CHAPTER 2

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2.1 FISH DIVERSITY

The term Biodiversity, describes the existence of variety of life forms on Earth. (Copp et al. 2005, Leveque et al. 2005, Lima-Junior et al. 2006, Mas-Marti et al. 2010) It includes all forms of terrestrial, aerial and aquatic flora and fauna, their genetic material and the ecosystem where they thrive. Global biodiversity is usually divided into three categories: genetic diversity, species diversity and ecosystem diversity. In 1977, Paine arranged lists of world ranking of mega-biodiversity countries. These countries have been ranked according to the species richness of mammals, birds and flowering plants of all the countries of the world. India is one of the mega biodiversity countries in the world. North-eastern region was declared as a biodiversity hotspot by the World Conservation Monitoring centre (Laffaille et al. 2005). The hills and valleys of this area give rise to numerous torrential hill streams and finally become part of Ganga-Brahmaputra-Barak-Chindwin-Kolodyne-Gomati-Meghna system (Kar et al. 2006).

Assessments of biodiversity and execution of conservation programme across the globe are hampered by slow progress in taxonomic research (Hoagland 1996a). The existing taxonomist groups are incapable to cope up with the overwhelming need for basic field surveys, species descriptions and systematic revisions to provide basic information for conservation planning. In addition, few taxonomists are able to distinguish critically between more than 1000 taxa (Costa and Carvalho 2007), but in reality there may be more species which are yet to be described (May and Beverton 1990). It is estimated that 1.4–1.8 million species have been described (Mace 2004, Stork 1988) out of a possible total of approximately 7–15 million (Mace 2004). Species and populations are going to extinct at an alarming pace due to various factors but at poorly understood rate (Hughes et al. 1997, Thomas et al. 2004). Many species may be going to extinct before they can be identified or described. This represents an issue for

conservation planning, because species that have not been identified cannot be protected effectively.

Fishes exhibit greater species diversity than any other group of vertebrates with over 32,000 recorded species (Froese and Pauly 2012). Fish are the group of organisms that are not monophyletic in origin, and therefore their process of evolution is not studied as a single event. Early fossil records are represented by a group of small, jawless, armoured fish known as Ostracoderms. Jawless fish lineages are mostly extinct. An extant clade, the Lampreys may approximate ancient pre-jawed fish. The diversity of jawed vertebrates may indicate the evolutionary advantage of a jawed mouth. It is unclear if the advantage of a hinged jaw is greater biting force, improved respiration, or a combination of factors (Lecointre and Le Guyader 2007). Over 10000 fish species live in fresh water (Lundberg et al. 2000); approximately 40% of global fish diversity and one quarter of global vertebrate diversity. The inventorying of fish diversity is very problematic due to a great range of divergence and is evident throughout the world. Taxonomic expertise is lacking even for major and commercially important groups. The few taxonomists who are working in developing countries, home to more than 95% of globally described species; find it difficult to access basic taxonomic information such as species descriptions. Where taxonomic keys are available, they are rarely revised and often inadequate to identify specimens unambiguously to the species level. This lacking in inventorying of fishes has been imposing threats to global fish biodiversity which can be grouped under five interacting categories: overexploitation; water pollution; flow modification; destruction or degradation of habitat; and invasion by exotic species (Allan and Flecker 1993, Jackson et al. 2001, Malimqvist and Rundle 2002, Naiman and Magnuson 1995, Naiman and Turner 2000, Postel and Richter 2003, Rahel 2002, Revenga et al. 2005). Environmental changes occurring at the global scale, such as nitrogen deposition, warming, and shifts in precipitation and runoff patterns (Galloway et al. 2004, Poff et al. 2002), are superimposed upon all of these threat categories.

International centre for living aquatic resource management (ICLARM) and Food and agricultural organisation (FAO) developed the FishBase project which is a database of fishes that provides information on nomenclature, distribution, ecology, growth, etc. FishBase is a global species database of fish species (specifically finfish). It is one of the most extensively accessed online databases on fish on the web. Over time it has evolved into a dynamic and versatile ecological tool, widely cited in scholarly publications (Stergiou and Tsikliras 2006). Rainer Froese encoded the beginnings of a software database in 1988. This database, initially confined to tropical fish, became the prototype for FishBase. FishBase was subsequently extended to cover all finfish, and was launched on the Web in August 1996. It is now the largest and most accessed online database for fish in the world (Palomares and Bailly 2011).

Over the last century, riverine ecosystems have suffered from intense human intervention resulting in habitat loss and degradation and as a consequence, many fish species have become highly endangered, particularly in rivers where heavy demand is placed on freshwaters. The main causes are habitat destruction and defragmentation, water abstraction, industries and private use (Dawson et al. 2003, Gibbs 2000, Ricciardi and Rasmussen 1999, Szollosi-Nagy 2004) exotic species introduction (Copp et al. 2005), pollution (Lima-Junior et al. 2006) and global climate change impacts (Leveque et al. 2005, Mas-Marti et al. 2010). Freshwater fish are one of the most threatened taxonomic groups (Darwall and Vie 2005) because of their high sensitivity to the quantitative and qualitative alteration of aquatic habits (Kang et al. 2009, Laffaille et al. 2005, Sarkar et al. 2010). As a consequence, they are often used as bio indicator for the assessment of water quality, river network connectivity or flow regime (Chovance and Hoffer 2003). Today the fish diversity and associated habitats management is a great challenge (Dudgeon et al. 2006). Conservation measures to mitigate the impact of the pressures have largely been slow and inadequate and as a result many of the species are declining rapidly.

At the level of the biogeographic realms and taking into account only fully freshwater fish families (i.e. the primary and secondary divisions), the largest

number of families by far (n=43) was found in the Neotropical region, with a high proportion of endemic families (33% or 77%) mainly belonging to the orders Characiformes and Siluriformes. This was followed by Oriental region (33 families, 15 endemic) and the Afrotropical region (32 families, 17 endemic). For strictly freshwater fishes, at the generic and species levels in the different biogeographic realms the overall pattern was quite similar to that at the family level with 4,035 species (705 genera) in the Neotropical region, 2,938 (390 genera) in the Afrotropical, 2,345 (440 genera) in the Oriental, 1,844 (380 genera) in the Palaearctic, 1,411 (298 genera) in the Nearctic, and 261 (94 genera) in the Australian. When taking into account the fresh and brackish-water fishes, the figures are, respectively, 4,231 species (769 genera) in the Neotropical region, 3,272 (542 genera) in the Afro-tropical, 2,948 (609 genera) in the Oriental, 2,381 (551 genera) in the Palaearctic, 1,741 (402 genera) in the Nearctic, and 580 (1,232 genera) in the Australian (Lévêque et al. 2008).

There have been extensive studies on taxonomy, biology and aquaculture of the Freshwater fishes of India, notably, Hamilton (1822), Jayaram (1999, 2010) and Talwar and Jhingran (1991). The marine fishes were distributed along the coastal sites and so far a total of 1740 species of marine fishes were described from India. Besides the marine fishes there were a total of 862 species freshwater fish species described so far of which 672 species are of primary freshwater group (Froese and Pauly 2013). Around 33% of Indian freshwater fishes were distributed along the North-Eastern Region.

The Ichthyofauna of the North-Eastern region of India has elements of the Indo-Gangetic region; and to some extent, of the Myanmar's and South-Chinese regions (Yadava and Chandra 1994). In 1982, it was reported that 172 species of fishes with reference to their economic importance while Sen 1985, reported 187 species of fishes from Assam and its environs. Singh in 1994 compiled a list of 230 species of fishes from north-eastern region of India. Nevertheless, another study in 1997,131 species of fishes was reported from the drainages in Arunachal Pradesh alone. Further, according to Sen 1982, 1985 and 2000, of the 806 species of fishes inhabiting the freshwaters of India (Talwar and

Jhingran 1991), North-Eastern region alone was found to represent 33.13% of the total Indian fresh water fish species. However, no detailed systematic fish inventory has been available on the Ichthyofauna of Mizoram, Tripura and Barak drainage in Assam even until early 21st century, except some works done by Kar and Dey, 2006.

2.2 MOLECULAR TAXONOMY IN FISH DIVERSITY

Species identification by molecular analysis has been used for many years. Initially, allozyme differences were used (Avise 1975), followed by mtDNA examination (Avise 1994). Genomic approaches to taxon diagnosis exploit diversity among DNA sequences to identify organisms (Kurtzman 1994, Wilson 1995). Bacterial identification system using SrRNA sequences was proved to be effective (Busse et al. 1996). DNA being less sensitive to degradation has a huge advantage over their protein-based counterpart methods (Hanner 2005). Moreover DNA can be accessed in all stages from egg to adult. Furthermore, synonymous mutations can be recognized in sequencing approaches, and polymerase chain reaction (PCR) amplification protocols make it possible to analyse minute amounts of tissue. Perhaps most importantly, DNA sequence data are easier to replicate and interpret across laboratories.

An increasing sophisticated realm of molecular techniques has been developed since the mid-1970's to study the molecular similarities of organism. These methods were preceded by protein sequencing and immunology, the widespread use of molecular techniques in fish systematic began with the discovery of allozyme polymorphisms. Allozyme and isozyme studies have been one of the most popular approaches in examining population genetic and stock divergence questions in fishes. They have also been useful in identifying cryptic species and in testing bio-geographic hypothesis. However the main disadvantage of using allozyme approaches is that bands (alleles) that have same electric charge and migrate to the same point in the gel may not be homologous. The scoring of gel is often subjective and bands are difficult to interpret when weak or close together (Kocher and Stepien 1997). Variants have traditionally been assumed to

be selectively neutral, enabling hypothesis of separation time to be tested. However, several studies have shown that allozyme variants are not neutral markers and are under selection (Avise 1994, Pogson et al. 1995, Powers and Schulte 1996).

Mitochondrial DNA regions have been well studied in fishes and knowledge of universal primer sequences (Kocher et al. 1989, Meyer et al. 1990, Palumbi 1996 and Simon 1994) for amplification by PCR and sequencing has made them very accessible. This has been used to address many different levels of taxonomic queries, depending on the region sequenced and the use of various correction factors for types and positions of substitutions. Silent sites of mitochondrial protein coding genes and non-transcribed control region are shown to be particularly useful for analysing relationships of recently diverged taxa such as among populations, species and genera. At higher taxonomic levels, more slowly evolving regions such as the 16S and 12S ribosomal regions were found to be more effective. The sequence evolution in mtDNA has been relatively well studied in fishes. Base substitution events occur relatively rapidly. MtDNA structure, gene order and secondary structure are largely conserved in fishes, as well as in other vertebrates.

Until early 2000, cytochrome b was probable the best studied mitochondrial gene in fishes (Block et al. 1993, Carr and Marshall 1991, Kocher and Stepien 1997, Meyer et al. 1990, Zhu et al. 1994). Although it has been widely used, some have questioned the ability of this sequence to resolve phylogenies (Graybeal 1993, 1994, Meyer 1994).

Mitochondrial ribosomal genes were often used to study more distantly related taxa. Substitutions in the small subunit (12S) accumulate relatively slowly, approximating the average for the entire mitochondrial genome. However, those in the large subunit (16S) evolve even more slowly (Simon 1994).

2.3 DNA BARCODING IN SPECIES IDENTIFICATION

From the University of Guelph, Ontario, Canada, was first established that the mitochondrial gene cytochrome c oxidase subunit 1 (*CO1)* can serve as the core of a global bio identification system for animals by discriminating 200 closely allied species of Lepidopterans and proposed the compilation of public library of *CO1* gene that would be linked to named specimens (Hebert et al. 2003a). In their following work, they suggested that the DNA-based identification system, founded on *CO1* can aid the resolution of this diversity. While the previous work validated the ability of *CO1* sequences to diagnose species in certain taxonomic groups, the next study extended these analyses across the animal kingdom. The results indicated that sequence divergences at *CO1* regularly enabled the discrimination of closely allied species in all animal phyla except the Cnidaria.

This success in species diagnosis reflected both the high rates of sequence change at *CO1* in most animal groups and constraints on intraspecific mitochondrial DNA divergence (Hebert et al. 2003c). Hebert et al. (2004b) sequenced *CO1* sequences of 260 of the 667 bird species that breed in North America. They found that every single one of the 260 species had a different *CO1* sequence. The sequences were either identical or were most similar to sequences of the same species. Hebert and his collegues thus reffered to *CO1* as potential barcode for species identification of animal kingdom.

The popularity of DNA barcoding was due to its inherent advantages over several previous methods. One advantage is its universality the standard DNA barcode region, a fragment of *CO1*, is very efficient for species identification and has good discrimination power for most animal groups. The universal primer, originally designed for marine invertebrates, proved effective for many animal phyla (Folmer et al. 1994, Hebert et al. 2004a, Hebert et al. 2003c).Thereafter, the Barcode of Life project was proposed to promote DNA barcoding as a global standard for sequence-based identification of eukaryotes. In 2004, this project was formally initiated by the establishment of the Consortium for the Barcode of Life

(CBOL), which aims to develop a standard protocol for DNA barcoding and to construct a comprehensive DNA barcode library (Stoeckle 2004).

A major study evaluating the efficacy of DNA barcoding was focused on the Neotropical skipper butterfly, *Astraptes fulgerator* at the Area Conservation de Guanacaste (ACG) in north-western Costa Rica. This species was already known as a cryptic species complex. The *CO1* genes of 484 specimens were sequenced from the ACG. The studies finally concluded that *Astraptes fulgerator* consisted of 10 different species in north-western Costa Rica (Hebert et al. 2004a).

The possibility of using DNA barcoding to assess the past diversity of the earth's biota was examined (Lambert et al. 2005). The *CO1* gene of a group of extinct ratite birds the moa, were sequenced using 26 sub fossil moa bones. As with Hebert's results, each species sequenced had a unique barcode and intraspecific *CO1* sequence variance ranged from 0 to 1.24

Ward et al. (2005) sequenced a 655 bp region of the mitochondrial *CO1* of 207 species of fish. All species could be differentiated by their *CO1* sequence, although single individuals of each of two species had haplotypes characteristic of a congener. Marine biologists also considered the value of the barcoding technique in identifying cryptic and polymorphic species and have suggested that the technique may be helpful when associations with voucher specimens are maintained.

In 2006, it was examined whether a *CO1* DNA barcode could function as a tool for identification and discovery for the 20 morphospecies of Belvosia parasitoid flies (Tachinidae) (Smith et al. 2006). Barcoding not only discriminated among all 17 highly host-specific morphospecies of Belvosia, but it also suggested that the species count could be as high as 32 by indicating that each of the three generalist species might actually be arrays of highly host-specific cryptic species.

In 2007, it was showed that short sequences of *CO1* DNA barcode can yield important, and surprisingly accurate, information about the composition of the entire genome (Min and Hickey 2007). Thus, for unsequenced genomes, the DNA barcodes can provide a quick preview of the whole genome composition.

In 2007, the application of DNA barcodes was introduced to forensics for wildlife crime investigation (Dawnay and Ogden 2007) which previously routinely involved genetic species identification based on DNA sequence similarity. They assessed the *CO1* gene for use in forensic analysis following published human validation guidelines. However, Borisenko et al. (2009) proposed efficient logistics of pre-laboratory specimen processing and seamless interfacing with molecular protocols for building a global library of DNA barcodes.

In 2009, DNA barcoding was used for identification of the commercially used puffer fishes (Cohen et al. 2009). In 2007, two individuals developed symptoms consistent with tetrodotoxin poisoning after ingesting home-cooked puffer fish purchased in Chicago. Both the Chicago retailer and the California supplier denied having sold or imported puffer fish but claimed the product was monkfish. However, genetic analysis and visual inspection determined that the ingested fish and others from the implicated lot retrieved from the supplier belonged to the family Tetraodontidae. Tetrodotoxin was detected at high levels in both remnants of the ingested meal and fish retrieved from the implicated lot. The investigation led to a voluntary recall of monkfish distributed by the supplier in three states and placement of the supplier on the U.S. Food and Drug Administration's Import Alert for species misbranding. This case of tetrodotoxin poisoning highlighted the need for continued stringent regulation of puffer fish importation by the U.S. Food and Drug Administration, education of the public regarding the dangers of puffer fish consumption, and raising awareness among medical providers of the diagnosis and management of food borne toxin ingestions and the need for reporting to public health agencies.

In 2011, a wonderful practical application of DNA barcoding by sequencing of a 658-bp 'barcode' fragment of *CO1* gene of forensically important Sarcophagidae (Diptera) from 85 specimens, representing 16 Australian species from varying populations (Meiklejohn et al. 2011). All species were resolved as reciprocally monophyletic, except *Sarcophaga dux*. The *CO1* 'barcode' sequence was found to be suitable for the molecular identification of the studied Australian Sarcophagidae.

In 2011, the importance of *CO1* DNA barcode was focused on in identification of two marine turtle leeches (*Ozobranchus margoi* and *Ozobranchus branchiatu*s) (McGowin et al. 2011). Using morphological taxonomy combined with distance- and character-based genetic sequence analyses, this study has established a DNA barcode for both species of Ozobranchus spp. leech and has shown that it can be applied successfully to the identification of leeches at earlier stages of development when morphological taxonomy cannot be employed.

In 2011, DNA barcoding of an invasive mammal species, the small Indian mongoose (*Herpestes javanicus*) in the Caribbean and Hawaiian Islands was carried out (Bennett et al. 2011). The work demonstrates the utility of using DNA barcoding approaches with mtDNA cytochrome b to discriminate between the two species and other sympatric members of the genus Herpestes (*Herpestes naso, Herpestes urva,* and *Herpestes edwardsii*).

In 2011, the effectiveness of *CO1* barcode in species identification of Tanzanian antelope species was evaluated (Bitanyi et al. 2011). A 470 base-pair region of the *CO1* gene was examined in 95 specimens representing 20 species of antelopes, buffalo and domestic Bovidae. All the Tanzanian species showed unique clades. Further they demonstrated that even short *CO1* fragments can efficiently identify antelope species.

In 2011, the role of DNA barcoding in the study of marine metazoan was reviewed (Bucklin et al. 2011), with concern that more than 230,000 known

species representing 31 metazoan phyla populate the world's oceans and perhaps another 1,000,000 or more species remain to be discovered. The campaign of barcoding increased with the involvement of more and more organisms (Bell et al. 2011, Clare et al. 2011, Janzen et al. 2011) to reveal the actual level of diversity among them.

In 2011, another group of workers developed and evaluated DNA barcodes for use in differentiating United States domestic and imported catfish species (Wong et al. 2011). They also suggest that as the United States heightens inspection and regulation requirements for sea food products, DNA barcoding will serve as an important tool in efforts to ensure consumer safety and fair international commerce.

In 2011, a barcode reference library for more than 80% of the named freshwater fish species of North America was established (April et al. 2011). This study demonstrates that 90% of known species can be delineated using barcodes. Moreover, it reveals numerous genetic discontinuities indicative of independently evolving lineages within described species, which points to the presence of morphologically cryptic diversity. From the 752 species analyzed, their survey flagged 138 named species that represent as many as 347 candidate species, which suggests a 28% increase in species diversity. In contrast, several species of parasitic and no parasitic lampreys lack such discontinuity and may represent alternative life history strategies within single species.

Bucklin et al. (2011) calculated an average retrieval of 2% new species in larger fish DNA barcoding studies, and they extrapolated this rate to about 600 overlooked or cryptic species to await discovery through similar studies. From the 31,000 species currently listed in the Catalogue of Fishes, about 4000 have been described new during the past 10 years (2000–2009), with 500 added in 2008 and 300 in 2009 (Eschmeyer et al. 2012).

Reid et al. (2011) assessed variability within the barcode region and the utility of both distance-based and character-based methods for species

identification for *CO1* barcode sequences (650 bp) for 174 turtle species. They suggested that complementing distance-based barcoding with character-based methods for identifying diagnostic sets of nucleotides provided better resolution in several cases where distance-based methods failed to distinguish species.

In 2012, barcoding approach for ornamental cyprinid fishes was presented a by expanding current barcode reference libraries (Collins et al. 2012); assessing barcode congruence with morphological identifications under numerous scenarios (e.g. inclusion of GenBank data, presence of singleton species, choice of analytical method); and providing supplementary information to identify difficult species. DNA barcoding offered a potentially attractive tool for quarantine inspection, as scrutinised for aquarium fishes. This was thus an efficient solution to the problem of poor regulation of international trade in ornamental fishes, which poses risks to both biodiversity and economic activity via invasive alien species and exotic pathogens. Border security officials need robust tools to confirm identifications, often requiring hard-to-obtain taxonomic literature and expertise in this context species identification through DNA barcoding provided an effective solution.

A comprehensive barcoding analysis of 22 Nassarius species was reported (Zou et al. 2012). They integrated the mitochondrial and nuclear sequences and the morphological characters to determine 13 Nassarius species and revealed four cryptic species and one pair synonyms. Distance, monophyly, and character–based barcoding methods were employed.

Vargas et al. (2012) developed a DNA-barcoding workflow capable of processing potentially large sponge collections and is routinely used for the Sponge Barcoding Project with success. Sponge specific problems such as the frequent co-amplification of non-target organisms have been detected and potential solutions are currently under development. The initial success of this innovative project has already demonstrated considerable refinement of sponge systematics.

The first comprehensive study was presented in 2012 targeting the entire reptile fauna of the fourth-largest island in the world (Nagy 2012), the biodiversity hotspot of Madagascar. Compared with available multi-gene phylogenies, DNA barcoding correctly assigned most samples to species, genus and family with high confidence and the analysis of fewer taxa resulted in an increased number of well supported lineages.

Taylor and Harris (2012) reviewed the DNA barcoding enterprise, its continued resistance to improvement and the implications of this on the future of the discipline. They anticipated the consistent failure of DNA barcoding to recognize its limitations and evolve its methodologies, reducing the usefulness of the data produced by the movement and throwing into doubt its ability to embrace NGS.

A DNA barcode data release for 3,400 specimens representing 521 species of fishes from 6 areas across the Caribbean and western central Atlantic regions (FAO Region 31) were represented (Weigt et al. 2012). Merged with their prior published data, the combined efforts resulted in 3,964 specimens representing 572 species of marine fishes and constitute one of the most comprehensive DNA barcoding ‘‘coverages’’ for a region reported to date. Kadarusman et al. (2012) assessed the diversity of the Papua rainbow fishes with DNA barcoding. Unexpected levels of cryptic diversity and endemism were detected since additional cryptic lineages were detected in several watersheds from the Vogelkop and the Lengguru massif.

DNA Barcoding also proved to be effective in characterizing Catfish from Northeast India (Bhattacharjee et al. 2012) where species range expansion, species synonymy, etc. and other taxonomic discrepancies were addressed.

Many large-scale barcoding projects have been initiated in 2013 with many of them exploring new avenues for application of DNA barcodes (Alcántar-Escalera et al. 2013, Brodin et al. 2013, Di Pinto et al. 2013, Galimberti et al.

2013, Hebert et al. 2013, KeskİN and Atar 2013, Maas et al. 2013, Pino-Bodas et al. 2013). Shen et al. (2013) used DNA barcoding to detect erroneous sequences in GenBank by evaluating deep intraspecific and shallow interspecific divergences to discover possible taxonomic problems and other sources of error.Galimberti et al. (2013) explored the effectiveness of DNA barcoding in food traceability, and to delineate some best practices in the application of DNA barcoding throughout the industrial pipeline. Porco et al. (2013) surveyed the occurrence and genetic structure of two major groups of soil invertebrates in both their native and introduced ranges: Collembola and earthworms. Their study established that invasive species surveys employing DNA barcoding gain additional resolution over those based on morphology as they allow evaluation of cryptic lineages exhibiting different invasion histories.

In 2013, a group of workers investigated herbal product integrity and authenticity with the goal of protecting consumers from health risks associated with product substitution and contamination (Newmaster et al. 2013). Product substitution occurred in 30/44 of the products tested and only 2/12 companies had products without any substitution, contamination or fillers. Some of the contaminants were found to pose serious health risks to consumers. In 2013 a major initiative was announced for formation of Cold Code, the international initiative to DNA barcode all species of these 'cold-blooded' vertebrates (Murphy et al. 2013).

In 2013, another breakthrough was made through DNA barcoding where more than 100 year Taxonomic perplexity was solved in Mahsheer fish (Laskar et al. 2013). Here in this case a species *Tor progenius* which was thought to be species under genus *To*r and was a matter of dispute among the taxonomist was ultimately found to be the true species of *Tor putitora.*

There are also evidences where *CO1* barcode sequence is proved to be useful in authentication of commercial biological items used in trade like Herbal food supplement (Little and Jeanson 2013), etc. and in addition for trade monitoring for threatened animals (Luo et al. 2013).

In 2013, a survey through DNA barcoding approach was made for mitochondrial DNA (mtDNA) variation in captive populations of 10 species of Neotropical amphibians maintained in an ex situ assurance programme at El Valle Amphibian Conservation Center (EVACC) in the Republic of Panama (Crawford et al. 2013). Even after Ten years of initiation of DNA barcoding and mass scale barcoding of earth's biota, many new groups are still left and or have been recently barcoded for the first time (Alonso et al. 2014, González-Varo et al. 2014, Leavitt et al. 2014, McFadden et al. 2014, Shokralla et al. 2014, Vargas et al. 2014, Vierna et al. 2014).

Amalgamation of NGS technology and DNA barcoding will be the new exciting turn point of barcoding in near future. Shokralla *et al.* 2014 demonstrated the potential application of next-generation sequencing platforms for parallel acquisition of DNA barcode sequences from hundreds of specimens simultaneously. Several studies have reviewed the methods and potential applications of DNA barcoding, most have focused on species identification and discovery, and relatively few have addressed applications of DNA barcoding data to ecology. These data, and the associated information on the evolutionary histories of taxa that they can provide, offer great opportunities for ecologists to investigate questions that were previously difficult or impossible to address. Joly et al. (2014) presented an overview of potential uses of DNA barcoding relevant in the age of eco-informatics, including applications in community ecology, species invasion, macroevolution, trait evolution, food webs and trophic interactions, meta communities, and spatial ecology. They also outlined some of the challenges and potential advances in DNA barcoding that lie ahead.

In 2014, the modified versions of three DNA extraction kits (i.e., Qiagen DNeasy Blood and Tissue Kit, Sigma-Aldrich Extract-N-Amp Kit; and Life Technologies Mag Max-96 DNA Multi-Sample Kit) and two polymerase chain reaction (PCR) setup methods (manual vs. automated) for use in DNA barcoding, with a focus on minimizing time, costs, and labor (Hellberg et al. 2014). Overall, the modified Extract-N-Amp Kit offered the greatest reduction in time and costs, while the DNeasy Blood and Tissue Kit produced sequences with the highest

quality and highest initial success rates. Automation of the PCR setup process resulted in slightly greater success (100%) compared to manual PCR setup.

Khedkar et al. (2014) described the species diversity of fishes of the Narmada River in India. Keskin (2014) used eDNA approach to investigate non-native freshwater fish species from fifteen different locations of Upper Sakarya Basin. They detected four of the most common invasive freshwater fish species. Their results clearly indicated that eDNA surveys could be used as an important molecular tool to monitor invasive fish species in freshwater ecosystems.

In 2015, the results of the FISH-BOL barcode project was collected and assessed the coverage for each family of bony shore-fishes and reef fishes from the tropical western Atlantic Ocean (Victor et al. 2015). The species from the public and private barcode lineages from the region on BOLD was identified; further identifications process is confirmed by vouchers, phylogeographic deduction, and the process of elimination. It has been estimated that 1029 of 1311 total bony shore-fish species in the region are barcoded (78.5%). For reef-associated fishes, 902 of 1083 species are barcoded (83.3%). About 70 of the 181 species not yet barcoded are endemic species from Florida/Gulf of Mexico or Venezuela, leaving about 90% of the central Caribbean reef fish species barcoded to date. Most species are represented by one barcode lineage, but among the gobioids and blennioids there are many more lineages (BINs) than species, indicating substantial cryptic diversity.

In 2015, an attempt to barcode the Lake Whitefish (*Coregonus clupeaformis*) (Overdyk et al. 2015) was made as it is a valuable species both commercially and recreationally in Lake Huron. As naturally the geographic and bathymetric separation of the three major basins in Lake Huron, the potential separation of Lake Whitefish within these basins, and the variation among life history (early and late spawning), it has been that Lake Huron might harbour cryptic lineages of Lake Whitefish at the basin level. To test this prediction, DNA barcodes of the based on *CO1* gene sequences were recovered from spawning phase Lake Whitefish (n = 5 per site), which were collected from sites (n = 28) around Lake Huron Fall during 2012. These sequences, combined with other

publically available DNA barcodes from the Barcode of Life Data System (BOLD), which thereafter helped to reveal twelve unique haplotypes across North America, with seven unique to Lake Huron.

In 2015, DNA barcoding approach for Seafood authentication which is a global concern was used (Naaum and Hanner 2015). As seafood consumption increases, so does public awareness of the associated nutritional and environmental issues related to seafood mislabeling. Cases of substitution continue to be observed, even after the adoption of DNA barcoding as a regulatory tool by the Food and Drug Administration in the United States in 2011. In this study high school students and educators were participated in a market survey using DNA barcoding to identify seafood. The Canadian Food Inspection Agency Fish List was used to determine whether any mislabeling had occurred. It was found that Twenty-three percent of samples were mislabeled, suggesting that the incidence of retail seafood mislabeling continues to be significant in Canada. Similarly, mislabeling in imported fish products were detected in China through the use of DNA barcoding (Yan et al. 2016), The fish were identified as smallmouth scad *Alepes apercna* (Perciformes, Carangidae) based on morphological characteristics as well as DNA barcode data, but were labeled as *Rastrelliger brachysoma.*

In 2016, Seafood mislabeling in the market of India in terms of economical and health concern was diagnosed (Nagalakshmi et al. 2016). Among different molecular markers, DNA barcoding has been successfully applied for seafood authentication. The study was aimed to find out the level of seafood mislabeling prevailing in India using DNA barcoding. A total of 100 seafood samples including fresh, frozen, ready-to-cook, ready-to-eat and canned products were collected from different geographical locations of India. Samples were authenticated by comparing the *CO1* gene sequences with public reference taxonomic databases. The present study reveals 22% of seafood mislabeling prevailing in Indian domestic market.

In 2016, a group studied the spawning area of the Atlantic eels and other fishesin Sargasso Sea in the North Atlantic subtropical gyre (Ayala et al. 2016). In

order to evaluate spatial variability of larval fish in the region, the group examined species diversity, composition and abundances at eight stations in the Subtropical Convergence Zone (STCZ) through combined approach of morphological and DNA barcode based identification. From a total of approximately 3500 collected specimens, minimum of 154 species from 50 families could be identified properly. The family Myctophidae had the highest species richness, with at least 32 species represented. The myctophids *Lepidophanes gaussi*, *Bolinichthys indicus, Notolychnus valdiviae* and *Ceratoscopelus warmingii* were the four most abundant species. Other common species included the three eels: *Nemichthys scolopaceus, Ariosomaba learicum* and *Anguilla anguilla*. Larval fish species composition differed substantially between the relatively closely spaced stations on either side of prominent hydrographic fronts in the study area, most probably because of the strong environmental gradients. Common eel species were concentrated between the fronts whereas common myctophids were of highest abundance at the outer edges of the fronts. The abundances of most species were generally enhanced in the vicinity of the fronts. The use of combined morphological and DNA-barcoding methods facilitated species identification, and documentation of the substantially higher levels and a larger degree of spatial variability in species diversity of fish larvae than previously shown for oligotrophic ocean areas.

In 2016, DNA barcoding for Ecological monitoring for the understanding of complex ecosystem functions was studied by a group of scientist (Jo et al. 2016). They used the diets of fish integrating indicators of environmental status. Nevertheless, to overcome the difficulties to visually identify items in gut contents to species level due to digestion of soft-bodied prey, etc. they used molecular approach to determine the species identities of consumed diet items of an introduced generalist feeder, brown trout (Salmo trutta), in 10 Tasmanian lakes and compared the results with those obtained from visual quantification of stomach contents. They obtained 44 unique taxa (OTUs) belonging to five phyla, including seven classes, using the barcode of life approach from cytochrome oxidase I (*CO1*). Compared with visual quantification, DNA analysis showed greater accuracy, yielding a 1.4-fold higher number of OTUs. Rarefaction curve

analysis showed saturation of visually inspected taxa, while the curves from the DNA barcode did not saturate. The OTUs with the highest proportions of haplotypes were the families of terrestrial insects Formicidae, Chrysomelidae, and Torbidae and the freshwater Chironomidae. Haplotype occurrence per lake was negatively correlated with lake depth and transparency. Nearly all haplotypes were only found in one fish gut from a single lake. This indicates that DNA barcoding of fish diets is a useful and complementary method for discovering hidden biodiversity.

In 2016, a study evaluates the usefulness of DNA barcoding to traditional morphology-based species identifications for the fish fauna of the north-eastern Congo basin (Decru et al. 2016). The *CO1* sequences of 821 samples from 206 morphologically identified species were compared for best match, best close match and all species barcoding analyses which ultimately resulted in a low identification success of 87.5%, 84.5% and 64.1%, respectively. The ratio 'nearest-neighbour distance/maximum intraspecific divergence' was lower than 1 for 26.1% of the samples, signifying possible taxonomic problems. In ten genera, belonging to six families, the number of species inferred from mtDNA data exceeded the number of species identified using morphological features; and in four cases indications of possible synonymy were detected. Finally, the DNA barcodes confirmed previously known identification problems within certain genera of the Clariidae, Cyprinidae and Mormyridae. The results accentuate the large number of taxonomic problems persistent in the taxonomy of the fish fauna of the Congo basin and illustrate the utility of DNA barcodes to compile a reliable taxonomic inventory of the Congo basin fish fauna.