

REVIEW ARTICLE

DNA-based identification of fishes: A review of barcoding methods and their efficiency assessment

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Abstract

The challenges of morphological identification of closely related species and larval stages of fishes led to increased demand for DNA-based methods to achieve precise species identification. The DNA-based technique has noteworthy applications in food authentication, detection of new species, understanding evolutionary history, and biomonitoring programs. With the advent of DNA barcoding and metabarcoding, many discrepancies in morphological identification can be overcome. DNA barcoding depends on the affirmation that conserved sequences can be utilized for species identification whereas metabarcoding implements advanced next-generation sequencing of diverse bulk samples or environmental samples for automated detection of multiple species. The present article reviews the applications of DNA barcoding and DNA metabarcoding and offers a fundamental understanding of fish identification through barcode information. The article also provides a brief insight into the differences between DNA barcoding and metabarcoding and cites the distinctions that make them different, yet reliable. The pitfalls of DNA barcoding also have been highlighted, especially when it comes to false identification in DNA barcoding using the Ticto Barb (*Pethia ticto*, Cyprinidae) as an example. Further, the study suggests the employment of various computational approaches for taxonomic validation, accurate and complete DNA libraries, and deeper research of molecular markers.

Keywords: COI, eDNA, Fish, False identification, Species identification

INTRODUCTION

Fish exhibit remarkable taxonomic variety and are an essential component of all aquatic ecological services and functions (Helfman et al. 2009). The fish biodiversity is disintegrating owing to relentless urbanization, the severe industrialization of agroecosystems, indiscriminate fishing, a lack of public awareness, and human activities, including harvest pressure, global warming, and water pollution (Ghosh et al. 2008; Bukola et al. 2015). Human interventions have not only destroyed habitats but also imbalanced the ecosystems by introducing non-native species into various geographic areas (Alpert 2006; Comtet et al. 2015). The introduction of aquaculture-reared allochthonous fish species can genetically deplete the population structure of native species by affecting their fitness and survival (Modeel et al. 2023). To comprehend how various species contribute to ecological systems and to conserve biodiversity, it

is crucial to have in-depth knowledge of the species composition and diversity of different geographical regions (Kim and Byrne 2006). Aquatic environments are home to a wide variety of species, including endangered, threatened, vulnerable, non-native, cryptic, and especially those species that have not yet been discovered due to inadequate sampling (Ghasemi et al. 2015; Bharti et al. 2023). It is essential to develop a reliable method for species identification in order to manage and protect the biodiversity of aquatic life.

Understanding the distribution, diversity, and ecological state of fishes requires an accurate identification system (Kirchhofer 1995). Majority of the studies identify fish species using morphological and morphometric characters such as body shape, size, numbers of rays, spines, fin shapes, and pigmentation, viz., Day (1888); Strauss & Bond (1990); Holden & Raitt (1974); Greenwood et al. (1966); Jayaram (1999); Negi et al. (2010); Betancur-R et al. (2013);

Nelson et al. (2016) and Eschmeyer et al. (2017). Species identification using morphological characters is often complicated, and errors can lead to incorrect assessment of ecological impacts on other species (Blaxter 2003; Hulley et al. 2018). In addition, there are changes in the morphology of some species through the developmental stages of their life cycle (Tillett et al. 2012). Some species have similar morphological characters but are genetically distinct and can only be distinguished through DNA-based methods (Hajibabaei et al. 2007; Iyiola et al. 2018). Considering these limitations, better species identification methods that not only take the phenotypic characters into account but also focus on genetic invariability are required.

Numerous techniques have been developed to improve species identification, including isoelectric focusing (IEF), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), enzyme-linked immunosorbent assay (ELISA), and DNA-based methods—(Pereira et al. 2008; Teletchea 2009). The DNA-based methods have become popular and reliable identification method as they gives opportunity to use non-invasive samples to reduce the scarification of animals and provides insights into genotypic variability at different taxonomic levels (Betancur-R et al. 2017). Genetic drift, ecological variables, and developmental plasticity, all contribute to the phenotypic heterogeneity within species in various geographic locations, resulting in population-level differentiation that can be understood using DNA-based identification approaches (Lostrom et al. 2015). DNA-based methods such as DNA hybridization, Random fragment length polymorphism (RFLP), Amplified fragment length polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), DNA microarrays, and DNA sequencing can be used for species identification (Zhang et al. 2004; Dooley et al. 2005; Aranishi et al. 2005; Maldini et al. 2006; Lakra et al. 2007; Kochzius et al. 2008; Pereira et al. 2008; Galal-Khallaf et al. 2017; Khedkar et al. 2014b; Coble et al. 2019). DNA barcoding and DNA metabarcoding are decisive identification methods based on

polymerase chain reaction and DNA sequencing of a standardized region of a selected gene labeled as the ‘barcode’ for species identification (Hebert et al. 2003b).

The use of sequences or functional taxonomic units in DNA barcoding and metabarcoding facilitates species detection along with ecological research and study of population structure for the conservation and monitoring of biodiversity. DNA barcoding also supports application of genetics in the field of fisheries to study divergence patterns and population structures in different groups (Bhattacharya et al. 2016). Barcoding techniques enhance our understanding of larval fish ecology and provide insights into population demographics and reproductive ecology (Hallerman 2021).

The current article seeks to discuss developments in the taxonomic identification of species using barcoding methods. The review provides detailed insight into the global status of DNA barcoding and its applications, a brief overview of environmental DNA (eDNA) metabarcoding and how it differs from DNA barcoding. The article also explores the limitations of DNA barcoding, especially arguing the use of a single mitochondrial gene for identification and existence of errors in databases leading to false identification.

DNA barcoding and its analysis: DNA barcoding uses standardized DNA sequences for the taxonomic identification of species and offers comprehensive and deep-rooted phylogenetic insights (Krishnamurthy et al. 2012). Hebert et al. (2003a) introduced DNA barcoding and suggested that mitochondrial genes such as cytochrome oxidase subunit-I (COI) can be universally used as a core identification system for animals. The process involves the extraction of DNA from specimens and PCR amplification using universal primers of a particular DNA barcode gene (Hebert et al. 2003a) (Fig. 1). The amplified PCR product is purified and sequenced, followed by a quality check of sequences. The obtained forward and reverse sequences are aligned, and sequence similarity is checked in response to which the software and the user assign a putative species identification (Spouge 2016). Some tools and software programs are also

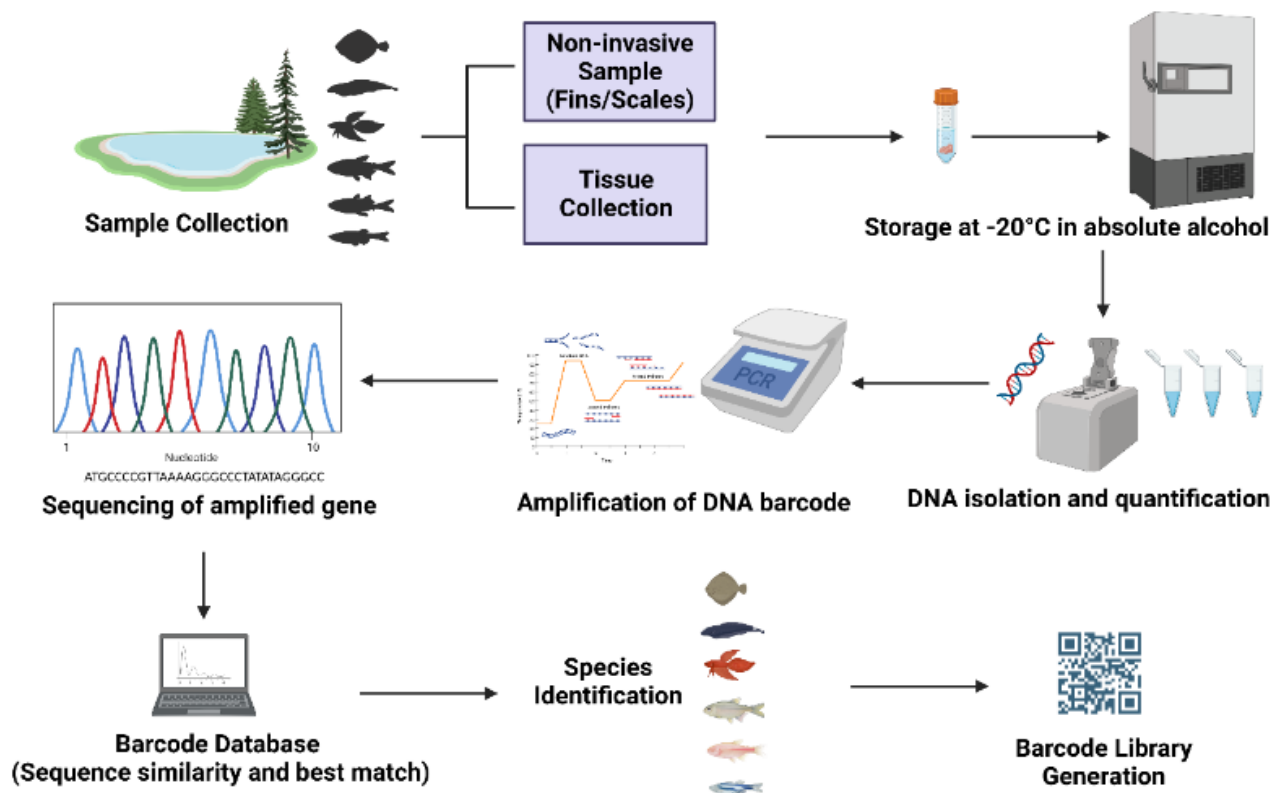


Fig.1. Representation of DNA barcoding methodology and its analysis.

available for sequence validation and quality check of sequencing to avoid ambiguity due to sequencing errors and quality of sequences, such as Sequencher v5.4 (<http://www.genecodes.com>), bioedit (Hall et al. 2011), PGDSpider (Lischer & Excoffier 2012), SeqTrace v0.9.0 (Stucky 2012), ClinQC (Pandey et al. 2016) etc. After validating the quality of sequences, the initial identification of the generated barcodes can be analyzed in BLAST (Basic local alignment search tool) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), BLAT (BLAST-like alignment tool) (<http://genome.ucsc.edu>), and BOLD-IDS (Barcode Of Life Data System-Identification System) (<https://www.boldsystems.org/>). Further, the sequences obtained are used to create a barcode library on BOLD (Barcode Of Life Data System) database and NCBI (National Center for Biotechnology Information).

With species identification, DNA barcoding also provides insights into phylogenetic and evolutionary perspectives of species (Hebert et al. 2003a). DNA barcodes can be analysed using cluster approaches

with different algorithms, where the most used algorithm is Neighbour-joining (NJ) (Hebert et al. 2003b; Ward et al. 2005; Lakra et al. 2011b). The Neighbour-joining (NJ) tree helps in constructing phylogenies by analyzing distances between each pair of sequences (Howe et al. 2002; Srivathsan & Meier 2012). Several species delimitation methods are also used for analysing DNA barcodes, such as Automatic Barcode Gap Discovery (ABGD) (Puillandre et al. 2012), the Generalized Mixed Yule Coalescent (GMYC) model (Pons et al. 2006), and Bayesian Poisson Tree Processes (bPTP) (Zhang et al. 2013).

DNA barcoding has been manifested in different taxa of animals such as neotropical Bats (Clare et al. 2007), amphibians (Vences et al. 2005), fishes (Ward et al. 2005), lepidopterans (butterflies and moths) (Hebert et al. 2003b), springtails (Hogg & Hebert 2004) and many more. The cytochrome oxidase subunit-I (COI) mitochondrial gene is generally used for DNA barcoding of animals (Hebert et al. 2003b). Mitochondrial genes are typically employed in DNA-based identification due to their maternal inheritance

and few insertions and deletions (Yacoub et al. 2015). Nuclear DNA is not considered a valid option for studying phylogenetic relations due to its shared ancestral polymorphism in closely related species (Simon et al. 1994). The other important factor for using mitochondrial DNA for studying divergence in species is its presence in higher copy number than nuclear DNA per cell (Desalle et al. 2019). Genes employed for taxonomic identification should have ~ 2% mutation rate for closely related species and low sequence variations in the flanking regions of barcode sequences to ease the PCR amplification (Ji et al. 2013). Other conserved gene sequences like 16S ribosomal DNA (16S-rDNA) in amphibians (Vences et al. 2005), cytochrome b (Cytb) in Turtles (Schoch et al. 2012), nuclear ribosomal ITS (Internal transcribed spacer) region in fungi (Schoch et al. 2012) and ribulose-1,5-bisphosphate carboxylase oxygenase gene (rbcL) in diatoms (a major group of algae) (Evans et al. 2007) are used as DNA Barcodes. The two most commonly validated genetic loci in species identification are cytochrome oxidase subunit-I (COI) and cytochrome b (Cytb) (Hebert et al. 2003b; Tobe et al. 2010). COI is a preferable DNA barcode because of its greater range of phylogenetic signal and the near-universal nature of its primers, which permit recovery of 5' end of the sequence easily (Hebert et al. 2003a). Waugh (2007) stated that COI has sufficient variation to differentiate species because of its low recombination rate, making it suitable for DNA barcoding. On the other hand, Cytb has better characterized deep evolutionary dynamics, and the levels of its genetic divergence are well associated within species and genera, making it efficient for phylogenetic analysis (Johns et al. 1998). Simmons et al. (2001) determined the phylogeny of tiger moths (Tribe Ctenuchini and Euchromiini; family Erebiidae) by comparing the phylogenetic signal of Cytb relative to COI and reported that Cytb had the same level of A/T bias and sequence variation as COI. Despite this for most of the experimentation, COI is extensively used by researchers for the identification of fish species because of its available reference data generated for this gene globally (Bhattacharya et al.

2016). DNA barcoding is dependent on the availability of reference data, and there are many species for which reference data is not available or not generated for geographically distant populations. Further, the universal set of COI primers does not amplify in all the species (with failure rates reported between 1 to 30%) and thus, it may be required to design species specific primers (Meier et al. 2006; Bhattacharya et al. 2016; Buckwalter et al. 2019; Panprommin et al. 2021).

DNA barcoding of fishes

Global status: Ward et al. (2005) barcoded 754 sequences from 207 Australian fish species for the first time and found different levels of genetic divergence within species (0.39%), genera (9.93%), families (22.18%), and orders (23.27%). Ivanova et al. (2007) developed universal primers and tested the efficiency of the COI and 16s rRNA genes in generating amplicons of DNA barcode sequences from 94 fish families. Furthermore, twenty one primers for amplifying the Cytb gene and nuclear rhodopsin gene in teleost fish species were developed and their efficiency was tested in 200 marine fish species (Sevilla et al. 2007). The study revealed several primer combinations and optimized the forward and reverse primers of the rhodopsin and Cytb genes to achieve success rates of greater than 99.9%. DNA barcoding has been attempted globally in different geographical regions with diverse numbers of species (Hubert et al. 2008; Ward et al. 2008; Holmes et al. 2009; Lara et al. 2010; Bucklin et al. 2011; Lakra et al. 2011a; Bhattacharjee et al. 2012; Muchlisin et al. 2013; Khedkar et al. 2014a; Afrand & Sourinejad, 2023; Modeel et al. 2024; Afrand et al. 2024). Papa et al. (2021) barcoded 1284 fishes from the Maroni River, Suriname (South America) using COI, and the results showed 199 fish species among which 25 were putative new candidate species. Three-hundred and thirty shark tissue samples were barcoded from fishing harbors and fish markets of Sri Lanka, reporting 17 shark species, among which 62% species were threatened with indication of cryptic species, potential interspecific introgression and ancestral polymorphism (Peiris et al. 2021). Some studies have

also targeted the mitochondrial 16s and 12s rRNA genes for DNA barcodes (Nguyen et al. 2006; Kochzius et al. 2010; Zeng et al. 2018; Cawthorn et al. 2012; Hossain et al. 2019). On the other hand, NAD (Nicotinamide adenine dinucleotide) genes also can be considered for identification of fish species. Species of family Sparidae were identified using NAD5 and NAD2 genes, which suggested better distinguishing properties of NAD genes than other markers (Ceruso et al. 2019; Ceruso et al. 2020). Recently, many studies have been done to authenticate the utility of DNA barcoding for identification of fishes, thus complementing the traditional taxonomic tools and creating a barcode library from diverse regions (Azmir et al. 2020; Liu et al. 2020; Ude et al. 2020; Wang et al. 2020b; Xing et al. 2020; Xiong et al. 2020; Adibah et al. 2020; Chen et al. 2022; Tang et al. 2023; Panprommin et al. 2023; Escobar Camacho et al. 2024). Figure 2 shows the genetic divergence levels within species, genera, and families based on different studies on fish DNA barcoding using COI gene.

A search for fish barcodes in the BOLD database revealed that at present (January, 2025) there are 327,433 barcodes for Actinopterygii, which represent 23,115 species, 24,290 barcodes for Elasmobranchii representing 1158 species, 730 barcodes for Petromyzonti which represent 53 species, 537 barcodes for 45 Holocephali species, 361 barcodes for 47 Myxini species, and 220 barcodes for Sarcopterygii, which represent 9 species. A collaborative endeavor spanning multiple global locations, the BOLD serves as a workbench overseeing all stages of DNA barcoding by offering a centralized bioinformatics platform for specimen and sequence record management (Ratnasingham & Hebert 2007). This allows for the study of species diversity, genetic variability, and evolution through the integration of data analysis.

DNA metabarcoding

An eDNA approach: DNA metabarcoding uses Next-generation sequencing (NGS) techniques and provides new opportunities to catalogue the multiple short reads of DNA sequences in single sample. DNA

metabarcoding can be performed with eDNA or bulk biodiversity samples for the automated identification of multiple species, facilitated by the use of high-throughput identification methods providing in-depth sequencing of uniquely tagged amplicons (Taberlet et al. 2012; Deagle et al. 2014). Environmental samples consist of DNA from various sources such as urine, saliva, skin cells, fecal matter, and body secretions (Bista et al. 2017). In case of aquatic organisms, the sample is usually in the form of water, and the results are based on the abundance of DNA molecules in the water sample. The survival of the DNA under different environmental conditions depends upon its stability under various chemical and physical factors such as temperature, pH, salinity, and biotic factors in the environment (Barnes & Turner 2016). The easily detectable DNA concentration in water reduces as soon as an organism is removed from the mesocosm because the persistence of extracellular DNA in the water samples depends upon the presence of organisms and factors affecting DNA degradation (Dejean et al. 2011). Maruyama et al. (2014) reported that the degradation rate of DNA is time-dependent and calculated 5.1 to 15.9% degradation per hour after removal of fish from water sample. The same study also showed that adult fishes release more eDNA than juveniles. Yamanaka et al. (2016) compared eDNA concentration between on-site filtered water samples and transported samples. The study found the presence of higher DNA concentration in on-site filtered samples, concluding that early filtration can reduce eDNA degradation. Therefore, efficacy and coverage of the metabarcoding vary greatly with preservation and sample collection methods.

One of the earlier studies that laid the foundation of species identification from water samples examined the accuracy of eDNA for detection of the American Bullfrog (*Rana catesbeiana*) in natural freshwater habitat and eighteen controlled environments (Ficetola et al. 2008). This was accomplished by amplifying 79 bp Cytb gene sequences, which successfully distinguished the presence or absence of the species in the water samples. Miya et al. (2015) developed MiFish primers based on the 12S rRNA

gene for metabarcoding of eDNA samples, which successfully detected 168 marine fishes among 180 in the Okinawa Churaumi Aquarium, Okinawa, Japan. The same study detected 93 species from natural sea waters near the aquarium using the MiFish primers. Evans et al. (2016) used eDNA metabarcoding to detect all vertebrate species of both fishes and amphibians, placed into a mesocosm and thus accurately measured species richness with even and skewed relative species abundances. Shaw et al. (2016) compared eDNA metabarcoding with traditional fish survey methods and detected all the fish species caught by the conventional fyke-net method using water samples. Stat et al. (2017) detected 287 eukaryotic families and recovered interspecific and intraspecific haplotypes of the emperor fishes, genus *Lathrinus*, from sea water samples of west Australia. Yamamoto et al. (2017) collected samples from Maizuru Bay, Japan and detected 128 fish species using MiFish metabarcoding primers. MitoFish is a fish mitochondrial genome database which can analyze eDNA amplified using MiFish primers, helpful in metabarcoding analysis (Sato et al. 2018). Milan et al. (2020) developed mini-barcodes (193 bp) of the 12S rRNA gene from the amplification of the full-length 12S marker (565 bp) and developed a 12S DNA reference database of 67 fish species. Apart from the 12S marker, eDNA metabarcoding has been done using 16S (Berry et al. 2017), Cytb (Minamoto et al. 2012) and COI (Leray et al. 2013) genes. Marques et al. (2020) developed an interactive web interface, “GAPeDNA”, for assessment of regional gaps in genetic databases of eDNA metabarcoding using 19 metabarcoding primers from the European Nucleotide Archive public reference database. Collins et al. (2021) generated a DNA reference library for metabarcoding of some marine and freshwater species which supports eight metabarcoding markers and thereby assists in species search and identification with a phylogeny quality control setup. Mariani et al. (2021) identified 19 elasmobranch taxa from Reunion Island in the Indian Ocean using elasmobranch-specific metabarcoding primers targeting a 171-bp 12S ribosomal fragment.

The eDNA approach has now become a proven approach to study species richness, biodiversity monitoring and anthropogenic effects on aquatic life (Miya 2022).

Comparison between DNA barcoding and metabarcoding: DNA metabarcoding is an extraordinary instance of barcoding that is applied to test the presence of more than one life form; thus, it is prone to taxonomic overlap between different groups (Dormontt et al. 2018). Several markers must be used to overcome issues with primer specificity and biasness when applying DNA metabarcoding for the diversity analysis of bulk DNA samples (Da Silva et al. 2019). The integration of information from different markers, particularly when they partially overlap in the amplified taxa may differ in taxonomic resolution (Leray and Knowlton 2015). Hence, metabarcoding for species-level identification requires more primer specificity, while DNA barcoding can successfully classify most organisms to the species level using universal primers (Cristescu 2014). In DNA barcoding, the reference data plays an important role in the identification of a species either from DNA barcoding or metabarcoding. DNA barcoding is based on DNA isolated directly from the species, whereas metabarcoding can supplement large-scale monitoring of fish species present in different environmental samples. Further, DNA quality also plays an important role in DNA metabarcoding; sometimes it becomes challenging to evaluate species composition, as quality and concentration of DNA can vary due to changes in procedures (Staats et al. 2016). Several environmental factors in the sampling location can cause eDNA to degrade. DNA metabarcoding depends upon the release of eDNA, which is affected by biomass and the life stage of an organism and its feeding niche (Ruppert et al. 2019). In this context, the study of several carnivorous species feeding on small fishes leads to the defecation of the undigested material, which can act as a source of eDNA in water. However, studying the feeding habits of fish species using eDNA obtained from water samples is very difficult due to the dispersal of DNA in flowing water.

Table 1. Brief comparison between DNA barcoding and DNA metabarcoding.

Characteristic	DNA barcoding	DNA metabarcoding
Sample type	• Invasive or non-invasive	• Non-invasive and environmental samples (urine, saliva, skin cells, fecal matter, and various body secretions).
Primer Specificity	• Species-specific/ universal primers are used.	• Multiple primer pairs or single primer pair with wide application used.
Sequencing	• Sanger sequencing is used.	• Next-Generation Sequencing techniques are used.
Sequence length	• Depends upon the primers used. Usually more than 500bp.	• Range from 100-400 bp depending upon sequencing technique and degradation of DNA.
Factors affecting	• Affected by sample storage conditions and quality of DNA isolated.	• Affected by temperature, pH, salinity, species abundance, DNA concentration in an environmental sample, and DNA degradation rate.
Applications	• Identification of species, food authentication, cryptic and invasive species detection. • Helpful in determining intra-species and inter-species genetic divergence. • Available sequences in databases can also be used to study the demography and phylogeography of a species.	• Biomass estimation, disease detection, diet analysis, and identification of invasive organisms, and studying interconnection of food webs. • Helpful in analyzing community structure and biodiversity estimates.
Limitations	• Drawbacks in online databases and vulnerability of technique to human errors. • Error in identification due to similar sequences and slow mutation rate among different organisms.	• Requires experimental validation. • Technically more challenging leading to taxonomic overlap and misidentification.

Metabarcoding can also be used in network ecology to study diet and prey-predator interaction (Cuff et al. 2022). The amount of eDNA copies obtained in water samples can be helpful in taxon-specific studies for the quantification of density and biomass (Pont et al. 2023). Interestingly, some studies have shown a positive association between the amount of species-specific eDNA present in a habitat and their abundance (density or biomass) (Takahara et al. 2012; Wilcox et al. 2016; Lacoursière-Roussel et al. 2016; Yates et al. 2019). The factors influencing eDNA concentrations depend upon the abundance of fish,

their size, reproductive phase, migration, and abiotic factors like water cycle, temperature, flow direction, and anthropogenic activities (Rourke et al. 2022). Metabarcoding involves massive sequencing and uses DNA barcoding databases for the identification of different species from mixed samples (Dormontt et al. 2018). Both techniques are interdependent; however, DNA barcoding involves well-curated individuals, whereas curation is impractical in metabarcoding (Cristescu 2014). A brief comparison between DNA barcoding and eDNA metabarcoding is summarized in Table 1.

Applications of barcoding methods: DNA barcoding has a wide range of applications including identification of species, authentication of food, study of evolution, and paleontological specimens from sources like dried rivers, non-perennial rivers, and underwater deposits in phreatic caves, sinkholes, and continental shelves (Kuppu et al. 2017; Louys 2018). Even a tiny amount of sample is enough to identify species, and using small amplicon size can provide information from degraded samples (Trivedi et al. 2014). Pauls et al. (2010) examined the applications of DNA barcoding in identification of cryptic species, and in association of life stages to the adult organisms. Moreover, larval stages in some fish species have distinct morphology and such species are difficult to identify due to the incomplete development of morphological keys (Ko et al. 2013). Buckwalter et al. (2019) collected and examined 393 fish larvae and created primers for mitochondrial COI, ND2 (NADH dehydrogenase), and Cytb regions and identified all larvae in a targeted genus to the species level. Hence, DNA barcoding is a useful method for such identification, as it does not rely upon the life stage and quality of the specimen. Identification is an important aspect of fish food industry processing, where uncooked and canned food needs to be inspected because of mislabeling, especially replacement of valuable species with less valuable ones (Civera 2003; Wong and Hanner 2008; Nagalakshmi et al. 2016; Xiong et al. 2020; Chang et al. 2021). DNA barcoding also can be helpful for wildlife forensic science and trade monitoring by detection of illegal supply of products from endangered and threatened species (Chapman et al. 2003; Johnson et al. 2014; Negi et al. 2016a). Following is an overview of a few of the most important applications for DNA barcodes.

Food Authentication and mislabeling: A significant surge in consumer demand for healthy meals has encouraged people to give greater consideration to their nutrition and the quality of the food they consume. Canned and uncooked food must be scrutinized due to mislabeling, particularly when precious species are substituted for cheaper varieties

(Xiong et al. 2020; Chang et al. 2021; Mat Jaafar et al. 2012). There is a greater chance of fraud, especially concerning mislabeling and the unreported substitution of expensive seafood. Several publications have highlighted this issue (Fernandes et al. 2021; Delpiani et al. 2020; Xiong et al. 2019; Muñoz-Colmenero et al. 2016; Pardo et al. 2016). This has increased the need for food authentication and the identification of counterfeit food items. Species identification is a crucial part of the processing of fish for the food industry (Pardo et al. 2020). Processed food products with misleading packaging cannot be identified using conventional speciation techniques like morphological identification since they might not have essential morphological characteristics (Khaksar et al. 2015). DNA barcoding has been used to authenticate processed fish food and seafood (Xiong et al. 2019; Chen et al. 2019; Adibah et al. 2020; Ghouri et al. 2020; Liu et al. 2020; Chen et al. 2021; Chang et al. 2021; Filonzi et al. 2021; Panprommin & Manosri 2022). To address the authenticity concerns elevated in processed fish food, DNA barcoding can be regarded as an important policy tool for species identification.

Genetic Diversity and cryptic species: Genetic diversity, a representation of the balance between the emergence and extinction of genetic variants, results in the development of distinct species as well as a wide range of populations within the same species (Ellegren and Galtier 2016). The rate of allele loss and fixation determines the observed variation in genetic diversity, which leads to mutation across the genome and between species (Lynch 2010). Molecular markers from both the nuclear and mitochondrial genomes have been used to study the genetic diversity and population structure of species (Gong et al. 2018; Berrebi et al. 2019; Popa et al. 2016; Fujimoto et al. 2017).

Divergent shifts in evolution and potential hidden deviations from the current taxonomic classifications have been uncovered by DNA barcoding (Lara et al. 2010). The existence of cryptic species with considerable genetic diversity but little morphological variability is one of the current issues in taxonomic

identification (Korshunova et al. 2019). In a relatively short order of time, cryptic species may arise by parallel evolution from recent divergence in distant or closely related taxa (Shin et al. 2023). The existence of cryptic species has been effectively established by a number of studies that have employed DNA barcoding for their taxonomic identification (Hubert et al. 2012; Mat Jaafar et al. 2012; Puckridge et al. 2013; Winterbottom et al. 2014; Hyde et al. 2014; Iyiola et al. 2018; Wang et al. 2020a). On the other hand, species complexes offer an ideal model to investigate the development of individual taxa and the genetic underpinnings of their divergence (Protas et al. 2023). Sometimes, at one or more developmental phases, members of a species complex show little to no phenotypic diversity, making them almost identical based on morphology (Sousa-Paula et al. 2021). It is significant to remember that within a species complex, distinct, severely separated lineages typically exhibit geographic separation (Conte-Grand et al. 2017). DNA barcoding offers a platform to distinguish complex species within a population (Imtiaz et al. 2017). A complex of five distinct species was revealed by mitochondrial COI sequencing of 480 specimens of the widely dispersed and commercially exploited species, *Lampris guttatus* (Hyde et al. 2014). The existence of the *Acanthocephala* species was discovered for the first time using the COI gene in another study conducted in coastal areas of Iran (Alavi-Yeganeh & Kishipourik 2024). In order to create a comprehensive snakehead reference barcode library, Conte-Grand et al. (2017) supported the existence of some species-level taxa in the genus *Channa* that represent species complexes rather than individual species. Consequently, DNA barcodes serve as an extremely adaptable, feasible, and beneficial framework for the identification of cryptic species and species complexes, as well as can improve taxonomic delimitation by serving as a link between systematics and identification.

Evolutionary timescale: The evolutionary processes connected to different populations or species can be measured using assessments of genetic diversity between them (Liu et al. 2019). DNA barcoding has

been useful in analyzing phylogenetically organized community data to look into the relationship between species and phylogenetic diversity, as well as the role of evolutionary history and functional traits in community structure (Gostel & Kress 2022). The molecular divergences between COI sequences are often used to study the phylogenetic relationship between species. The molecular clock, a technique based on the mutation rate of biomolecules assumes that genetic change occurs at a constant pace throughout lineages. Estimations of these changes can be used to determine when evolutionary divergence events occurred across the Tree of Life (Ho 2008). Many tools and techniques have been employed by researchers to study the evolution of mitochondrial genes and the divergence pattern of fish species (Ward et al. 2005; Bhattacharya et al. 2016). One of the main applications investigated by several research is the haplotype diversity to study the inherited mutations in genes and the demographic analysis to study the population growth and decline with evolutionary processes (Keskin et al. 2013; Shen et al. 2016; Yadav et al. 2020; Ren et al. 2018; Modeel et al. 2023).

Wildlife trafficking: For a long time, only considered an environmental and ecological issue, wildlife trafficking is one of the most significant categories of organized international crime (Smart et al. 2021). Using DNA barcoding technology to quickly identify species could make it easier to keep an eye on the animal product trade (Nougoue 2012). DNA barcoding has exposed the wildlife trafficking of important sources of animal proteins and other products (Khan et al. 2018; Formentão et al. 2021; Dipita et al. 2022). Global fish populations are declining as a result of the illicit trade. Holmes et al. (2009) examined shark fins from illegal fishing in Australian waters and found many of these species belonged to the IUCN red list. Asis et al. (2016) detected dried ray and shark as well as live juvenile eels from illicit commerce from the Philippines. Even though many aquatic species are regulated, the trafficking of juvenile species and the transportation of dried or processed goods continue to be a part of illegal trafficking.

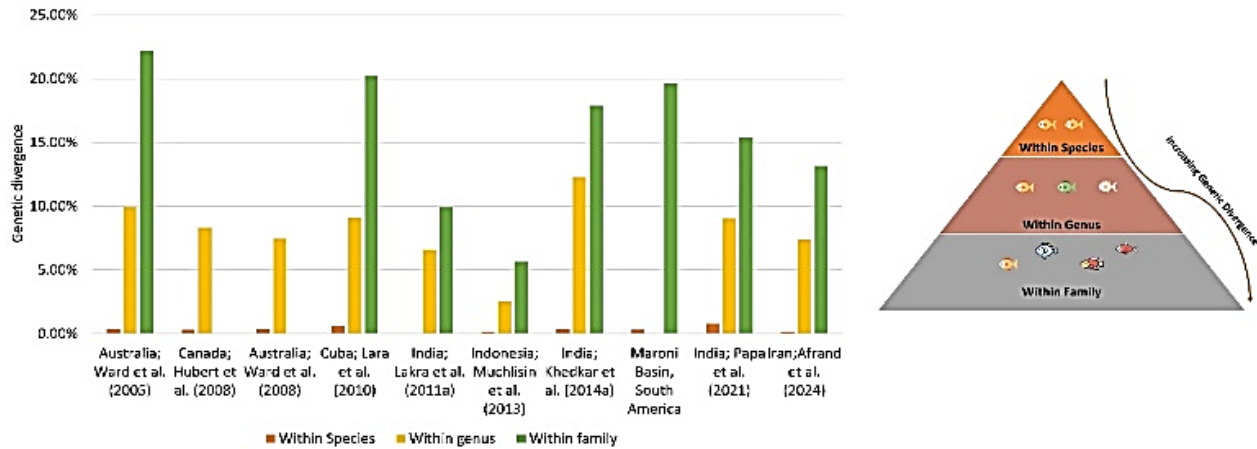


Fig.2. Graphical representation of genetic divergence levels within species, genera, and families based on different studies on fish DNA barcoding.

Biodiversity and conservation: One of the biggest issues is biodiversity loss because of lack of research on how much biodiversity is present regionally and worldwide, as well as how quickly it is being lost as a result of human activities (Krishna Krishnamurthy et al. 2012). DNA barcoding offers the chance to recognize, catalog, and examine specimens with the goal to assess genetic variability within species and comprehend species variety within an ecosystem. When it comes to evaluating biodiversity, DNA barcoding has several applications. Barcodes have the potential to be valuable tools for identifying invasive species within an environment, tracing fish larvae to adult species to provide insights into biodiversity, and identifying sibling species that may have been diverged due to geographical isolation or human-made obstacles like dams (Trivedi et al. 2016). When combined with DNA barcoding, genomic research can be a highly useful tool for assessing biodiversity worldwide and assisting conservationists in developing preservation and monitoring strategies.

On the other hand, DNA metabarcoding permits the use of high-throughput techniques to support high probabilities of detection of species with low abundance even at hard-to-access or unsafe sampling locations (Coble et al. 2019). DNA metabarcoding is a multi-specific approach which can identify many species from an environmental sample without any prior knowledge of the species (Valentini et al. 2016). The eDNA approach has the potential to study various

processes of ecosystem and community changes in relation to biodiversity. It offers the ability to identify endangered species, invasive species, and modified organisms, along with characterizing the relative abundance of a species (Bohmann et al. 2014). Furthermore, metabarcoding can also provide information about the feeding habits of various species from stomach or gut content (De Barba et al. 2014; Berry et al. 2015; Guilleraut et al. 2017; Granquist et al. 2018). A number of studies on detection of fish communities using eDNA isolated from water samples have been conducted to point out the utility of metabarcoding for quantifying relative abundance of species, conservation and monitoring, diet analysis, invasive-species and wildlife disease detection (Valentini et al. 2016; Hänfling et al. 2016; Civade et al. 2016; Thomas et al. 2016; Thomsen et al. 2016; Fujii et al. 2019; Miya et al. 2020). eDNA analysis combined with metabarcoding has the ability to study the complete fish diversity of a waterbody using water and sediment samples (Taberlet et al. 2012). Biodiversity patterns including alpha-diversity (diversity within location) and beta-diversity (change in diversity of species) also can be determined by eDNA metabarcoding (Li et al. 2018). Applications of barcode data obtained from DNA barcoding and DNA metabarcoding are summarized in Figure 3.

Challenges and future prospects: Along with so many positive attributes, DNA barcoding has numerous downsides in the use of taxonomic classification.

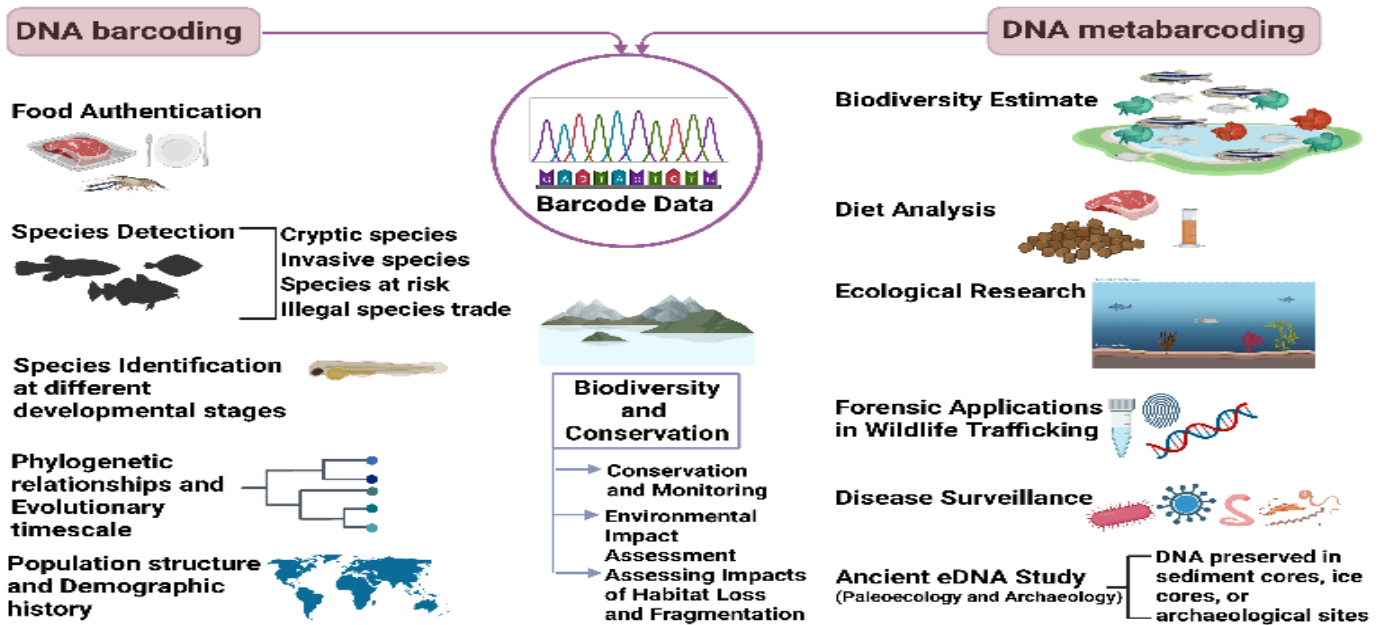


Fig. 3. Schematic diagram of applications of DNA barcoding and metabarcoding.

Many different biological phenomena may impede DNA barcoding such as mitochondrial heteroplasmy, paternal leakage, hybridization and introgression between different species, polyploidization, incomplete lineage sorting, and errors in creating DNA reference libraries for specimen identification (Sachithanandam et al. 2012). One of the main concerns is that classification of species on the basis of a single mitochondrial gene is not always appropriate (Stoeckle & Thaler 2014). Another limitation is that human mistakes and vulnerability in creating libraries can lead to clashing results with multiple labels on the same species (Collins & Cruickshank 2013). (Negi et al. 2016a) suggested that the use of different computational methods is mandatory to overcome the drawbacks of online databases, as single methods can provide false results. Bhattacharya et al. (2016) pointed out that some species diverge only by a single base pair because of the very slow rate of mutations. Such similar species can complicate issues of species identification using DNA barcoding. Another issue concerned with DNA barcoding is the lack of sequences for all species in databases (Peoples et al. 2017). In DNA metabarcoding, sequencing of uniquely tagged amplicons with NGS has the potential to yield a tremendous amount of information, however the high

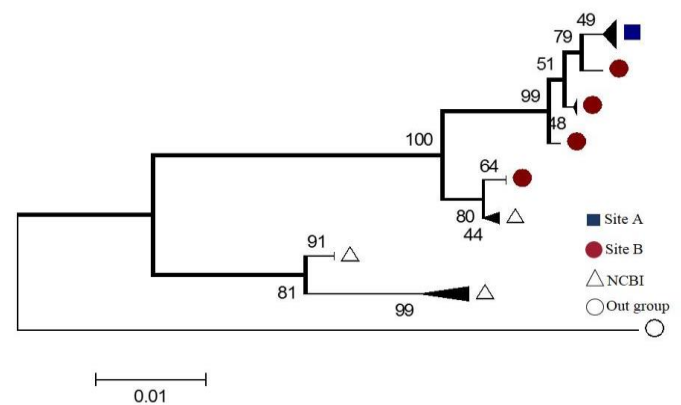


Fig. 4. *Pethia ticto* sequences for two highly divergent clades with >6% sequence divergence for the same species.

cost of NGS remains as a challenge (Liu et al. 2012). It is very difficult and expensive to prevent, detect, and correct errors and biases when combining hundreds of PCR replicates (Bohmann et al. 2022).

On the other hand, there are many species from different locations which show high genetic distances, for example, such as the Ticto barb (*Pethia ticto*) which may exist in a species complex or there may be cryptic species diversity even in the same habitat as well as from adjoining regions. We constructed a NJ phylogenetic tree using sequences of the COI gene of *Pethia ticto* in Joshi (2017), Negi et al. (2016b), and Negi et al. (2018) from different parts of India. We found high intra-species genetic divergence (>6%), which was greater than the inter-species sequence

divergence, but the lineages are named as a single species; thus, appropriate taxonomic reconsideration is needed. In support of this, two highly divergent clades of *P. ticto* were observed in which sequences from the same location clustered with samples of distant locations, indicating the presence of cryptic species diversity or different evolutionary significant units (Figs. 3 and 4). In addition, we also found that 17 sequences of *Pethia ticto* submitted to NCBI are not clustered with the original sequences. This finding was similar to those of Hallerman (2021), in which previously defined single species showed high intra-species sequence divergence (20%) from adjoining region or pool of the same habitat and suggested cryptic species. Genetic characterization of many species has not been thoroughly characterised in order to ascertain whether they are genetically variable than others or the misidentification of species is merely due to the use of a single computational method which resulted in the appearance of high divergence between species. Therefore, existing data sets of online repositories need to highlight data which has high potential to be false-positive for a particular species. Along with this, more rigorous species dilemma resolving methods should be used (such as Bayesian Phylogenetics and Phylogeography) so that better taxonomic assignments can be achieved (Rannala & Yang 2003). Molecular phylogenetics has seen a huge increase in the use of Bayesian approaches as a result of the accessibility of user-friendly software programs such as BEAST (Bouckaert et al. 2019) and MrBayes (Ronquist et al. 2012) that uses complex evolutionary models to create posterior probability of trees (Nascimento et al. 2017). Another software, SpedeSTEM allows researchers to perform a species delimitation analysis by calculating the maximum likelihood species tree using intraspecific genetic data (Ence & Carstens 2011). Vitecek et al. (2017) used different species delimitation tools on 14 easily differentiable species of Caddisflies Trichoptera, Family Limnephilidae and found STACEY (Species Tree And Classification Estimation, Yarely) as most reliable tool. Thus, to withstand these limitations, there is a need for more accurate and complete DNA

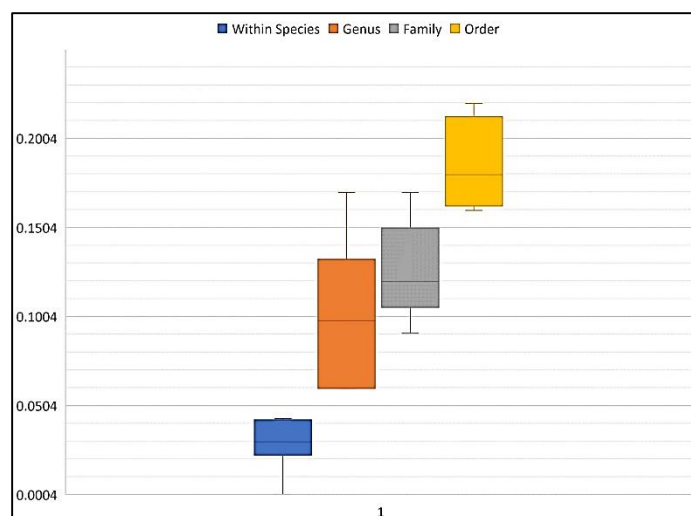


Fig.5 Sequence divergence ranges between different operational taxonomic units.

libraries for reference data with fully correct sequences and better analysis techniques.

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مقاله مروری

شناسایی ماهیان مبتنی بر DNA: مروری بر روش‌های بارکدینگ و ارزیابی کارایی آنها

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چکیده: چالش‌های شناسایی مورفولوژیکی گونه‌های نزدیک و مراحل لاروی ماهیان منجر به افزایش تقاضا برای روش‌های مبتنی بر DNA جهت دستیابی به شناسایی دقیق گونه‌ها شد. تکنیک مبتنی بر DNA کاربردهای قابل توجهی در تشخیص مواد غذایی، گونه‌های جدید، درک تاریخچه تکاملی و برنامه‌های نظارت زیستی دارد. با ظهور بارکد DNA و metabarcoding، می‌توان بر بسیاری از اختلافات در شناسایی مورفولوژیکی غلبه کرد. بارکد DNA به تأیید این موضوع بستگی دارد که توالی‌های حفاظت شده را می‌توان برای شناسایی گونه‌ها استفاده کرد، در حالی که metabarcoding توالی‌یابی پیشرفته نسل بعدی نمونه‌های با تعداد زیاد و متنوع یا نمونه‌های محیطی را برای تشخیص گونه‌های مختلف اجرا می‌کند. مقاله حاضر به بررسی کاربردهای بارکد DNA و metabarcoding می‌پردازد و درک اساسی از شناسایی ماهی از طریق اطلاعات بارکد ارائه می‌دهد. همچنین دید مختصری در مورد تفاوت‌های بین بارکد DNA و metabarcoding ارائه می‌کند و به اختلافاتی اشاره می‌کند که آنها را متفاوت و در عین حال متکی به هم نشان می‌دهد. مشکلات بارکدگذاری DNA نیز برجسته شده است، به عنوان مثال، هنگامی که صحبت از شناسایی نادرست در بارکدگذاری DNA با استفاده از Ticto Barb (*Pethia ticto*, Cyprinidae) می‌شود. علاوه بر این، این مطالعه استفاده از روش‌های محاسباتی مختلف برای اعتبارسنجی تاکسونومیک، اطلاعات جامع و دقیق DNA و تحقیقات عمیق‌تر نشانگرهای مولکولی را پیشنهاد می‌کند.

کلمات کلیدی: COI، eDNA، ماهی، شناسایی نادرست، شناسایی گونه