the 27 sequences generated

The nucleotide composition were analyzed further for the three genus namely, *Channa, Labeo* and *Lepidocephalus*. They are explained as follows:

In the genus *Channa*, three other sequences downloaded from database were included to further explain the genus specific amino acid composition along with our four generated sequences. The frequency of G is lower compared to other nucleotides in all cases and the frequency of C is high in all the cases. As also explained by the Neighbor joining tree, nucleotide composition analysis also shows the same explanation. SGJB-BF1 representing *Channa striata* is placed in a different branch in the neighbor joining tree while SGJB-BF3 representing *Channa orientalis* clustered together with FJ459481.1 which is a partial mitochondrial COI sequence of *Channa orientalis* downloaded from database and two sequences of *Channa punctatus* clustered together with EU417795.1 and EU417796.1 which are sequences of *Channa punctatus* downloaded from database. Likewise the nucleotide composition analysis shows that SGJB-BF2, SBJB-FO1A, EU417795.1 and EU417796.1 shows similar frequency pattern of all the four nucleotides which is in agreement with what is explained by the NJ tree. Other observations are also in agreement with the NJ tree's branching pattern.

In the genus *Labeo*, unlike the genus *Channa*, the frequency of T(U) is higher than other three in all the cases which can be regarded as genus specific nucleotide composition character. But likewise in the genus *Channa*, the frequency of G is lower in all the cases with lowest frequency (15.8% only) in case of SGBK-DF1 representing *Labeo bata.*The frequencies of all the four nucleotides are more or less similar and shows conserved nature within the four groups, 1st consisting of SGBK-DF1, HM147890.1, HM147889.1, HM147888.1, HM147886.1 and HM147887.1., 2nd group (SGBK-DF3, EU417800.1, EU417801.1, EU417802.1, HQ645092.2 and HQ645094.2), 3rd group (SGBK-AUJF39, GU195094.1, GU195093.1, GU195089.1, GU195092.1, GU195095.1, GU195091.1, GU195096.1 and GU195090.1) and 4th group (SGBK-DF2). The characters are all in accordance with the neighbor joining tree.

In case of the genus *Lepidocephalus*, right from the morphological analysis, there has been some confusion whether our samples are *Lepidocephalus guntea* or other species. The nucleotide composition analysis shows that T and C is present most abundantly in all the sequences. The total nucleotide composition shows significantly that the frequency of G in *Lepidocephalus guntea* is higher than 15 but in our generated sequences which are expected to be *Lepidocephalus berdmorei* the frequency of G is less than 15 in all four cases. In *Lepidocephalus guntea* sequences the frequency of C is always higher than T but in case of *Lepidocephalus berdmorei* it is the opposite i.e., the frequency of T is higher than that of C in all cases. These findings clearly state that our generated sequence of *Lepidocephalu*s is not *guntea*.

4.3.7 Amino acid composition and Codon usage

The variation in amino acid composition of each ornamental fishes were studied. The frequency of the amino acid Leucine which is a non-polar amino acid is highest in all the fishes which is clearly visible in the figure 11. The average amino acid composition analysis among the ornamental fishes under study clearly shows that in an average the frequency of

Trp is lowest (1.11% only) while the frequency of Leu is highest (17.96%). In case of *Sisor rabdophorus* (SGBK-AUFO27), Leu is richest with a frequency of 25.97% while the frequencies of Arg and Val are lowest (0.64%). The amino acid Cys is absent in all the fishes of the genus Labeo, *Lapidocephalus*. Ala is absent in *Rasbora daniconius*, *Puntius sophore* and *Puntius chola*. Asn is absent in *Amblyceps mangois*. Arg is absent in case of *Barbonymus gonionotus*, *Rasbora daniconius* and *Esomus danricus* and *Wallago attu*. Trp is absent in case of *Wallago attu*, *Labeo gonius*, *Barbonymus gonionotus*, *Labeo boga*, *Labeo bata*, *Cirrhinus mrigala*, *Puntius sophore* and *Esomus danricus*.

The pattern of codon usage in representative ornamental fish species' DNA barcode region was studied. The nucleotide composition is biased to codon usage. Since the barcode region is rich in AT nucleotide, the amino acid is more biased to AT rich codon. Table 8 shows codon usage patterns in the ornamental fishes under study. All frequencies are averages over all taxa. Relative synonymous codon usage is given in parenthesis following the codon frequency.

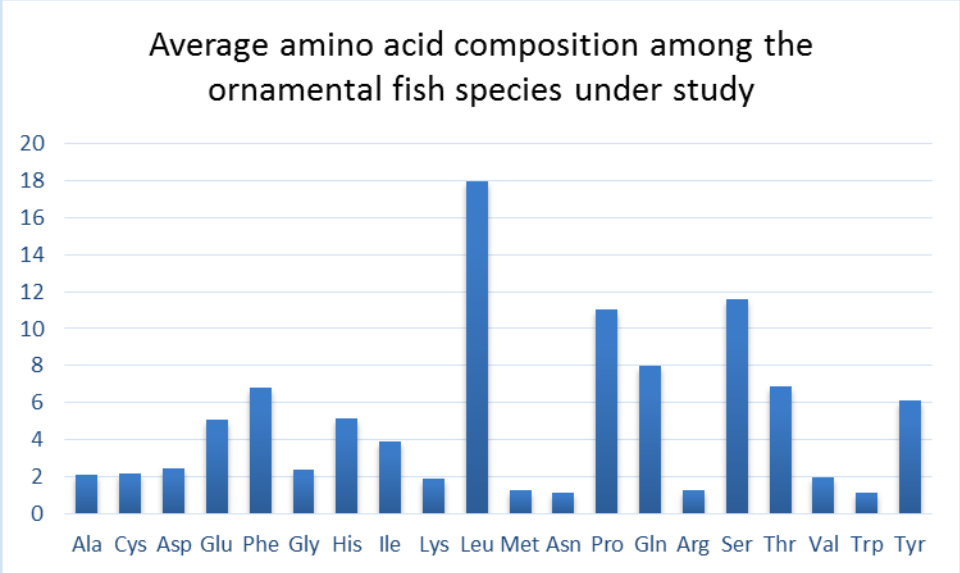


Figure 12. Chart showing average amino acid composition for the ornamental fishes under study.

Table 7. Frequency of codons in COI sequences of the ornamental fishes under study

Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU
UUU(F)	5.4	1.05	UCU(S)	6.7	2.17	UAU(Y)	3.1	0.69	UGU(C)	1.1	0.67
UUC(F)	4.8	0.95	UCC(S)	5.4	1.75	UAC(Y)	6	1.31	UGC(C)	2.1	1.33
UUA(L)	8.4	1.76	UCA(S)	2.9	0.95	UAA(*)	9.9	1.55	UGA(*)	1.8	0.28
UUG(L)	6.6	1.39	UCG(S)	3.3	1.05	UAG(*)	7.6	1.18	UGG(W)	1.7	1
CUU(L)	2.7	0.58	CCU(P)	3.5	0.79	CAU(H)	3.9	0.93	CGU(R)	0.2	0.6
CUC(L)	2.8	0.58	CCC(P)	6	1.36	CAC(H)	4.5	1.07	CGC(R)	0.7	1.8
CUA(L)	2	0.42	CCA(P)	3.1	0.71	CAA(Q)	4.5	0.69	CGA(R)	0.1	0.4
CUG(L)	6.1	1.27	CCG(P)	5	1.14	CAG(Q)	8.5	1.31	CGG(R)	0.9	2.5
AUU(I)	1.3	0.56	ACU(T)	2.2	0.72	AAU(N)	0.8	0.91	AGU(S)	0.2	0.06
AUC(I)	3	1.34	ACC(T)	4.5	1.45	AAC(N)	0.9	1.09	AGC(S)	0.1	0.02
AUA(I)	2.5	1.1	ACA(T)	3.5	1.14	AAA(K)	1.8	1.28	AGA(R)	0.2	0.6
AUG(M)	1.9	1	ACG(T)	2.1	0.68	AAG(K)	1	0.72	AGG(R)	0	0.1
GUU(V)	0.3	0.34	GCU(A)	1.1	1.3	GAU(D)	2	1.08	GGU(G)	0.4	0.42
GUC(V)	0	0	GCC(A)	0	0	GAC(D)	1.7	0.92	GGC(G)	0	0
GUA(V)	0.8	1.06	GCA(A)	0.9	1.08	GAA(E)	4.6	0.96	GGA(G)	0.8	0.77
GUG(V)	2	2.6	GCG(A)	1.3	1.62	GAG(E)	4.9	1.04	GGG(G)	3	2.82

4.4 Development of Phylogenetic relationship between the different species of ornamental fishes identified

4.4.1 Neighbor Joining tree

A neighbor-joining tree was constructed using K2P distance of the 27 developed sequences of ornamental fishes and other 46 other sequences downloaded from GenBank as shown in figure. 12. The neighbor joining tree of these sequences clearly showed that all the species of ornamental fishes were separated into distinct clusters. In most of the cases the individuals within a species clustered together confirming their species allocation. It is clearly observed that *Labeo calbasu* and *Labeo gonius* clustered very closely hence they may be closely related species. However, *Lepidocephalus guntea* showed deep conspecific divergence whch may be due to the presence of haplotype diversity or may be due to the reason that they are of different species which is further detailed in discussion. The ornamental fishes *Labeo calbasu,Cirrhinus mrigala, Barbonymus gonionotus, Puntius*

sophore, Rasbora daniconius, Esomus danricus, Mystus vittatus, Channa orientalis, Channa punctatus, Glossogobius giuris, Wallago attu, Amblyceps mangois and *Sisor rabdophorus* were all clustered separately in their distinct clades with a bootstarp support of 100%. This point clearly indicate that these ornamental fishes belong to different species. Interestingly the ornamental fish *Labeo bata* which was supposed to be in the distinct cluster with other sequences of *Labeo bata* from the database was placed separately in the tree whereas *Labeo boga* was placed along with the sequences of *Labeo bata*.

4.4.2 Model selection for analysis of Maximum likelihood based approach

Phylogenetic analysis based on Maximum likelihood approach is one of the best method which clearly explains the phylogenetic relationship between different species. For the construction of Maximum likelihood tree, the first thing which is a must to develop a perfect tree is to choose the best model. The optimal model that best explain the evolution of the sequences were strained through goodness-of-fit test of each model measured through Bayesian Information Criterion (BIC) and Akaike Information Criterion (AIC). Non-uniformity of evolutionary rates among sites may be modeled by using a discrete Gamma distribution (+*G*) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+*I*).This result is an evaluation of 24 models for nucleotide substitutions. The model with the lowest Bayesian Information Criterion (BIC) value are considered to describe the substitution pattern the best. In this study the model General Time Reversible (GTR) with invariant sites (+I) along with Gamma rate categories were selected to best interpret the phylogeny.

Table 8. Model test for phylogenetic analysis by Maximum Likelihood generated by putting the sequences used in this study using MEGA 5.0.

Model	Parameters	BIC	AICc	*lnL*	(+*I*)	(+*G*)	*R*	*f*(A)	*f*(T)	*f*(C)	*f*(G)	*r*(AT)	*r*(AC)	*r*(AG)	*r*(TA)	*r*(TC)	*r*(TG)	*r*(CA)	*r*(CT)	*r*(CG)	*r*(GA)	*r*(GT)	*r*(GC)
GTR+G+I	61	10869.470	10406.705	-5142.094	0.49	0.74	4.85	0.258	0.298	0.273	0.171	0.056	0.025	0.053	0.048	0.331	0.006	0.024	0.361	0.002	0.080	0.010	0.004
GTR+G	60	10892.669	10437.482	-5158.491	n/a	0.17	6	0.258	0.298	0.273	0.171	0.041	0.025	0.043	0.036	0.355	0.006	0.024	0.388	0.003	0.066	0.010	0.004
TN93+G+I	58	10900.727	10460.698	-5172.115	0.46	0.52	5.63	0.258	0.298	0.273	0.171	0.025	0.023	0.052	0.021	0.337	0.014	0.021	0.368	0.014	0.078	0.025	0.023
TN93+G	57	10916.742	10484.291	-5184.920	n/a	0.16	6.38	0.258	0.298	0.273	0.171	0.022	0.020	0.039	0.019	0.359	0.013	0.019	0.392	0.013	0.059	0.022	0.020
HKY+G+I	57	10956.453	10524.002	-5204.775	0.50	0.72	3.52	0.258	0.298	0.273	0.171	0.034	0.031	0.133	0.029	0.211	0.019	0.029	0.231	0.019	0.200	0.034	0.031
HKY+G	56	10999.896	10575.025	-5231.294	n/a	0.19	4.4	0.258	0.298	0.273	0.171	0.028	0.026	0.139	0.024	0.221	0.016	0.024	0.242	0.016	0.209	0.028	0.026
T92+G+I	55	11076.191	10658.899	-5274.239	0.49	0.75	3.28	0.278	0.278	0.222	0.222	0.032	0.026	0.171	0.032	0.171	0.026	0.032	0.214	0.026	0.214	0.032	0.026
T92+G	54	11120.943	10711.231	-5301.412	n/a	0.19	4.24	0.278	0.278	0.222	0.222	0.026	0.021	0.180	0.026	0.180	0.021	0.026	0.225	0.021	0.225	0.026	0.021
GTR+I	60	11121.778	10666.592	-5273.046	0.56	n/a	2.23	0.258	0.298	0.273	0.171	0.090	0.049	0.091	0.078	0.224	0.010	0.047	0.244	0.004	0.137	0.018	0.007
K2+G+I	54	11148.925	10739.212	-5315.403	0.51	0.96	2.92	0.250	0.250	0.250	0.250	0.032	0.032	0.186	0.032	0.186	0.032	0.032	0.186	0.032	0.186	0.032	0.032
TN93+I	57	11201.393	10768.942	-5327.245	0.56	n/a	2.14	0.258	0.298	0.273	0.171	0.049	0.045	0.087	0.043	0.216	0.028	0.043	0.235	0.028	0.131	0.049	0.045
K2+G	53	11203.170	10801.037	-5347.323	n/a	0.22	2.81	0.250	0.250	0.250	0.250	0.033	0.033	0.184	0.033	0.184	0.033	0.033	0.184	0.033	0.184	0.033	0.033
HKY+I	56	11214.380	10789.509	-5338.536	0.56	n/a	2.09	0.258	0.298	0.273	0.171	0.049	0.045	0.115	0.042	0.183	0.028	0.042	0.200	0.028	0.173	0.049	0.045
T92+I	54	11317.001	10907.289	-5399.441	0.56	n/a	2.03	0.278	0.278	0.222	0.222	0.046	0.036	0.149	0.046	0.149	0.036	0.046	0.187	0.036	0.187	0.046	0.036
K2+I	53	11353.716	10951.583	-5422.596	0.56	n/a	2.03	0.250	0.250	0.250	0.250	0.041	0.041	0.168	0.041	0.168	0.041	0.041	0.168	0.041	0.168	0.041	0.041
JC+G+I	57	11677.366	11244.915	-5565.232	0.54	1.91	0.5	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
JC+G	56	11714.576	11289.705	-5588.634	n/a	0.22	0.5	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
JC+I	56	11810.230	11385.359	-5636.461	0.56	n/a	0.5	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
GTR	59	12643.468	12195.860	-6038.688	n/a	n/a	1.69	0.258	0.298	0.273	0.171	0.087	0.079	0.088	0.075	0.198	0.009	0.074	0.216	0.011	0.132	0.016	0.017
TN93	56	12760.285	12335.413	-6111.489	n/a	n/a	1.7	0.258	0.298	0.273	0.171	0.057	0.052	0.083	0.049	0.196	0.033	0.049	0.214	0.033	0.124	0.057	0.052
HKY	55	12773.605	12356.313	-6122.946	n/a	n/a	1.69	0.258	0.298	0.273	0.171	0.056	0.051	0.107	0.049	0.170	0.032	0.049	0.185	0.032	0.161	0.056	0.051
T92	53	12841.823	12439.690	-6166.649	n/a	n/a	1.69	0.278	0.278	0.222	0.222	0.051	0.041	0.140	0.051	0.140	0.041	0.051	0.176	0.041	0.176	0.051	0.041
K2	52	12877.774	12483.222	-6189.423	n/a	n/a	1.69	0.250	0.250	0.250	0.250	0.046	0.046	0.157	0.046	0.157	0.046	0.046	0.157	0.046	0.157	0.046	0.046
JC	55	13294.839	12877.547	-6383.563	n/a	n/a	0.5	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083

4.4.3 Phylogenetic analysis based on Maximum Likelihood and Maximum Parsimony approach

Both the Maximum likelihood tree and Maximum Parsimony tree gave more or less same topology and hence describe similar phylogenetic relationship between the different species under study. Based on an automatically generated initial tree by MEGA 5.0, a final Maximum Likelihood tree with the highest log likelihood value of -6025.7339 was constructed and it is shown in Figure 13. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site.

From the ML tree it is clearly visible that all the fishes belonging to a particular family were clustered distinctly along with their relevant species. All the 11 ornamental fishes belonging to the family Cyprinidae namely, *Labeo calbasu* (SGBK-DF2), *Labeo gonius* (SGBK-DF3), *Labeo boga* (SGBK-AUJF39), *Cirrhinus mrigala* (SGBK-AUFO35), *Barbonymus gonionotus* (SGBK-BF8), *Puntius chola* (SGBK-AUFO5), *Puntius sophore* (SGBK-FO2B and SGBK-OF4), *Labeo bata* (SGBK-DF1), *Rasbora daniconius* (SGJB-AUECO1) *and Esomus danricus* (SGJB-AUECO2), they all are clustered within the same main clade. Likewise all other fishes were clustered within the family specific clades. Interestingly SGBK-DF1 representing the ornamental fish *Labeo bata,* although it is a fish of the genus Labeo, it is placed far from their congeners near *Rasbora daniconius*. Within the Channid fishes, it is clearly seen that, *Channa orientalis* (SGJB-BF3) is more closely related to *Channa punctatus* (SGJB-BF2 and SGJB-FO1A) than *Channa striata* (SGJB-BF1). *Nandus nandus* (SGBK-BF6) and *Badis badis* (SGBK-OF29F) were clustered together in a clade which shows that they are somehow very closely related phylogenetically.

The Maximum Parsimony tree was obtained by using the close-neighbor-interchange algorithm with search level 1 in which the initial trees were obtained with the random addition of sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The consistency index of the tree is 0.334742, the retention index is 0.540840 and the composite index is 0.189361 for all sites and parsimony informative sites. Under a maximum parsimony analysis trees are scored according to the degree to which they imply a parsimonious

distribution of the character data. The MP tree also shows more or less the same pattern of phylogenetic relationship between the ornamental fishes except in the case that fishes of the family Siluridae and Channidae appear to be more close here. The MP tree also indicates that *Rasbora daniconius* and *Esomus danricus* are closely related, morphologically these two are more or less similar. Similarly, *Labeo calbasu*, *Labeo gonius*, *Labeo boga* clustered very close to each other. They may be closely related species.

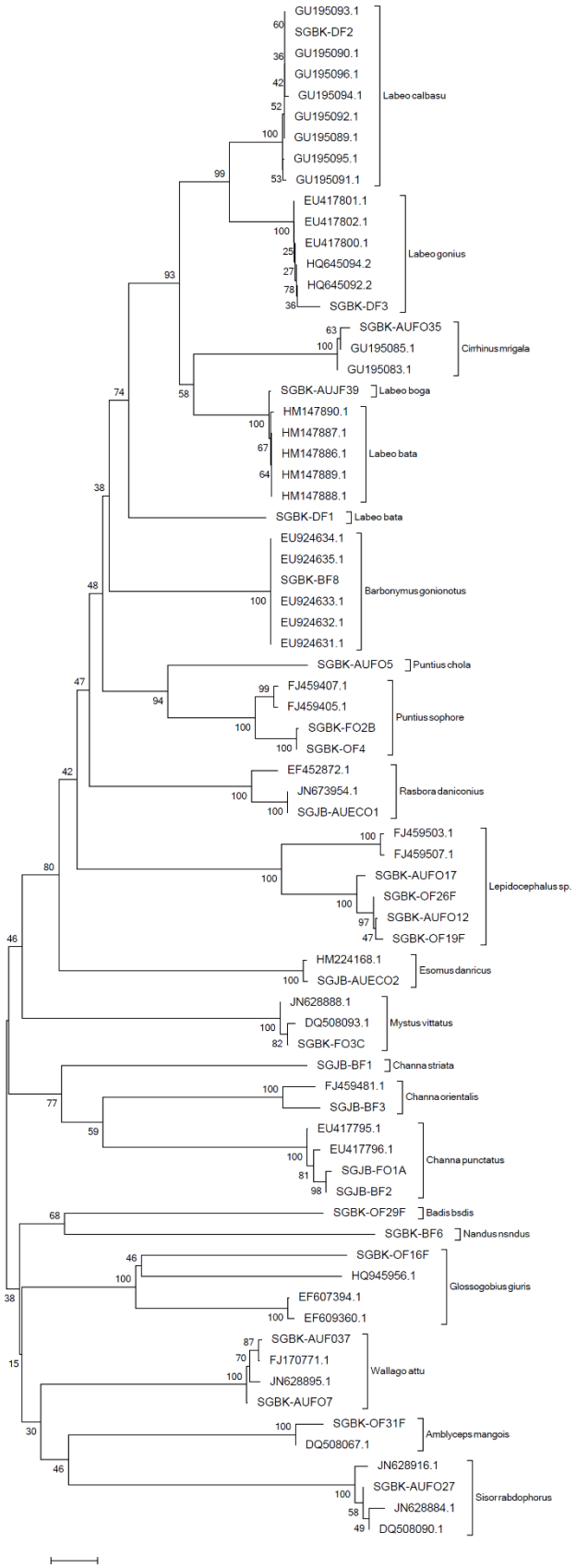


Figure 13. Neighbor-joining tree developed by using K2P distances generated by our generated sequences of ornamental fishes along with sequences from GenBank.

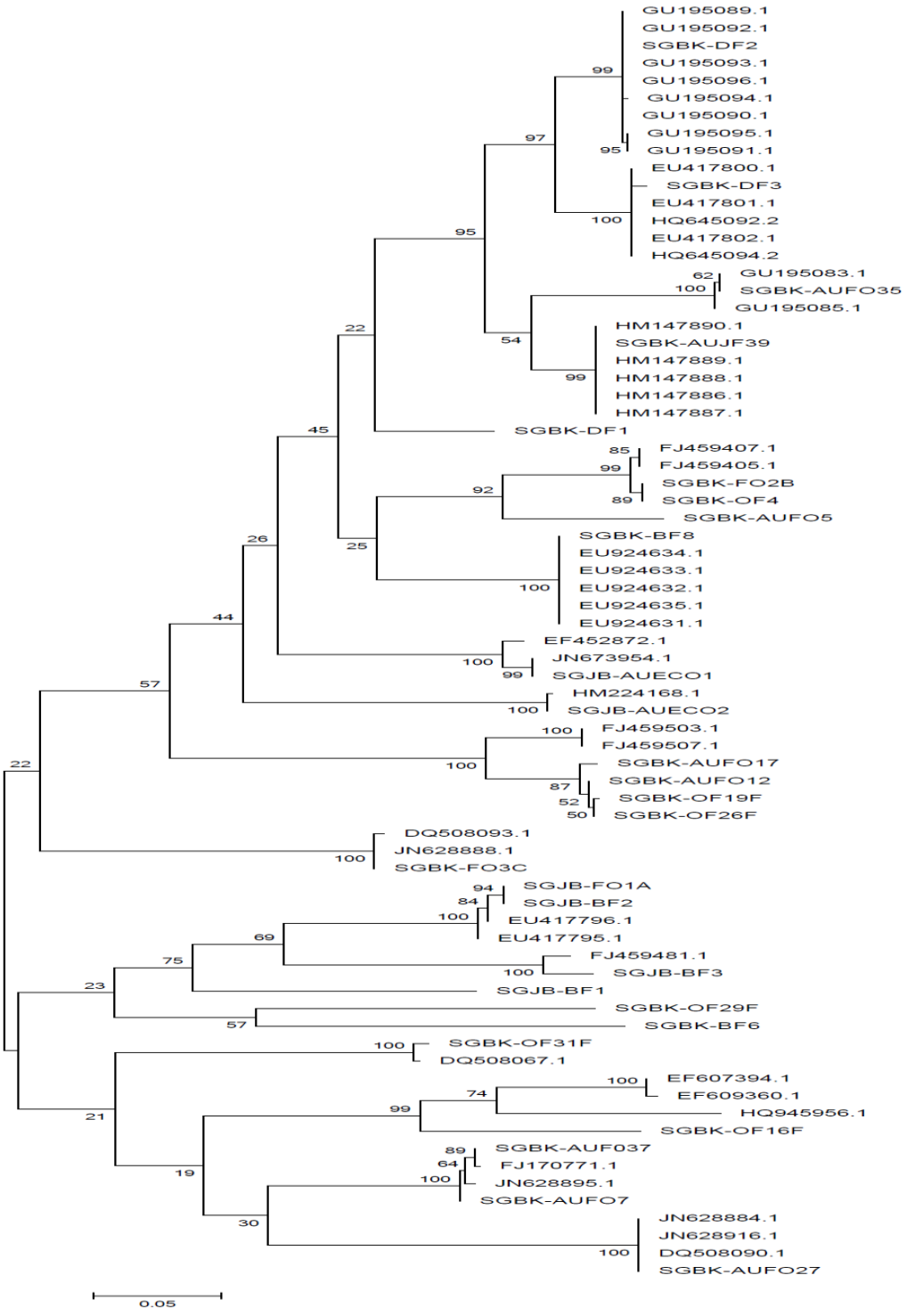


Figure 14. Maximum likelihood tree generated from the ornamental fish sequences by adopting GTR+G+I model.

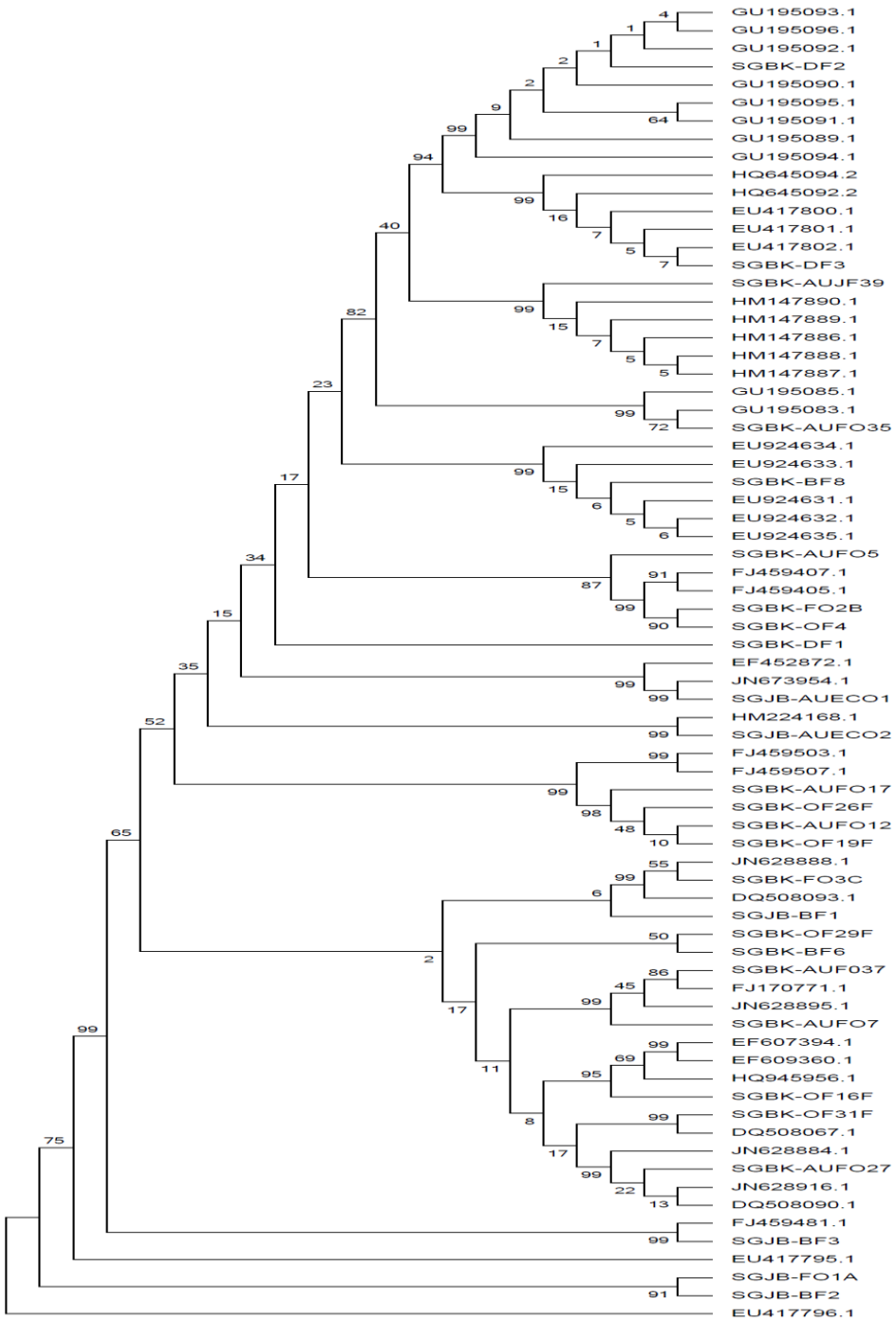
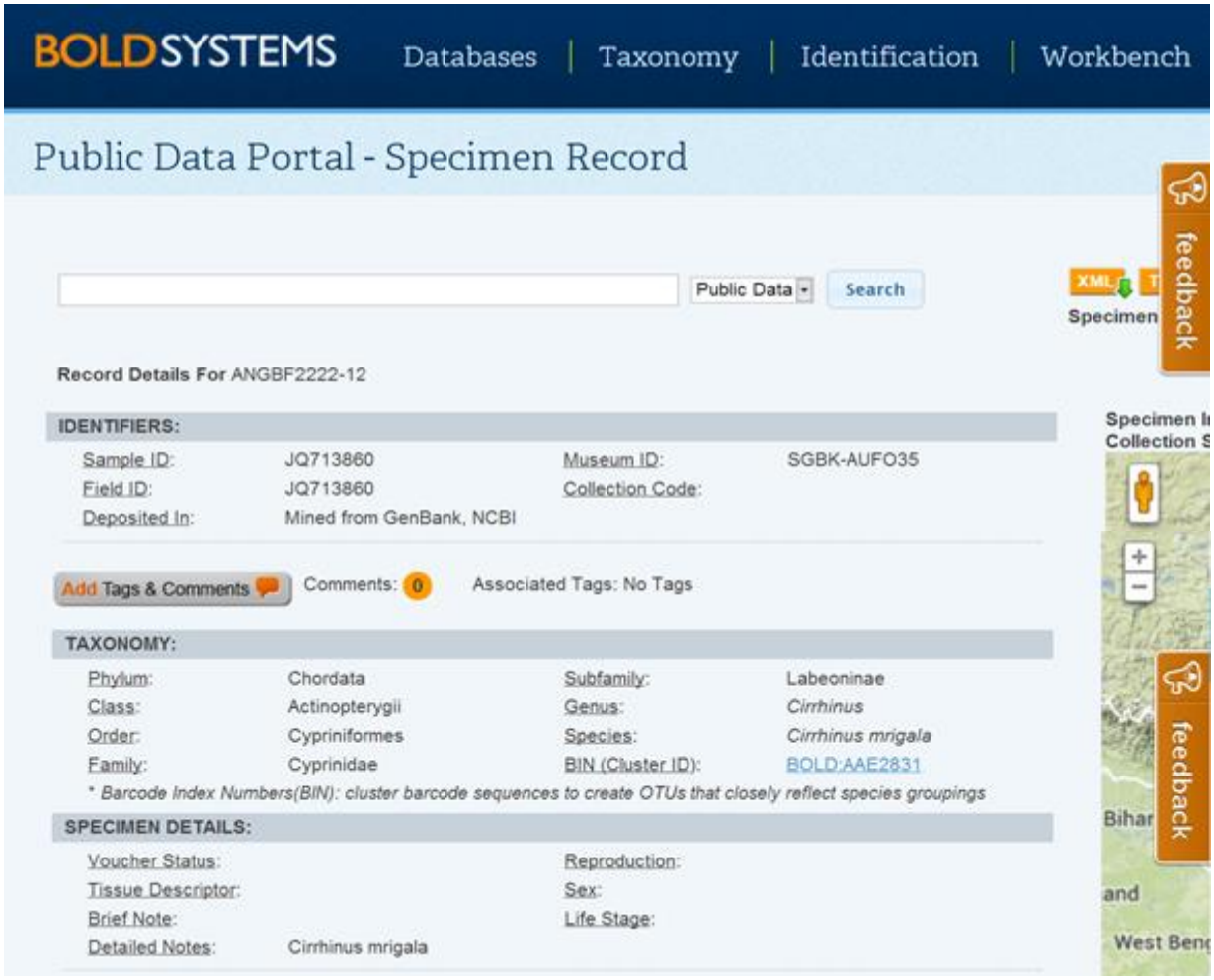


Figure 15. Maximum parsimony tree developed from the sequences under study.

4.5 Development of reference barcode library of the ornamental fishes from Manipur

We have developed a reference barcode library of the major ornamental fishes of Manipur, India by generating the species specific DNA barcode of 21 species of ornamental fishes which acts as passport for each species and submitting the details into the two major databases GenBank and BOLD. An example of the barcode of an ornamental fishes in the library is given in the figure 15.



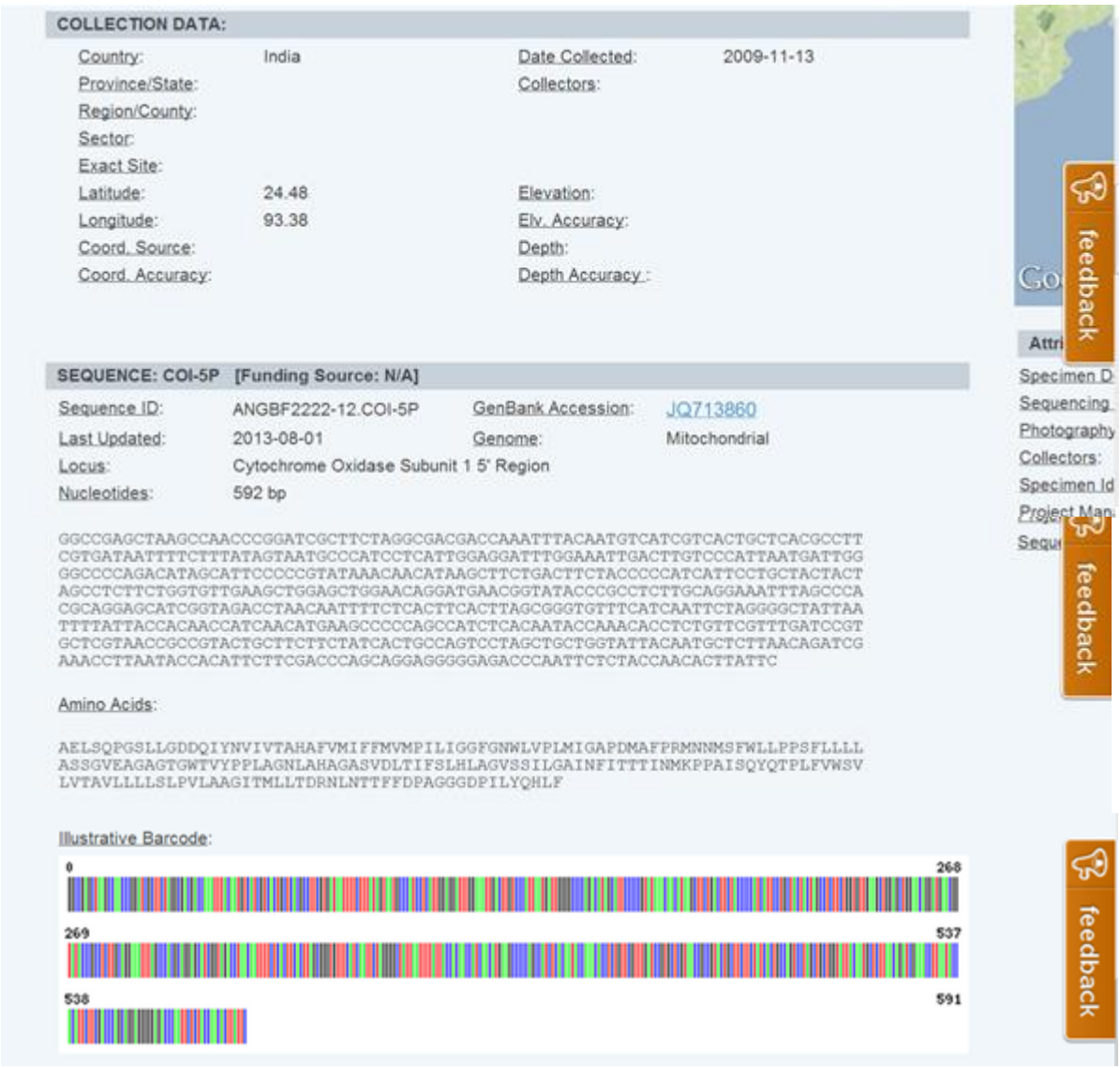


Figure 15. DNA barcode of the ornamental fish, SGBK-AUFO35 which represents *Cirrhinus mrigala.*