Unlocking Nature's Genetic Secret: The Power of DNA Barcoding to identify mislabeled fish species with reference to Upper Lake, Bhopal

ABSTRACT

Fish comprise slightly more than half of all vertebrates, with India accounting for 7.7% of the world's fish variety. Because of their great diversity and phenotypic flexibility, it can occasionally be challenging to identify them only based on morphological features in their various developmental phases. DNA barcoding has emerged as a transformative tool in biological research, revolutionizing the way we identify and classify species. This review explores the profound impact of DNA Barcoding in elucidating biodiversity of Upper Lake, Bhopal (M.P) and addressing challenges of misrepresentation and ambiguity in species classification. By employing short, standardized DNA sequences from specific gene regions as a molecular barcodes, DNA barcoding offers rapid, accurate and objective species identification transcending the limitation of traditional morphological methods. From biodiversity discovery and ecological monitoring to applications in agriculture, food safety, conservation and human health, DNA barcoding has broad-range which highlight its pivotal role in advancing our understanding of the living world and its potential to address pressing global challenges. Through a synthesis of key findings and advancements in the field, this review underscores the power of DNA barcoding in unlocking nature's genetic and shaping the future of biological research.

Keywords: COI Gene, DNA Barcoding, Fish Biodiversity, Taxonomy, Species Identification, Upper Lake

INTRODUCTION

Fishes exhibit the greatest species diversity, with over 32,300 fish species documented worldwide (Froese and Pauly, 2018). In India, there are approximately 3,157 fish species, comprising 892 purely freshwater species, 1,545 purely marine species, and 17 purely brackish water species (NBFGR, 2020). The most vulnerable ecosystems are freshwater ecosystems (Sala *et al.*, 2000). Upper Lake, a peaceful haven nestled in the heart of Bhopal, Madhya Pradesh, also known as "Bada Talab," this expansive water body is one of the most picturesque and iconic landmarks of the city. Spanning over an area of approximately 31 square kilometers, Upper Lake is not only a source of natural beauty but also holds immense cultural and historical significance. Steeped in history, Upper Lake dates back to the 11th century when it was built by Raja Bhoj, the legendary king of Malwa. Over the centuries, it has served as a lifeline for the city, supplying water for irrigation, fishing, and recreational activities. Upper Lake was designated as a Ramsar site on August 2002. Ramsar sites are wetlands of international importance designated under the Ramsar Convention, an international treaty adopted in 1991 to protect wetlands around the

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world. Upper Lake is not only a scenic attraction but also a hotspot of biodiversity, including various species of fish.

Fish species diversity: Upper Lake supports a diverse range of fish species, (Table 1) providing a habitat for both native and introduced fish varieties. These species have adapted to the lake's unique ecosystem and contribute to its ecological balance.

Introduced Species: Over the years, several exotic fish species have been introduced to Upper Lake, either intentionally or accidentally. These introduced species may include various types of tilapia, trout, and others.

Roles in the Ecosystem: Fish play a crucial role in the ecosystem of Upper Lake. They contribute to nutrient cycling, control populations of aquatic organisms, and serve as prey for various predators, thus maintaining the ecological balance of the lake.

Challenges: Upper Lake faces various challenges related to fish biodiversity, including habitat degradation, pollution, invasive species, and overfishing. These factors can have detrimental effects on the fish populations and overall health of the lake ecosystem.

This Lake supports a rich diversity of fish species (Figure 1), encompassing both native and introduced taxa. The distribution patterns of fish species within the lake vary spatially and temporally, influenced by factors such as water quality, habitat structure, and anthropogenic disturbances. Sahadevan and and Shrivastava (2000) are credited with providing the first information about the ichthyofaunal assemblance of the lake, recording 43 species of fish. Kodarkar (2009) has identified 42 species. Mishra (2007) found that there are 43 species in 19 genera. The fish fauna of Upper Lake Bhopal has also been examined by Joshi (1992), Shrivastava (1994) and Valecha (1995). Tamot and Awasthi (2010) focused on Indigenous fish species, while Napit (2013) documented 43 different species in the same context.

Sharma *et al*., 2014 documented 56 species within the lake, with 36 fish species displaying Ornamental characteristics. Among these ornamental species, the dominant order was Cypriniformes, comprising 15 species, followed by Perciformes with 12 species, Siluriformes with 5 species, Synbranchiformes with 2 species, and Beloniformes and Osteoglossiformes each contributing 1 species. Within the dominant order Cypriniformes, the family Cyprinidae exhibited prominence, consisting of 12 genera, with the genus Channa being notably dominant with 4 species. Hussain *et al*. (2017 further contributed by recording 35 fish species in the Upper Lake of Bhopal. (Singh and Gupta, 2019) reported the presence of 27 fish species belonging to 4 orders namely Cypriniformes (21), Ophiocephaliformes (3), Perciformes (2), Mestacembeliformes (1) and nine families Cyprinidea (17 species), Ophiocephalidae (3 species), Cobitidae, Heteropneustidae, Siluridea, Bagrigae, Mestacembelidea, Gobidae, Ambassidae (1

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species) and 16 genera. Species identification and classification have historically fallen within the specialized domain of taxonomists, serving as the foundation for nomenclature and facilitating various biological investigations (Frezal and leblois, 2008). In recent years, a significant environmental challenge revolves around the diminishing biodiversity, with variations observed across different taxonomic groups. This issue has garnered attention at multiple levels. However, efforts to conserve biodiversity have faced uncertainty due to the lack of precise global baseline data and the unknown rate of loss, particularly concerning fundamental knowledge (Dirzo and Raven, 2003). As articulated by Dayrat (2005), correctly delineating species boundaries and identifying species are fundamental to uncovering life's diversity, as they ascertain whether distinct individual organisms belong to the same entity or not. A DNA barcode is a unique pattern of DNA sequence that has the ability to identify every living things, much like the distinctive bar pattern in a universal product code (UPC) identifies every consumer goods (figure 2).

The accurate identification of species is fundamental to various disciplines, including ecology, evolutionary biology and conservation. Traditional taxonomical approaches relying solely on morphological characteristics often face challenges due to morphological variations within species, cryptic species complexes, and limited taxonomical expertise. These challenges lead to misrepresentation and ambiguity in fish identification impacting our understanding of biodiversity patterns and hindering conservation efforts. DNA barcoding, a molecular technique based on short standardized DNA sequences, offers a promising solution to these challenges by rapid, accurate and objective means of species identification. Due to morphological similarities between nearby species, genetic identification of biodiversity is now necessary. Certain organisms, particularly fish exhibit phenotypic plasticity in response to environmental changes (Hutchings *et al*., 2007). DNA barcoding could play a significant role in the biological conservation and management of biodiversity and fisheries in Upper Lake Bhopal.

Outline of DNA Barcoding

DNA Barcoding is a diagnostic technique in which short DNA sequences can be used for species identification. Hebert *et al*. (2003) original proposed the use of cytochrome c oxidase gene (COI) as a universal animal barcode for the purpose to differentiating across Lepidopteran species in the context of DNA barcoding (Hebert *et al*., 2003). DNA barcoding is able to identify organisms in both their early stages and adulthood. Ko *et al*. (2013), for instance, identified 100 fish larvae specimens with a success rate of >65% at the species level using DNA barcoding technology. Similarly, Naim *et al*. (2012) effectively classified about 60 mud crab individuals into four species using the COI gene. The fundamental concept of DNA barcoding lies in the observation that there is greater variation between species, allowing for species differentiation through the use of short, standardized nucleotide sequences. While the utilization of DNA barcoding is very controversial for using a single mitochondrial gene for species identification, it is believed to be a powerful tool (Ward *et al*., 2009). In addition to distinguishing between

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species, DNA barcoding can also occasionally differentiate between higher taxonomic levels such as genera, families, orders, and classes, which are attributed to the continual augmentation in genetic variation (Hubert *et al*., 2008). At present, utilizing barcode analysis represents a cost-efficient choice for species identification under certain circumstances, and this trend is expected to grow as reference libraries are established and analytical protocols are simplified (Hajibabaei *et al*., 2005). Furthermore, DNA barcodes have been acquired for over 8000 fish species, with the COI sequences stored in the Barcode of Life Data Systems (BOLD) online platform and repository (Ratnasingham and Hebert, 2007).

COI Gene: Species Identification

The cytochrome c oxidase subunit 1 (COI) gene (Figure 3) has emerged as a cornerstone in DNA barcoding for species identification. This gene, found in the mitochondrial genome, exhibits considerable variation between species while maintaining a relatively conserved sequence within species. This unique property allows scientists to accurately distinguish between different species by sequencing the COI gene and comparing it to reference database. The COI gene sequence is deemed appropriate for this task due to its typically rapid mutation rate, which enables the differentiation of closely related species. Additionally, the sequence of this gene remains relatively consistent among individuals within the same species. Despite initial skepticism regarding the ability of COI sequence differences to distinguish closely related species, it has been observed that a sequence divergence of more than 2% is commonly identified between such organisms, indicating the effectiveness of the barcode.

Potential of DNA Barcoding in Upper Lake Bhopal:

In the context of Upper Lake Bhopal, DNA Barcoding holds immense potential to address the challenges associated with species identification and biodiversity assessment. By generating DNA barcode libraries encompassing the Lake's Fauna, researchers can create a comprehensive reference database for species identification. This database can aid in the detection of invasive species, monitoring of endangered taxa, and assessment of ecosystem heath. Moreover, DNA Barcoding enables the identification of larval or cryptic life stages, which are often overlooked using traditional morphological approaches.

Procedure of DNA Barcoding for Fish Identification

Hundreds of laboratories worldwide have participated in the barcoding endeavor to catalog the world's biodiversity. As a result, novel techniques for Polymerase Chain reaction (PCR) and DNA extraction have been created increasingly in tandem with the creation of barcoding primers (Handly *et al*., 2017).

Most widely utilized techniques for Extracting DNA

To successfully extract pure DNA, cells/tissues must be effectively disrupted, nucleoprotein complexes denatured, nucleases inactivated and contaminants removed. The most common procedures include:

- Whether organic extraction (several phenol/chloroform methods) or a multi-step liquid chemical technique can be used. Cells are lysed in the presence of a detergent (CTAB: cetyltrimethylammonium bromide; SDS: sodium dodecyl sulfate) in this traditional, extensively used method, and cell debris is typically removed by centrifugation. After that, proteins are precipitated with organic solvents like phenol and chloroform after being denatured or digested by a protease. Centrifugation is used to remove the protein precipitate after separation. Typically, ethanol or isopropanol precipitation is used to extract purified DNA. RNase is added at a later stage of the procedure to digest RNAs. Organic separation is still commonly utilized despite the toxicity of phenol and the possibility of leftover phenol or chloroform affecting downstream uses (figure 4).

- Silica-based technologies are widely used in commercially available kits. DNA preferentially binds to silica membranes, beads, or particles at a certain pH and in the presence of particular salts. The washing process eliminates the cellular pollutants. Elution buffers, or low salt buffers, are used to elute DNA. The kit buffers contain chaotropic salts to help with DNA extraction, and it can be automated. It can also be used in spin columns and microchips (Tan and Yiap, 2009).

Quantification of extracted DNA

The yield of isolated DNA in mg/µl from the sample is measured by a Nano Spectrophotometer at a wavelength of 260/280 nm. The purity of the DNA is determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. The ratio at 260/280 nm is commonly used to assess the purity of DNA concerning protein contamination and RNA impurities. The DNA sample is considered pure when the 260 to 280 ratio comes near 1.8. However, a DNA sample ratio of 1.5-1.9 can be easily used for PCR.

Primer Synthesis

The most crucial element of selective amplification is primer design. COI (cytochrome c oxidase 1) primer is designed to specifically target and amplify the COI gene, which is widely used genetic marker for DNA barcoding and species identification. Cytochrome c oxidase will be amplified using universal sets of primers, namely Fish F1 and Fish R1 primer. For primer deigning some prior DNA sequence information from the target DNA is required. Certain rules for primer designing are important to consider.

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PCR Amplification

The amplification is carried out following the program revealed by Garg *et al.* (2023). This program consists of 35 cycles with an initial denaturation at 95ºC for 5 minutes, annealing at

95ºC for 30 seconds, annealing at 53ºC for 60 seconds, and extension at 72ºC for 60 seconds per cycles, with a final extension at 72ºC for 10 minutes and holding temperature at 4ºC for 10 minutes (Table 3). In a 25µl reaction mixture, amplification is carried out comprising 9.50µl of distilled water, 12.50µl of 2X PCR Master mix, 1µl primer (F), 1µl of primer (R), and 1µl of Template DNA (Table 2). The PCR amplification is analyzed by loading PCR- amplified products electrophoresis with a COI marker, such as 1-kilo base pairs (kbp) DNA ladder, the PCR amplification is examined. A brilliant band of he anticipated size indicates the presence of the PCR product, which will be purified.

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Tools for analysis include bioinformatics and DNA sequencing

The commercially available kit from HiMedia, India is used for the purification of PCR products, cycle sequencing, and Sanger sequencing. The sequencing analysis 5.2 version software (Applied Biosytems Inc., CA, USA), ORF finder (https://www.ncbi.nlm.nih.gov), and online nucleotide BLAST (https://blast.ncbi.nlm.nih.gov) are used to trim low-quality reads and gaps from forward chromatograms. To create a final collection, all generated and publically available database genomes are aligned using Clustal-X software (Garg and Dohre, 2023).

Establishing a Genetic Reference Library

The BOLD website and the National Centre of Biotechnology Information (NCBI) website can be used to examine sequencing findings and find the maximal identity of an organism using template sequences. Unknown sequences are compared by BOLD (figure 5) to the most similar sequences in the database. The output from this database is presented as a percentage of resemblance. In order to create a barcode record researchers needed the species name, voucher specimen data (i.e., place, date, specimen repository, photos), sequence data, PCR primers and trace files (sequencer's original outputs).

DNA Barcoding Implementation

DNA barcoding has many implementation in numerous sectors, including the reservation of natural resources, the protection of endangered species, the management of agricultural pests, the identification of disease vector, the monitoring of water quality, the authenticity of natural health products, and the identification of medicinal plants, have found numerous uses for DNA barcoding. (Panprommin *et al.*, 2020) utilized DNA barcoding with the COI gene to identify larval fish species in the lower Ing River, Thailand. The study successfully identified 35 species with high accuracy. The genetic distance within species was low. While most species were classified as least concern, the findings underscore the potential of DNA barcoding for precise larval fish species identification. In forensic sciences, drug authenticity can be verified using DNA samples (Carvalho *et al.*, 2015). The importance of using both human and non-human DNA analysis in criminal investigations is rising (Barcaccia *et al.*, 2015) highlighted DNA barcoding as a molecular tool to combat mislabelling and food piracy. The technique, involving standardized target regions in extra-nuclear genomes, is effective for species identification in

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agriculture. It is increasingly used in the food industry to ensure accurate food labelling, detect food piracy and maintain food safety. Standardized protocols make this technology suitable for routine analyses, supporting agencies in safeguarding food quality. The barcode of Life Data Systems (BOLD) database has a high number of cytochrome c oxidase I gene (COI) sequences, some authors have selected the primary DNA barcode as the first instrument for calibrating fish species diversity (April *et al.*, 2011).

DNA barcoding and mini-barcoding were used by (Xing *et al.*, 2020) to assess the authenticity of animal-derived food in the Chinese market. Out of 52 samples (meat, poultry, and fish), 94% generated barcode sequences. The survey revealed a 23% mislabelling rate, with undeclared species found in most cases. The findings highlight the need for regulatory measures and ongoing monitoring to address mislabelling in animal-derived products.

In situations where taxa were easily misidentified because of the prevalence of cryptic species, barcodes have also complemented morphological investigations (Ward *et al.*, 2008). Since mitochondrial DNA markers are inherited from a single mother (maternal inheritance), they are not diploid and can therefore be used with similar effectiveness in population-level research (Avise, 2004). The COI gene offers data on genetic variation within a single species population, and this data can be used to infer genetic drift and migration phenomena in fish populations (Mohammad Geba *et al*., 2016).

Wilson *et al.* (2016) compared the efforts at DNA barcoding in Tropical East Asia to those in other tropical regions and propose that DNA barcodes, or metabarcodes derived from next-generation sequencers, could be particularly helpful in characterizing and linking species-level biodiversity units in inventories that include taxa for which there is no formal description (especially arthropods), as well as in large-scale, low- impact methods of vertebrate population assessments and monitoring using secondary sources of DNA (environmental DNA and DNA derived from invertebrates).

Barcoding allows for the prosecution of illegal dealing in endangered species. For instance, several coastal African nations face a serious threat to their biodiversity from the illegal shark-fin trade. Two major ways that DNA barcoding supports conservation policy are by expediting local biodiversity assessments to prioritize conservation areas or assess the effectiveness of conservation action and by furnishing data regarding evolutionary histories and phylogenetic diversity (Rubinoff, 2006).

Significance of DNA Barcoding

DNA barcodes are quite useful for identification of both fresh specimens and market products such as frozen fish. The fish diversity in marine and continental waters can be effectively cataloged through the use of DNA barcode studies, which aid in the identification of potentially

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new species and genera, furnish data on species that serve as excellent models for comparative biogeographical research, and impart knowledge on the molecular systematic of fish species complexes. These findings enhance estimates of the richness of local species, aid in the delineation of taxonomic units for conservation initiatives, and enable the clear discrimination of commercially important fish species (Beheregaray, 2008; Carvalho *et al*., 2011).

DNA barcodes can be useful tool for classifying new or previously recognized species with previously unknown species based on barcode sequences. In instances where morphologic characteristics are absent or deceptive, it can also be used to classify specimens to known species. According to Williams *et al*. (2013), DNA barcoding may be crucial to the conservation of species that have already been investigated and the enforcemental laws when combined with a reference database.

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CONCLUSION

In conclusion, DNA barcoding emerges as a valuable tool for resolving misrepresentation and ambiguity in species identification in Upper Lake Bhopal, a Ramsar site of international importance. By harnessing the power of molecular genetics, DNA barcoding offers a standardized and reliable approach to biodiversity assessment, conservation planning, and ecological management. Continued research efforts, capacity building, and interdisciplinary collaborations are crucial to fully realize the potential of DNA barcoding in safeguarding the ecological integrity and biodiversity of Upper Lake Bhopal.

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Table 1. List of Fish Biodiversity recorded in Upper Lake Bhopal, (MP)

S No.	Fish species name	Author's name (year)									
		Mukesh Kumar Napit (2013)	Showkathussainet *al.* (2017)	Bilquees *et al.* (2010)	Nidhi Singh *et al.* (2022)	Praveen Tamot *et al.* (2010)	Thomas *et al.* (2013)	Khushwinder Singh *et al.* (2019)	Tahir mmalla *et al.* (2009)	ShaziaTabassum Shah *et al.* (2023)	RekhaChauhan *et al.* (2014)
1.	*Gudusiachapra*	✓	✓								
2.	*Notopterusnotopterus*	✓	✓			✓					
3.	*Barilus bola*	✓									
4.	*Catlacatla*	✓									
5.	*Cirrhinusmrigala*	✓	✓			✓					
6.	*Cirrhinusreba*	✓	✓								
7.	*Garragotyla*	✓	✓					✓			
8.	*Escomusdanricus*	✓	✓								
9.	*Labeobata*	✓	✓					✓			
10.	*Labeocalbasu*	✓	✓			✓		✓			
11.	*Labeodussumeri*	✓	✓								
12.	*Labeogonius*	✓	✓			✓					
13.	*Labeorohita*	✓	✓								
14.	*Osteobramacotio*	✓	✓								
15.	*Oxygasterbacaila*	✓	✓								
16.	*Puntiussophore*	✓	✓					✓			
17.	*Puntiusticto*	✓	✓			✓		✓			
18.	*PuntiusSarana*	✓	✓			✓		✓			
19.	*Rasboradaniconius*	✓	✓					✓			
20.	*Cyprinuscarpio*	✓	✓		✓	✓					
21.	*Maccognathusaculeatum*	✓									

22.	*Lepulocephalichthyx*	✓									
23.	*TrichogasterfasciatuS*	✓									
24.	*Nemacheilusbotia*	✓									
25.	*Ompokbimuculatus*	✓	✓			✓		✓			
26.	*Mystusbleekeri*	✓	✓			✓					
27.	*Mystusaor*	✓	✓								
28.	*MystusSeenghala*	✓	✓			✓					
29.	*MystusCavasius*	✓	✓					✓			
30.	*Heteropneustesfossilis*	✓	✓					✓	✓		
31.	*Clariasbatrachus*	✓	✓		✓	✓					
32.	*Xenentodoncancila*	✓	✓			✓					
33.	*Channamarulius*	✓	✓								
34.	*Channaleucopunctatus*	✓									
35.	*Channapunctatus*	✓	✓					✓			
36.	*Channastraitus*	✓	✓					✓		✓	
37.	*Channagachua*	✓	✓					✓			
38.	*Chandanama*	✓	✓								
39.	*Chandaranga*	✓						✓			
40.	*Nandusnandus*	✓	✓								
41.	*Glossigobiusgiuris*	✓				✓		✓			
42.	*Mastacembelusarmatus*	✓				✓		✓			
43.	*Mastacembeluspancalus*	✓									
44.	*Catlacatla*		✓			✓					
45.	*Bariliusbendelisis*		✓								
46.	*Parambassisranga*		✓								
47.	*Wallagoattu*					✓					
48.	*Hypophthalmichthysmolitrix*					✓					

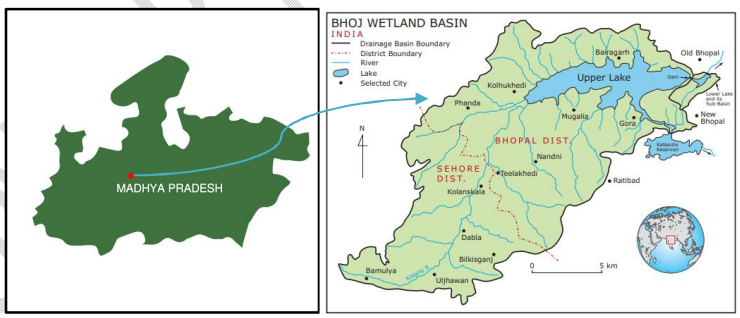
49.	*Ompokpabda*					✓					
50.	*Nemacheilusbotia*							✓			
51.	*Chela labuca*							✓			
52.	*Puntiusconchonius*							✓			
53.	*Puntiusfilamentosus*							✓			
54.	*Puntiuschola*							✓			
55.	*Puntiuschrysopterus*							✓			
56.	*Amblypharyngodonmola*							✓			
57.	*Amblypharyngodonmicrolepis*							✓			
58.	*Daniodevario*							✓			
59.	*Labeoboga*							✓			
60.	*Aspidopariajaya*							✓			
61.	*Ctenopharyngodonidella*			✓							
62.	*Carrasiusauratus L.*										✓

Table 2.Composition of PCR Master Mix

S.No.	Chemicals	Configuration	Amount
1.	PCR Master Mix	2X	12.5 µl
2.	Primer (F)	10 picomol	1.00 µl
3.	Primer (R)	10 picomol	1.00 µl
4.	Distilled water	Molecular Grade	9.50 µl
5.	Template DNA	40-60 ng/µl	1.00 µl
			TOTAL 25.00 µl

Table 3. PCR program cycles for amplification of *cox1* gene for DNA barcoding

Denaturation	Amplification (×35 cycles)			Extension
	Denaturation	Annealing	Extension	
95°C	95°C	53°C	72°C	72°C
5 min.	30 sec	60 sec	60 sec	10 min



https://iasbaba.com/wp-content/uploads/2023/01/bw.png

Figure 1. Map showing Upper Lake Bhopal, Madhya Pradesh (India)

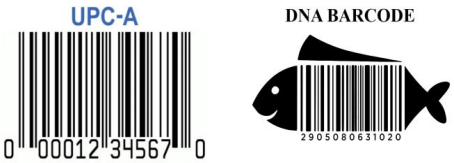


Figure 2. DNA based Identification system

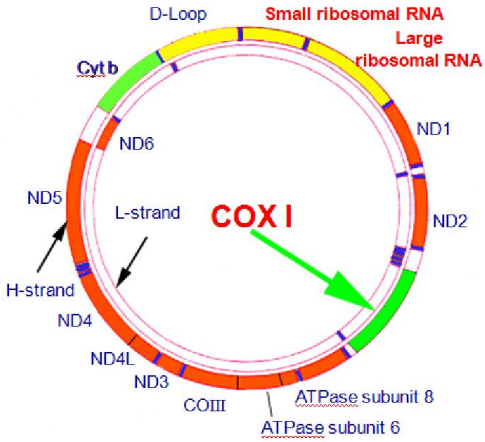


Figure 3. Mitochondrial gene (COI or Cox1)

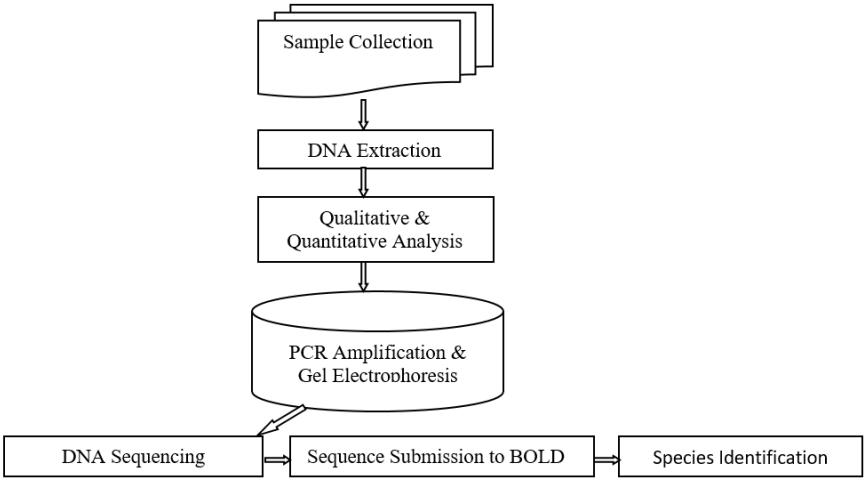


Figure 4. Flow Chart of DNA Barcoding

Figure 5. Barcode of life data system (BOLD System)