DNA BARCODES

CHAPTER 4

RESULTS

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RESULTS

Overview

Collection of ornamental fish resourse as State-of-Arts from different drainage systems of Northeast India and collection points was prepared following the baseline information from earlier studies and the sampling and morphological identification done in this study and detailed in **chapter-4.1 "*Vouchering of the ornamental fish samples*"**.

Species specific genetic marker as DNA barcode was developed for the studied samples of ornamental fishes and the sequences were analyzed to assess the species and detailed in **chapter-4.2 "*Species specific genetic marker as DNA barcode sequences*"**.

Combined approach of Morphology and DNA barcoding was undertaken to identify the actual traded ornamental fish from Northeast India along with the species status and detailed in **chapter-4.3 "*DNA barcoding approach to detect traded ornamental fishes from Northeast India*"**

Nucleotide composition analyses including transition-transversion analysis, codin usage, amino acid composition, etc. were made. Mini-barcode was designed for the ornamental fishes for ease of the identified samples for rapid field based identification and detailed in **chapter-4.4 "*Barcode sequence analysis*"**.

Finally, phylogenetic relationship of different ornamental fishes of Northeast was inferred by analyzing the DNA barcode sequences and detailed in **chapter-4.5 "*Phylogenetic analysis of different ornamental fishes*"**.

CHAPTER 4.1
VOUCHERING OF THE ORNAMENTAL FISH SAMPLES

4.1.1 MORPHOLOGY BASED IDENTIFICATION

In this study a total of 130 specimens of ornamental fishes were collected from various locations of the North-eastern states, India. The first hand field identification based on 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 Jayaram (1999) and "Inland fishes of India and adjacent countries" by Talwar and Jhingran (1991). And also from the original described research papers. Based on this study, following the standard family level taxonomic keys, the specimens were categorized under 18 families under 6 orders. The comparisons revealed straightforward identification of the specimens and the important features with respect to identification of the studied specimens under each family are explained below. The pictures of each of the species identified in this study were shown from the representative specimens.

Table 4.1 The field identification of the ornamental fish under different orders and families based on available checklist

Sl No.	Orders	No. of Families	No. of Species
1.	Cypriniformes	3	23
2.	Siluiformes	5	14
3.	Perciformes	7	11
4.	Synbranchiformes	1	1
5.	Osteoglossiforfes	1	2
6.	Tetraodontiformes	1	1

4.1.1.1 ORDER CYPRINIFORMES

4.1.1.1.1 FAMILY CYPRINIDAE

A total of 52 different specimens (among our studied fishes) fall under this family. The specimens represented by various common or generic trade names

such as barbs, Hill trouts, Danio, carplets, etc which were first hand identified to their respective species. The diagnostics keys to each of the identified species is described in Appendix 2.

4.1.1.1.1.1 Barbs

The specimen named Pool barb (ID-SGBD-DOF4) and Spotfin barb (ID-SGBK-OF4) were identified to 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 specimens of Green Swamp barb (ID- SGBD-DOF82, SGBK-AUFO5) and Swamp barb (ID-SGBD-DOF25, SGBD-DOF26) was identified as *Puntius chola*. Its morphological characters perfectly match with the one described by 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 Barb (ID-SGBD-S1, SGBD-S2 and SGBD-S1) were identified to *Puntius manipurensis.* Its body colour is dark to silvery with two black blothes one at near the operculum and the other near the caudle peduncle less distinct than its congener *P. ticto*. The fins and the caudal peduncle are scarlet red in colour.

The specimen Silver barb (ID- 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 Slender rasbora (ID-AUECO1) and Flying barb (ID-SGBD-N1, SGBD-N2, SGBD-Y3) were identified as *Rasbora daniconius* and *Esomus danricus* respectively. *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 dody. 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.

4.1.1.1.1.2 Carps

The specimens Minor carp (ID- SGMC-MOF28, SGMC-MOF35, SGMC-MOF33, SGMC-MOF30, SGMC-MOF29, SGMC-MOF34), Labeo (ID-OFISH057-12) and Miniscale shark (ID- SGBK-DF2) were identified as *Labeo bata*, *Labeo calbasu* and *Labeo gonius* 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 threatened species by IUCN (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*.

Another specimen of this category named Kalabans (ID- SGBD-A1 to SGBD-A5) were identified as *Labeo dero*. It has snout without any lateral lobe, pores on snout are generally present with a distinct groove, the dorsal fin higher than body depth and is fin inserted above last quarter of pectoral fin which makes it distinct from the other congeners.

4. 1.1.1.1.3 Danio

The specimen named Giant Danio (ID- SGBD-BOF43, SGBD-BOF44, SGBD-BOF45) and Turquoise Danio (ID- SGBD-BOF12) were identified as *Devario aequipinnatus* and *Devario devario* respectively. The lateral line scales in *Devario aequipinnatus* was observed to be 37 with a short barbels where as it is

found to be 17 in case of *Devario devario*. Moreover, barbels in *D. devario* were also absent, which is a dintinguishing characters from the former.

4.1.1.1.1.4 Gangetic latia

The specimen named Gangetic latia (ID- SGBD-BOF49, SGBD-BOF50, SGBD-BOF51 and SGBD-DOF62) were identified as *Crossocheilus latius*. Its upper half of the body is greyish with irregular dark punctuations. Faint longitudinal stripes on flanks which broaden near the base of caudal fin, Gill rackers 37, presence of maxillary and rostral barbels, rostral barbels often short and rudimentary dorsal and caudal fins yellowish grey in colour, scales moderate.

4.1.1.1.1.5 Indian Hill Trout

The specimen named Indian Hill trout (ID- SGBD-BOF23, SGBD-BOF56, SGBD-BOF55, SGBD-BOF53, SGBD-DOF40, SGBD-BOF54 and SGBD-BOF57) is identified as members of the genus *Barilius.* It had tubercules on snout and lower jaw large and well developed in some cases where as in many cases its poorly developed. Body has colour dull green to silvery with greyish back and had 7-12 dark bands descending towards lateral lines.

4.1.1.1.1.6 Garra

The specimen Garra (ID- SGBD-BOF30, SGBD-BOF31, SGBD-BOF32, SGBD-BOF33 and SGBD-BOF34) is identified as *Garra arupi.* Body had Transverse row of Tubercles on snout, 7 Stripes on caudal Peduncle. It can be distinguished from the other members of congeners by the absence of W- shaped Band on Caudal fin and Rostral Lobe on Snout.

4.1.1.1.1.7 Kingfish

The specimen kingfish (ID- SGBD-BOF15 and SGBD-BOF36) is identified to as *Semiplotus semiplotus.* the meristic counts revealed 25 Dorsal soft rays, Anal spines were 2, Anal soft rays were 7, the last simple dorsal fin ray spine was not serrated and transverse row of 10-12 open pores (5-6 on each side) across the snout directed posterior toward the middle of orbit were observed.

4.1.1.1.1.8 Mola carplet/Pale carplet

The specimen Mola carplet (ID- SGBD-DOF15) and the Pale carplet (ID-SGBD-DOF16) is identified as *Amblypharyngodon mola.* Body is elongate Small scales, lateral line incomplete, golden yellow with a broad silvery lateral band on body.

4.1.1.1.1.9 Neon Hatchet

The specimen Neon Hatchet (ID- SGBD-DOF63 and SGBD-DOF64) is identified as *Chela cachius.* The body is deep and compressed, mouth is slightly oblique and lateral line is complete with 53 scales observed. The colour is mostly shining silver, translucent; a shining greenish longitudinal from level of dorsal fin is seen.

4.1.1.1.2 FAMILY PSILORHYNCHIDAE

A total of only one specimen (among our studied fishes) fall under this family. The common trade name of the specimen under stydy is Torrent stone carp (ID- SGBD-BOF18). This is identified as *Psilorhynchus homaloptera* based on described morphologic keys. Body brown on back, abdomen pale and with 7-10 dark spots along lateral line. Observed key morphological characters of the specimen is detailed in Appendix 2.

4.1.1.1.3 FAMILY COBITIDAE

A total of 11 specimens (among our studied fishes) fall under this family. The species are mostly knows as differents loachetclike ladder loach, Bengal loaches, stripped loaches, etc etc which were first hand identified to their respective species. The diagnostics keys to each of the identified species is described in Appendix 2.

4.1.1.1.3.1 Queen loach and Bengal Loach

The specimen Queen loach (ID- SGBD-DOF119, SGBD-DOF121, SGBD-DOF122, SGBD-Z1) and Bengal loach (ID- SGBD-DOF97, SGBD-DOF123) is identified as *Botia dario.* It possesses 7 vertical bars on the body with

narrower interspaces and lacks any distinct markings on the interspaces which make it different from its other congeners.

4.1.1.1.3.2 Ladder Loach and Gangetic Loach

The specimen Ladder loach (ID- SGBD-BOF10) and Gangetic loach (ID-SGBD-BOF11) is identified as *Botia rostrata.* The body had bars that are interconnected like a network and pale spots within the dark vertical bars.

4.1.1.1.3.3 Moosefaced Loach

The specimen Moosefaced loach (ID- SGBD-DOF68) is identified as *Canthophrys gongota.* It is the only species described of its genus. The colour of the body is white on the sides, above clouded with dotted spots, and below silvery, the lateral line runs straight above the middle.

4.1.1.1.3.4 Zipper loach and Striped loach

The specimen Zipper loach (ID- SGBD-DOF49) and Striped loach (ID- SGBD-DOF49) is identified as *Acanthocobitis botia.* It is distinguished from the congeners by the absence of a suborbital flap in male, the flap being replaced by a suborbital groove; lateral line reaches at least to anus.

Table 4.1.1 Vouchering of the ornamental fish samples (Cypriniformes) from Northeast India

Sl No	Order	Family	Trade name	Species Identified	Latitude Longitude	Drainage
1	Cypriniformes	Cyprinidae	Pool barb	*Puntius sophore*	92.45 E 24.5 N	River Sunai in Assam
2			Spotfin barb	*Puntius sophore*	93.39 E 24.48 N	River Barak in Manipur
3			Green Swamp barb	*Puntius chola*	92.37 E 24.49 N	River Dholeshwari in Hailakandi
4			Swamp barb	*Puntius chola*	93.01 E 24.46 N	River Barak in Assam
5			Barb	*Puntius manipurensis*	93.849 E 24.714 N	Merakhong River in Manipur
6			Silver barb	*Barbonymus gonionotus*	92.54 E 24.45 N	River Katakhal
7			Flying barb	*Esomus danricus*	93.922 E 24.804 N	River Nambul in Manipur

8			Slender rasbora	*Rasbora daniconius*	93.922 E 24.804 N	River Nambul in Manipur
9			Minor carp	*Labeo bata*	92.836 E 24.761 N	River Barak
10			Labeo	*Labeo calbasu*	92.45 E 24.57 N	River Kushiara near Bangladesh
11			Miniscale shark	*Labeo gonius*	92.44 E 24.53 N	River Kushiara near Bangladesh
12			Kalabans	*Labeo dero*	93.899 E 24.719 N	River Nambul in Manipur
13			Giant Danio	*Devario aequipinnatus*	95.69 E 28.149 N	River Lohit in Arunachal Pradesh
14			Turquoise Danio	*Devario devario*	96.079 E 27.511 N	River Noadihang in Arunachal Pradesh
15			Gangetic latia	*Crossocheilus latius*	95.69 E 28.149 N	River Lohit in Arunachal Pradesh
16			Indian Hill trout	*Barilius cf bendelisis*	96.079 E 27.511N, 95.706 E 28.258 N	River Lohit in Arunachal Pradesh
17			Garra	*Garra arupi*	95.709 E 28.26 N	River Lohit in Arunachal Pradesh
18			Kingfish	*Semiplotus semiplotus*	96.079 E 27.511 N	River Noadihang in Arunachal Pradesh
19			Mola carplet	*Amblypharyngodon mola*	93.01 E 24.46 N	River Barak
20			Pale carplet	*Amblypharyngodon mola*	93.01 E 24.46 N	River Barak
21			Neon Hatchet	*Chela cachius*	92.776 E 25.033 N	River Jatinga in Assam
22		**Psilorhynchidae**	Torrent stone carp	*Psilorhynchus homaloptera*	96.079 E 27.511 N	River Noadihang in Arunachal Pradesh
23		**Cobitidae**	Queen loach	*Botia Dario*	92.37 E 24.49 N	River Sunai in lower Assam
24			Bengal loach	*Botia dario*	92.45 E 24.5 N	River Sunai in lower Assam
25			Ladder loach	*Botia rostrata*	96.079E 27.511 N	River Noadihang in Arunachal Pradesh
26			Gangetic loach	*Botia rostrata*	96.079E 27.511 N	River Noadihang in Arunachal Pradesh
27			Moosefaced loach	*Canthophrys gongota*	92.45 E 24.5 N	River Sunai

28			Zipper loach	*Acanthocobitis botia*	92.948E 25.113N	River Jatinga
29			Striped loach	*Acanthocobitis botia*	92.948E 25.113N	River Jatinga

4.1.1.2 ORDER SILURIFORMES

4.1.1.2.1 FAMILY BAGRIDAE

The species are mostly, Striped dwarf catfish and long whiskers catfish etc which were first hand identified to their respective species. The diagnostics keys to each of the identified species is described in Appendix 2.

4.1.1.2.1.1 Striped dwarf catfish

The specimen Striped dwarf catfish (ID- SGMC-MOF3) was identified as *Mystus vittatus* and the observed diagnostic characters match well with described characters. The body had 3 or 4 longitudinal colour bands above and below. The adipose fin is short and inserted after an interspace behind rayed dorsal fin. The longitudinal groove extends to base of occiput.

4. 1.1.2.1.2 Long Whiskers Catfish

The specimen Long Whiskers Catfish (ID- SGMC-MOF9) was identified as *Mystus gulio*. The head and the back of the body are bluish brown in colour, maxillary barbels extended to the end of pelvic fin. The longitudinal groove is short and do not extends to base of occipital processes.

4. 1.1.2.2 FAMILY SCHILBEIDAE

The species are River catfish and Gangetic ailia which were first hand identified to their respective species. The diagnostics keys to each of the identified species is described in table Appendix 2.

4.1.1.2.2.1 River Catfish

The specimen River Catfish (ID- SGBD-J1 and SGBD-J2) is identified as *Eutropiichthys murius*. Most of the key features used to distinguish this species

from its congener are difficult to diagnose in early life stage. The length of nasal barbels as a taxonomic key is confusing with the other congeners, while presence of cleft of mouth only provides easy diagnosis of both the species. Teeth on palate in a band narrower than premaxillary band or just equal to, nasal barbels reach a short distance behind posterior edge of eyes.

4.1.1.2.2.2 Gangetic ailia

The specimen Gangetic ailia being represented by the codes (ID- SGMC-MOF6) were identified as *Ailia coila.* This genus is distinguishable due to the absence of dorsal fin, number of anal fin rays count which is very important for species identification. The body colour is silvery to dull brown without any black blotch on caudal fin base or along side of body.

4.1.1.2.3 FAMILY SISORIDAE

A total of 15 specimens (among our studied fishes) fall under this family. The species are Copper Cat fish, Whiptail Catfish Giantmoth Catfish, etc. which were first hand identified to their respective species. The diagnostics keys to each of the identified species is described in Appendix 2.

4.1.1.2.3.1 Copper Catfish

The specimen Copper catfish (ID- SGJB-DOF77 and SGJB-DOF78) were identified as *Glyptothorax telchitta.* This species is distinguishable from other congeners except *G. gracile* and *G. platypogonoides* due to rough body. But in later two species the dorsal fin is distinctly higher than depth of body. Body is darkish brown above and sides, yellow below; head, sides and fins mottled with dark spots. Skin on head is tuberculated.

4.1.1.2.3.2 Gagata and Indian Gagata

The specimens Gagata (ID- SGBD-G1, SGBD-W3, SGBD-W1, SGBD-W2, SGBD-H1 and SGBD-H2) is found to be comprised of two species the one is *Gagata dolichonema* and the other is *Gagata cenia.* This is distinguished from *Gogangra, Bagarius* and Sisor by having a compressed (vs. depressed) head, and

by having the outer and inner mental barbels close together with their origins nearly parallel (vs. widely separated, origin of the inner barbels anterior in Gogangra and Bagarius, and origin of the inner barbels posterior in Sisor).

The dorsal part of thoracic region of *Gagata dolichonema* is observed with 4 black stripes, median longitudinal groove extending to end of occipital process. Maxillary barbel is longer than head. Dorsal-fin spine produced into long filament. Pectoral-fin spine is without filamentous projection. On the other hand the specimen of Gagata and Indian Gagata (ID- SGBD-G2) were identified to be *Gagata cenia.* The maxilary barbel is shorter than head and Pectoral fins without any filamentous prolongation.

4.1.1.2.3.3 Indian Whiptail catfish

The specimen Indian Whiptail catfish (ID- SGJB-DOF80) were identified as *Sisor rhabdophorus.* The body elongate with a long tapering tail, a series of bony plates from base of dorsal fin to base of caudal fin are present, adipose dorsal fin is in the form of a spine, 67-70 Lateral line ossicles were present, rayed dorsal fin is serrated anteriorly.

4.1.1.2.3.4 Suckerthroat

The specimen Suckerthroat (ID- SGBD-BCF22, SGBD-BCF23 and SGBD-BCF24) is identified as *Pseudecheneis sulcata.* The body is greyish brown in colour with irregular blotches. thoracic adhesive apparatus with 12-14 transverse laminae; separate pelvic fin, slender caudal peduncle, larger eye. Skin was observed to be smooth with tuberculate in some areas as per description. Lateral line complete and midlateral. Barbels was flattened, and present in four pairs. Maxillary barbel with the ventral surface is densely covered with papillae and pointed tip, barbel extending about two-thirds of distance between its base and base of first pectoral-fin element.

4.1.1.2.3.5 Giant Moth Catfish

The specimen Giant Moth Catfish (ID- DOF69) is identified as *Erethistes pussilus. Erethistes* is distinguished from its sister genus *Erethistoides* by its more

tough body and by the direction of the serrations on the anterior margin. The body is moderate ventrally flattened. Barbels present in 4 pairs, maxillary barbels is broader at the base. Dorsal spine is serrated distinctly. Pectoral spine had divergen 9-12 serrations on the anterior margin.

4.1.1.2.4 FAMILY SILURIDAE

A total of 2 specimens (among our studied fishes) fall under this family. The species are Butter Catfish and Two Stripe Gulper Catfish. which were first hand identified to their respective species. The diagnostics keys to each of the identified species is described in Appendix 2.

4.1.1.2.4.1 Butter catfish

The specimens Butter catfish being represented by the codes (ID- SGMC-MOF8) were identified as *Ompok bimaculatus.* The colour of the body is silvery, dorsally dark greyish brown in colour, al large spot on the shoulder on lateral line, a small black spot on the caudal peduncle. Body is elongate and compressed. Barbels were present in two pairs, maxillary barbells were long and extended beyond the anal fin origin, caudal fin deeply forked. The comparison of the length of maxillary barbels is one of the helpful criteria to distinguish all the congeners.

4.1.1.2.4.2 Two Stripe Gulper Catfish

The specimens Two Stripe Gulper Catfish being represented by the codes (ID- SGBD-M1) were identified as *Ompok pabda.* The body is silvery-grey with a touch of yellow, dark on back. Barbels present in two pairs, mandibular barble was found extended up to posterior border of eye. Maxillary barbles much shorter extend only to the middle or tip of the pectoral fin.

4.1.1.2.5 FAMILY CLARIIDAE

One specimen (among our studied fishes) fall under this family. The species is Walking catfish which was first hand identified to their respective species. The diagnostics keys to each of the identified species is described in Appendix 2.

The specimen of Walking catfish (ID- SGMC-MOF5) is identified as *Clarias batrachus.* Its body was elongate, head depressed, barbells were found in four pairs. Maxillary barbel was found to be extending near to base of first dorsal-fin ray. Nasal barbel was observed extending near to tip of occipital process. The dorsal and lateral surfaces of head and body were grey to dark grey, fading to pale grey on the ventral surfaces. An irregular row or two of white spots sometimes present on body dorsal to anal-fin base. Dorsal and caudal fins grey to dark grey with very thin hyaline distal margin.

Table 4.1.2 Vouchering of the ornamental fish samples (Siluriformes) from Northeast India

Sl No	Order	Family	Trade name	Species Identified	Latitude Longitude	Drainage
30	**Siluriformes**	**Family Bagridae**	Striped dwarf catfish	*Mystus vittatus*	91.861E 26.254N	River Brahmaputra
31			Long Whiskers Catfish	*Mystus gulio*	88.313E 22.547 N	River Hoogli
32		**Schilbeidae**	River Catfish	*Eutropiichthys murius*	93.101 E 24.779 N	River Jiri
33			Gangetic ailia	*Ailia coila*	88.313 E 22.547N	Hoogli River
34		**Sisoridae**	Copper catfish	*Glyptothorax telchitta*	88.313 E 22.547N	Hoogli River
35			Gagata	*Gagata dolichonema*	93.889 E 24.616 N	Imphal river
36			Gagata	*Gagata cenia*	93.101 E 24.779 N	River Jiri
37			Gagata	*Gogangra viridescens*	92.948E 25.113N, 93.101E 24.779N	River Jatinga, River Jiri
38			Indian Gagata	*Gagata cenia*	93.101 E 24.779 N	River Jiri
39			Indian Whiptail catfish	*Sisor rhabdophorus*	93.101 E 24.779 N	River Jiri
40			Suckerthroat	*Pseudecheneis sulcata*	95.709 E 28.26 N	River Dibang in Arunachal Pradesh
41			Giant Moth Catfish	*Erethistes pussilus*	93.101 E 24.779 N	River Jiri
42		**Siluridae**	Butter catfish	*Ompok bimaculatus*	88.313 E 22.547 N	River Hoogli
43			Two Stripe Gulper Catfish	*Ompok pabda*	93.953 E 24.847 N	River Imphal
44		**Clariidae**	Walking catfish	*Clarias batrachus*	88.313 E 22.547 N	River Hoogli

4.1.1.3 ORDER PERCIFORMES

4.1.1.3.1 FAMILY AMBASSIDAE

A total of 4 specimens (among our studied fishes) fall under this family. The species is Glass fish which was first hand identified to their respective species. The diagnostics keys to each of the identified species is described in Appendix 2.

The specimen of Glass fish (ID- SGBD-DOF11, SGBD-DOF23, SGBD-DOF13 and SGBD-DOF12) is identified as *Pseudambassis ranga*. The body is deep compressed with mouth oblique. The appearance of the body is transparent with a trace of greenish-yellow and a silvery stripe on the side of the body, a dusky spot on the shoulder is also seen.

4.1.1.3.2 FAMILY NANDIDAE

A total of 3 specimens (among our studied fishes) fall under this family. The species is Glass fish which was first hand identified to their respective species. The diagnostics keys to each of the identified species is described in Appendix 2.

4.1.1.3.2.1 Mud perch and Leaf fish

The specimens of Mud perch (ID- SGBD-DOF74, SGBD-DOF75) and Leaf fish (ID- SGBK-BF6) is identified as *Nandus nandus*. Body is rhomboidal and fairly deep, compressed. Dorsal and anal spines are strong and the second spine is the longest, caudal fin is round in shape. Body colour is greenish-brown in nature; three vertical patchy bloches are seen. Scales ctenoid in nature

4.1.1.3.3 FAMILY MUGILIDAE

One specimen (among our studied fishes) fall under this family. The species is Corsula mullet which was first hand identified to their respective species. The diagnostics keys to each of the identified species is described in Appendix 2. The specimen (ID- SGBD-DOF113) is identified to *Rhinomugil*

corsula. The identification keys matched with the described keys. The colour of the body was dull brown on the dorsal side and silvery on the ventral.

4.1.1.3.4 FAMILY GOBIIDAE

A total of 5 specimens (among our studied fishes) fall under this family. The species is Tank goby which was first hand identified to their respective species. The diagnostics keys to each of the identified species is described in Appendix 2.

The specimens of Tank goby (ID- SGBD-DOF45, SGBD-DOF46, SGBD-DOF47, SGBD-DOF48 and SGBK-OF16,) are identified to *Glossogobius guiris*. The identification keys matched with the described keys. The colour of the body was yellowish brown and had five blotches. The dorsal, pectoral nd caudal fins were mottled with dark spots.

4.1.1.3.5 FAMILY ANABANTIDAE

A total of 2 specimens (among our studied fishes) fall under this family. The species were Climbing perch and Climbing gourami which was first hand identified to their respective species. The diagnostics keys to each of the identified species is described in Appendix 2.

Climbing perch and Climbing gourami

The specimens Climbing perch (ID- SGBD-B1) and Climbing gourami (ID- SGBD-B3) is identified to *Anabas testudineus* based on the describe taxonomic keys. The body is greenish to dark grey in colour on its dorsal side and pale yellow on its ventral side, a dark spot on the base of the caudal fin.

4.1.1.3.6 FAMILY BADIDAE

A total of 4 specimen (among our studied fishes) fall under this family. The species were Blue Badis and Chameleon Fish which was first hand identified to their respective species. The diagnostics keys to each of the identified species is described in Appendix 2.

Blue Badis and Chameleon Fish

The specimen Blue Badis (ID- SGBD-DOF72, SGBD-DOF73) and Chameleon Fish (SGBD-DOF89, SGBK-OF29) is identified to *Badis badis*. The specimen had conspicuous dark blotch covering superficial part of cleithrum above the base of pectoral fin. Lacks a dark blotch on caudal peduncle. It had a series of prominent dark blotches along dorsal fin base and middle of dorsal fin; and also had indistinct bars on side.

4.1.1.3.7 FAMILY CHANNIDAE

A total of 14 specimens (among our studied fishes) fall under this family. The species were traded with a generic name of snakeheads like Bullseye snakehead, spotted snakehead, hekered snake fish, etc which was first hand identified to their respective species. The diagnostics keys to each of the identified species is described in Appendix 2.

4.1.1.3.7.1 Bullseye snakehead

The specimen of bullseye snakehead (ID- SGBD-BOF19) is identified as *Channa marulius*. The observed taxonomic characters matched with the described characters. A large black ocellus on upper caudal fin base is observed, longitudinal striae were present anterior to isthmus, presence of 5-6 dark oval blotch on flank which terminate below lateral line.

4.1.1.3.7.2 Walking snakehead and Ceylone snakehead

The specimen of Walking snakehead (ID- SGBD-DOF112, SGBD-DOF116) and Ceylone snakehead (ID- SGBD-DOF117, SGBD-DOF118) were identified to *Channa orientalis*. The dorsal side and flanks are green; pectoral fins were with a series of blue and faint orange vertical bands. A large ocellus on the last part of the dorsal fin rays wasobserved.

4.1.1.3.7.3 Spotted snakehead or Green snakehead or Chekered snake fish

The specimen of Spotted snakehead, Green snakehead or Chekered snake fish (ID- SGBD-C1, SGBD-C2) is identified as *Channa punctatus*. The colour

varies from green to black on its dorsal sides and flanks, several dark blotches and black spots on the body can be observed.

4.1.1.3.7.4 Black spot grouper

The specimen Black spot grouper (ID- SGBD-BOF35) is identified as *Channa melanostigma* which was newly described species from northeast India. The diagnostic keys and meristics counts matched with the original description.

4.1.1.3.7.5 Snakehead murrel, Banded snakehead and Striped snakehead

The specimen Snakehead murrel or Banded snakehead (ID- SGBD-D2), and Striped snakehead (ID- SGBD-D3) is identified as *Channa striata*. The body is greyish green to dark green in colour on upper side and pale yellow from the middle. There were also presences of angular bands that run obliquely from snout to edge of gill cover.

Table 4.1.3 Vouchering of the ornamental fish samples (Perciformes) from Northeast India

Sl No	Order	Family	Trade name	Species Identified	Latitude Longitude	Drainage
45	Perciformes	**Ambassidae**	Glass fish	*Pseudambassis ranga*	93.01 E 24.46 N	River Barak
46		**Nandidae**	Mud perch	*Nandus nandus*	92.37 E 24.49 N	River Sunai
47			Leaf fish	*Nandus nandus*	92.37 E 24.49 N	River Sunai
48		**Mugilidae**	Corsula mullet	*Rhinomugil corsula*	93.01 E 24.46 N	River Barak in Lower Assam
49		**Gobiidae**	Tank goby	*Glossogobius guiris*	92.776 E 25.033 N	River Jatinga in Assam
50		**Anabantidae**	Climbing perch	*Anabas testudineus*	93.929 E 24.707 N	River Imphal in Manipur
51			Climbing gourami	*Anabas testudineus*	93.929 E 24.707 N	River Imphal in Manipur
52		**Family Badidae**	Blue Badis	*Badis badis*	92.37 E 24.49 N	River Sunai
53			Chameleon Fish	*Badis badis*	92.37 E 24.49 N	River Sunai
54		**Channidae**	Bullseye snakehead	*Channa marulius*	95.869 E 28.157 N	River Lohit in Arunachal Pradesh
55			Walking snakehead	*Channa orientalis*	92.37 E 24.49 N	River Sunai
56			Ceylone snakehead	*Channa orientalis*	93.01 E 24.46 N	River Barak in Lower Assam

57			Spotted snakehead	*Channa punctatus*	93.929 E 24.707 N	River Imphal in Manipur
58			Green snakehead	*Channa punctatus*	93.929 E 24.707 N	River Imphal in Manipur
59			Chekered snake fish	*Channa punctatus*	93.929 E 24.707 N	River Imphal in Manipur
60			Black spot grouper	*Channa melanostigma*	95.709 E 28.26 N	River Dibang in Arunachal Pradesh
61			Snakehead murrel	*Channa striata*	93.94 E 24.781 N	River Imphal
62			Banded snakehead	*Channa striata*	93.94 E 24.781 N	River Imphal
63			Stripped Snakehead	*Channa striata*	93.94 E 24.781 N	River Imphal

4.1.1.4 OTHER ORDERS

4.1.1.4.1 FAMILY MASTACEMBELIDAE

A total of 4 specimen (among our studied fishes) fall under this family. The species were traded with a generic name of eels like Tire track eel, spiny eel, etc which was first hand identified to their respective species based on available literature. The diagnostics keys to each of the identified species is described in Appendix 2.

4.1.1.4.1.1 Barred Spiny eel

The specimen Barred Spiny eel (ID- SGBD-DOF39 and SGBD-DOF65) is identified to *Macrognathus pancalus* based on available taxonomic keys. Body is compressed eel like, devoid of tooth plate. Dorsal fin was observed to have 25 spines. The body is olive green on dorsal with many yellowish spots on flanks and also had dark brown vertical stripes to the posterior of the body. Caudal fin with numerous black spots was observed.

4.1.1.4.1.2 Tire track eel

The specimen Tire track eel (ID- SGBD-P and SGBD-DOF38) is identified to *Mastacembelus armatus*. Body is slender with rich brown in colour and zig-zag lines, sometimes connected to form network. A row of black spots is

also visible along the base of dorsal fin. Dorsal and anal fin broadly joined to caudal fin.

4.1.1.4.2 FAMILY NOTOPTERIDAE

A total of 4 specimens (among our studied fishes) fall under this family. The species were traded with a trade name of clown knifefish, bronze or grey feather back, etc which was first hand identified to their respective species based on available literature.

4.1.1.4.2.1 Clown knifefish

The specimen of clown knifefish (ID- SGBD-E1) is identified as *Chitala chitala*. The body is compressed maxilla extends considerably beyond the posterior edge of the eye, the colour of the body is coppery brown with 15 transverse silvery bars on the back which makes it easy identification from its other congeners.

4.1.1.4.2.2 Grey featherback and Bronze featherback

The specimen of Grey featherback (ID- SGBD-DOF37) and Bronze Featherback (ID- SGBD-F1, SGBD-F2) is identified as *Notopterus notopterus*. The body is oblonge in shape and compressed, mouth is moderate with maxilla extending to middle of the eye. The colour of the body is silvery with no transverse bars on the back.

4.1.1.4.3 FAMILY TETRAODONTIDAE

One specimen (among our studied fishes) fall under this family. The species were traded with a trade name of Ocellated pufferfish (ID- SGBD-DOF81) which was first hand identified to their respective species based on available literature.

Ocellated pufferfish

The specimen was identified to *Tetraodon cutcutia*. It had 11 dorsal fin rays, the body colour was olive green and yellowish at the flanks with dark

reticulated markings. A dark ocellus was observed just in front of the dorsal and anal fin which makes it easy to identify. The morphologic keys are described in Appendix 2.

Table 4.1.4 Vouchering of the ornamental fish samples (Other Orders) from Northeast India

Sl No	Order	Family	Trade name	Species Identified	Latitude Longitude	Drainage
64	Synbranchif-ormes	Mastacembelidae	Barred Spiny eel	*Macrognathus pancalus*	92.45 E 24.5 N	River Sunai
65			Tire track eel	*Mastacembelus armatus*	93.889E 24.616N, 92.776E 25.033 N	Imphal River, River Jatinga
66	Osteoglossif-ormes	Notopteridae	Clown knifefish	*Chitala chitala*	93.953 E 24.847 N	Imphal river
67			Grey featherback	*Notopterus notopterus*	93.101 E 24.779 N	River Jiri
68			Bronze Featherback	*Notopterus notopterus*	93.101 E 24.779 N	River Jiri
69	Tetraodo-ntiformes	Tetraodontidae	Ocellated pufferfish	*Tetraodon cutcutia*	92.37 E 24.49 N	River Sunai

4.1.2. CHECKLIST OF THE ORNAMENTAL FISH

The morphological features helped us to first hand identify, the ornamental fish resources of Northeast India and the checklist of the samples under study was prepared and presented in Table 4.1.1-4.1.4 that demonstrated the specimen belonging to 53 species within 38 genus and 18 families, however bewilderment with regard to accurate species status exists in few cases and it was sought to identified the species with combined approach of morphology and molecular study (detailed in Appendix 2).

CHAPTER 4.2
SPECIES SPECIFIC GENETIC MARKER AS DNA BARCODE SEQUENCES

4.2.1 GENOMIC DNA

The extracted genomic DNA was of high molecular weight (~10-20kb). The concentration of the DNA were high and revealed purity in the range of 1.45 – 1.7 in terms of ratio of the absorbance at 260/280 nm in UV-VIS Spectrophotometer.

4.2.1.1 FROM BLOOD

The concentration of the DNA were in the range of 400 – 500 ng/µl that revealed purity in the range of 1.65 – 1.7 in terms of ratio of the absorbance at 260/280 nm. The blood samples showed relatively ease in terms of digestion and cell lysis in comparison to tissue samples. Furthermore, the integrity of the genomic DNA extracted from blood samples in most cases were intact and therefore were less degraded. Few representative of genomic DNA extracted from blood were exhibited in Figure 4.2.1.

4.2.1.2 TISSUE

The extracted genomic DNA was of high molecular weight (~20 kb). The concentration of the DNA were in the range of 400 – 2300 ng/µl that revealed purity in the range of 1.45 – 1.60 in terms of ratio of the absorbance at 260/280 nm. The tissue samples showed relatively difficult in terms of digestion and cell lysis and required fine homogenization. Furthermore, the genomic DNA extracted from tissue samples in most cases were showed smear, which may be primarily due to hydrodynamic stress and long term treatment of samples taken during the extraction process. Few representatives of genomic DNA extracted from blood, tissue and preserved samples were exhibited in Figure 4.2.1.

4.2.1.3 FIN

The genomic DNA extracted was of high molecular weight (~10-20 kb). The concentration of the DNA were in the range of 400 – 2300 ng/µl that revealed

purity in the range of 1.45 – 1.60 in terms of ratio of the absorbance at 260/280 nm. The tissue samples showed relatively difficult in terms of digestion and cell lysis and required fine homogenization. Furthermore, the genomic DNA extracted from tissue samples in most cases were showed smear, which may be primarily due to hydrodynamic stress and long term treatment of samples taken during the extraction process. Few representatives of genomic DNA extracted from blood, tissue and preserved samples were exhibited in Figure 4.2.1.

4.2.2 UV-VIS SPECTROPHOTOMETRIC DETERMINATION OF GENOMIC DNA

Optical densities (OD) were measured at in UV spectrophotometer (Biophotometer, Eppendrof) against nuclease free water as blank. The yield and purity of DNA samples were estimated from the OD values which are tabulated as follows **-**

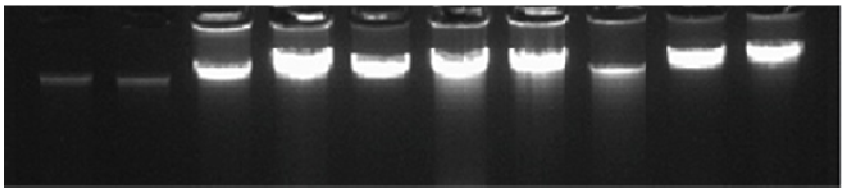
Table: 4.2.1 Concentration of different representative genomic DNA isolated from ornamental fish sample

Sample	Concentration (ng/ul)	Sample	Concentration (ng/ul)	Sample	Concentration (ng/ul)
SGBD-DOF49	1790	SGBD-DOF41	1190	SGBD-C2	574
SGBD-DOF50	1131	SGBD-BOF54	731	SGBD-C1	698
SGMC-MOF6	956	SGBD-BOF57	966	SGBD-D3F	1267
SGBK-OF31F	559	SGBD-DOF83	759	SGBD-D2F	872
SGBD-DOF16	2290	SGBD-DOF119	1290	SGBD-DOF64	598
SGBD-DOF15	1800	SGBD-DOF121	1900	SGBD-DOF63	1567
SGBD-B3	1560	SGBD-Z1	1056	SGBD-E1F	884
SGBD-B1	1323	SGBD-DOF123	923	SGMC-MOF18	627
SGMC-MOF2	1110	SGBD-DOF122	711	SGBD-DOF4	878
SGBD-DOF72	1662	SGBD-DOF97	762	SGMC-MOF5	671
SGBK-OF29F	1588	SGBD-BOF11F	788	SGBD-BOF50	879
SGBD-DOF73	1445	SGBD-BOF10F	652	SGBD-BOF51	561
SGBD-DOF89	1313	SGBD-DOF68	363	SGBD-BOF49	543
SGBK-BF8	535	SGBD-BOF35F	878	SGBD-DOF62	899
SGBD-BOF23	1364	SGBD-BOF19F	1236	SGBD-BOF15	764
SGBD-BOF56	1118	SGBD-DOF117	618	SGBD-BOF36	1608
SGBD-BOF55	439	SGBD-DOF112	849	SGBD-BOF45	849
SGBD-BOF53	1987	SGBD-DOF116	1987	SGBD-BOF44	992
SGBD-DOF40	2334	SGBD-DOF118	1214	SGBD-BOF43	2014

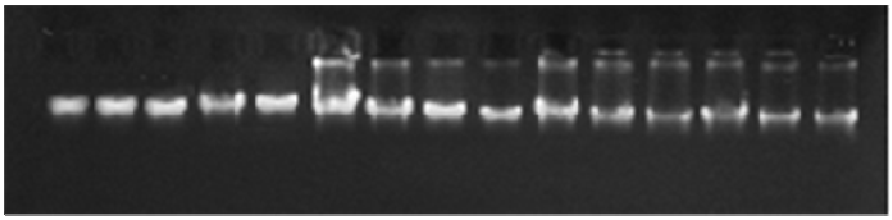
SGBD-BOF12	889	SGBD-H1	589	SGBD-DOF75	785
SGBD-Y3F	723	SGBD-H2	752	SGBK-BF6	962
SGBD-N2	1181	SGMC-MOF28	1281	SGBD-F2	1281
SGBD-N1	2145	SGMC-MOF35	2040	SGBD-F1	1132
SGBD-J1	890	SGMC-MOF33	491	SGBD-DOF37	1191
SGBD-J2	1298	SGMC-MOF30	1298	SGMC-MOF8	1308
SGBD-G2F	1156	SGMC-MOF29	1119	SGBD-M1	1129
SGBD-G1F	1023	SGMC-MOF34	1023	SGBD-DOF60	1023
SGBD-W3	1113	SGBK-DF2	1313	SGBD-DOF11	415
SGBD-W1	1211	SGBD-A1F	871	SGBD-DOF23	871
SGBD-W2	1161	SGBD-A5F	661	SGBD-DOF13	871
SGBD-BOF33	890	SGBD-A3F	903	SGBD-DOF12	910
SGBD-BOF31	1326	SGBD-A2F	826	SGBD-BCF24	926
SGBD-BOF30	945	SGBD-A4	645	SGBD-BCF23	545
SGBD-BOF34	1003	SGBK-DF3	703	SGBD-BCF22	813
SGBD-BOF32	776	SGBD-DOF65	1707	SGBD-BOF18	1659
SGBD-DOF47	1298	SGBD-DOF39	1007	SGBD-DOF82	1007
SGBD-DOF45	1108	SGBD-P	1217	SGBD-DOF25	1178
SGBK-OF16F	987	SGBD-DOF38	579	SGBK-AUFO5	560
SGBD-DOF46	1219	SGMC-MOF9F	769	SGBD-DOF26	959
SGBD-DOF48	1182	SGMC-MOF3F	882	SGBD-S2	772
SGBD-DOF95	853	SGBD-DOF74	953	SGBD-S1	667

4.2.3 PCR AMPLIFICATION OF *CO1* DNA BARCODE FRAGMENT

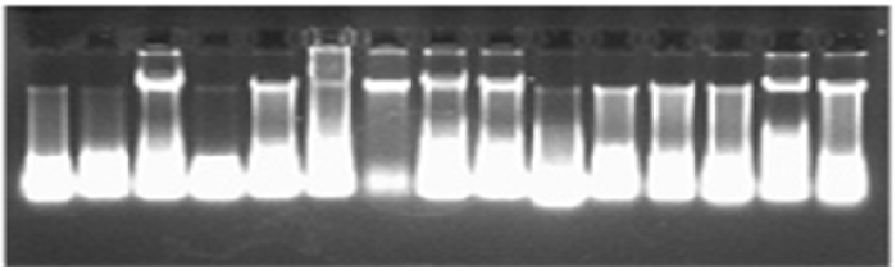
The primer pair mentioned in the methodology for amplification of full-length (655 bp) barcode region led successful PCR amplification in all the events. A single uniform band of length 655 bp was amplified that carried traces of primers that migrated further by passing the PCR products. After purification, none of the PCR amplicons got degraded and therefore no smearing observed. Likewise, there were no traces of primers on the purified PCR products that are indispensable for getting good sequencing results. The amplified PCR products were shown in Figure 4.2.2.



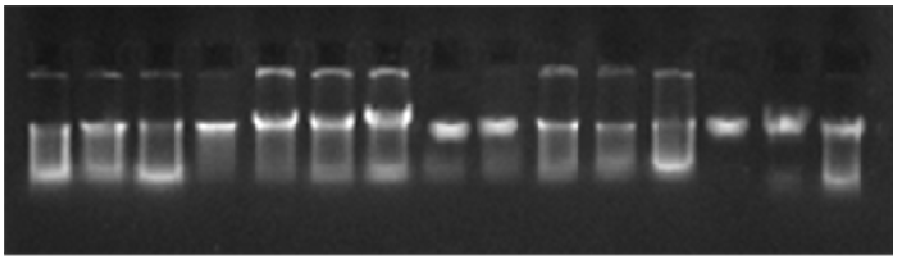
(a)



(b)



(c)



(d)

Figure 4.2.1 Few representatives Gel electropherogram of genomic DNA extracted from Fish blood samples (fig a,b), tissue sample (fig c) and Fin (fig d)

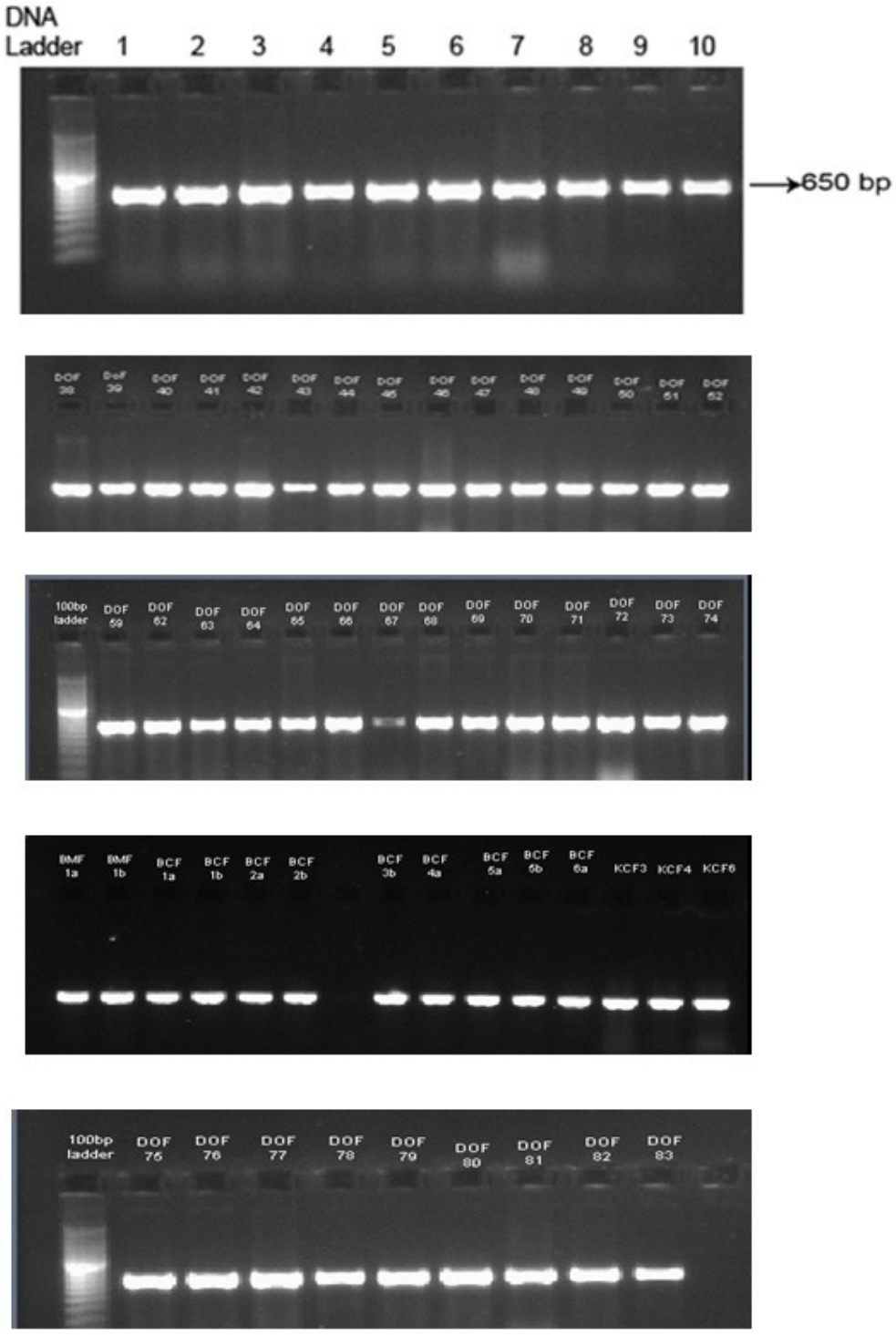


Figure 4.2.2 Lane 1 – 37 contains representative *CO1* PCR amplicons of ornamental fish samples which are of 655 bp length, as been confirmed by comparison with standard 100 bp DNA Ladder on 1.5% agarose gel

4.2.4 DNA SEQUENCING BY AUTOMATED DNA SEQUENCER

The sequence data output were recorded in terms of 4 colour peaks (for 4 nucleotides A, T, G and C) called sequence chromatograms in .abi format. The chromatogram is interpreted into respective DNA sequences in Applied Biosystem Seq Scape and ABI 3500 Data collection Software.

4.2.4.1 RAW SEQUENCES ANALYSIS

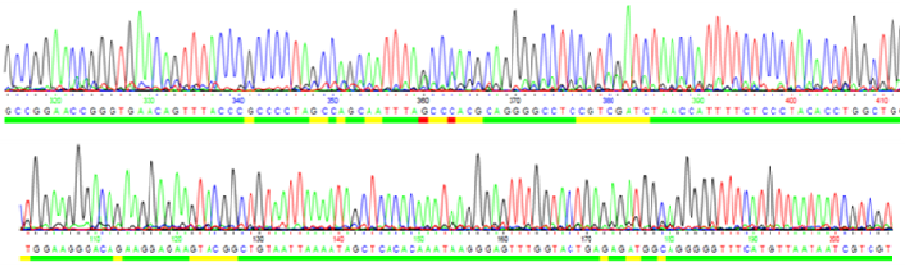
The sequencing results were obtained in the course of two chromatograms for each sample; one for the forward strand and another for the reverse strand. The software Sequence Scanner (Applied Biosystems, USA) outputted the chromatograms in the shape of the original sequence. The Quality for basecall of each of the sequences were checked with SeqScanner and found to be in the score of 40-50 QV (Quality Value) in all the cases, which confirmed that the sequences being 99.99% accurate. The Peak quality (height, breadth, etc) of the sequences chromatogram were checked position by position using BioEdit (Ibis Biosciences, Carlsbad; USA) software and were found to be clean and without any background noise which were then confirmed with the reference library dataset interms of querry coverage and % identity and found to be highly accurate.

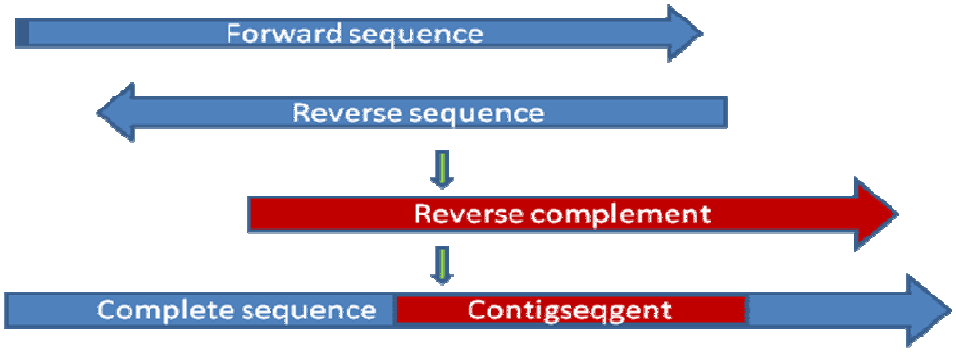
Furthermore, the accuracy of the sequences was confirmed by the amino acid sequences of the gene by NCBI ORF FINDER.

4.2.4.2 FINAL SEQUENCES AFTER ANNOTATION

As both the forward and reverse sequences represented the same gene location of the same sample, the reverse sequence being transformed in the reverse complement form (transformation of sequence of a particular strand into reverse complement form brings it in the format of a sequence of the same location of the other strand with which the sequence is complementary) and match with the forward sequence revealed 100% alignment with out any gap or index. This 100% aligned sequence was studied the last sequence and its transformation and protein BLAST result revealed 100% homology with a partial amino acid array of the mitochondrial *CO1* gene, that confirmed the sequences being correct.

Figure 4.2.3 was provided as an example of raw sequence annotation of the sample SGBD-DOF8.





>DOF11 (612 bp)Ready
GGCACAGCTTTAAGCCTTCTTATCCGAGCAGAACTTAGCCAACCCGGCGCCCTTCTAGG
GGACGACCAGATCTACAATGTTATTGTTACGGCCCACGCCTTTGTAATAATTTTCTTTA
TAGTAATACCAATCATGATTGGAGGCTTTGGAAACTGATTAATTCCACTAATGATCGGA
GCCCCCGATATAGCATTTCCTCGAATGAATAATATGAGCTTCTGGCTCTTACCCCCTTC
CTTTCTCTTACTTTTAGCCTCTTCAGGTGTCGAAGCTGGGGCTGGGACTGGCTGAACAG
TCTACCCTCCGTTAGCCGGAAACCTTGCCCACGCTGGAGCATCCGTTGACTTAACTATT
TTTTCACTTCATTTAGCAGGGGTCTCGTCAATTCTAGGAGCAATTAATTTTATTACTAC
TATTATTAATATGAAACCTCCCGCAGTCTCAATATATCAAATTCCCCTCTTTGTTTGAG
CTGTCCTAATTACGGCCGTCCTTCTTCTCCTATCTCTCCCAGTCTTAGCCGCCGGCATT
ACAATGCTTTTAACTGACCGAAACCTGAACACTGCATTCTTTGACCCGGCAGGAGGTGG
AGACCCCATCCTTTATCAGCACTTATTCTGA

>DOF11| Partial Protein Sequence 1 ORF:1..606 Frame +1
MVGTALSLLIRAELAQPGALLGDDQIYNVILTAHAFVMIFFMVMPIMIGGFGNWLVPLM
IGAPDMAFPRMNNMSFWLLPPSFLLLLASSGVEAGAGTGWTVYPPLAGNLAHAGASVDL
TIFSLHLAGVSSILGAINFITTIINMKPPAISQYQTPLFVWAVLITAVLLLLSLPVLAA
GITMLLTDRNLNTTFFDPAGGGDPH

Figure 4.2.3 Example of sequence editing. Sequences are shown as raw data's in the form of peaks above. The red and blue peaks represents Thymine and Cytosine respectively while the black and green represents Guanine and Adenine respectively.

4.2.5 BARCODE SEQUENCE CHARACTERISTICS

Both the PCR amplified products and their corresponding DNA sequences were larger than 600 bp and without any indels and coherent amino acid codes with a partial fragment of mitochondrial *CO1* gene confirmed the sequences being correct and assured that no NUMTs being amplified. After trimming of noisy ends of the sequences more than 600 bp product were yielded in each case. The BLASTN result of sequence alignment showed 100% alignment with the partial coding sequence of fish mitochondrial *CO1* gene with no gap or indels (insertion/deletions). In most instances, the sequence quality values were above 40. Furthermore, all the sequences aligned with ClustalX2 also showed no indels among themselves. The sequence translation revealed coherent partial amino acids with fish mitochondrial CO1 protein without any stop codon. Thus, it was affirmed that the generated sequences were fragments of mitochondrial *CO1* gene.

4.2.6 SEQUENCES SUBMITTED IN GENBANK AND BOLD

All the annotated sequences were finalized and submitted to GenBank by the name as identified through morphological identification mentioned in the first chapter of Result were found correct and obtained valid accession numbers. The sequences were also submitted to BOLD under the BOLD project console having code name 'OFISH' Figure 4.2.4 also found the sequences being correct and stored all the sequences and assigned process ID's for each of the samples. The details of BOLD project, which stores the sequences, were given in Table 4.2.2-4.2.5. As stated in the previous chapter, the collected ornamental fishes are categorized into 6 orderds as per the available checklist and related morphological parapeters. The barcode sequences generated of the specimens are submitted under the respective families which are the following

ORDER CYPRINIFORMES:

A total of 67 specimens belonging to 23 species identified under this order are submitted in the global database. The accession details of the specimens are

summarized in the following table

Table 4.2.2 Barcode sequence generated and Submitted from order Cypriniformes in Global database as state-of-arts and Strengthening of Global databases -Genbank, NCBI and BOLD.

Sl No	Order	Name of the Ornamental fish	Sequences generated	
			BOLD Process ID	(Genbank Acc. No) (size in bp)
1	Cypriniformes	Pool barb	OFISH001-12	JN815267 (654bp)
2		Spotfin barb	OFISH064-12	JQ713844 (624 bp)
3		Green Swamp barb	OFISH039-12, OFISH060-12	JN815309 (639 bp), JQ713852 (655 bp)
4		Swamp barb	OFISH014-12, OFISH015-12	JN815285 (620 bp), JN815286 (624 bp)
5		Barb	OFISH101-13, OFISH100-13, OFISH102-13	KF511528 (625 bp), KF511529 (625 bp), KF511530 (625 bp)
6.		Silver barb	OFISH056-12	JQ713846 (655 bp)
7.		Flying barb	OFISH078-13, OFISH077-13, OFISH076-13	JN673955 (655 bp), KF511504 (622 bp), KF511505 (622 bp), KF511506 (622 bp)
8.		Slender rasbora	-	
9.		Minor carp	OFISH136-13, OFISH134-13, OFISH135-13, OFISH137-13, OFISH138-13, OFISH139-13	KF511569 (620 bp), KF511570 (620 bp), KF511571 (620 bp), KF511572 (620 bp), KF511573 (620 bp), KF511574 (620 bp)
10		Labeo	OFISH057-12	JQ713848 (624 bp)
11		Miniscale shark	OFISH058-12	JQ713849 (576 bp)
12		Kalabans	OFISH103-13, OFISH107-13, OFISH106-13, OFISH105-13, OFISH104-13	KF511531 (620 bp), KF511532 (624 bp), KF511533 (624 bp), KF511534 (624 bp), KF511535 (626 bp)
13		Giant Danio	OFISH071-13, OFISH070-13, OFISH069-13	KF511497 (628 bp), KF511498 (628 bp), KF511499 (626 bp)
14		Turquoise Danio	OFISH052-12	JX105479 (624 bp)

15		Gangetic latia	OFISH027-12, OFISH074-13, OFISH072-13, OFISH073-13	JN815299 (626 bp), JX105481 (618 bp), KF511500 (624 bp), KF511501 (624 bp), KF511502 (627 bp)
16		Indian Hill trout	OFISH053-12, OFISH121-13, OFISH120-13, OFISH119-13, OFISH019-12, OFISH122-13, OFISH123-13	KF511547 (629 bp), JN815290 (661 bp), JN815291 (660 bp), KF511548 (629 bp), KF511549 (628 bp) KF511550 (624 bp), KF511551 (624 bp)
17		Garra	OFISH111-13, OFISH112-13, OFISH109-13, OFISH110-13, OFISH113-13	KF511537 (629 bp), KF511538 (616 bp), KF511539 (631 bp), KF511540 (620 bp), KF511541 (557 bp)
18		Kingfish	OFISH129-13, OFISH108-13	KF511536 (617 bp), KF511557 (565 bp)
19		Molacarplet	OFISH010-12	JN815277 (617 bp)
20		Pale carplet	OFISH011-12	JN815278 (600 bp)
21		Neon Hatchet	OFISH028-12, OFISH029-12	JN815300 (631 bp), JN815301 (634 bp)
22		Torrent stone carp	OFISH132-13	KF511560 (594 bp)
23		Queen loach	OFISH048-12, OFISH049-12, OFISH050-12, OFISH128-13,	JX105468 (627 bp), JX105475 (633 bp), JX105478 (618 bp), KF511556 (620 bp)
24		Bengal loach	OFISH042-12, OFISH051-12	JX105476 (637 bp), JX105477 (618 bp)
25		Ladder loach	OFISH126-13	KF511554 (568 bp)
26		Gangetic loach	OFISH127-13	KF511555 (568 bp)
27		Moosefaced loach	OFISH032-12	JX105467 (565 bp)
28		Zipper loach	OFISH130-13	KF511558 (601 bp)
29		Striped loach	OFISH131-13	KF511559 (588 bp)

ORDER SILURIFORMES:

14 species identified under this order are submitted in the global database. The accession details of the specimens are summarized in the following table

Table 4.2.3 Barcode sequence generated and submitted from order Siluriformes in Global database as state-of-arts and Strengthening of Global databases - Genbank, NCBI and Barcode of Life Datasystems (BOLD).

Sl No	Order	Name of the Ornamental fish	Sequences generated	
			BOLD Process ID	(Genbank Acc. No) (size in bp)
1	Siluriformes	Striped dwarf catfish	OFISH142-13	KF511563 (620 bp)
2		Long Whiskers Catfish	OFISH143-13	KF511564 (620 bp)
3		River Catfish	OFISH095-13, OFISH096-13	KF511523 (629 bp), KF511524 (629 bp)
4		Gangetic ailia	OFISH145-13	KF511566 (620 bp)
5		Copper catfish	-	JN628914 (619bp)
6		Gagata	OFISH090-13, OFISH094-13, OFISH092-13, OFISH093-13, OFISH088-13, OFISH089-13	KF511520 (627 bp), KF511521 (627 bp), KF511522 (627 bp)
7		Gagata		KF511518 (624 bp)
8		Gagata		KF511516 (629 bp), KF511517 (629 bp), KF511553 (552 bp)
9		Indian Gagata	OFISH091-13	KF511519 (629 bp)
10		Indian Whiptail catfish	OFISH133-13	KF511561 (625 bp)
11		Suckerthroat	OFISH099-13, OFISH098-13, OFISH097-13	KF511525 (622 bp), KF511526 (622 bp), KF511527 (603 bp)
12		Giant Moth Catfish	-	JN628913 (619 bp)
14		Butter catfish	OFISH144-13	KF511565 (620 bp)
15		Two Stripe Gulper Catfish	OFISH116-13	KF511544 (629 bp)
16		Walking catfish	OFISH146-13	KF511567 (620 bp)

ORDER PERCIFORMES:

A total of 33 specimens representating 11 species identified under this order are submitted in the global database. The accession details of the specimens are summarized in the following table

Table 4.2.4 Barcode sequence generated and submitted from order Perciformess formes in Global database as state-of-arts and Strengthening of Global databases - Genbank, NCBI and Barcode of Life Datasystems (BOLD).

Sl No	Order	Name of the Ornamental fish	Sequences generated	
			BOLD Process ID	(Genbank Acc. No) (size in bp)
1	Perciformes	Glass fish	OFISH007-12, OFISH013-12, OFISH009-12, OFISH008-12	JN815274 (534bp), JN815275 (584 bp), JN815276 (607 bp), JN815283 (639 bp)
2		Mud perch	OFISH036-12, OFISH037-12,	JN815306 (643 bp), JN815307 (643 bp)
3		Leaf fish	OFISH055-12	JQ713845 (614 bp)
4		Corsula mullet	OFISH044-12	JX105471 (623 bp)
5		Tank goby	OFISH024-12, OFISH022-12, OFISH065-12, OFISH021-12, OFISH023-12	JN815293 (537 bp), JN815296 (623 bp), JQ713857 (632 bp), JN815294 (604 bp), JN815295 (619 bp)
6		Climbing perch	OFISH086-13	KF511514 (625 bp)
7		Climbing gourami	OFISH087-13	KF511515 (629 bp)
8		Blue Badis	OFISH034-12, OFISH035-12	JN815304 (631 bp), JN815305 (622 bp)
9		Chameleon Fish	OFISH04112, OFISH066-12	JN815311 (628 bp), JQ713858 (569 bp)
10		Bullseye snakehead	OFISH124-13	KF511552 (582 bp)
11		Walking snakehead	OFISH124-13, OFISH043-12	JN245991 (655 bp), JX105470 (623 bp)
12		Ceylone snakehead	OFISH046-12, OFISH045-12, OFISH047-12	JX105472 (635 bp), JX105473 (570 bp) JX105474 (637 bp)
13		Spotted snakehead	-	JN245992 (624 bp)
14		Green snakehead	-	JN245990 (655 bp)
15		Chekered snake fish	OFISH084-13, OFISH085-13	KF511512(622 bp), KF511513 (622 bp)
16		Black spot grouper	OFISH117-13	KF511545 (626 bp)
17		Snakehead murrel	-	JN245989 (655 bp)
18		Banded snakehead	OFISH079-13	KF511507 (622 bp)
19		Stripped Snakehead	OFISH080-13	KF511508 (655 bp)

OTHER ORDERS:

A total of 9 specimens covering 3 orders representing 4 species are submitted in the global database. The accession details of the specimens are summarized in the following table

Table 4.2.5 Barcode Sequence Generated and Submitted from order Synbranchiformes, Osteoglossiformes and Tetraodontiformes in Global database as state-of-arts and Strengthening of Global databases -Genbank, NCBI and Barcode of Life Datasystems (BOLD).

Sl No	Order	Name of the Ornamental fish	Sequences generated	
			BOLD Process ID	(Genbank Acc. No) (size in bp)
1	**Synbranchiformes**	Barred Spiny eel	OFISH030-12, OFISH018-12,	JX105465 (602 bp), JN815289 (631 bp)
2		Tire track eel	OFISH118-13, OFISH017-12	JN815288 (654 bp), KF511546 (627 bp)
3	**Osteoglossiformes**	Clown knifefish	OFISH083-13	KF511511 (558 bp)
4		Grey featherback	OFISH016-12	JN815287 (624 bp)
5		Bronze Featherback	OFISH082-13, OFISH081-13	KF511509 (619 bp), KF511510 (626 bp)
6	**Tetraodontiformes**	Ocellated pufferfish	OFISH038-12	JN815308 (637 bp)

Figure 4.2.4 BOLD sequence submission project summary page that contains details of the Ornamental fish samples and their corresponding sequences.

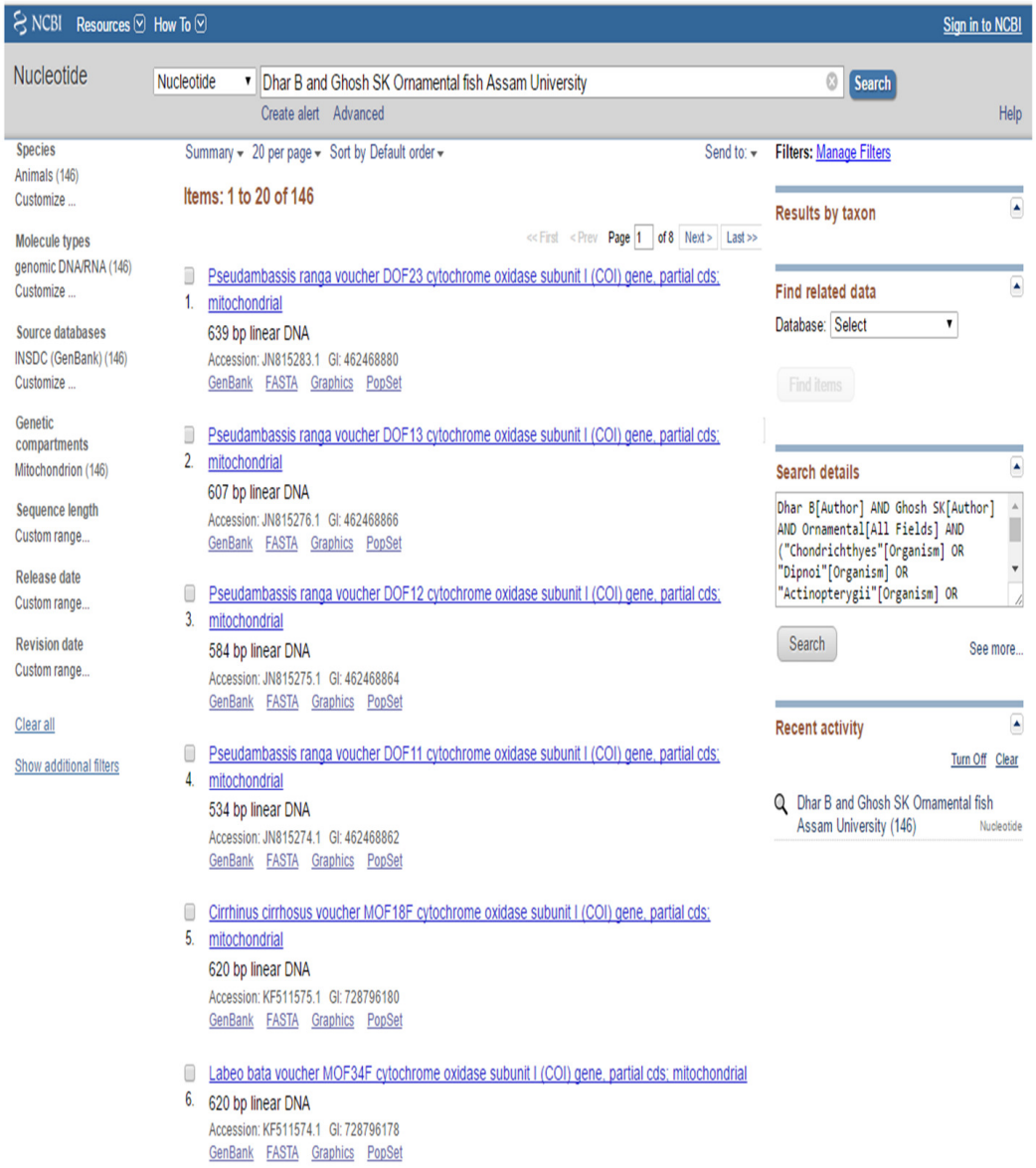


Figure 4.2.5 Sequence of the ornamental fish samples submitted in NCBI with their corresponding sequences.

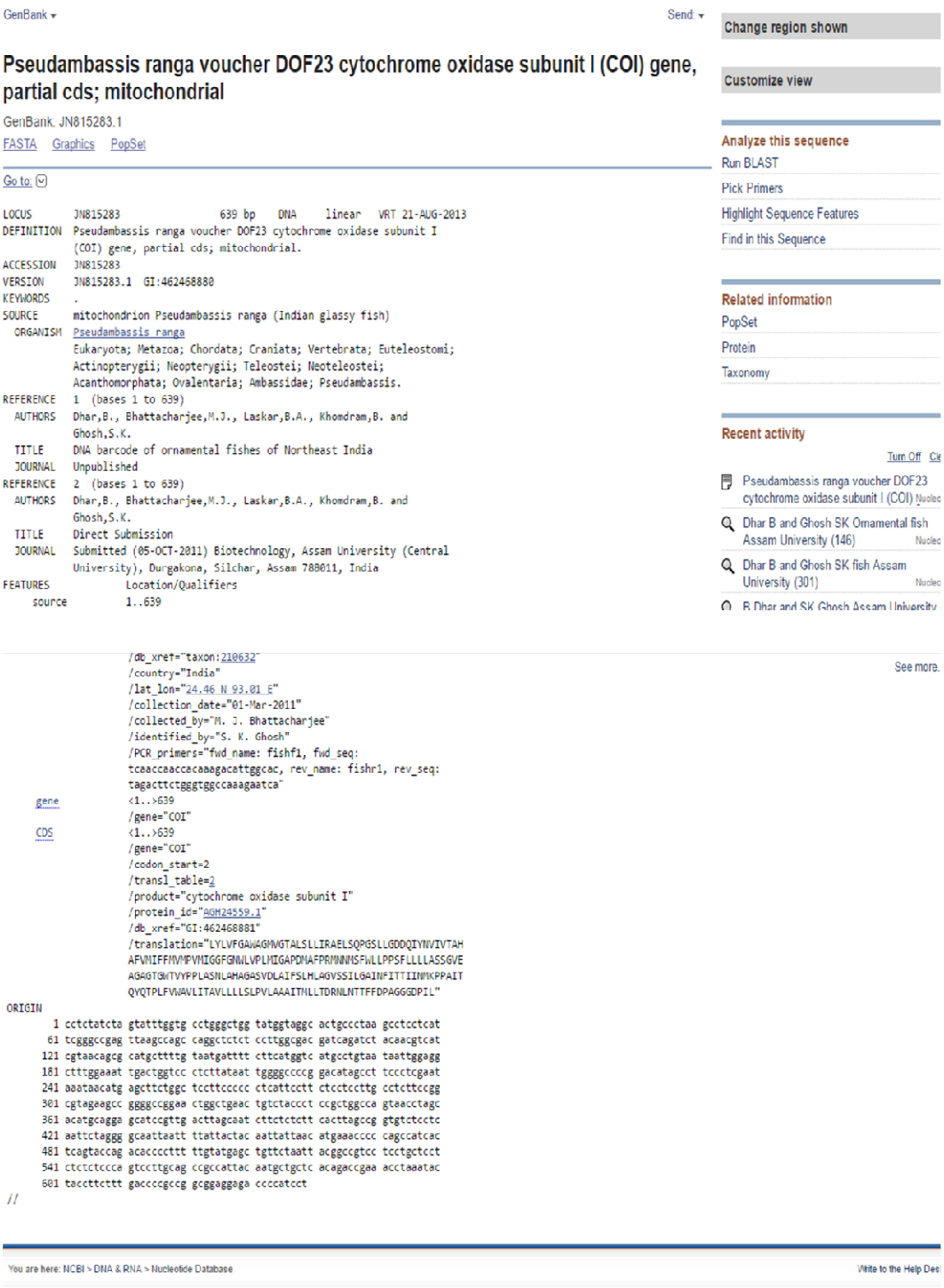


Figure 4.2.6 Representative of the Sequence pagein Genbank (NCBI) with the details of the sequence submitted

CHAPTER 4.3
DNA BARCODING APPROACH TO DETECT TRADED ORNAMENTAL FISH FROM NORTHEAST INDIA

4.3.1 DNA BARCODE BASED IDENTIFICATION

It was investigated, through DNA Barcoding and morphological assessment for the identification of 130 ornamental fish specimens exported from different exporters of North East India. The collected ornamental fishes were barcoded and submitted in the Genbank as well as in the BOLD under the project, "DNA barcoding of ornamental fishes of North-East India [OFISH]". The DNA barcode database provides a system of species identification based upon the finding of the closest match of the query sequences with database reference sequences. The extensive species identification based on the consensus of the similarity match on BOLD based on Species Level Barcode Records and Public Record Barcode Database as well as Genbank, for the studied ornamental fish specimens revealed straightforward identification for 86 specimens belonging to 37 species (Table 4.3.1) which are further confirmed by morphologic keys. Among them, the samples bearing single trade names like Gangetic latia, Neon Hatchet, Tank goby, Glass fish, Barred Spiny eel, Labeo, Miniscale shark, Flying barb, Silver barb and Corsula mullet, Giant Danio, Clown knifefish, Sucker throat, Indian Hill Trout**,** Indian Whiptail catfish, Long Whiskers Catfish, Striped dwarf catfish, Butter catfish, Gangetic ailia, Walking catfish, Gagata, River catfish, Turquoise Danio, Tire track eel, bullseye snakehead, etc. showed significant similarity with each of the distinct species. However, in a few instances, the samples with multiple trade names showed the closest match with a particular species. For example, all the sequences of Pool barb, Spot fin barb showed 97-99% similarity with *Puntius sophore,* Mola carplet, Pale carplet showed 99% similarity with *Amblypharyngodon mola,* Mud perch, Leaf perch showed 99% similarity with *Nandus nandus*, Blue badis, Chameleon fish showed 98-99% similarity with *Badis badis*, Spotted snakehead, Green snakehead fish or Chekered snake fish showed 99% similarity with *Chana punctatus,* Grey featherback*,* Bronze Featherback showed 99% similarity with *Notopterus*

notopterus, Climbing perch, Climbing gourami showed 98% similarity with *Anabas testudineus*. Some of the specimens showed a range of 96-99% similarity with the database sequence like Tire track eel with *Mastacembelus armatus* (96-97%), Snakehead murrel, Striped snakehead, Banded snakehead with *Channa striata* (96-99%), Walking snakehead, Ceylone snakehead with *Channa orientalis* (96-99%).

On the other hand few specimens could not be identified to respective species based on barcoding alone, those were solved and identified using morphology which are discussed in the later part under case study (Table 4.3.2). Comprehensive species identification of the studied ornamental fishes based on BOLD identification system and GenBank databases is depicted in Table 4.3.1.

Table 4.3.1 Straight forward identification of the studied ornamental fishes based on similarity match with BOLD Identification System and GenBank. The match is exclusively based on similarity of developed sequences with database sequences.

Fish specimen with trade name	Closest match with species in BOLD-IDS		Close match in Genbank (BLASTN Similarity in %)	Identified as species
	Species Level Barcode Records (Process ID)	Public Record Barcode Database (Process ID)		
Gangetic latia	*Crossocheilus latius* (ANGBF9951-12, ANGBF9956-12)	*Crossocheilus latius* (ANGBF9951-12, ANGBF9956-12)	*Crossocheilus latius (99)*	*Crossocheilus latius*
Neon Hatchet	*Chela cachius*	*Chela cachius* (GBGC4871-08)	*Chela cachius (97)*	*Chela cachius*
Tank goby	*Glossogobiusguiris*	No match	*No match (99)*	*Glossogobius guiris*
Glass fish	*Parambassis ranga*	No match	*Pseudoabassis ranga (99)*	*Parambassis ranga*
Barred Spiny eel	*Macrognathus pancalus*	*Macrognathus pancalus* (GBGC4234-08, GBGC4233-08)	*Macrognathus pancalus (99)*	*M. pancalus*
Labeo	*Labeocalbasu* (GBGC4252-08, ANGBF7333-12)	*Labeocalbasu* (GBGC4252-08, ANGBF7333-12)	*Labeo calbasu (100)*	*Labeo calbasu*
Flying barb	*Esomusdanricus*	*Esomusdanricus* (ANGBF6124-12, ANGBF6125-12)	*Esomus danricus(99)*	*Esomus danricus*
Silver barb	*Barbonymus gonionotus* (GBGC6590-09, GBGC6591-09, GBGC6592-09, ANGBF5730-12)	*Barbonymus gonionotus* (GBGC6590-09, GBGC6591-09, GBGC6592-09, ANGBF5730-12)	*B.gonionotus (100)*	*B. gonionotus*
Corsula mullet	*Rhinomugil corsula*	*Rhinomugil corsula*	*Rhinomugil corsula (98)*	*Rhinomugil corsula*

Giant Danio	*Devario aequipinnatus*	*Devario aequipinnatus* (RCYY280-11, RCYY334-11, ANGBF6126-12)	*Devario equipinnatus (98-99)*	*D. aequipinnatus*
Clown knifefish	*Chitala chitala* (ANGBF6042-12, ANGBF6132-12, ANGBF6043-12)	*Chitalachitala* (ANGBF6042-12, ANGBF6132-12, ANGBF6043-12	*Chitala chitala (99-100)*	*Chitala chitala*
Suckerthroat	*Pseudecheneis sulcata*	*Pseudecheneis sulcata* (GBGC8471-09)	*Pseudecheneis sulcata (99)*	*Pseudecheneis sulcata*
Indian Hill Trout	*Barilius bendelisis* (CYTC3711-12, CYTC4266-12)	*Barilius bendelisis* (CYTC3711-12, CYTC4266-12)	*Barilius bendelisis (100)*	*Barilius bendelisis*
Indian Whiptail catfish	*Sisor rabdophorus*	*Sisorrabdophorus* (CFISH028-12, CFISH027-12)	*Sisor rabdophorus (99)*	*Sisor rabdophorus*
Long Whiskers Catfish	*Mystus gulio*	*Mystus gulio* (GBGCA2888-13)	*Mystus gulio (99)*	*Mystus gulio*
Striped dwarf catfish	*Mystus vittatus*	*Mystus vittatus* (CFISH009-12)	*Mystus vittatus (99)*	*Mystus vittatus*
Giant Moth Catfish	*Erethistes pussilus*	*Erethistes pussilus*	*Erethistes pussilus (98)*	*Erethistes pussilus*
Copper catfish	*Glyptothorax telchitta*	*Glyptothorax telchitta*	*Glyptothorax telchitta (98)*	*Glyptothorax telchitta*
Butter catfish	*Ompokbimaculatus*	*Ompokbimaculatus*	*Ompokbimaculatus (98)*	*Ompok bimaculatus*
Gangetic ailia	*Ailia coila* (ANGBF6053-12, ANGBF6054-12, GBGC4011-08)	*Ailia coila* (ANGBF6053-12, ANGBF6054-12, GBGC4011-08)	*Ailia coila (100)*	*Ailia coila*
Walking catfish	*Clariasbatrachus*	*Clariasbatrachus* (ANGBF2196-12, , CFISH068-12)	*Clariasbatrachus (100)*	*Clariasbatrachus*
Gagata	*Gagata dolichonema* (GBGC9465-09)	*Gagata dolichonema* (GBGC9465-09)	*Gagata dolichonema (99)*	*Gagatad olichonema*
River Catfish	*Eutropiichthys murius* (CFISH012-12, CFISH013-12 DBFN023-11)	*Eutropiichthys murius* (CFISH012-12, CFISH013-12 DBFN023-11)	*Eutropiichthys murius (99-100)*	*E. murius*
Turquoise Danio	*Devario devario*	*Devario devario* (GBGC4896-08)	*Devario devario (100)*	*Devario devario*
Tire track eel	*Mastacembelusarmatus*	No match	*Mastacembelus armatus (96-97)*	*M. armatus*
Bullseye snakehead	*Channa marulius*	*Channa marulius* (DBFN124-11)	*Channa marulius (99)*	*Channa marulius*
Pool barb	*Puntius sophore*	*Puntius sophore* (RCYY481-11)	*Puntius sophore (97-99)*	*Puntius sophore*
Spot fin barb	*Puntius sophore*	*Puntius sophore* (RCYY481-11)	*Puntius sophore (97-99)*	*Puntius sophore*
Molacarplet	*Amblypharyngodon mola*	*Amblypharyngodon mola* (DBFN339-12)	*Amblypharyngodon mola (99)*	*Amblypharyngodo nmola*
Pale carplet	*Amblypharyngodon mola*	*Amblypharyngodon mola* (DBFN339-12)	*Amblypharyngodon mola (99)*	*Amblypharyngodo nmola*
Mud perch	*Nandus nandus* (DBFN050-11)	*Nandus nandus* (DBFN050-11)	*No match*	*Nandus nandus*

Leaf fish	*Nandus nandus* (DBFN050-11)	*Nandus nandus* (DBFN050-11)	*Nandus nandus*	*Nandus nandus*
Spotted snakehead	*Channa punctatus*	*Channa punctatus* (DSCHA078-13, ANGBF2441-12)	*Channa punctatus (99)*	*Channa punctatus*
Green snakehead	*Channa punctatus*	*Channa punctatus* (DSCHA078-13, ANGBF2441-12)	*Channa punctatus (99)*	*Channa punctatus*
Chekered snake fish	*Channa punctatus*	*Channa punctatus* (DSCHA078-13, ANGBF2441-12)	*Channa punctatus (99)*	*Channa punctatus*
Blue Badis	*Badis badis*	*Badis badis* (ANGBF6048-12, ANGBF6138-12)	*Badis badis (98-99)*	*Badis badis*
Chameleon Fish	*Badis badis*	*Badis badis* (ANGBF6048-12, ANGBF6138-12)	*Badis badis (98-99)*	*Badisbadis*
Grey featherback	*Notopterus notopterus* (ANGBF6016-12)	*Notopterus notopterus* (ANGBF6016-12)	*Notopterus notopterus (99)*	*N. notopterus*
Bronze Featherback	*Notopterus notopterus* (ANGBF6016-12)	*Notopterus notopterus* (ANGBF6016-12)	*Notopterus notopterus (99)*	*N. notopterus*
Climbing perch	*Anabas testudineus*	No match	*Anabas testudineus (98)*	*Anabas testudineus*
Climbing gourami	*Anabas testudineus*	No match	*Anabas testudineus (98)*	*Anabas testudineus*
Barb	*Puntius manipurensis*	*Puntius manipurensis* (RCYY470-11, RCYY471-11)	*Puntius padamya (97)*	*Puntius manipurensis*
Snakehead murrel	*Channa striata*	*Channa striata* (ANGBF2417-12, ANGBF2419-12)	*Channa striata (96-99)*	*Channa striata*
Banded snakehead	*Channa striata*	*Channa striata* (ANGBF2417-12, ANGBF2419-12)	*Channa striata (96-99)*	*Channa striata*
Striped snakehead	*Channa striata*	*Channa striata* (ANGBF2417-12, ANGBF2419-12)	*Channa striata*	*Channa striata*
Walking snakehead	*Channa orientalis*	*Channa orientalis* (DSCHA009-07, ANGBF2437-12)	*Channa orientalis (96-99)*	*Channa orientalis*
Ceylone snakehead	*Channa orientalis*	*Channa orientalis* (DSCHA009-07, ANGBF2437-12)	*Channa orientalis (96-99)*	*Channa orientalis*
Ocellated pufferfish	*Tetraodon cutcutia* (GBGCA5150-13, CYTC3695-120)	*Tetraodon cutcutia* (GBGCA5150-13, CYTC4343-12)	*Tetraodon cutcutia (100)*	*Tetraodon cutcutia*

The remaining specimens could not be identified to species based on barcoding alone and showed contradictory results in the respective database and grouped as Category 1 for the 12 specimens who could not be distinguished between several highly similar species. The Category 2 for the leftover 32 specimens which do not have pre-existing conspecific sequences in the database with which similarity match is to be made was presented in Table 4.3.2.

Table 4.3.2 Confused species status of the studied ornamental fishes based on similarity match with the database. The developed sequences of the specimen that revealed similarity with the sequences of multiple species in BOLD-IDS and Genbank as Category 1 and the remaining samples with the lack of conspecific sequences in the database are grouped as Category 2. Further analyzed with Morphology, NJ clustering approach and K2P distance based method for confirmation of those species.

Specimen with trade name	Sequences generated (Genbank Acc. No)	Close match in with species in BOLD-IDS		Close match in Genbank (BLASTN Similarity %)
		Species Level Barcode Records (Process ID)	Public Record Barcode Database (ProcessID)	
Green Swamp barb	JN815309 , JQ713852	*Puntius conchonius* *P. chola*	*Puntius conchonius* (RCYY532-11) *P. chola* (SRFBI033-11)	*Puntius conchonius (99)* *P. chola (99)* *P. fraseri (98)*
Swamp barb	JN815285, JN815286	*Puntius conchonius* *P. chola*	*Puntius conchonius* (RCYY532-11) *P. chola* (SRFBI033-11)	*No match (0)*
Gagata	KF511518	*Gagata cenia, G. gagata, Nemapteryxmacron otacantha*	*Gagata cenia (*CFISH035-12,CFISH037-12, GBGC9466-09*), G. gagata* (ANGBF6120-12)	*Gagata cenia (99-100)*
Indian Gagata	KF511519	*Gagata cenia, G. gagata, Nemapteryx macronotacantha*	*Gagata cenia (*CFISH035-12,CFISH037-12, GBGC9466-09*), G. gagata* (ANGBF6120-12)	*Gagata cenia (99-100)*
Miniscale shark	JQ713849	*Labeo gonius, L. fimbriatus*	*Labeo gonius* (GBGC4209-08, GBGC4210-08 *L. fimbriatus* (GBGCA2387-13)	*Labeo gonius (99-100)*
Minor carp	KF511569, KF511570, KF511571, KF511572, KF511573, KF511574	*Labeo bata, Labeo boga*	*Labeo bata, Labeo boga*	*Labeo bata (100), Labeo boga (100)*
Queen loach	JX105468, JX105475, JX105478, KF511556	No match	No match	*Botia almorhae (99)*
Bengal loach	JX105476 , JX105477	No match	No match	*Botia almorhae (99)*
Moosefaced Loach	JX105467	No match	No match	*Canthophrys gongota (96)*

Gagata	KF511516, KF511517, KF511553	No match	No match	*Gogangra viridescens (97)*
Kalabans	KF511531, KF511532 , KF511533, KF511534, KF511535	No match	No match	*Bangana sp (100)*
Kingfish	KF511536 , KF511557	No match	No match	*Capoeta antalyensis (91), Schizothorax sinensis (91)*
Garra	KF511537, KF511538, KF511539, KF511540, KF511541	No match	No match	*Garra tengchongensis, Garra orientalis (92)*
Two Stripe Gulper Catfish	KF511544	No match	No match	*Ompok bimaculatus(95)*
Black spot grouper	KF511545	No match	No match	*Channa aurantimaculata (93)*
Indian Hill trout	KF511550, KF511551	No match	No match	*Raiamas bola (87)*
Ladder loach	KF511554	No match	No match	*Botia dario (92)*
Gangetic loach	KF511555	No match	No match	*Botia dario (92)*
Zipper Loach	KF511558	No match	No match	*Acanthocobitis botia (87)*
Striped Loach	KF511559	No match	No match	*Acanthocobitis botia (87)*
Torrent stone carp	KF511560	No match	No match	*Psilorhynchus homaloptera (98)*

4.3.1.1 CATEGORY 1: HIGHER-LEVEL SIMILARITY WITH MULTIPLE SPECIES

The 13 specimens under six trade names in the category 1 were identified into four species, where the specimens of Swamp barb, Green Swamp barb were identified to be *Puntius chola* (Hamilton 1822, Vishwanath and Laisram 2004), Gagata and Indian Gagata were identified to be *Gagata cenia* (Figure 4.3.1.A1) (Hamilton 1822, Roberts and Ferraris 1998) and Minor carp, Miniscale shark was identified as *Labeo bata* and *L. Gonius* respectively (Talwar and Jhingran 1992).

4.3.1.2 CATEGORY 2: NEWLY GENERATED SEQUENCES

The remaining 31 specimens under category 2 with 15 trade names which are the newly generated sequences were identified to 12 species. The specimens, like Gagata is identified to *Gogangra viridescens* (Figure 4.3.1. A2). The dorsal fin

rays were 6; Pectoral fin rays were 8; Anal fin rays were 8 and most importantly, outer and inner mental barbles were found widely separated from the origin of inner barbles anterior to the origin of the outer barbles while the maxillary barble extending to the pectoral fin base (Roberts and Ferraris 1998). Queen loach, Bengal Loach was identified to be *Botia dario*. The specimen had 7 vertical bars across the body with narrower interspaces (Figure 4.3.1. B1) and lacks any distinct markings on the interspaces unlike *Botia udomritthiruji*, a species described from Southern Myanmar, which has 5 vertical bar and have markings on the interspaces. Two specimens viz. Ladder loach and Gangetic loach were identified to be *Botia rostrata* by morphological parameters (HEOK HEE 2007). The studied specimens had dorsal fin with i9 rays, Anal fin with i5 rays, Pecrtoral fin rays was ii10; Pelvic fin with i7 rays; Caudal fin rays is found to be i,9+8,i, Lateral line scale is complete, there are pale spots within the dark vertical bars and all the bars are interconnected like a network (Figure 4.3.1. B2). The specimens of Garra are identified to be *Garra arupi* (Figure 4.3.1. C), a new species described from Arunachal Pradesh of India (Nebeshwar et al. 2009), Kingfish is identified as *Semiplotus semiplotus*, the meristic counts revealed 25 Dorsal soft rays, Anal spines were 2, Anal soft rays were 7, the last simple dorsal fin ray spine was not serrated and transverse row of 10-12 open pores (5-6 on each side) across the snout directed posterior toward the middle of orbit were observed (Figure 4.3.1. D) (Vishwanath and Kosygin 2000). In case of Indian Hill trout, the morphological parameters were recorded and found to be within the range of description, among the other characters the barbles were absent (Figure 4.3.1. E1, E2), which confirmed our specimens to be *Barilius barna* (Dishma and Vishwanath 2012). Black spot grouper is analyzed by morphology and identified as *Channa melanostigma*, a new snakead reported from NE India as compared with original description (Geetakumari and Vishwanath 2011). Similarly, Zipper Loach, Striped loach is identified as *Acanthocobitis botia* (Kottelat 1990), Two Stripe Gulper Catfish is identified as *Ompok pabda* (Hamilton 1822, Talwar and Jhingran 1992), Torrent stone carp as *Psilorhynchus homaloptera* based on described morphologic keys (Shangningam et al. 2013), Moosefaced Loach is identified as *Canthophrys gongota* based on original description (Hamilton 1822),

the specimen of Kalabans is recognised as *Labeo dero* (Hamilton 1822, Talwar and Jhingran 1992) the morphological characters were detailed in Appendix 2.

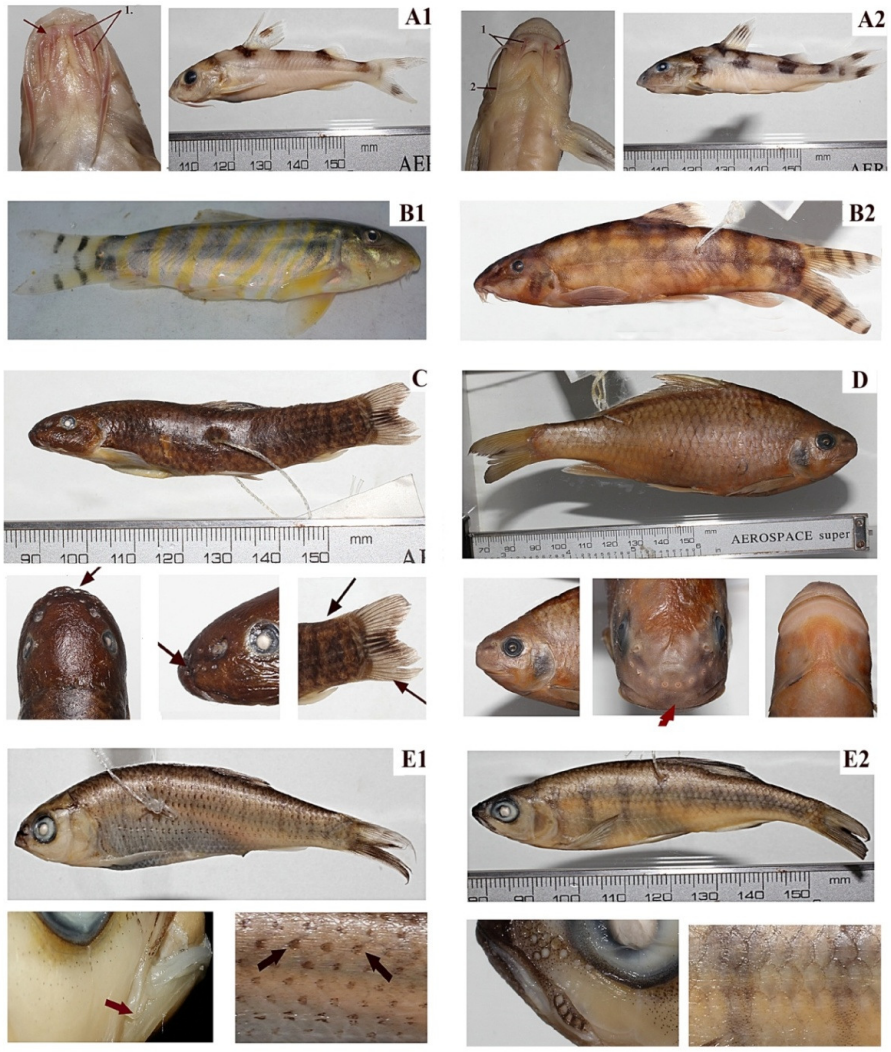


Figure 4.3.1 A1 showing *Gagata cenia* and **A2** *Gogangraviridiscens*, 1. Inner and outer mental barbles, 2. Maxillary barbles. *Gogangra viridiscens*is identified by the presence of both the mental barbles that were widely separated from the origin as shown by an arrow, is distinguished from *G. cenia*. **B1** showing *Botia dario* and **B2** *Botia rostrata.* **C** showing *Garra arupi* with transverse row of tubercles on the snout, stripes on caudal peduncle and absence of W- shaped band on caudal fin as showed by an arrow. **D** showing *Semiplotus semiplotus* with transverse row of 10-12 open pores across the snout. **E1** is illustrating *Barilius bendelisis* and **E2** *B. barna.* Among the diagnostic characters observed were the presence of barbles, black spot on the scales in *B. bendelisis* while it is absent in *B. barna* as shown by an arrow.

4.3.2 NEIGHBOUR-JOINING AND K2P DISTANCE

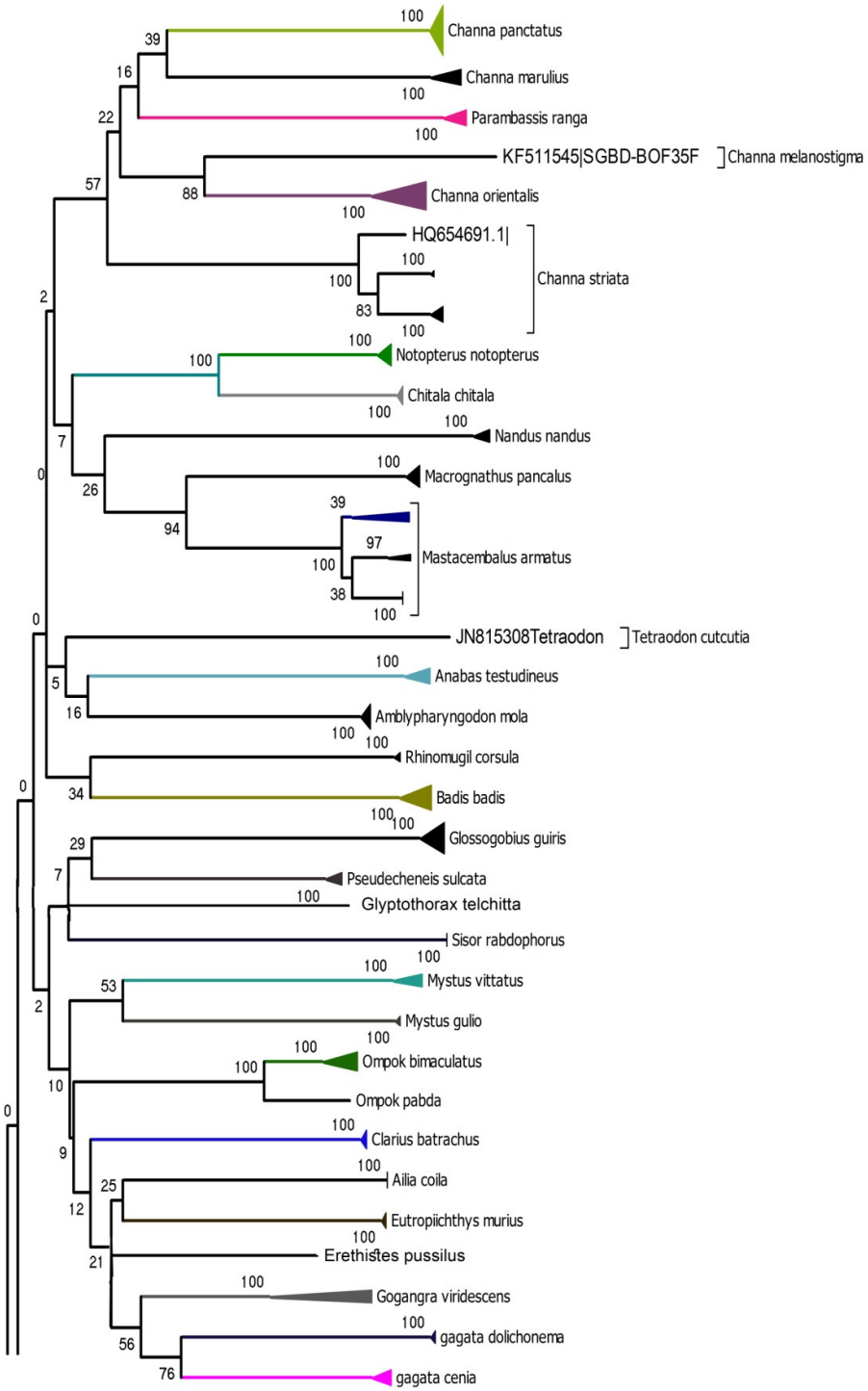
The NJ tree and the K2P distance were calculated taking altogether the generated *CO1* sequences along with the database sequence with which the similarity was matches as barcode replicated (Table 4.3.3). The 86 specimens which showed significant similarity with 37 species clustered cohesively with the matched species and distinctly with respect to others in the NJ-tree. Our data, DNA barcode based studies showed intraspecies individuals that showed similarity in the range of 97-100% clustered cohesively with each other while distinct with respect to interspecies (Bhattacharjee et al. 2012). The remaining 44 specimens which were identified into 16 species by morphology, also showed cohesive clusters with the individuals of the same species and distinctly with the other identified species (Figure 4.3.2). The specimens of Green Swamp barb/ Swamp barb, showed higher similarity with the multiple species like *Puntius chola, P. conchonius, P. fraseri* in the database. The morphology revealed our specimens to be *P. chola*. This is also a consensus with NJ clustering where the database sequence of *P. chola, P. conchonius, P. fraseri* clustered with the generated sequences of *P. chola* as a cohesive unit. Similarly, Gagata/Indian Gagata, and Minor carp are confirmed to be *Gagata cenia and Labeo bata* respectively. Such anomalies may arise due to the presence of mislabelled sequence in the database (Meier et al. 2006).

Within the clusters, the mean K2P distance was found to be 0.0084 $\pm$ 0.002, and maximum K2P distance was 0.045. While, between the clusters, mean K2P distance was observed as 0.252 $\pm$ 0.024 with Minimum K2P distance of 0.056. The K2P distance table is summarized in Appendix 3. Among them, 11 specimens representing four species showed deep conspecific divergence; concordant with the similarity match and are detailed in Table 4.3.4. The barcode gap, taken as the least inter-specific distance (Bhattacharjee et al. 2012, Meier et al. 2008) between the distinctly clustered congeners was straight forward in comparison to the cohesively clustered conspecies (Figure 4.3.3). As the above facts were evident to the 130 sequences, therefore, all the specimens were identified belonging to 53 species.

Table 4.3.3 Details of database sequences taken in this study

Species	**GenBank accession number**
Puntius sophore	JQ667571.1, HM057186.1, HM057188.1, JX260957.1, JX260953.1, FJ459404.1, FJ459407.1, FJ459405.1, FJ459403.1, HE650141.1, FJ459406.1
Amblypharyngodon mola	HM224137.1, JX260820.1, JX260818.1, JX260821.1, JX260822.1, JX260819.1
Crossocheilus latius	HM798581.1, HM798579.1, HM798580.1
Chela cachius	EF452891.1
Glossogobius guiris	JX260875.1, JX260876.1, JX260874.1, JX260877.1, JN815293.1
Nandus nandus	JX260922.1
Parambassisranga	JQ667560.1
Channa punctatus	FJ459409.1, FJ459408.1, FJ459410.1, EU417796.1, EU417795.1, HM117201.1, HM117200.1, HM117199.1, HM117198.1, HM117197.1, EU342202.1 , EU342201.1
Badis badis	AY662746.1, FJ459454.1, FJ459453.1, FJ459452.1
Notopterus notopterus	FJ459527.1, FJ459526.1, JQ667556.1, FJ459525.1,
Macrognathus pancalus	EU417777.1, EU417778.1, FJ459513.1, EU417776.1, FJ459512.1
Mastacembelus armatus	JX260912.1, JX260910.1, JX260909.1, EU417806.1, EU417807.1, JX260911.1, JQ667549.1
Barbonymus gonionotus	EU924635.1, EU924634.1, EU924633.1, EU924632.1, JQ346157.1, JN896650.1, JN896649.1, JQ661378.1, JN896651.1, JQ661381.1
Labeo calbasu	GU195096.1, GU195092.1, GU195090.1, GU195089.1, GU195088.1, GU195087.1, EU417759.1, GU195093.1
Labeo gonius	JX946409.1, HQ645094.2, HQ645092.2, JX946417.1, JX983345.1
Esomus danricus	FJ459489.1, FJ459487.1, HM224168.1, FJ459490.1
Rhinomugil corsula	JX983482.1, JX983483.1
Devario aequipinnatus	FJ459485.1, HM224154.1, HQ141076.1

Chitala chitala	JX891538.1, JX891537.1, JX891536.1, FJ459468.1, FJ459466.1
Anabas testudineus	JX983214.1, JX983213.1, JX260824.1
Gagata dolichonema	DQ846701.1
Eutropiichthys murius	JX983291.1, JN228956.1, JX983292.1
Pseudecheneis sulcata	DQ508087.1
Barilius bendelisis	FJ459422.1, FJ459420.1
Sisor rabdophorus	JN628883.1, JN628917.1, DQ508090.1
Mystus gulio	FJ384684.1, KC595988.1, GU132994.1
Ompok bimaculatus	JX901504.1, FJ230067.1, FJ230068.1, JX983419.1, JX983418.1
Ailia coila	FJ459444.1, FJ459442.1, EU911003.1, EU911002.1
Clarias batrachus	JQ699208.1, JQ699204.1, JQ699206.1, GQ466399.1, GQ466403.1
Mystus vittatus	JN228949.1, JN228950.1, JN228953.1
Puntius conchonius	JN965201.1
P. fraseri	JX260949.1
Canthophrys gongota	FJ459532.1
Channa striata	HM117206.1, HM117205.1, HQ654691.1, EU342203.1, EU342204.1
Channa orientalis	FJ459484.1, FJ459481.1, FJ459482.1, FJ459483.1
Devario devario	EF452866.1
Gogangra viridescens	DQ508078.1
Gagatacenia	JN628893, EU417769.1, EU417770.1
Channa marulius	JX260841.1, JX260840.1, JX983244.1, JX983243.1
Psilorhynchus homaloptera	DQ026436.1
Labeo bata	KC757216.1, HM147883.1, HM147881.1, HM147884.1


Channa panctatus
Channa marulius
Parambassis ranga
KF511545|SGBD-BOF35F
Channa melanostigma
Channa orientalis
HQ654691.1|
Channa striata
Notopterus notopterus
Chitala chitala
Nandus nandus
Macrognathus pancalus
Mastacembalus armatus
JN815308Tetraodon
Tetraodon cutcutia
Anabas testudineus
Amblypharyngodon mola
Rhinomugil corsula
Badis badis
Glossogobius guiris
Pseudecheneis sulcata
Glyptothorax telchitta
Sisor rabdophorus
Mystus vittatus
Mystus gulio
Ompok bimaculatus
Ompok pabda
Clarius batrachus
Ailia coila
Eutropiichthys murius
Erethistes pussilus
Gogangra viridescens
gagata dolichonema
gagata cenia

Continued

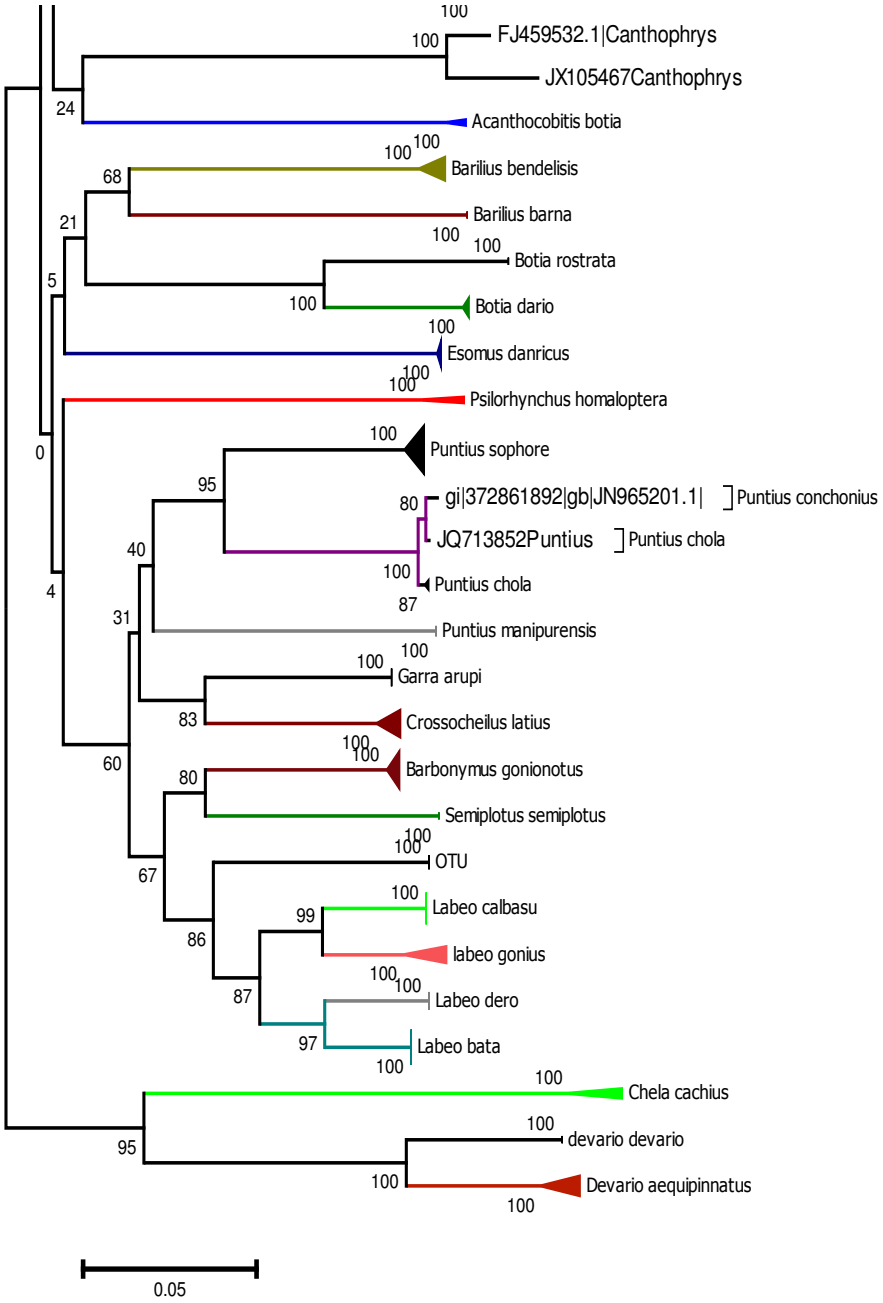


Figure 4.3.2 Neighbour-Joining tree of *CO1* gene sequences derived from 130 ornamental fish species under trade using K2P distance parameter. The numbers at the nodes are the bootstrap values based on 1000 replicates.

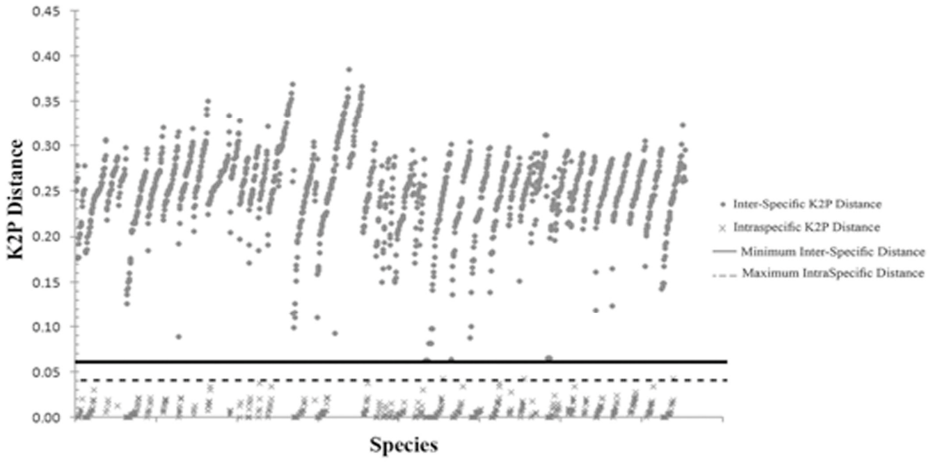


Figure 4.3.3 The scatter plot of genetic distances within species against genetic distances between the species, the red cross marks showing intraspecific K2P distance and the blue dots showing interspecific K2P distance. A straight line is at minimum interspecific K2P distance with a dotted line at maximum intraspecies distance. The barcode gap, taken as the minimum interspecific distance was straightforward in delineating all the species taking on the study.

Table 4.3.4: Minimum Interspecific and Maximum Intraspecific K2P genetic distance, NJ-Cluster with bootstrap values of the specimens that revealed similarity in the range of 96% - 100% with the database sequences.

Specimens	**Identified to be**	**NJ-Cluster of the developed and database sequences (bootstrap) from Fig 4.3.3**	**Max K2P Distance within the same species**	**Min K2P Distance with other species**
Striped snakehead Banded snakehead Snakehead murrel	*Channa striata*	Clustered as a three groups (82, 99) under a single node (99)	0.044	0.191
Walking snakehead Ceylone snakehead	*Channa orientalis*	Formed two sub-clusters (99, 99) under a single node (100)	0.042	0.172
Tire track eel	*Mastacembalus armatus*	Formed three sub-clusters (50, 70) under a single node (100)	0.037	0.152
Moosefaced loach Gongota loach	*Canthophrysgongota*	Clustered under single node (99)	0.043	0.236

4.3.3 SPECIES STATUS OF THE ORNAMENTAL FISH TRADED FROM NORTHEAST INDIA

The combined approach of *CO1* DNA barcoding and morpho-taxonomy were employed to identify the collected 130 specimens into respective species, as *CO1* barcode region is already proven to be gold standard for discriminating animal species. The study readily delineates 130 specimens to 53 species. In a few cases, although the specimens have different trade names, they represented same species. Furthermore, it was evident throughout this study that, many threatened species were traded and for a few species, the IUCN (International Union for Conservation of Nature) and CAMP (Conservation Assessment and Management plan), which followed IUCN Red list Criteria for the assessment of species status in the wild, revealed contradictory species status (Figure 4.3.4). Among them, the species status of *Rhinomugil corsula*, *Pseudecheneis sulcata*, *Mystus vittatus* in IUCN is 'Least Concern' but the same is 'Vulnerable' as per CAMP, *Anabas testudineus,* the species status in IUCN is 'Data Deficient' but the same is 'Vulnerable' as per CAMP, similarly, the species status of *Puntius manipurensis, Glossogobius guiris* is 'Vulnerable' in IUCN, but the status of the same is 'Not Available' in CAMP. Therefore, species status of many fish appeared to be not accurately evaluated and needs revision. In this study, 53 species were identified from the traded samples. Among them, 14 species had multiple trade name, four species had single trade names. Importantly, 17 species belong to the threatened category and 31 species belong to Lower risk near threatened/Least concern and the rest are deficient in data.

Identified Species	Species Status												
	International Union for Conservation of Nature, IUCN (2013)						Conservation Assessment and Management Plan, CAMP (1998)						
	Threatened Category			LC	DD	NA	Threatened Category			LRnt	LRlc	DD	NA
	EN	VU	NT				EN	VU	NT				
Puntius sophore				■						■			
Amblypharyngodon mola				■							■		
Crossocheilus latius				■								■	
Chela cachius				■									■
Tetraodon cutcutia				■						■			
Glossogobius guiris		■											■
Nandus nandus				■						■			
Parambassis ranga				■									■
Channa punctatus						■				■			
Badis badis				■									■
Notopterus notopterus				■						■			
Macrognathus pancalus						■				■			
Mastacembelus armatus				■									■
Barbonymus gonionotus				■									■
Labeo calbasu)				■						■			
Labeo gonius				■						■			
Esomus danricus				■							■		
Rhinomugil corsula				■				■					
Devario aequipinnatus				■						■			
Chitala chitala			■				■						
Anabas testudineus					■			■					
Gagata dolichonema				■									■
Eutropiichthys murius				■						■			
Pseudecheneis sulcata				■				■					
Puntius manipurensis		■											■
Barilius bendelisis				■						■			
Sisor rabdophorus				■			■						
Mystus vittatus				■				■					
Mystus gulio				■									■
Ompok bimaculatus			■				■						
Ailia coila			■					■					
Clarias batrachus				■				■					
Puntius chola				■				■					
Botia dario				■									■
Canthophrys gongota				■									■
Channa striata				■							■		
Channa orientalis				■				■					
Devario devario				■									■
Gogangra viridescens				■									■
Gagata cenia				■									■
Labeo dero				■				■					
Semiplotus semiplotus		■											■
Garra arupi						■							■
Ompok pabda			■				■						
Channa melanostigma						■							■
Barilius barna				■						■			
Channa marulius				■						■			
Botia rostrata		■								■			
Acanthocobitis botia				■									■
Psilorhynchus homaloptera													
Labeo bata				■						■			

Figure 4.3.4 The lists of the identified fish species is being traded from Northeast India as ornamental fish and their status in both IUCN and CAMP. Among the 130 fish samples 17 species belong to threatened category as compare to IUCN and CAMP. EN: Endangered, VU: Vulnerable, LC: Least Concern, LRnt: Lower Risk near threatened, LRlc: Lower Risk least concern, DD: Data deficient, NT: Near Threatened, NA: Not available.

CHAPTER-4.4
BARCODE SEQUENCE ANALYSIS

4.4.1 NUCLEOTIDE COMPOSITION OF *CO1* SEQUENCES OF ORNAMENTAL FISHES

Nucleotide composition at all codon position of the ornamental fish species was analyzed. The base frequencies for each sequence and for total barcode length were calculated by MEGA 6.2. The analysis revealed that nucleotide composition at third codon position showed significant variation while for first and second codon position nucleotide composition was almost uniform across different species (Figure 4.4.1). The nucleotide composition of the ornamental fish species showed similar kind of pattern across full length barcode and 1st and 2nd codon position. At 3rd codon position, all the species showed variation in all four nucleotide. A tendency toward low G content was observed and at 3rd codon position, it was significantly lower in all the collected species. At the 2nd and 3rd codon position, there was a bias towards AT over GC (Figure 4.4.2). Comparing the correlation of average GC content with GC content at each codon position, in 6 orders of ornamental freshwater fishes, GC content at the 3rd codon position was strongly positively correlated (r = 0.99) to the overall GC content of the barcode region (Figure 4.4.3). At second codon position, the GC content was positively correlated (r = 0.81) to overall GC content and at first codon position, it was positively correlated (r = 0.11) to the overall GC content. To obtain a closer perception of GC content distribution, the correlation between GC content at each codon position and average GC content in species of different orders of ornamental fishes was plotted.

Table 4.4.1 Nucleotide composition of the ornamental fish species along with the conspecific sequences from database with references to 3 major orders

Orders	T	C	A	G	T-1	C-1	A-1	G-1	T-2	C-2	A-2	G-2	T-3	C-3	A-3	G-3
Cypriniformes	29.3	27.1	26.2	17.3	19.0	25.6	24.9	30.6	41.8	28.9	15.2	14.3	27.5	26.8	38.6	7.1
Perciformes	28.9	29.2	23.8	18.0	18.2	25.4	26.3	30.0	42.0	29.0	15.0	14.0	26.5	33.3	30.1	10.1
Siluriformes	30.0	27.1	25.5	17.4	17.9	25.6	25.5	31.1	42.1	29.0	15.2	13.7	30.1	26.6	35.7	7.6
Synbranchiformes	28.1	28.4	26.6	16.8	18.2	26.0	26.4	29.5	42.2	29.1	14.7	14.2	24.2	30.2	38.7	6.8
Osteoglossiformes	29.0	26.1	27.7	17.4	18.0	25.2	25.7	30.8	42.3	28.2	14.9	14.6	26.0	24.8	42.4	6.7
Tetraodontiformes	26.2	31.3	25.1	17.4	18.0	26.5	25.6	30.3	41.0	29.4	15.2	14.7	20.0	37.9	34.6	7.1

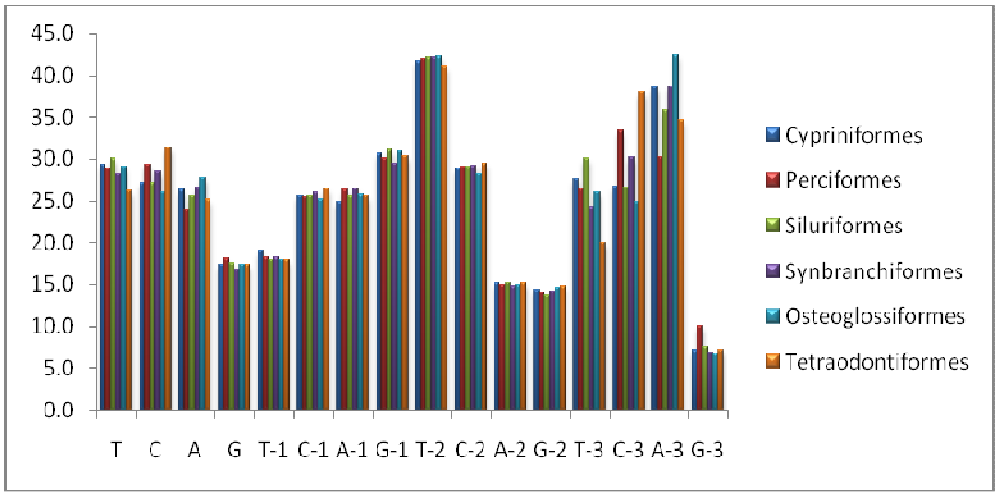


Figure 4.4.1 Nucleotide Composition of the *CO1* barcode sequences of the ornamental fishes

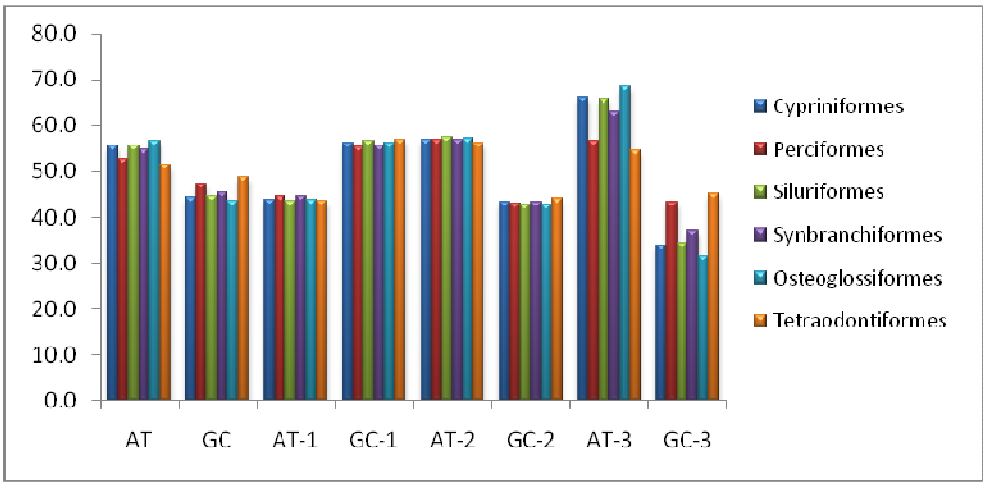


Figure 4.4.2 Nucleotide Bias in *CO1* barcode sequences of ornamental fishes. At the 2nd and 3rd codon position, there was a bias towards AT over GC

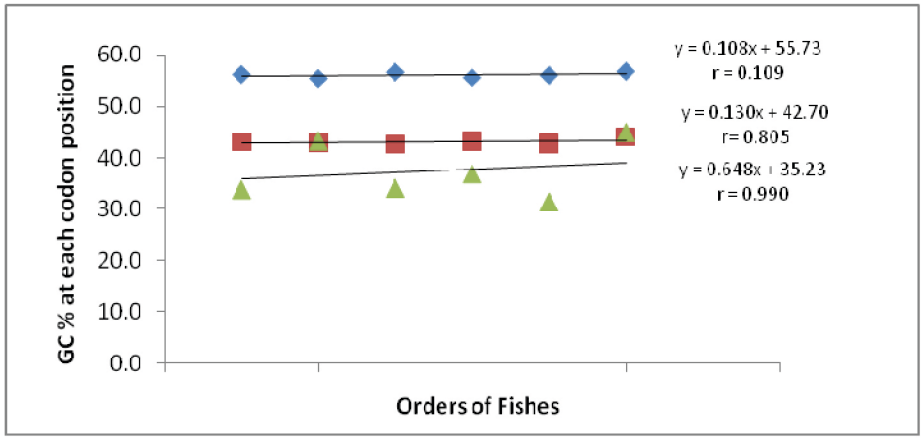


Figure 4.4.3 Correlation of average GC content with GC content at each codon position, in 6 orders of Indian freshwater fishes.

Each point represents correlation of average GC content in an order at a given codon position with average GC content. Each codon position is marked with a different marker as given in the figure legend.

In addition to AT-GC content, mitochondrial genomes also vary in their patterns of strand asymmetry (usually measured as GC skew and AT skew). Figure 4.4.4 shows the plot of AT and GC skew for different ornamental fish species at each codon position in the barcode and for the total barcode region. Strand asymmetry in the total barcode region showed a different pattern than in each codon position. Complete barcode region of all the studied species (Figure 4.4.4) showed overall negative GC skew, in most of the cased AT showed mostly negative skew whereas positive AT skew only for few samples. At first codon position, (Figure 4.4.5) both AT and GC showed positive skew while at second codon position, (Figure 4.4.6) AT and GC skew showed negative values. Third codon position (Figure 4.4.7), showed a positive and negative AT skew whereas GC skew showed negative values.

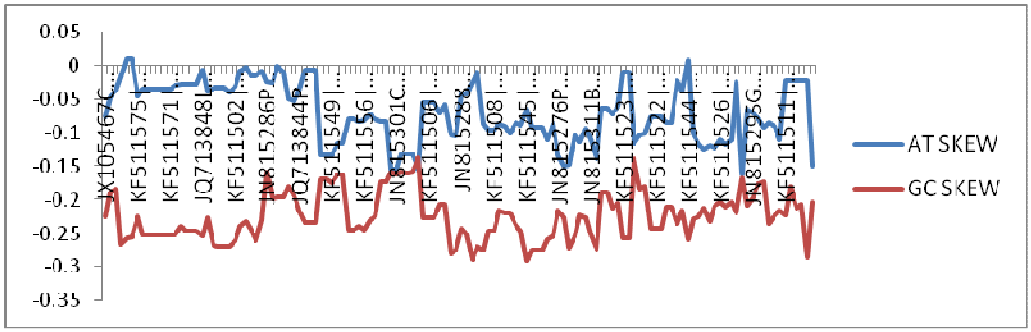


Figure 4.4.4 Average AT and GC Skew of entire *CO1* barcode region of different ornamental fishes

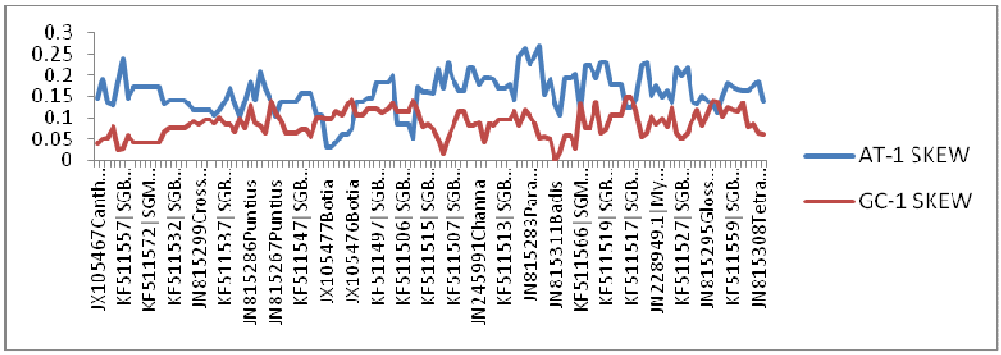


Figure 4.4.5 Average AT and GC Skew of 1st codon of *CO1* barcode region of different ornamental fishes

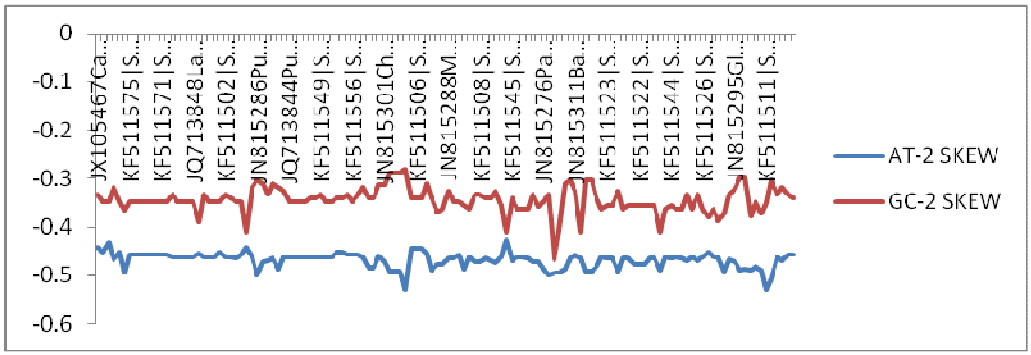


Figure 4.4.6 Average AT and GC Skew of 2nd codon of *CO1* barcode region of different ornamental fishes

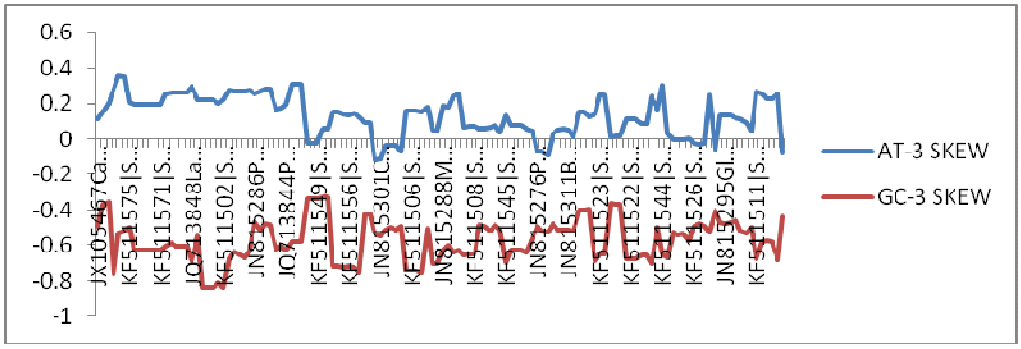


Figure 4.4.7 Average AT and GC Skew of 3rd codon of *CO1* barcode region of different ornamental fishes

4.4.2 AMINO ACID COMPOSITION

The amino acid composition of the *CO1* barcode region was calculated using MEGA.6.2 for all the ornamental fish species collected along with the global dataset as barcode replicates. The study showed highest frequency of Leucine followed by Alanine and Glycine among the studied samples as given in Figure 4.4.8

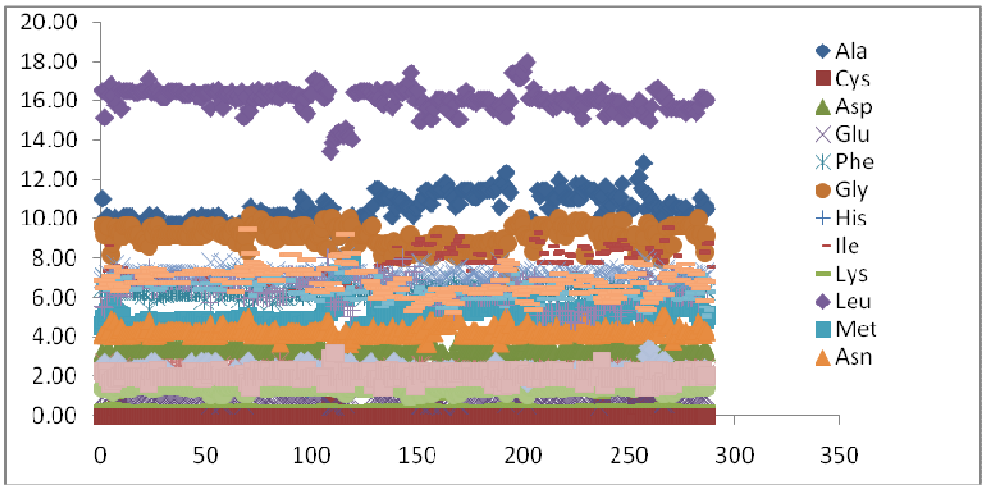


Figure 4.4.8 Amino acid composition of the different ornamental fishes based on *CO1* barcode sequences

4.4.3 CODON USAGE

There are 64 possible codons that code for 20 amino acids (and stop signals) so one amino acid may be encoded by several codons (e.g., serine is encoded by six codons in nuclear genes). It is therefore interesting to know the

codon usage for each amino acid. The numbers of the 64 codons used in a gene is computed for all examined sequences. In addition to the codon frequencies, the relative synonymous codon usage (RSCU) statistic is also computed. Many amino acids are coded by more than one codon; thus multiple codons for a given amino acid are synonymous. However, many genes display a non-random usage of synonymous codons for specific amino acids. A measure of the extent of this non-randomness is given by the Relative Synonymous Codon Usage (RSCU).

The RSCU for a particular codon (i) is given by **RSCUi = Xi / åå Xi/n.** Where, Xi is the number of times the i^{th} codon has been used for a given amino acid, and n is the number of synonymous codons for that amino acid.

The codon usage is calculated (Table 4.4.2) using vertebrate mitochondrial codon table as template for the fish specimen. The sequences under analysis did not contain any stop codon. Differences in the frequency of occurrence of synonymous codons in coding sequences were observed as summarized in table. Among the codons Leucine preference was seen followed by codons for serine in the studied specimens.

Table 4.4.2 Codon Usage of *CO1* barcode sequences of ornamental fishes

Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU
UUU(F)	5.7	0.86	UCU(S)	3.1	1.43	UAU(Y)	2.5	1.16	UGU(C)	0	0.83
UUC(F)	7.6	1.14	UCC(S)	2.6	1.18	UAC(Y)	1.8	0.84	UGC(C)	0	1.17
UUA(L)	6.3	1.12	UCA(S)	4	1.85	UAA(*)	0	0	UGA(W)	4.2	1.81
UUG(L)	0.5	0.1	UCG(S)	0.4	0.18	UAG(*)	0	0	UGG(W)	0.5	0.19
CUU(L)	8.9	1.59	CCU(P)	3	0.81	CAU(H)	1.4	0.74	CGU(R)	0.3	0.39
CUC(L)	5.2	0.93	CCC(P)	4.9	1.31	CAC(H)	2.4	1.26	CGC(R)	0.1	0.18
CUA(L)	9.9	1.76	CCA(P)	6.2	1.66	CAA(Q)	3.9	1.66	CGA(R)	2	2.72
CUG(L)	2.9	0.51	CCG(P)	0.8	0.21	CAG(Q)	0.8	0.34	CGG(R)	0.5	0.71
AUU(I)	10.9	1.37	ACU(T)	2.5	0.75	AAU(N)	4.3	0.97	AGU(S)	0.4	0.2
AUC(I)	5	0.63	ACC(T)	3	0.91	AAC(N)	4.6	1.03	AGC(S)	2.6	1.17
AUA(M)	7.7	1.43	ACA(T)	7.4	2.24	AAA(K)	1	1.92	AGA(*)	0	0
AUG(M)	3.1	0.57	ACG(T)	0.4	0.11	AAG(K)	0	0.08	AGG(*)	0	0
GUU(V)	4.1	1.16	GCU(A)	4.6	0.83	GAU(D)	2.1	0.62	GGU(G)	3.7	0.77
GUC(V)	2.4	0.68	GCC(A)	9.9	1.81	GAC(D)	4.7	1.38	GGC(G)	2.8	0.58
GUA(V)	6.6	1.85	GCA(A)	6.7	1.21	GAA(E)	1.5	1.53	GGA(G)	8.8	1.85
GUG(V)	1.1	0.31	GCG(A)	0.8	0.15	GAG(E)	0.5	0.47	GGG(G)	3.9	0.81

4.4.4 TRANSITION/TRANSVERSION IN *CO1* BARCODE SEQUENCE

Transition was found to be dominant over transversion for entire barcode length. Similarly transition to transversion ratio was found to be 1.09 and 1.03 at 2nd and 3rd codon positions respectively. However, at 1st codon position transition was found to be significantly high with R (transition/transversion) of 3.07 (Table 4.4.3).

Substitutions of nucleotides were found to be more prominent in 3rd codon position than in 1st and 2nd codon position. Second codon position exhibited least number of substitutions for all nucleotide pairs.

Table 4.4.3 Transition/Transversion ratio in *CO1* barcode sequences

Domain	Identical (ii)	Transition (si)	Transversion (sv)	R=si/sv
Position	Avg	490.00	67.00	56.00
1st Codon	187.00	13.00	4.00	3.07
2nd Codon	201.00	2.00	1.00	1.09
3rd Codon	102.00	52.00	51.00	1.03

4.4.5 SELECTION OF MINI-BARCODE FRAGMENT

The full-length *CO1* barcode of the species that revealed straightforward identification as shown in chapter 4.3 of result section were selected for design of mini-barcode. The dataset therefore includes 287 full-length *CO1* barcode sequences of 53 ornamental fish species; of them 130 sequences were self developed and rest of the replica sequences were acquired from the GenBank to make at least 4 sequences per species wherever possible.

In general 287 sequences belonging to the orders (Cypriniformes, Siluriformes and Perciformes, etc) of fresh water ornamental fishes were used for the analysis of *CO1* barcodes (generated as well as conspecific sequences mined from Database). The sequence based demarcation of species has its roots on the variable regions present between the sequences and the variation mainly includes transition and transversion. Inter-specific transition and transversion substitutions were calculated for each of the 654 base pair positions. The comparison of

Interspecific K2P distance with average Interspecific transition and transversion in terms of correlation coefficient (i.e. K2P versus transition and K2P versus transversion) thereby predicts the role involved separately of each type of nucleotide substitution (transition and transversion) in species differentiation. The comparison showed the genetic divergence between the species is positively correlated with both transition and transversion (Pearson correlation, $R = 0.92$, $p<0.001$ and $R = 0.95$, $p<0.001$ for transition and transversion respectively).

Figure 4.4.9 shows substitution pattern of the nucleotides across the full length barcodes. As seen in the figure, transitions and transversions follow a distinct pattern of distribution in all the three orders of fishes. Overall, transitions (represented by +1) are dominant over transversions (represented by -1). Transversion biased sites are found to be scattered randomly throughout the full-length barcode.

Table 4.4.4 Comparison of the nucleotide properties of mini-barcode region (171 bp) to full-length barcode (655 bp).

Selected *CO1* segments' characteristics			**Divergence parameter characteristics**		
Segments	Length (bp)	Variable site%	Transition/ Transversion bias (R)	Avg. K2P distance between species	Avg. K2P distance within species
Full-length Barcode	655	40	1.612	0.252± 0.024	0.0084 ± 0.002
261 to 432	171	57.3	0.84	0.235 ± 0.05	0.006 ± 0.003

Both mini-barcode and full length barcode have similar trend of K2P genetic distance, however, mini-barcode have higher number of transversion substitution in relation to transition thereby revealing lower transition/transversion bias in comparison to the full length barcode. The graph points out the presence of a continuous stretch of transversion dominant segment lying between base pair positions BP_{261} –BP_{432} (position calculated from BLAST SEARCH against full length *CO1*), in all the studied orders of fishes. In this segment, average 40% of the nucleotide sites showed transversions which was higher than the average 28%

transversion sites seen in the full length barcode. This 171bp long transversion 'hotspot' was selected as a potential region for selecting a minibarcode.

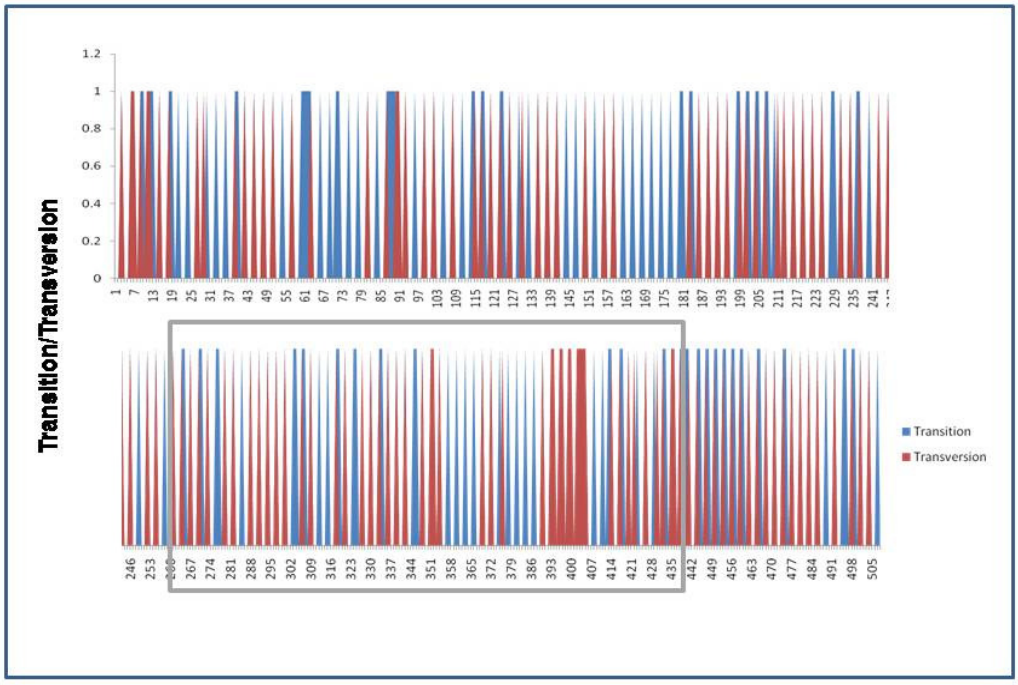
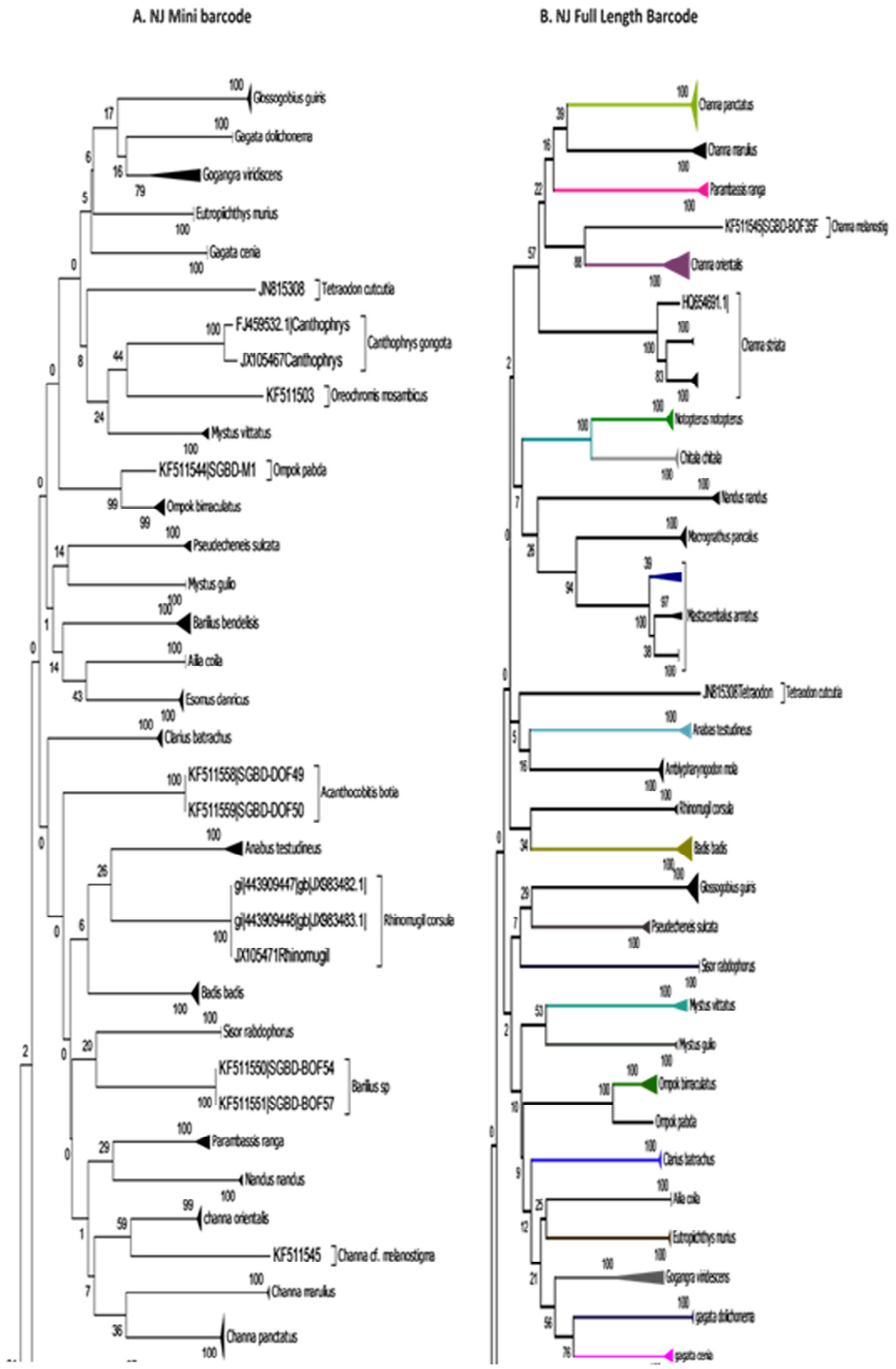


Figure 4.4.9 Distribution patterns of transitions and transversion across full-length barcode (654bp) of 287 sequences.

4.4.5.1 VALIDATION OF THE PROPOSED SEGMENT FOR USE IN SPECIES DELIMITATION: TREE BASED APPROACH

This 171bp segment of transversion hotspot region was retrieved for all the species of the three orders. A NJ tree (Figure 4.4.10), based on the K2P divergences of the nucleotides of the minibarcode region, showed that in most cases conspecific sequences clustered together and were distinct from the remaining species. Comparison of the two NJ trees, constructed from full length barcode and minibarcode (171bp), revealed similar clustering pattern for most of the sequences with a high bootstrap support.


A. NJ Mini barcode
Glossogobius guiris
Gegata dolichonema
Gogangra viridescens
Eutropiichthys murius
Gegata cenia
JN815308 Tetraodon cutcutia
F.J459532.1|Canthophrys
JX105467Canthophrys
Canthophrys gongota
KF511503 Oreochromis mosambicus
Mystus vittatus
KF511544|SGBD-M1 Ompok pabda
Ompok bimaculatus
Pseudecheneis sulcata
Mystus gulio
Barilius bendelisis
Aila coila
Esomus danricus
Clarius batrachus
KF511558|SGBD-DOF49
KF511559|SGBD-DOF50
Acanthocobitis botia
Anabus testudineus
gi|443909447|gb|JX983482.1|
gi|443909448|gb|JX983483.1|
JX105471Rhinomugil
Rhinomugil corsula
Badis badis
Ssor rabdophorus
KF511550|SGBD-BOF54
KF511551|SGBD-BOF57
Barilius sp
Parambassis ranga
Nandus nandus
channa orientalis
KF511545 Channa cf. melanostigma
Channa marulius
Channa panctatus
B. NJ Full Length Barcode
Channa panctatus
Channa marulius
Parambassis ranga
KF511546|SGBD-BOF35F Channa melanostig
Channa orientalis
HQ654691.1|
Channa striata
Notopterus notopterus
Chitala chitala
Nandus nandus
Macrognathus pancalus
Mastacembalus armatus
JN815308Tetraodon Tetraodon cutcutia
Anabas testudineus
Anableplarynogodon mola
Rhinomugil corsula
Badis badis
Glossogobius guiris
Pseudecheneis sulcata
Ssor rabdophorus
Mystus vittatus
Mystus gulio
Ompok bimaculatus
Ompok pabda
Clarias batrachus
Aila coila
Eutropiichthys murius
Gogangra viridescens
gegata dolichonema
gegata cenia

Continued

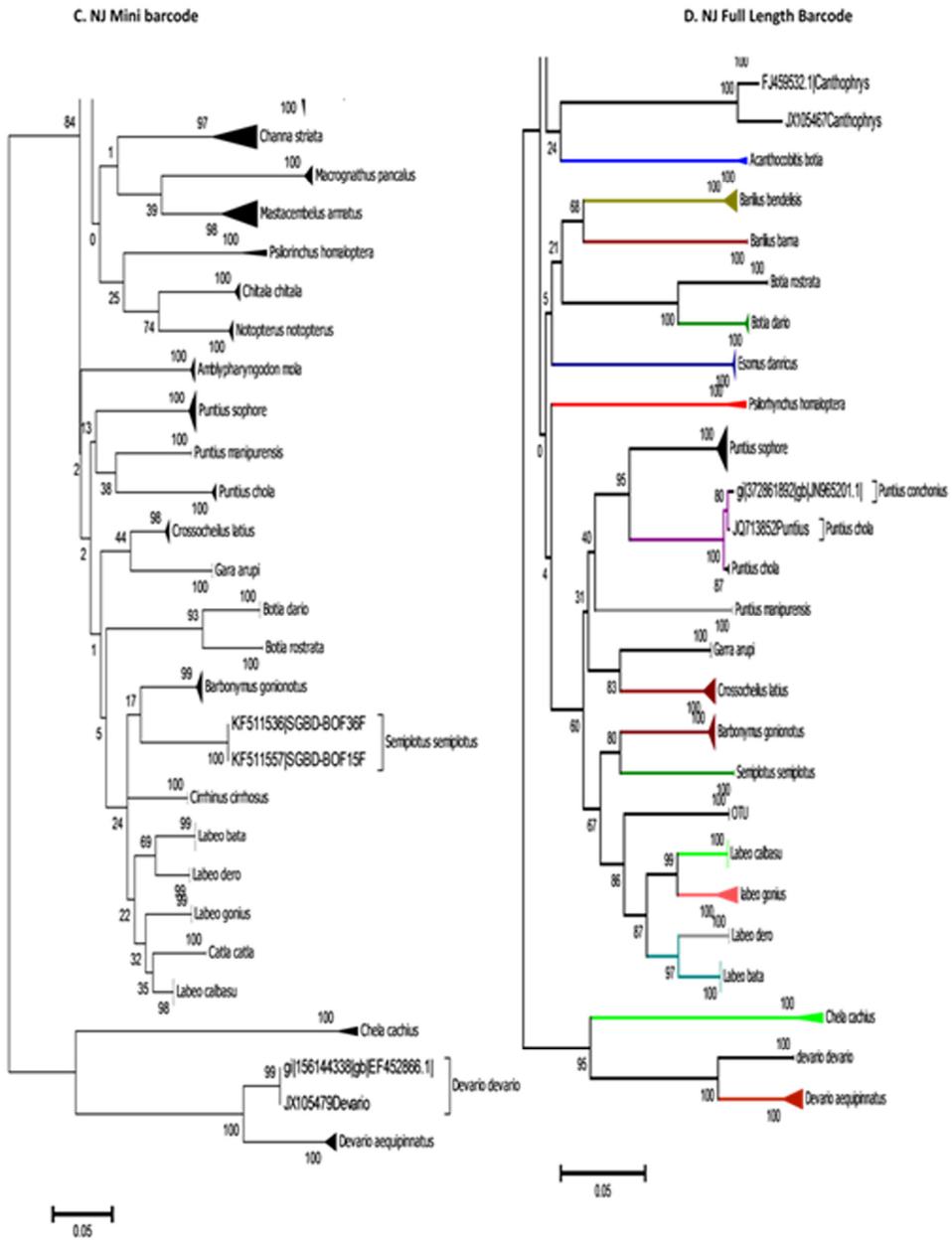


Figure 4.4.10 Neighbour joining (NJ) tree developed using K2P distance among 187 mini-barcode sequences (171 bp). All the mini-barcode sequences show straightforward species delineation comparable to full length (654 bp), where the conspecifics have clustered cohesively with respect to the distinctly clustered congeners. (A, C) are NJ Tree based on mini-barcode, (B, D) NJ Tree based on full length *CO1* barcode sequences.

4.4.5.2 K2P DISTANCE: MINI-BARCODE VS FULL LENGTH BARCODE

As presented in previous chapter, the mean K2P distance within the clusters using full length barcode, was found to be 0.0084 ± 0.002, and maximum K2P distance was 0.045. While, between the clusters, mean K2P distance was observed as 0.252 ± 0.024 with Minimum K2P distance of 0.056. Using similar workflow, the mean K2P distance using mini-barcode was found to be 0.006 ± 0.003, and maximum K2P distance was 0.046. While, between the clusters, mean K2P distance was observed as 0.294 ± 0.05 with Minimum K2P distance of 0.056.

Table 4.4.5 Mean value of intergeneric and conspecific K2P divergence

K2P genetic distance	Barcode region	Min	Mean	Max	SE
Within species	Full length (650bp)	0.00	0.0084	0.045	0.0024
Between species		0.056	0.252	0.386	0.024
Within species	Mini-Barcode (171bp)	0.00	0.006	0.046	0.003
Between species		0.056.	0.294	0.671	0.05

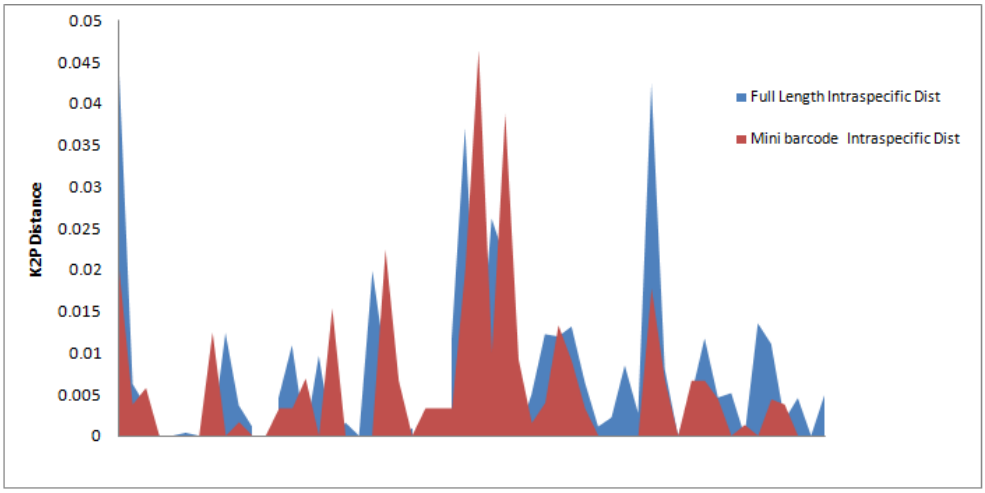


Figure 4.4.11 Comparison of intraspecies distance between full length *CO1* barcode and mini-barcode. Each line represents intraspecific distance between species pairs. The blue peak represents K2P distance between

conspecies based on full length *CO1* barcode. The red peak represents K2P distance between conspecies based on mini *CO1* barcode.

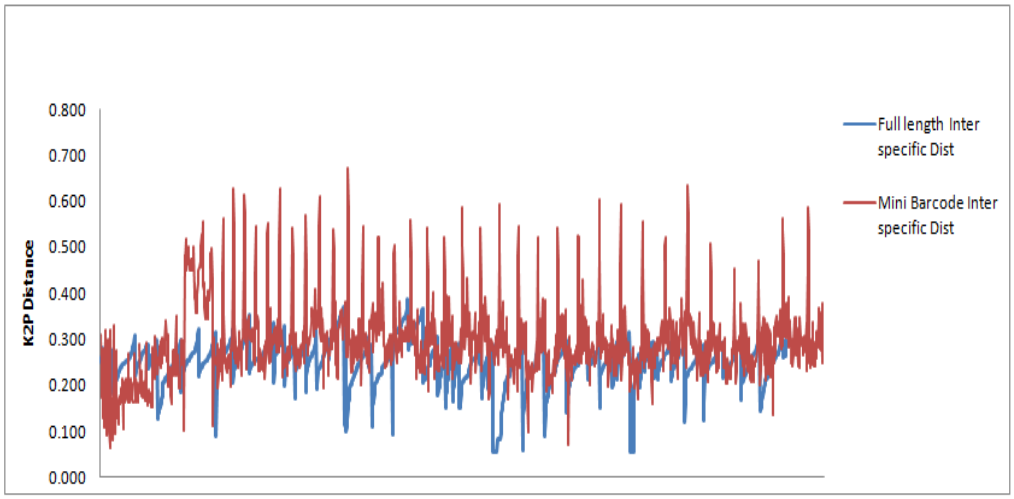


Figure 4.4.12 Comparison of inter-species distance between full length *CO1* barcode and mini-barcode. Each line represents inter-specific distance between species pairs. The blue line represents K2P distance between different species based on full length *CO1* barcode. The red line represents K2P distance between different species based on mini *CO1* barcode

4.4.5.3 PRIMERS FOR AMPLIFICATION OF MINI BARCODE

The primers were designed from the flanking region of the transversion rich barcode motif. The multiple sequence alignment of the complete barcode region of the 6 orders showed that the sequences upstream and downstream of the "transversion hotspot" loci were less variable across species within an order. So, by considering the less variable site and taking consensus nucleotide from the alignment with a few degenerate nucleotides, the primer pairs common for each of the orders as well as order specific primers were finally designed as:

Fish Com F- 5/ - GCNTTCCCNCGAATRAANAACAT- 3/ and
Fish Com R- 5/ - GATNGTNGTGATGAAGTTNAT - 3/.
Cypri F- 5/ - GGCRTTCCCNCGWATAAACAAC - 3/ and
Cypri R- 5/ - GGTGGTWGTAATGAARTTAAT - 3/
Siluri F- 5/ - GCATTCCCYCGAATRGAYAACA - 3/ and
Siluri R- 5/ - GATDGTTGTGATGAAGTTGAT - 3/

Perci F- 5′ - CCTTYCCTCGAATRAACAACAT - 3′ and

Perci R- 5′ - ATGGTKGTGATGAAGTTGAT - 3′

The primer pairs designed with the help of *in silico* tools, Oligocalc and Sequence manipulation suite, abided by the recommended parameters (Tm, specificity, secondary structure, GC clamp, etc.) and were found to be optimum. Finally, the primer pairs were subjected to MEGA BLAST in the global database for checking its specificity. The representative sequences from the orders were generated through *in-silico* PCR. The result showed product of about 221bp for all the 6 orders of ornamental fish. Finally, the 171bp nucleotide segment lying between BP_{267} –BP_{421} comprising the mini-barcode was recovered, thus leaving sufficient upstream and downstream sequence to avoid noisy data in the target region. The primers properties are summarized below:

4.4.5.4 PCR AMPLIFICATION OF THE MINI BARCODE FRAGMENT

The specificity and the robustness of the designed Primers in amplifying the target sequences were tested by PCR of the representative sequences from the respective 6 orders of Ornamental fish under study. The PCR was performed by varying the annealing temperature from lower to higher stringency with an aim to recover the specific fragment of the desired size. It was observed that the primer sets worked best at 48°C for all the orders, where a distinct single band of desired fragment size was found as detailed in (Figure 4.4.13). In all the cases a positive control of 650bp standard barcode sequence was kept for further validation of the experiment. Similarly, a clean distinct band of desired amplicon size was observed in-case of the common primers targeting all the 3 orders.

Finally, designed primer set was tested in a randomly collected fish for the amplification of the desired fragment. The affirmative result showed the efficacy of the primers in amplifying the target sequences.

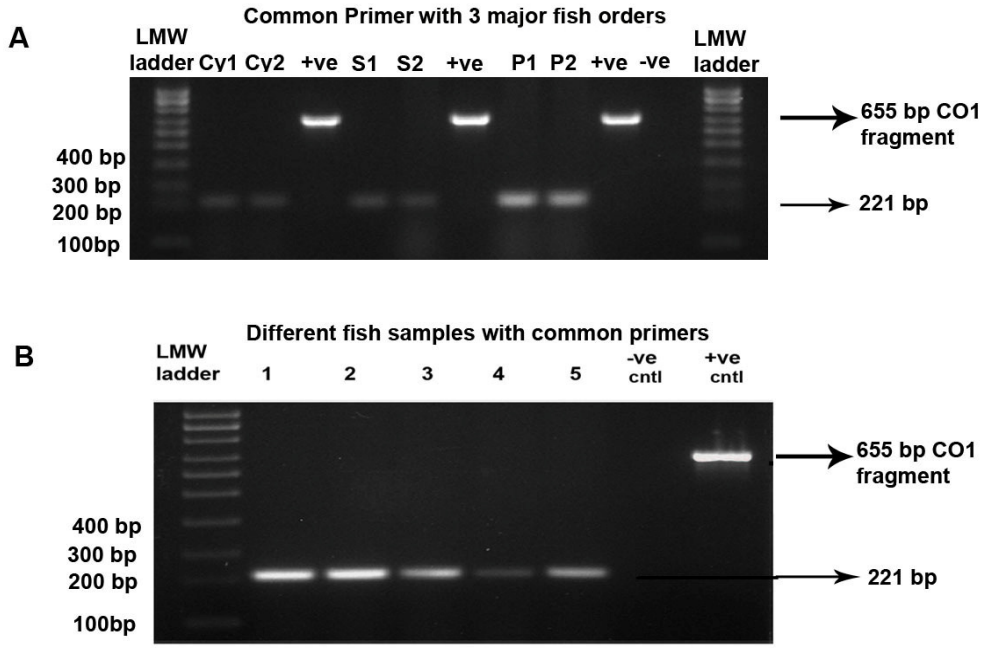


Figure 4.4.13 PCR amplicons of the mini-barcode using consensus primers. A. PCR amplicon size of 221bp for Cypriniformes and Siluriformes and 223bp for Perciformes was observed with the designed motif primer for the 3 orders of fishes, B. PCR amplification of the randomly selected fishes representing different orders with the consensus primers.

4.4.5.5 SEQUENCING OF THE PCR PRODUCTS

The sequence chromatogram of the generated fragment (Barcode motif) showed clean data with Quality Value (QV) greater than 40, which confirmed that the sequences were 99.99% accurate. The BLAST result showed significant similarity with the previously developed sequences in the database. Moreover, the partial amino acid sequences of the target region also showed homology with standard barcode region. This confirmed the accuracy of the primers. The initial ~30bp from both the ends were trimmed apprehending the presence of primers sequences. The rest of the portion, containing the transversion rich mini-barcode, was aligned pairwise for all the orders under study and compared with the standard barcode region.

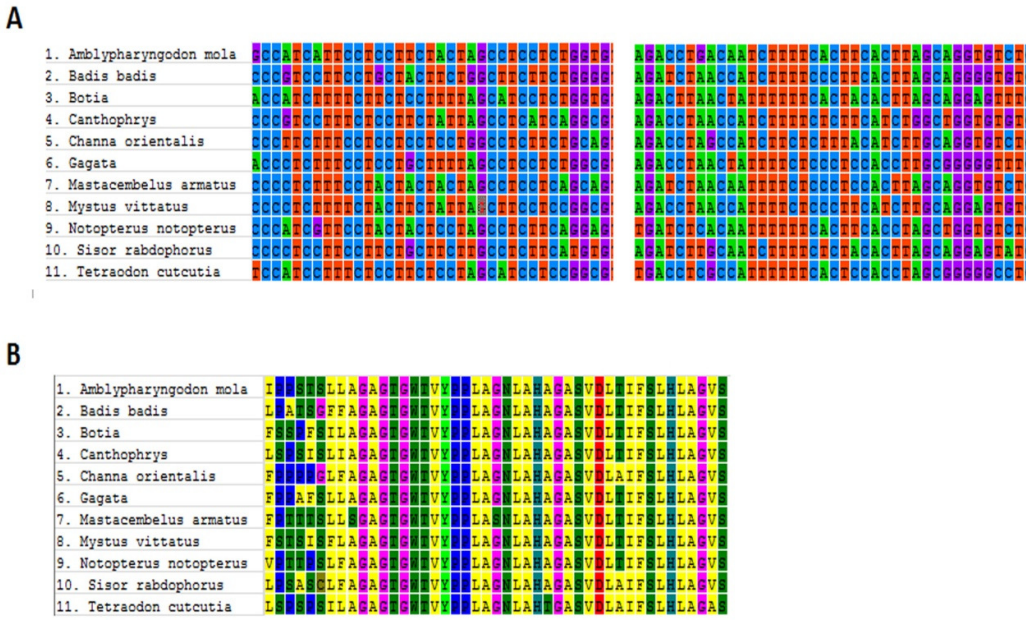


Figure 4.4.14 Mini-barcode sequences of the representative samples of ornamental fishes from the 6 orders. A. Representation of multiple sequence alignment of the mini-barcode region. B. Amino acid sequence of the selected mini-barcode region.

CHAPTER 4.5
PHYLOGENETIC ANALYSIS OF DIFFERENT ORNAMENTAL FISHES

As already showed in the previous section, *CO1* gives better resolution of species delineation. So here, *CO1* sequences for species identification and phylogenetic analysis was employed.

4.5.1 MAXIMUM LIKELIHOOD APPROACH

A maximum likelihood method for inferring evolutionary trees from DNA sequence data was developed by Felsenstein in 1981. In evaluating the extent to which the maximum likelihood tree is a significantly better representation of the true tree, it is important to find out the variance of the difference between log likelihood of different tree topologies.

4.5.1.1 MODEL TEST FOR MAXIMUM LIKELIHOOD ANALYSIS

The optimal model that best explain the evolution of the sequences or in other words the model that fits for the sequences to be used for phylogenetic analysis for ML analysis were filtered through goodness-of-fit test of each model measured through Bayesian Information Criterion (BIC) and Akaike Information Criterion (AIC) with and without assuming the existence of evolutionary rates among sites modelled by discrete gamma distribution (+G) and allowance of the presence if invariant sites (+I). This result is an evaluation of 24 models for nucleotide substitutions. For each of these models, MEGA 6.2 provided the estimated values of shape parameter of the Gamma distribution, the proportion of invariant sites, and the substitution rates between bases or residues, as applicable and presented in Table 4.5.1. Depending on the model, the assumed or observed values of the base frequencies used in the analysis were also provided. Both BIC and AIC selected substitutions models that was more complex than the true model. By rules, the true model was among the top three when BIC was used and among the top five when AIC was used. However, the models with the lowest BIC scores are considered to describe the substitution pattern the best and as barcodes sequences showed extreme variable sites.

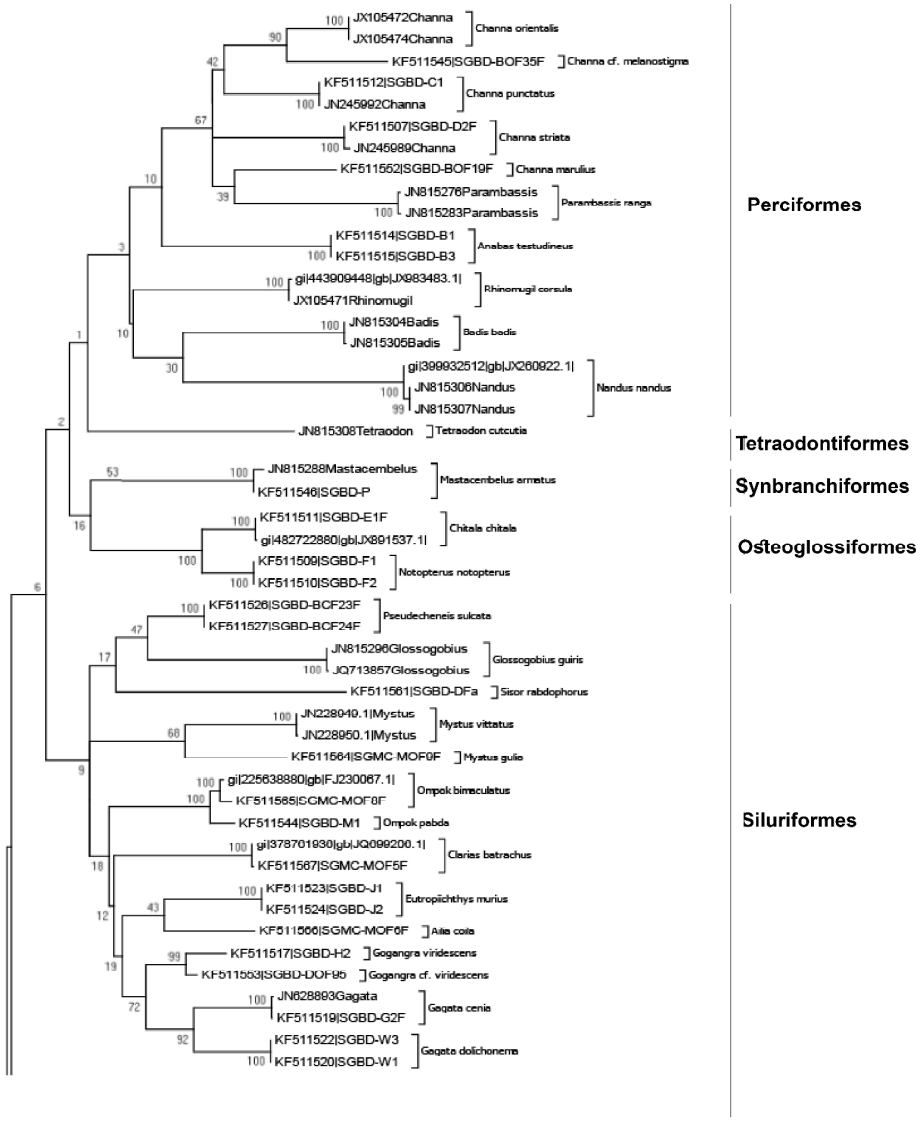
Table 4.5.1: Model Test for selection of best fit model based on BIC (Bayesian Information Criteria) for reconstruction phylogenetic tree based on Maximum Likelihood

Model	#Param	BIC	AICc	lnL	Invariant	Gamma	R	Freq A	Freq T	Freq C	Freq G	A=>T	A=>C	A=>G	T=>A	T=>C	T=>G	C=>A	C=>T	C=>G	G=>A	G=>T	G=>C
GTR+G+I	183	23511.06	21919.04	-10775.76	0.43	0.69	2.85	0.25	0.30	0.27	0.17	0.05	0.04	0.10	0.04	0.24	0.03	0.03	0.26	0.00	0.14	0.06	0.00
TN93+G+I	180	23605.23	22039.29	-10838.91	0.44	0.75	2.14	0.25	0.30	0.27	0.17	0.05	0.04	0.11	0.04	0.19	0.03	0.04	0.21	0.03	0.16	0.05	0.04
GTR+G	182	23608.70	22025.37	-10829.94	n/a	0.27	3.09	0.25	0.30	0.27	0.17	0.05	0.03	0.08	0.04	0.25	0.04	0.03	0.28	0.00	0.12	0.07	0.00
HKY+G+I	179	23611.72	22054.47	-10847.51	0.44	0.76	2.06	0.25	0.30	0.27	0.17	0.05	0.05	0.11	0.04	0.18	0.03	0.04	0.20	0.03	0.17	0.05	0.05
TN93+G	179	23731.27	22174.01	-10907.28	n/a	0.31	2.06	0.25	0.30	0.27	0.17	0.05	0.05	0.10	0.04	0.19	0.03	0.04	0.22	0.03	0.15	0.05	0.05
HKY+G	178	23742.61	22194.05	-10918.31	n/a	0.31	2.06	0.25	0.30	0.27	0.17	0.05	0.05	0.11	0.04	0.18	0.03	0.04	0.20	0.03	0.17	0.05	0.05
T92+G+I	177	23823.39	22283.52	-10964.05	0.44	0.81	1.96	0.28	0.28	0.22	0.22	0.05	0.04	0.15	0.05	0.15	0.04	0.05	0.18	0.04	0.18	0.05	0.04
T92+G	176	23953.96	22422.78	-11034.69	n/a	0.31	1.96	0.28	0.28	0.22	0.22	0.05	0.04	0.15	0.05	0.15	0.04	0.05	0.18	0.04	0.18	0.05	0.04
K2+G+I	176	23962.61	22431.44	-11039.02	0.44	0.82	1.98	0.25	0.25	0.25	0.25	0.04	0.04	0.17	0.04	0.17	0.04	0.04	0.17	0.04	0.17	0.04	0.04
K2+G	175	24099.35	22576.87	-11112.74	n/a	0.31	1.98	0.25	0.25	0.25	0.25	0.04	0.04	0.17	0.04	0.17	0.04	0.04	0.17	0.04	0.17	0.04	0.04
GTR+I	182	24774.31	23190.98	-11412.74	0.47	n/a	2.11	0.25	0.30	0.27	0.17	0.08	0.06	0.10	0.07	0.20	0.01	0.06	0.23	0.01	0.15	0.02	0.01
TN93+I	179	25013.49	23456.24	-11548.40	0.47	n/a	1.75	0.25	0.30	0.27	0.17	0.06	0.05	0.10	0.05	0.18	0.03	0.05	0.20	0.03	0.14	0.06	0.05
HKY+I	178	25020.75	23472.19	-11557.38	0.47	n/a	1.74	0.25	0.30	0.27	0.17	0.06	0.05	0.11	0.05	0.17	0.03	0.05	0.19	0.03	0.16	0.06	0.05
JC+G+I	175	25117.20	23594.72	-11621.67	0.45	1.02	0.50	0.25	0.25	0.25	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
T92+I	176	25167.99	23636.81	-11641.71	0.47	n/a	1.99	0.28	0.28	0.22	0.22	0.05	0.04	0.15	0.05	0.15	0.04	0.05	0.19	0.04	0.19	0.05	0.04
JC+G	174	25206.13	23692.33	-11671.48	n/a	0.26	0.50	0.25	0.25	0.25	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
K2+I	175	25242.78	23720.30	-11684.46	0.47	n/a	1.97	0.25	0.25	0.25	0.25	0.04	0.04	0.17	0.04	0.17	0.04	0.04	0.17	0.04	0.17	0.04	0.04
JC+I	174	26239.87	24726.08	-12188.35	0.47	n/a	0.50	0.25	0.25	0.25	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
GTR	181	28102.76	26528.12	-13082.32	n/a	n/a	1.74	0.25	0.30	0.27	0.17	0.09	0.08	0.10	0.07	0.19	0.01	0.07	0.21	0.01	0.15	0.02	0.01
HKY	177	28406.88	26867.01	-13255.80	n/a	n/a	1.74	0.25	0.30	0.27	0.17	0.06	0.05	0.11	0.05	0.17	0.03	0.05	0.19	0.03	0.16	0.06	0.05
TN93	178	28407.22	26858.66	-13250.62	n/a	n/a	1.75	0.25	0.30	0.27	0.17	0.06	0.05	0.10	0.05	0.18	0.03	0.05	0.20	0.03	0.14	0.06	0.05
T92	175	28524.20	27001.72	-13325.17	n/a	n/a	1.75	0.28	0.28	0.22	0.22	0.05	0.04	0.14	0.05	0.14	0.04	0.05	0.18	0.04	0.18	0.05	0.04
K2	174	28584.58	27070.79	-13360.71	n/a	n/a	1.74	0.25	0.25	0.25	0.25	0.05	0.05	0.16	0.05	0.16	0.05	0.05	0.16	0.05	0.16	0.05	0.05
JC	173	29508.00	28002.90	-13827.78	n/a	n/a	0.50	0.25	0.25	0.25	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08

In this study for ML based phylogenetic interpretation, the model General Time Reversible Model (GTR) along with discrete gamma rate categories (+G) and invariant sites (+I) were favored.

4.5.1.2 PHYLOGENETIC ANALYSES BASED ON MAXIMUM LIKELIHOOD APPROACH

The ML analysis in MEGA 6.2 started with an initial tree that was automatically generated as default tree by MEGA by the Neighbor- Joining (NJ) and BioNJ algorithm using a matrix of pair wise distances estimated under Tamura and Nei model for nucleotide sequences. Based on the automatically generated initial tree a final ML tree with the highest log likelihood value of -2169.5372 with well supported bootstrap support proportion was produced and shown in Figure 5.



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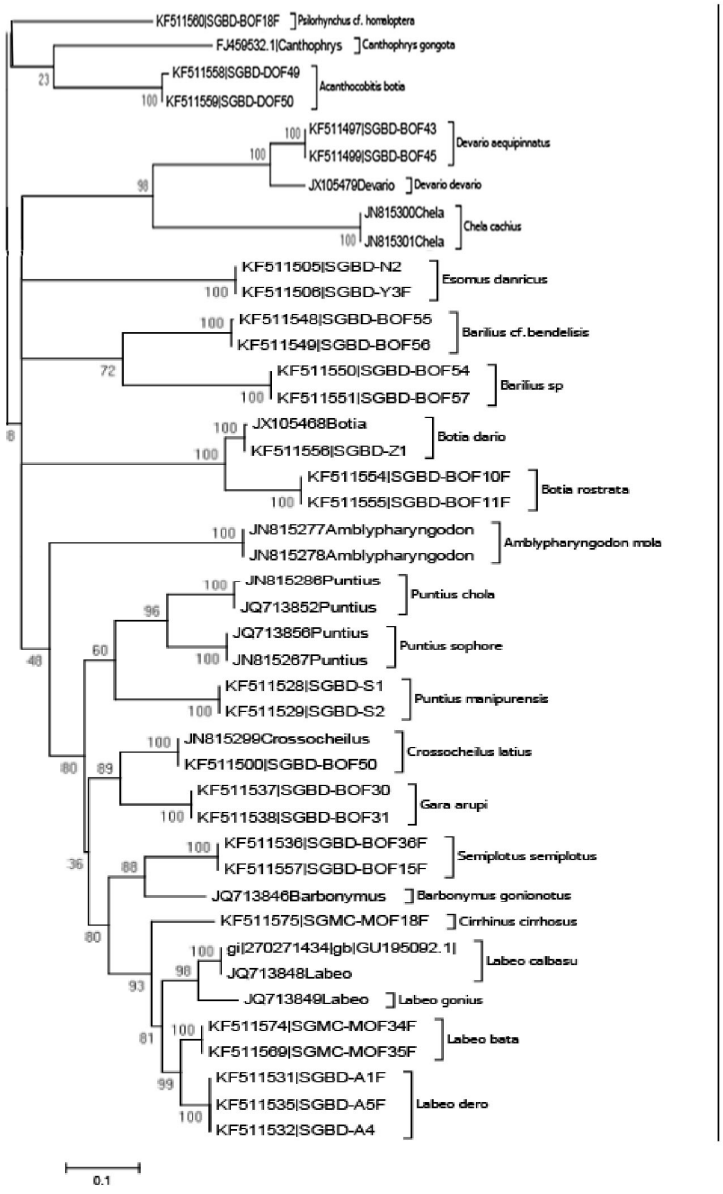

Figure 4.5.1: ML Tree of different ornamental fish of Northeast India. The entire ML tree was constructed based on 128 sequences belonging to different species. All species clustered cohesively distinct from other species with a strong bootstrap support of 100. The species belonging to same order clustered separately from the other orders.

In congruence with both the NJ and the ML tree, all the conspecific individuals had clustered cohesively under a single node while congeners had clustered distinctly with a strong bootstrap support of >95%. Again, the congeners belonging to a particular order clustered distinctly originating from a common ancestral node (Figure 4.5.1) which were in concurrence with the taxonomic rank. However, the representative sequences of the species *Glossogobius guiris* (accession numbers JN815293, JN815296, JQ713857, JN815294, JN815295) that revealed ambiguous clustering in the ML tree. The said specimen showed clustering near Siluriformes instead of clustering near Perciformes.

ORDER PERCIFORMES:

Order Perciformes comprising of 11 species (*Channa panctatus, C. striata, C. marulius, C. melanostigma, C. orientalis, Anabus testudineus, Pseudambassis ranga, Nandus nandus and Rhinomugil corsula*) under 7 genera and 7 families (Table 4.1.1) showed distinct cluster pattern as shown in Figure 4.5.2. All the members of a species clustered cohesively and distinctly from other species and originated from a single node, similarly all the genera clustered distinctly with a common ancestral node.

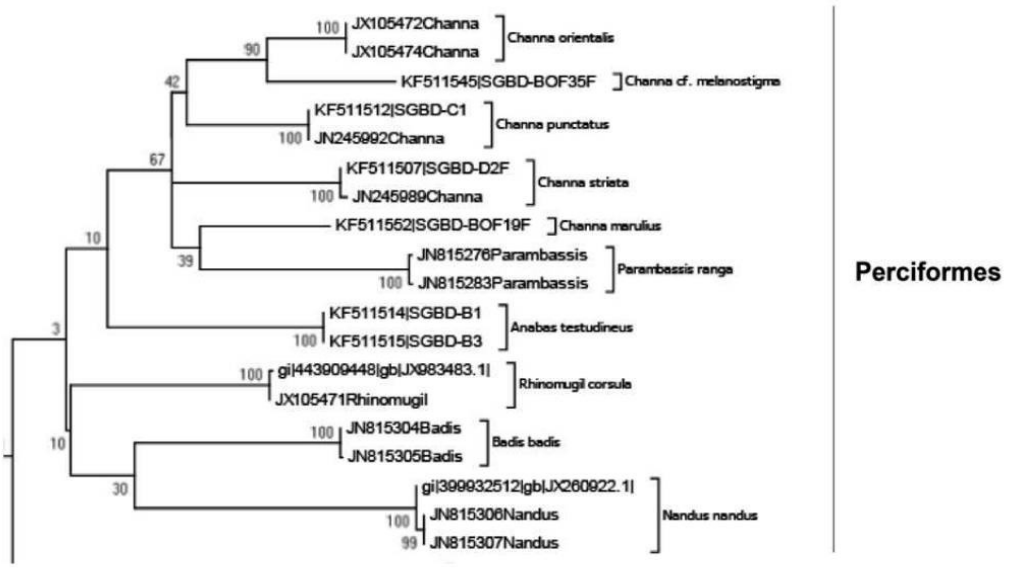

Figure 4.5.2 ML tree showing distinct clustering of the species under the order Perciformes.

ORDER SILURIFORMES:

Order Siluriformes comprising of 14 species (*Mystus vittatus, M. gulio, Eutropiichthys murius, Ailia coila, Glyptothorax telchitta, Gagata dolichonema, G. cenia, Gogangra viridescens, Sisor rhabdophorus, Pseudecheneis sulcata, Erethistes pussilus, Ompok bimaculatus, O. pabda and Clarias batrachus*) under 11 genera and 4 families (Table 4.1.2) showed distinct cluster pattern as shown in Figure 4.5.2. All the members of a species clustered cohesively and separately with respect to other species, similarly all the genera clustered distinctly with a common ancestral node. However *G. guiris* (order perciformes) clustered separately with a common ancestral under Siluriformes as shown in Figure 4.5.3.

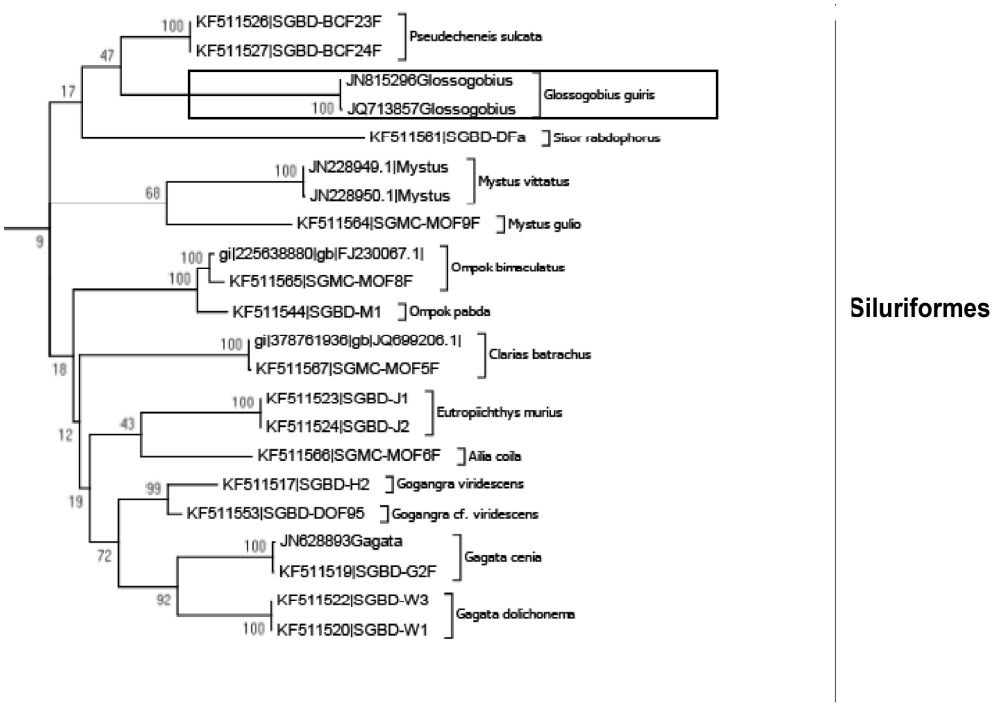

Figure 4.5.3.ML tree showing distinct clustering of the species under the order Siluriformes.

ORDER CYPRINIFORMES

Order Cypriniformes comprising of 24 species under 16 genera and 3 families (Table 4.1.1) showed distinct cluster pattern as shown in Figure 4.5.4. All the members of a species clustered cohesively and separately from other species originating from a single node, similarly all the genera clustered distinctly from other genera. All the taxa clustered distinctly under common ancestral node of Cypriniformes.

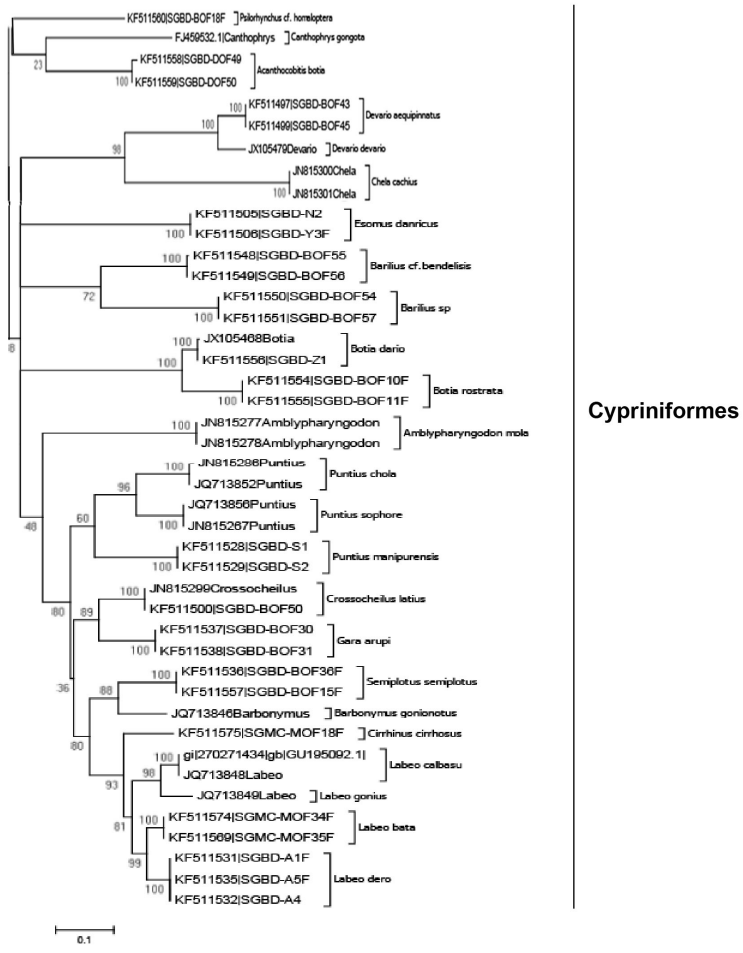

Figure 4.5.4 ML tree showing distinct clustering of the species under the order Cypriniformes.

4.5.2 MOLECULAR CLOCK IN ESTIMATING TIME OF EVOLUTION: ML APPROACH

For estimation of divergence time, the molecular clock uses the mutation rate of DNA/Amino acid sequence of organism to deduce the time of divergence or evolution of two organisms from a common ancestor. This is achieved by performing a Maximum Likelihood test of the molecular clock hypothesis for a given tree topology and sequence alignment. The molecular clock must first be calibrated against independent evidence about dates, such as the fossil records.

4.5.2.1 TESTING THE MOLECULAR CLOCK HYPOTHESIS

The molecular clock test was performed by taking into consideration the *CO1* gene of mt DNA by comparing the ML value for the given topology with and without the molecular clock constraints under General Time Reversible model (+G+I) from model test (table 4.5.1). Differences in evolutionary rates among sites were modeled using a discrete Gamma (G) distribution (shape parameter shown) and allowed for invariant (I) sites to exist (estimate of percent invariant sites shown). The null hypothesis of equal evolutionary rate throughout the tree was rejected at a 5% significance level (P = 0.0009). The Likelihood ratio of the test is summarized in table 4.5.2. The analysis involved 95 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 508 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Table 4.5.2 Results from a test of molecular clocks using the Maximum Likelihood method

	***ln*L**	**Parameters**	**(+G))**	**(+I)**
With Clock	-10107.424	58	0.629	0.43
Without Clock	-10036.171	105	0.59	0.42

4.5.2.2 ESTIMATION OF DIVERGENCE TIME

The time of divergence of different orders of fishes was estimated by generating a ML tree using representative fish *CO1* DNA sequences from the 3 major orders under study under GTR G+I following Baysian Scores. A strict calibration constrains in LRMD method were put at the MRCA (Most Recent Common Ancestor) of the genus *Channa* and *Parachanna* based on the previous fossil records which is 84-110 MYA (Million Year Ago). Another alternate Calibration was made at the node of the emergence of the genus *Channa* as per fossil record which is ~50 MYA. The emergence of different species occurred in different time interval during geological process Figure 4.5.5.

ORDER CYPRINIFORMES:

Order Cypriniformes emerged from the Perciformes and Siluriformes during 213.26 – 69.53 MYA. The different members of Cypriniformes under the current study evolved during 130.04 – 12.31 MYA (Million Year Ago). The mean divergence time or Time to Most Recent Common Ancestor (TMRCA) between *Labeo bata* and *L. dero* is 15.94 MYA. The mean divergence time between *Labeo gonius* and *L. clabasu* is 12.31 MYA. The mean TMRCA for *Cirrhinus cirrhosus* and *Barbonymus* is 33.02 and 59.65 MYA respectively. The *C. latius* and *G. arupi* diverged from a common ancestor around 48.07 MYA, *Puntius manipurensis* has TMRCA of 59.69, where as *P. chola* and *P. sophore* has TMRCA of 34.51 MYA. The mean divergence time for *Botia dario* and *B. rostrata* is calculated to be 17.81 MYA. Similarly, mean the divergence time for *Barilius bendelisis* and *B. barna* is calculated to be 66.31 MYA. *Esomus danricus* and *Chela cachius* divergence from common ancestor (TMRCA) in around 120.73 MYA whereas *Devario devario* and *D. aequipinnatus* in 14.18 MYA.

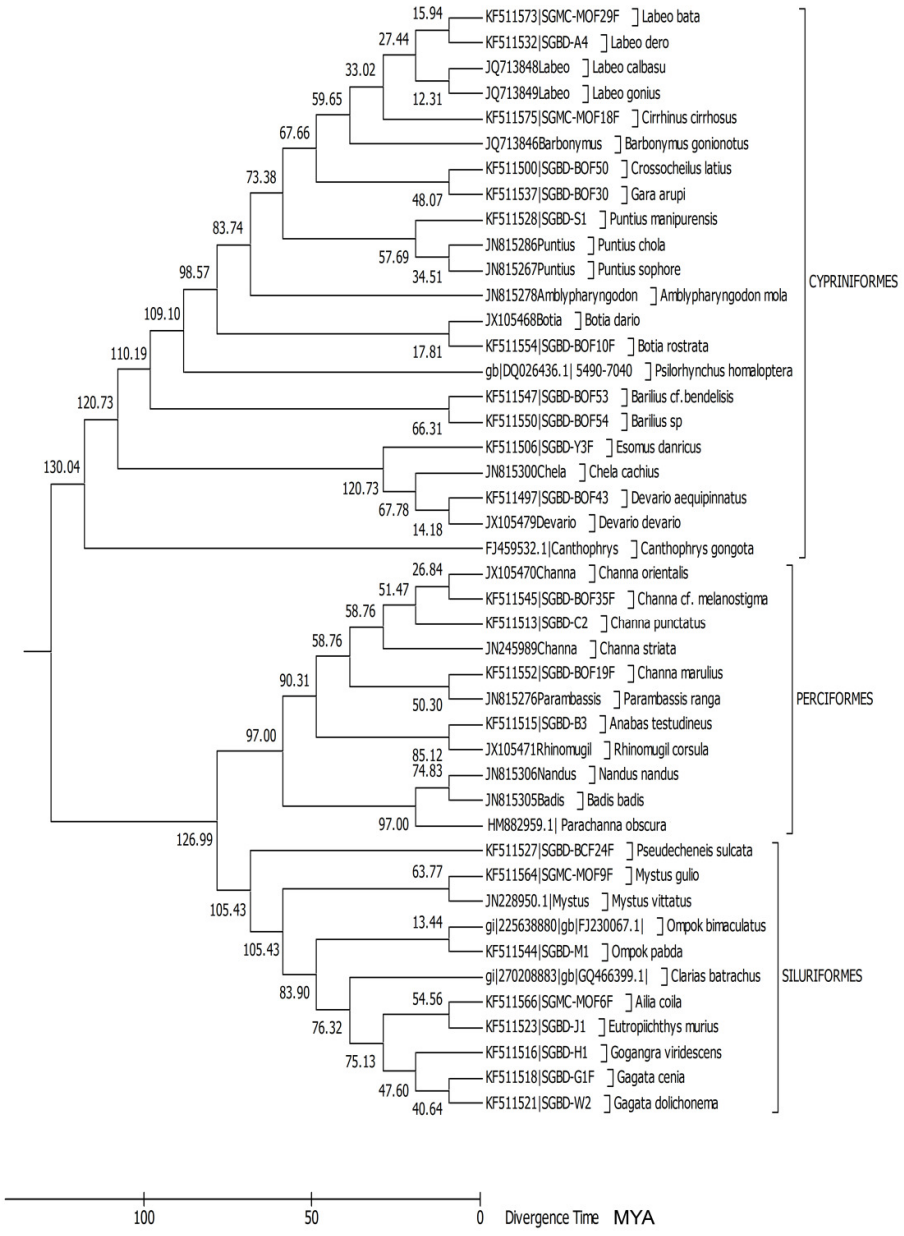

Figure 4.5.5 Time tree of different ornamental fish based on ML approach. The emergence of different species occurred in different time interval during geological process. The calibration constrains in LRMD method were put at the MRCA (Most Recent Common Ancestor) of the genus *Channa* and *Parachanna* based on the previous fossil records.

ORDER PERCIFORMES

Order Perciformes emerged from the other orders during 194 – 59.97 MYA. The different members of Perciformes under the current study evolved during 97– 26.84 MYA. The genus *Channa* diverged from its nearest genus *Parachanna* in around 97 MYA (Calibration point). The mean Time to Most Recent Common Ancestor (TMRCA) between *Channa orientalis* and *C. melanostigma* is 26.84 MYA whereas C. panctatus diverged from its other congeners in 51.47 MYA. The mean TMRCA for *C.striata and C. marulius* is 58.76 MYA. The mean TMRCA for *Parambassis ranga is* found to be 50.3 MYA. The mean divergence time for *Anabas testudinues* and *Rhinomugil corsula* is calculated to be 85.12 MYA. Similarly, mean the divergence time for *Badis badis* and *Nandus nandus* is calculated to be 74.83 MYA.

ORDER SILURIFORMES

The different members of Siluriformes under the current study evolved during 105 – 13.44 MYA. The mean Time to Most Recent Common Ancestor (TMRCA) between *Gagata dolichonema* and *G. cenia* is 40.64 MYA whereas *Gogangra viridiscens* diverged from its nearest genus *Gagata* in around 47.6 MYA of mean TMRCA. The mean TMRCA for *Eutropiichthys murius and Ailia coila* is 54.56 MYA. The mean TMRCA for *Clarias batrachus is* found to be 76.32 MYA. On the other hand the mean divergence time for *Ompok bimaculatus* and *O. pabda* is calculated to be 13.44 MYA. Similarly, the mean divergence time for *Mystus vittatus* and its other congener *M. gulio* is calculated to be 63.77 MYA whereas *Pseudecheneis sulcata* diverged from the other members of Sisoridae with a mean TMRCA of 105.43 MYA.