

CHAPTER-1
INTRODUCTION

1.1 Fishes

The term fish most precisely describes any non-tetrapod craniate (i.e. an animal with a skull and in most cases a backbone) that has gills throughout life and whose limbs, if any, are in the shape of fins (Nelson, 2006). Most fish are ectothermic ("cold-blooded"), allowing their body temperatures to vary as ambient temperatures change, though some of the large active swimmers like white shark and tuna can hold a higher core temperature (Goldman KJ, 1997). Fish are abundant in most bodies of water. They can be found in nearly all aquatic environments, from high mountain streams (e.g., char and gudgeon) to the abyssal and even hadal depths of the deepest oceans (e.g., gulpers and anglerfish). Fishes exhibit greater species diversity than any other group of vertebrates with more than 32,000 recorded species (Froese and Pauly, 2012).

Many types of aquatic animals commonly referred to as "fish". In earlier times, even biologists did not make a distinction- sixteenth century natural historians classified also seals, whales, amphibians, crocodiles, even hippopotamuses, as well as a host of aquatic invertebrates, as fish (Cleveland *et al.*, 2001). However, according the definition above, all mammals, including cetaceans like whales and dolphins, are not fish. In some contexts, especially in aquaculture, the true fish are referred to as finfish (or fin fish) to distinguish them from other animals. A typical fish is ectothermic, has a streamlined body for rapid swimming, extracts oxygen from water using gills or uses an accessory breathing organ to breathe atmospheric oxygen, has two sets of paired fins, usually one or two (rarely three) dorsal fins, an anal fin, and a tail fin, has jaws, has skin that is usually covered with scales, and lays eggs.

1.2 Ornamental fishes

Ornamental fishes usually mean eye-catching colourful and interesting fishes of various characteristics, which are kept as pets in confined space of an aquarium or a garden pool for fun and fancy. They are usually kept in glass aquarium and hence popularly known as 'Aquarium Fishes'. These living jewels need not always have bright colors, as sometimes their peculiar characteristics such as body color, morphology, mode of taking food etc. may also add to their attractiveness. Most of the small food fish which are treated as unwanted for conventional farming have good potency as ornamental

fishes. Generally the ornamental fishes are selected based on their body color (preferably attractive), body shape (unique shape compared to food fishes), and aquarium suitability. Ornamental fish are tropical species; including finfish, such as goldfish, aquatic invertebrates, and amphibians, common in the aquaria trade and not used for sport fishing purposes or raised for food.

Ornamental fishes can be broadly categorized into two categories, classified and non-classified ornamental fishes (Mahapatra *et al.*, 2003). The small fish like *Botia derio*, *Danio dangila*, *Puntius shalynius* etc are classified types of ornamental fish, which can be reared in an aquarium throughout their lifespan. On the other hand, some larger food fishes like, *Rita rita*, *Channa marulius*, *Labeo gonius* etc. are treated as ornamental fish in their juvenile stage and are termed as non-classified ornamental fish.

1.3 Importance of ornamental fishes

Ornamental fish keeping is one of the most popular hobbies in the developed countries of the world and is gaining popularity in many developing markets of the world too. With the increasing popularity of household aquariums in many countries, ornamental fish play an important role in the international fish trade, providing employment opportunities to the rural population and considered as an earner of foreign exchange for many developing countries. However, the scope of this sector and the impact on human and aquatic communities are often inaccurately known and unappreciated. Statistics reported to FAO from member States indicate that the world export value in 1998 of ornamental fish was US\$174 million, with imports valued at US\$257 million. Since 1985 the value of international trade in exports of ornamentals has increased at an average growth rate of approximately 14% per year. According to FAO (2004), export earnings from ornamental fish trade is US \$ 251 million and more than 60% of the production comes from the households of developing countries. The wholesale value of the global ornamental fish trade is estimated to be US\$ 1 Billion while the retail value is US\$6 Billion. More than 2,500 species are traded and some 30-35 species of fresh water fish dominate the market. The trade with an annual growth rate of 8 percent offers a lot of scope for development. The entire industry, when non-exported product, wages, retail sales and associated materials are considered, has been estimated to be worth around US\$15 billion. Such a vast and important industry has the

potential to contribute to the economic growth of states concerned and the sustainable development of aquatic resources.

1.3.1 India's share in global ornamental fish trade

India's share in global ornamental fish trade is negligible and at present the ornamental fish export from India is dominated by the wild caught species. The unit value of ornamental fish is higher than the food fish. Hence this sector offers good opportunity for rural and urban households to augment income and link them to the International trade (MPEDA, ornamental fish assistance schemes).

The popularity and trade of ornamental fish in India is dominated by the exotic species that are bred in captivity. Throughout the world, the export of the ornamental fishes is mostly encircled around the South-East Asian countries, but India's share in this aspect is negligible. West Bengal is a pioneering state in respect to the ornamental fish trade in India. The world trade of ornamental fishes in 2006-2007 was estimated to be nearly Rs. 3,950 crores of which India's shares is estimated to be less than Rs. 6 crores which is only 0.15 per cent of the global trade. Singapore has over 20% share in the world market while Malaysia and Sri Lanka command 7% and 5% respectively. USA is the largest market of ornamental fishes, followed by the European Union and Japan. India's domestic trade is Rs. 10 crores and is growing at the rate of 20% annually. The export of ornamental fishes from India at present is mainly confined to freshwater varieties.

1.4 Distribution of ornamental fishes-World

Freshwater ornamental fishes comprise a diverse group, with up to 150 families reported (Hensen *et al.*, 2010). One of the common families is the Cyprinidae (Teleostei: Cypriniformes), and Hensen *et al.*, (2010) recorded 333 species of this group in the aquarium trade. The global diversity is far higher, however, at over 2,400 species (Nelson, 2006). Many, such as the barbs, danios and rasboras are popular aquarium and pond fishes, being ubiquitously available at low prices from aquarium and general pet-retailers. In particular, the danios and barbs are frequently promoted as being suitable for beginner aquarists.



Fig. 1. Global distribution of ornamental fishes. The map shows the main locations in the world where ornamental fishes and world's largest ornamental fish farms are located. The information is based on the items of the OFI (Ornamental Fish International) journal. OFI was founded on 1st June 1980 in Rome and this organization deals with the state of the international ornamental aquatic industry.

1.4.1 India

Our country has a rich and unique biodiversity with a variety of indigenous ornamental fishes. There are a total of 34 biodiversity hotspots across the world. Out of these 3 are located in India. A biodiversity hotspot is a bio-geographic region with a significant reservoir of biodiversity that is under threat from humans. Indian waters possess a rich diversity of Ornamental fishes, with over 300 varieties of indigenous species. North-East India forms a part of two of the 34 biodiversity hotspots listed by conservation International, the Himalayas and Indo-Burma (Roach, 2005). The Himalayas are the home of the world's highest mountains and deepest gorges. The mountains rise abruptly, resulting in a diversity of eco-systems. Indo-Burma also called the Indo-China Bioregion includes the portions from Eastern India to Vietnam. The state of Manipur belongs to the Indo-Burma region (Myers *et al.*, 2000). This region is endowed with a variety of brilliantly colored ornamental fishes. The region, North-East India, is distinctive in having certain endemic genera of fishes, viz., *Aborichthys*, *Akysis*, *Badis*, *Bangana*, *Chaca*, *Conta*, *Erethistoides*, etc, (Goswami, UC *et al.*, 2012).

Due to the diversity of topographic and climatic features of North-Eastern states of India, this region is rich in endemic fish. These endemic species are attracting hobbyists both locally and globally. The up-to-date inventory of the fish species of North-Eastern hill region 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) (Mahapatra *et al.*, 2007).

1.4.2 Manipur

Manipur which is located in the extreme North Eastern part of India has an area of 22327 square kilometres and is subdivided into 9 districts. After the world famous Shiroi Lily and the Sangai, matter has now come to light that Manipur is also home to a number of fish species which are highly prized for their ornamental values in Europe and the United States. A total of 139 species of ornamental fishes are found in Manipur including both the classified and non-classified ones (Mahapatra *et al.*, 2007). Within the state of Manipur, there are two major river basins, viz. the Barak River Basin and the Manipur River Basin. The Barak River originates from the northern hills of Manipur and is joined by a number of tributaries such as Irang, Maku, Tuivai, etc. and thereafter enters Cachar District of Assam. The Manipur river basin has eight major rivers such as Imphal, Iril, Nambul, Sekmai, Chakpi, Thoubal and Khuga. These rivers are home for many endemic and highly prized ornamental fishes. The Khuga River is famous for having the beautiful endemic ornamental fish, *Puntius khugae*. The rivers draining the Manipur Hill Area are comparatively young due to the hilly terrain through which they flow. These rivers are corrosive in nature and assume turbulent form in rainy season. Important rivers draining the western area include Maku, Barak, Jiri, Irang and Leimatak. These rivers are noted for being the home of many endemic ornamental fishes like, *Barilius bendelisis*, *Acanthocobitis botia*, *Badis badis*, *Sisor raddophorus* etc. Rivers draining the eastern part of the State include Chamu, Khunou and other short streams.

Varieties of indigenous fishes are found in Manipur and these fishes possess characteristics of ornamental fishes. Ngamu is a fine example of such kind of fishes. The North East India is the leading source of ornamental fish in India. This region contributes

85% of the ornamental fish export from India. Among the North Eastern states, Manipur has a vast potential of fishery resources (report of Manipur Science aquarium).

1.5 Problem in species identification

The present status of ornamental fish industry in Manipur is very hopeful. This state is endowed with rich resource of native type of ornamental fish species. In spite of this ornamental fish treasure possess by this region, there are various problems that stand against making headway in this state. Ornamental fishery resources face a range of challenges: the need for their conservation and sustainable use; the need to ensure that benefits are equitably shared; problems caused by habitat loss and degradation, harmful fishing practices (over-fishing and destructive fishing, such as the use of cyanide); and changes in international trade patterns and concerns about the introduction of exotic species (FAO, fisheries and aquaculture department).

In addition, the aquarium hobbyists are always looking for new varieties in the market since they will prove to be something new and gain major interest to others. The term “need of new items or new species in aquariums” is the motivation of our work, since we know that despite of having such a great number of local ornamental fishes in Manipur, all of them are not known to the aquarists in the international market of ornamental fishes. And the main problem faced by the aquarists is the proper identification of these fishes. If an ornamental fish species is properly identified, that very species could be properly conserved and could solve all the related problems. Though many research works has been done for proper identification of the endemic fishes of Manipur (Vishwanath *et al.*, 1985, 1998, 2000, 2004, 2005, 2007, 2012), their work were solely based on morphology. Morphology-based identifications of fishes are time consuming and problematic for several reasons. For some fishes, it is difficult or impossible to identify juveniles. DNA barcoding will assist in both distinguishing species with similar morphologies as well as in resolving the status of sub species. DNA barcoding protocol, has been demonstrated as an effective fish identification tool in situations including consumer protection and fisheries management/conservation (Hebert *et al.*, 2003).

1.6 Mitochondrial DNA with special reference to COI genome

More than 40 years ago, starch gel electrophoresis of proteins was first used to identify species. Nearly 30 years ago, single gene sequence analysis of ribosomal DNA as well as other genes, rDNA, allozymes etc. was being used to investigate evolutionary relationships at a high level (Woese and Fox, 1977), and mitochondrial DNA approaches dominated molecular systematics in the late 1970s and 1980s (Avise, 1994).

Mitochondrial DNA (mtDNA) is the DNA located in organelles called mitochondria, structures within eukaryotic cells that convert the chemical energy from food into a form that cells can use, adenosine triphosphate (ATP). The mitochondrion is an organelle which contains its own DNA encodes 37 genes. These are 13 genes for subunits of respiratory complexes I, III, IV and V, 22 for mitochondrial tRNA and and 2 for rRNA. In animals, mitochondrial genome is regarded as the best target of analysis than the nuclear genome because of many advantageous features like lack of introns, haploid mode of inheritance and its limited exposure to recombination. The mitochondrial DNA has a relatively fast mutation rate, which results in a significant variation in gene sequences between species.

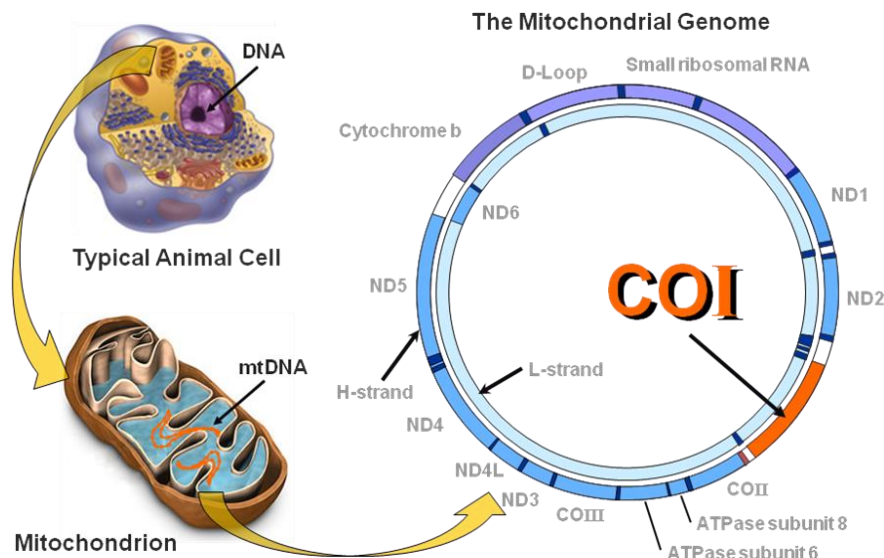


Figure 2. Mitochondrial genome showing the different arrangements of genes with the COI portion being highlighted in orange. A partial fragment of COI is considered as DNA barcode for animals.

The mitochondrial DNA (mtDNA) has a relatively fast mutation rate, which results a significant variation in mtDNA sequences between species and, in principle, a comparatively small variance within species. Robust primers also enable the routine recovery of specific segments of the mitochondrial genome.

1.6.1 DNA barcoding

DNA barcoding involves sequencing a 650 base pair fragment of the mitochondrial gene COI (cytochrome c oxidase I). There is some controversy over the reason for and applications of barcoding. Various authors have proposed different purposes for DNA barcoding, but the most prevalent concept of barcoding is the creation of a library of sequences that can be used to identify previously described taxa (Meusnier *et al.*, 2008; Rubinoff, 2006).

Because barcoding relies on mitochondrial DNA (mtDNA) it has inherent advantages and disadvantages. The mitochondrial genome is known for having relatively well conserved regions that are excellent for primer creation. Mitochondrial DNA, unlike nuclear DNA, has no introns, rarely experiences recombination, and is maternally inherited in a haploid manner (Hebert *et al.*, 2003; Rubinoff, 2006; Ballard & Rand, 2005). Phylogenetics often utilizes mitochondrial DNA because it is useful when studying species-level relationships and recently diverged taxa (Hebert *et al.*, 2003; Rubinoff, 2006). However, the quick rate of evolution that makes mitochondrial DNA so useful for recent divergences becomes problematic when divergences dating to the Mesozoic or earlier are examined (Mitchell, 2008). This rapid rate of evolution of mitochondrial DNA can lead to homoplasy since frequent base pair changes might result in convergent similar sequences in two unrelated taxa (Rubinoff, 2006). The barcoding region is a gene segment within a protein-coding region of the mitochondrial genome. Protein-coding regions of DNA have specific constraints that can be useful or detrimental to this application. First of all, since a change in nucleotides will often have an effect on the amino acids and hence the protein that is produced, sequences can only experience limited changes. Fortunately, the third positions of codons are not under strong selection to remain constant because of the redundancy of the amino acid coding system. Therefore, one-third of the nucleotide sites have a higher potential to change

once species diverge. Another advantage to using protein-coding regions instead of genes encoding RNA is the relative rarity of indels (Hebert *et al.*, 2003). In protein coding genes indels are partially constrained by the necessity of avoiding frame shifts. There are advantages of using the gene COI for barcoding. The mitochondrial genome is often associated with well-conserved primers and the COI gene is particularly rich in highly conserved primers. Hebert *et al.*, (2003) reported that the primers have been functional with “representatives of most, if not all, animal phyla”. This gene has added advantage of both being rapid enough (at silent sites) to differentiate between phylogeographic groups within a species and slow enough (at amino acid replacement sites) to determine deeper phylogenetic relationships (Hebert *et al.*, 2003).

1.7 Applications of DNA Barcoding

Utilizing barcodes for routine species identifications is the most widely accepted of the potential applications. Suggestions have also been made to use DNA barcodes for species descriptions, phylogenetic analysis and conservation efforts although these applications are highly controversial (Rubinoff, 2006).

The original application of DNA barcoding was species identification. Hebert *et al.*'s (2003) plans for barcoding include making a database of all COI barcoding sequences so that future specimens may be identified. This database would become part of a Global Biodentification System (GBS) designed to help solve many of the problems associated with morphological taxonomy and help reduce misidentifications. Will & Rubinoff (2004) point out that the barcodes can successfully identify a specimen only when its barcode sequence is an exact match of an identified specimen that is already in the database. If the sequence is not identical to one already included in the profile then a researcher will have no sure way of identifying the specimen using barcoding. Genetic diversity within a species is a problem when only a single representative of each species is included in the profile (Rubinoff, 2006).

Researchers must assume that intraspecific variation is significantly less than interspecific variation within the barcoding sequence region (Meyer and Paulay, 2005; Langhoffet *et al.*, 2009; Ward, 2009; Lukhtanov *et al.*, 2009). One proposed method to differentiate between species is to set a standard threshold for the percentage of variation that is tolerated for specimen identification. Setting such a boundary is not a solution

because the proposed divergence between species (3% for invertebrates and 2% for mammals and birds) is not successful in delineating between all species (Rubinoff, 2006).

Mitchell (2008) agreed and promoted the use of barcodes to discover new species as long as subsequent morphological and molecular analyses are performed. He cites a study where a species of moth was originally discovered by barcoding and subsequently verified. The use of DNA barcodes to highlight areas of traditional taxonomy that should be re-evaluated has been gaining popularity in recent years (Kerr *et al.*, 2009; Packer *et al.*, 2009). Mitchell (2008) also suggests that barcoding sequences could be used as placeholders when new species are discovered but not yet formally described. This application would facilitate consistent identification of undescribed taxa.

Another of the proposed uses for DNA barcoding is to quickly catalogue all of the biodiversity on the planet before it disappears and to help determine where to focus conservation efforts (Rubinoff, 2006).

1.7.1 Integration of taxonomy and molecular approaches

Taxonomy, the science of assigning names to species and higher taxa, is crucial to other fields of science. Unfortunately, the number of taxonomists is in decline and there are already insufficient numbers of specialists in this field to handle the existing workload (Rubinoff, 2006; Hebert *et al.*, 2003). Hebert *et al.*, (2003) estimate that “since few taxonomists can critically identify more than 0.01% of the estimated 10-15 million species, a community of 15,000 taxonomists will be required, in perpetuity, to identify life if our reliance on morphological diagnosis is to be sustained.” In addition to the large number of taxonomists, a significant time investment is needed – estimates from 940 years to several millennia have been proposed– to describe all existing species if the traditional methods are applied (Meier *et al.*, 2006). Mitchell (2008) agrees that the shortage of taxonomists is difficult to remedy because taxonomic techniques are time consuming and highly specialized. Clearly the sheer magnitude of the problem puts a limit on the capabilities of taxonomy.

Barcoding can utilize the expertise of current as well as former taxonomists, since previously identified museum specimens should be used to produce barcodes whenever possible. This is particularly useful because there is an unfortunate decline in the

popularity of taxonomy and some groups of organisms no longer have expert taxonomists. The barcode database will help preserve taxonomic information in a novel format and allow laboratories without morphology experts to identify relevant species (Hebert *et al.*, 2003). Taxonomists will benefit because with fewer samples to identify they will have more time to pursue research on their specialty taxa (Mitchell, 2008). In addition to the large specialized workforce needed to perform species identifications, morphological taxonomy has other serious challenges. Morphology is not consistent due to phenotypic plasticity and genetic variability within species. In many species, there are also significant differences in morphology between the genders and different life stages. Cryptic species, by definition, are often impossible to identify by morphological characteristics alone. All of these obstacles in species identification often lead to incorrect identification. Another reason for incorrect species identification is that some researchers attempt to use keys without the appropriate level of expertise. Furthermore, some of the taxonomic keys in use are flawed and such keys are rarely revised since to do so is a major undertaking. The current method of species identification is also in need of improvement because it is costly both financially and in terms of time (Hebert *et al.*, 2003; Mitchell, 2008).

Some proposed alternatives to the traditional method of morphological taxonomy involve DNA technology. Researchers have attempted to take advantage of the variability present in the genome to identify species (Hebert *et al.*, 2003). There is a precedent of using mitochondrial DNA sequences in addition to morphology to resolve difficult species identifications (Mitchell, 2008).

1.8 Success of DNA Barcoding

Various studies and analyses of those studies have been performed to determine the success of DNA barcoding for species identification. Meusnier *et al.*, (2008) report barcoding success levels over 97% in studies involving birds, mammals, fishes, and arthropods. Hebert *et al.*, (2003) created a profile of one hundred species from seven diverse animal phyla and then attempted to identify newly analyzed taxa using this profile. This experiment resulted in a 96% success rate of correctly assigning the taxa to the appropriate phylum (it is important to note that this is not species identification). Furthermore, each species had a different COI sequence for the barcoding region. This

process was repeated with a different data set including eight orders of insects and 50 newly analyzed taxa were correctly assigned to each order. Hebert *et al.*, (2003) repeated this experiment once more making a profile for two hundred species that have recently diverged from one another. The 150 newly analyzed individuals were all assigned the correct species identification. Hebert *et al.*, (2003) stated that the majority of the individuals fell into monophyletic groups that reflect their accepted taxonomic relationships. They believe that the barcoding study was exceptionally successful in terms of species identification: ‘test’ taxa were always either genetically identical to or most closely associated with their conspecific in the profile (Hebert *et al.*, 2003). Furthermore, they came to the conclusion that COI was capable of handling this application, even when it comes to deeper divergences (Hebert *et al.*, 2003). The majority of barcoding studies have success rates similar to those determined by Hebert *et al.*, (2003). However, there are some studies that have shown a higher rate than 5% failure to determine species assignment by barcoding alone (Mitchell, 2008). As with any other system, DNA barcoding has its share of flaws which are often more informative than the successes. Hebert *et al.*, (2003) admit that some misidentifications did occur during their study. Errors were thought to be due to hybridization, introgression, polyploidization, incomplete lineage sorting, *Wolbachia* infections (in invertebrates) and “numts” (paralogous copies of mitochondrial genes that are inserted into nuclear DNA) (Hebert *et al.*, 2003; Mitchell, 2008; Ward, 2009). Other studies cite problems with discovering new species using the distance-based methods that are typical of barcoding (Mitchell, 2008). DNA barcoding also encounters problems common to any type of molecular analysis: degradation may make it impossible to amplify a sequence and primers can never be truly universal due to the potential to develop mutations in the primer binding regions (Meusnier, 2008; Mitchell, 2008). Mitochondrial DNA often provides different relationships between taxa than nuclear DNA. A group of researchers analyzed relevant literature and discovered that there were differences among relationships assigned by nuclear and mitochondrial DNA in more than 20% of the studies they surveyed. Due to the nature of the study, this estimate may be conservative which would mean that the barcoding region could provide information that is inconsistent with relationships based on nuclear genes over 20% of the time (Rubinoff, 2006). Some researchers view this abundance of flaws as a reason for evolutionary

biologists to reject the widespread use of DNA barcoding. Others believe that barcoding is the best system we have at present and that almost all systems have some problems associated with them. It seems to be a matter of opinion whether the obstacles that potentially prevent correct identifications are more significant than the benefits to be gained from this novel application of mitochondrial DNA sequencing.

1.9 State of Art of the problem

In spite of the ornamental fish treasure possess by Manipur, there are various problems that stand against making headway in this state. Until now no specific checklist of all the ornamental fishes were made or developed, most of the studies were usually based on some 1 or 2 species belonging to some specific river or stream or other. So there is a need to develop one checklist for the ornamental fishes of Manipur from which one can easily derived the specific fishes' genus, the order and family it belongs etc. Many popular aquarium are now available in the markets in high prices but using the same varieties of ornamental fishes is not so preferred by the aquarists. The aquarium hobbyists are always looking for new varieties in the market since they will prove to be something new and gain major interest to others. The term “need of new items or new species in aquariums” is the motivation of our work, since we know that despite of having such a great number of local ornamental fishes in Manipur, all of them are not known to the aquarists in the international market of ornamental fishes. And the main problem faced by the aquarists is the proper identification of these fishes. Though many research works has been done for proper identification of the endemic fishes of Manipur (Vishwanath *et al.*, 1985, 1998, 2011, 2012), their work were solely based on morphology. Morphology-based identifications of fishes are time consuming and problematic for several reasons. For some fishes, it is difficult or impossible to identify juveniles. DNA barcoding will assist in both distinguishing species with similar morphologies as well as in resolving the status of sub species and morphologically cryptic species.

1.9 Aim and objective of the current research

1. Molecular identification and characterization of the different species of ornamental fishes found in Manipur based on DNA barcode.
2. Sequence analysis of the DNA barcode region of the ornamental fishes.
3. Development of Phylogenetic relationship between the different species identified.
4. Development of reference barcode library of all the ornamental fishes from Manipur.