CHAPTER- 5:

DISCUSSION

5.1 Ornamental fish resources of Manipur, North East India and their status

The state Manipur is one of the 12 mega biodiversity rich zones of the world and forms a distinctive part of the Indo-Burma Biodiversity Hotspot of the world. After the world famous Shiroi Lily and the Sangai (the Brow Antlered Deer), matter has now come to light that Manipur is also a home to a number of fish species which are highly prized for their ornamental values in Europe and the United States. The up-to-date inventory of the fish species of North East India showed 250 potential ornamental fish species. Out of this, the highest number recorded from Assam (187), followed by Arunachal Pradesh (165), Meghalaya (159), Manipur (139), Tripura (103), Nagaland (71), Mizoram (46), and Sikkim (29). Conservation status of native ornamental fishes have shown that out of 250 sp., 10 are critically endangered, 28 are endangered, 49 are vulnerable, 45 are lower risk near threatened, 8 are lower risk least concern, 3 are data deficient and 107 are not evaluated (Tandon *et al.,* 2007). Review of literature indicates that only limited information is available on fish resources of north east India with special reference to its potential as cultivable, sport and ornamental fishes (Ponniah and Sarkar, 2006). Manipur is blessed with the presence of mild climate and abundance of ornamental fishes in nature and contributes the lion's share of total ichthyospecies in the North East India. Presence of diverse natural water bodies is also an added advantage. However, there is vast unexplored potential for indigenous ornamental fishes in this state. Scientific and systematic exploration of this potential will definitely ensure a significant place of the state in this sphere, besides employment generation and earning of foreign exchange. As a result fish stocks particularly those dwelling in inland open water areas, have gradually become endangered. Accurate and unambiguous identification of these ornamental fishes is important in order to overcome the possible future threats of being totally devastated and to assist in managing fisheries for long term sustainability as well as to improve ecosystem research and conservation.

Recent estimates suggested that worldwide 20% of all freshwater fish species are extinct, endangered or vulnerable (Maclean and Jones, 1995). In the IUCN Red List, out of the 21 different species of ornamental fishes studied, *Sisor rabdophorus* is placed in **EN** (**Endangered**) status. Three species *Channa orientalis*, *Badis badis* and

Glossogobius giuris are placed in **VU** (**Vulnerable**) category. *Barbonymus gonionotus, Rasbora daniconius, Labeo gonius, Puntius chola, Puntius sophore, Lepidocephalus guntea, Amblyceps mangois, Mystus vittatus* and *Nandus nandus,* these nine species of ornamental fishes are **Least concern** (**LC**). *Cirhinus mrigala, Labeo bata, Labeo calbasu, Wallago attu, Channa striata* and *Channa punctata* are **LRnt** and only one ornamental fish, *Esomus danricus* is categorized as **LRlc**. According to a new study in the journal *Biological Conservation,* the hobby of keeping ornamental fishes may be causing some of the most beautiful species on earth to become extinct. The study looks at the trade from just one country, India from 2005 to 2012 and it reports that dealers there exported 1.5 million freshwater fish in at least 30 threatened species, including a dozen that are endangered. Just within the red line torpedo barbs, a colorful species complex, more than 3000,000 individual fish were shipped to the United States and a half dozen other countries (Raghavan *et al.,* 2013). Among the 21 different ornamental fishes, only 17 species are categorized as classified ornamental fishes and remaining 4 species namely, *Barbonymus gonionotus, Puntius chola, Puntius sophore* and *Labeo boga* are categorized as non-classified. But recently aquarists are demanding these fishes because of the bright and beautiful coloration along with their strange and extraordinary behavior and characters of these fishes. The fishes under study were being selected based on their specific and strange characters which lead them to be used as aquarium fishes by various aquarists. These features are mentioned briefly in the following table.

Table 9. The ornamental fishes under study mentioning their ornamental characters because of which they are being used by aquarists

Sl no.	Ornamental fish	Ornamental characters
1	*Cirrhinus mrigala*	Charming predatory habit
2	*Nandus nandus*	Calm behaviour
3	*Barbonymus gonionotus*	shining silver coloration
4	*Labeo bata*	Charming predatory habit
5	*Labeo calbasu*	Charming predatory habit
6	*Labeo gonius*	Charming predatory habit
7	*Amblyceps mangois*	body coloration and shape
8	*Labeo boga*	Charming predatory habit
9	*Puntius chola*	Body coloration

10	*Puntius sophore*	Body coloration
11	*Sisor rabdophorus*	Amazing shape and strange color
12	*Mystus vittatus*	Strange feeding habit
13	*Wallago attu*	Strange feeding habit
14	*Glossogobius giuris*	Charming predatory habit
15	*Badis badis*	Chamelionic habit
16	*Lepidocephalus sp.*	Amazing shape and strange color
17	*Channa striata*	Charming predatory habit
18	*Channa punctata*	Charming predatory habit
19	*Channa orientalis*	Charming predatory habit
20	*Rasbora daniconius*	Jumping behaviour
21	*Esomus danricus*	Jumping behaviour

5.2 Morphology based identification of the ornamental fishes and development of DNA barcodes

During the brief morphological identification of the specimens, most of the observed characters were in agreement with the already described characters as per Jayaram (1999) and Talwar and Jhingran (1991). The *Lepidocephalus* fishes were having some different morphological features. Diversified ornamental criteria like beautiful color pattern, stripes and bands extraordinary behaviours etc. were recorded and an inventory is being developed based on their ornamental value and is given in appendix.

The extraction of genomic DNA by Phenol Chloroform extraction method yielded good amount of genomic DNA in all the cases. The concentration of the DNA were in the range of 50 – 250 ng/µl. The fish primers as used in DNA barcoding of Australia's fish species (Ward *et al.,* 2005) successfully amplified the COI gene. The figures 4.1.1. and 4.1.2. clearly showed the above said results. The amplicons were further purified before sequencing to get good quality sequences. After annotation of the sequences they were subjected to ClustalX and MEGA 5.0 softwares for further analysis of the barcode regions. Sequences were submitted to the databases, GenBank and BOLD.

Both the databases GenBank and BOLD revealed definitive identity matches for consensus sequences of 12 species in the range of 98.37% - 100%. One sample, AUFO5

(*Puntius chola*) has 99.38% homology with *Puntius chola* in BOLD but has 95% homology with *Puntius brevis* in GenBank. So we consider this sample as *Puntius chola*. We encountered several difficulties in ascertaining the accuracy of BOLD and NCBI (GenBank) records that illustrate current shortcomings in these systems. BOLD data records and sequences often lack transparency for all but the most common species (Wong, L. L., 2011). Two samples have some problem in similarity search process. The two samples were OF29F and AUECO2 which matched 98% with *Badis badis* and 100% with *Esomus danricus* in NCBI (GenBank) respectively were unable to match any records in BOLD.

5.3 Sequence analysis

One crucial barcoding criteria is that congeneric divergence should be higher than conspecific divergence (Hubert *et al.,* 2008). In our study, the mean value of congeneric and conspecific divergence were found to be 0.217 and 0.006 respectively i.e., 21.7% and 0.6% respectively. Genetic diversity was generally lower within species than between species, with 95% of total intraspecific variation less than 5.48% K2P distance (Collins *et al.,* 2012). The divergence gap between the species is clearly visible in comparison to the divergence within the species and thereby helps in easy discrimination of the species. The pairwise divergence between the species showed that most diverged species-pair among all the analyzed sequences was *Sisor rabdophorus* and *Channa punctata* with a distance of 0.325.

Although barcode analysis seeks only to delineate species boundaries, there is clearly some phylogenetic signal in *COI* sequence data. Congeneric species always clustered together and in most cases so did confamilial species (Ward, R. D. *et al.,* 2005). The Neighbor-joining tree has reaffirmed that the each species of ornamental fishes formed a single tight cluster of *COI* mtDNA variants distinct from the clusters of closely related species. However, one sequence in *Labeo bata* (SGBK-DF1) formed a single distinct clade with greater than 2% K2P distance as proposed by Hebert *et al*. (2003) from their conspecific sequences. This sequence must be interpreted with caution and required further research. Most of the sequences belonging to the same genera were found to be more closely related with a bootstrap support of 77-99%. However, the species *Labeo*

bata was found to be more closely related with *Cirrhinus mrigala* than their other congeneric individuals. High bootstrap support for species nodes in this study and in other animal groups suggested neighbor-joining analysis of *COI* barcode sequences will be widely effective.

The overall nucleotide composition depicted in figure 4.2.2.clearly shows that in all the cases the frequency of G is lowest in comparison to the other three nucleotides i.e., T(U), C and A. Each species have their own way of nucleotide composition which when studied thoroughly could be used as species specific character for each species. The nucleotide composition is almost conserved in 1st codon position but shows biases in 2nd and 3rd codon positions. It is most biased in 2nd codon position. In second codon position, the frequencies of G is lower than 10% in all the ornamental fishes except SGBK-BF6 (*Nandus nandus*), SGBK-OF31F (*Amblyceps mangois*), SGBK-OF29F (*Badis badis*) and SGBK-OF16F (*Glossogobius giuris*). The frequencies of the nucleotide A is very variable among all the ornamental fishes. Likewise the frequencies of other two nucleotide frequencies were also far variable among the fishes. The nucleotide composition analysis results within the genus *Channa* were also in agreement with the interpretation given by the Neighbor joining tree. The nucleotide composition analysis indicates that SGJB-BF2, SBJB-FO1A, EU417795.1 and EU417796.1 showed similar frequency pattern of all the four nucleotides which is in agreement with what is explained by the NJ tree. In the NJ tree these fishes are clustered together. Other observations regarding nucleotide composition pattern are also in agreement with the NJ tree's branching pattern. For the genus *Labeo,* the frequencies of all the four nucleotides are more or less similar and shows conserved nature within the four groups, 1st consisting of SGBK-DF1, HM147890.1, HM147889.1, HM147888.1, HM147886.1 and HM147887.1., 2nd group (SGBK-DF3, EU417800.1, EU417801.1, EU417802.1, HQ645092.2 and HQ645094.2), 3rd group (SGBK-AUJF39, GU195094.1, GU195093.1, GU195089.1, GU195092.1, GU195095.1, GU195091.1, GU195096.1 and GU195090.1) and 4th group (SGBK-DF2). Here also all the observations were in agreement with the results obtained from Neighbor joining tree.

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

5.4 Phylogenetic assessment by Maximum likelihood and Maximum parsimony approach

For phylogenetic studies both Maximum parsimony and Maximum likelihood approaches were adopted. Maximum Likelihood analysis functions much like Maximum Parsimony analysis in that the trees are scored based on a character data set, and the tree with the best score is selected. An ML tree has meaningful branch lengths. These lengths are usually interpreted as being proportional to the average probability of change for characters on that branch. On a branch length of 1 we would expect an average of one change per character.

The MP tree indicated that *Rasbora daniconius* and *Esomus danricus* are closely related, morphologically these two are more or less similar. Similarly, *Labeo calbasu*, *Labeo gonius* and *Labeo boga* clustered very close to each other. They may be closely related species.

Before undergoing phylogenetic test the best model which suits the data was tested through model test in MEGA-5 program. Normally the model with the lowest Bayesian information criterion (BIC) value is selected for undergoing the phylogenetic test. The model test is followed by construction of maximum likelihood (ML) phylogenetic tree under the selected model. Both the ML and MP topology were almost congruent. The most striking observation found is that *Nandus nandus*, *Glossogobius giuris and Badis*

badis. Though they belong to the order Perciformes but clustered close to the Siluriformes fishes whereas *Channid* fishes are clustered separately. All the individuals of *Glossogobius, Nandus, Badis, Channa* belong to the order Perciformes, but few of them (*Nandus nandus, Glossogobius giuris and Badis badis*) clustered separately with the Silurid fish but not with the *Channa*. This may be due to the fact that the order perciformes are polyphyletic origin. However a detailed Morphology as well as molecular study are required in this aspect.

The construction of comprehensive reference libraries is essential to foster the development of DNA barcoding as a tool for monitoring biodiversity and detecting invasive species (deWaard *et al.,* 2011). By developing DNA barcodes of 27 specimens of ornamental fishes found in Manipur and submitting to two public databases, NCBI (GenBank) and BOLD, we provided an important tool for future fish researchers to get easy access to some of the ornamental fishes of Manipur, India.