DNA BARCODING IN INDIAN MARINE FISHES: *Leiognathus equulus* and *Priacanthus prolixus*

Project work by

APARNA S

AB20ZOO003

Under the guidance of

Dr. REEMA KURIAKOSE, Associate Professor

Department of Zoology, St Teresa's College (Autonomous) Ernakulam

Kochi - 682011

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CERTIFICATE

This is to certify that the project report entitled "**DNA BARCODING IN INDIAN MARINE FISHES: *Leiognathus equulus* and *Priacanthus prolixus*** "submitted by Ms. **Aparna S**, Reg No:- **AB20ZOO003** in partial fulfilment of the requirement of Bachelor of Science degree of Mahatma Gandhi University, Kottayam, is a bonafide work under my guidance and supervision and to my best knowledge, this is her original effort.

Dr. Reema Kuriakose
Associate Professor
Department of Zoology
St. Teresa's College (Autonomous)
Ernakulam

Dr. Soja Louis
Head of Department
Department of Zoology
St. Teresa's College (Autonomous)
Ernakulam

EXAMINERS

1)

2)

DECLARATION

I, hereby declare that this project work entitled "**DNA BARCODING IN INDIAN MARINE FISHES: *Leiognathus equulus and Priacanthus prolixus***" is submitted to St. Teresa's College (Autonomous), Ernakulam affiliated to Mahatma Gandhi University, Kottayam in partial fulfilment of the requirements of Bachelor of Science degree in Zoology. This work has not been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in this report are entirely my own.

NAME: APARNA S SIGNATURE

REGISTRATION NUMBER: AB20ZOO003

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ABSTRACT

The present study reflects DNA barcoding in nine Indian Marine fishes. DNA barcoding involves sequencing a fragment of the mitochondrial cytochrome oxidase I (COI) gene, known as DNA barcodes, from taxonomically unknown specimens. The following study has been undertaken to research and analyze the barcode of Indian Marine fishes and their DNA sequences. DNA barcoding is a method of species identification using a short section of DNA from a specific gene. This study focuses on barcoding the DNA of 9 genera of fishes collected from Beypore Fishing harbour, Kozhikode. The study was completed in 4 phases. The first two phases were collection and preservation of specimens for later analysis. Third phase consisted of DNA isolation from the collected samples and amplification of the sequence. Since DNA extraction is a sensitive process, buffers are added to stabilize the pH over cell lysis and isolation. The final phase consisted of PCR and barcoding the isolated DNA samples. From the 21 samples collected, fishes belonging to 9 genera were identified which are Cynoglossus, Nemipterus, Decapterus, Selar, Mene, Pomadasys, Carangoides, Leiognathus and Priacanthus. Out of the 9 genera the specifically analyzed species were *Leiognathus equulus*, *Priacanthus prolixus*.

INTRODUCTION

Identification of fishes contributes to the classification of each fish and the crucial function they play in the biological world, as well as the discovery of important food and medicinal ingredients from them. Fishes are the primary food source of humans, hence research on them has a significant impact on human life. Various approaches can be used to identify an unknown specimen. Identifying it with the use of morphology was a typical method employed previously, but morphology only provides external structural features, fishes have undergone multiple ontogenetic development, convergent and divergent evolution, identifying them only based on their morphological characteristics will be very challenging. As a result, morphological features alone are not sufficient for unknown specimens; in these cases, DNA barcoding is used.

DNA barcoding, or sequence-based specimen identification, was developed by Paul Hebert in 2003 to identify a broad range of taxa by sequencing a standardized short DNA fragment, the “DNA barcode”. DNA Barcoding is a technique for identifying different species. It was first used in fishes by Ward in 2005. It functions by examining a specific DNA region and this specific area is known as the DNA barcode. For animal identification, the most broadly used barcode marker is mitochondrial Cytochrome C Oxidase subunit I (COI), which is highly conserved across species employing oxidative phosphorylation for metabolism. After that, the sequence of this DNA barcode is compared to a reference library that contains information on many different species and their barcodes. DNA Barcoding consists of the following steps: DNA Isolation, Amplifying the isolated DNA using Polymerase Chain Reaction,

Gel Electrophoresis and Sequence Analysis. DNA isolation is a key step because, without high quality DNA, the PCR amplification will not be optimal. The following statements are some of the benefits of DNA barcoding over previous classification systems: DNA barcoding help in accurately distinguishing some species that are similar in morphology, cryptic species are indistinguishable biological groups that are incapable of interbreeding and cannot be distinguished using traditional methods of classification because traditional methods classify cryptic species as a single group, even though these species show genetic variation. (Lakra et al., 2011).

They can be easily distinguished through DNA Barcoding; Barcoding methods can often give information without causing harm to the animal studied. In cases of morphological ambiguity, such as with larval stages, DNA barcoding technology can help identify species. The applications of DNA barcoding includes ecological monitoring, early detection, control and removal of non-indigenous species, fisheries management, food safety and protection of endangered species. The results generated by DNA barcoding are limited or biased to the frequency of occurrence, and quantifying fish abundance from molecular data is another key problem for this approach. There are several different types of DNA extraction methods. A few of them include Phenol Chloroform Isoamyl Alcohol, Proteinase K, CTAB Method, Spin Column-based Methods and Magnetic Bead-based Technique. However, the method implied on each specimen depends on sample type and purity and the yield of DNA that is to be obtained. DNA extraction is completed in 4 steps: lysis, separation, precipitation, and purification. There are 2 main types of DNA sequencing methods. The classical method is known as Chain Termination method or Sanger Sequencing Method. Other modern methods that can process a

large number of DNA molecules swiftly are collectively called High-Throughput sequencing (HTS) and NextGeneration sequencing methods (NGS). In the conducted study, DNA isolation was done using the DNeasy Blood & Tissue Kit and respective buffers namely Proteinase K, Buffer ATL, Ethanol, Buffer AL, Buffer AW1, Buffer AW2, Buffer AE and Buffer AP. Polymerase Chain Reaction (PCR) is a powerful method for amplifying particular segments of DNA, distinct from cloning and propagation within the host cell. Out of the various PCR techniques, amplification of the isolated sequence was done using normal Polymerase Chain Reaction. DNA sequencing was done to the amplified sequence using Sanger sequencing method. It is done to determine the nucleic acid sequence or order of nucleotides in DNA. Lastly, a phylogenetic tree was drawn with the help of the software MEGA 11 to identify the species of the specimen.

AIM AND OBJECTIVE

AIM

- To identify 2 species of marine fishes by using DNA Barcoding

OBJECTIVE

- To barcode the various types of Indian Marine Fishes.
- To provide the statistics, characteristics, and presence of types of fishes in certain areas.
- To compare and classify the varieties of fishes in marine habitat.

REVIEW OF LITERATURE

DNA Barcoding is a molecular diagnostic method used for identification of species by using a standardized DNA sequence or genetic region which acts as the 'barcode'. 'DNA barcoding' is a new identification tool proposed by Hebert et al. (2003), and is a valuable addition to the taxonomic tool box. They advocated the use of short DNA sequences from the specified region of the genome termed as DNA barcode for biological identification. It implies sequencing of a standard DNA locus as a tool for identifying species. An ideal DNA barcode should be easily retrievable with a single primer pair, be amenable to bidirectional sequencing and effectively provides high discrimination among species.

According to Savoleinen et al., (2005), the scientific benefits of DNA barcoding include:(i) enabling species identification, including any life stage or fragment, (ii) facilitating species discoveries based on cluster analyses of gene sequences, (iii) promoting development of handheld DNA sequencing technology that can be applied in the field for biodiversity inventories and (iv) providing insight into the diversity of life.

Based on the works of Jeremy C. Andersen et al., (2019), the collection of DNA barcode sequences from unidentified specimens provides useful genomic data and at the same time DNA barcoding techniques are being used with increasing frequency to guide management decisions, particularly for the identification of alien invasive species (Dejean et al., 2012).

This study utilizes the standard Cytochrome C Oxidase subunit I (COI) which is found in most eukaryotes and highly conserved and so can be copied from unknown organisms. They also have less intraspecific (within species) variation than interspecific (between species) variation, known as the "Barcoding Gap". When fully developed, a COI identification system will provide a reliable, cost-effective, and accessible solution to the current problem of species identification. Its assembly will also generate important new insights into the diversification of life and the rules of molecular evolution (Hebert et al., 2003). The mitochondrial genome of animals is a better target for analysis than the nuclear genome because of its lack of introns, its limited exposure to recombination and its haploid mode of inheritance (Saccone et al., 1999). Robust primers also enable the routine recovery of specific segments of the mitochondrial genome (Folmer et al., 1994).

The major goal of DNA-barcoding efforts is to aid identification of specimens by matching sequences to a sequence library. The revolution introduced by DNA barcoding resides in the molecularization, computerization and standardization of taxonomic approach. The identification and then the interpretation of molecular entities is the main goal of DNA barcoding that could be reached only by users with a sound theoretical background on what is identifiable by this technique (Casiraghi et al., 2010). Many authors have proposed DNA barcoding as an integrated approach with classical taxonomy for species identification and authentication. Modifications in extraction methods, primer sequences, use of an engineered polymerase and even the combining of barcodes from multiple loci has been used successfully to clear any issues related to DNA Barcoding in vertebrates.

In order to test the utility of DNA Barcoding in forensic vertebrate species identification, COI sequences from previously identified samples from humans and a variety of domestic and wild specimens of Brazilian mammals, birds, fishes were compared against the Barcode of Life Database (BOLD). BOLD provided a correct species-level identification for 12 out of the 20 queried sequences (60%) and presented the correct species as the best matched one for 17 out of 18 samples morphologically identified to this level (94%). (Carvalho, 2014)

Barcoding can be used as an alternative to traditional sampling methods in fish research. Barcoding procedures can often give information without causing harm to the animal being investigated. Hebert et al., (2003) proposed using DNA barcoding to help fish identification, which prompted the formation of Fish Barcode of Life (FISH-BOL), which aims to barcode all taxonomically documented fish species (Ward et al., 2009). The FISH-BOL project began in 2005, and roughly 8,000 of the 31,000 fish species recognised have been barcoded for the COI gene. According to the initial report, around 98 percent and 93 percent of marine and freshwater species may be distinguished using barcodes, respectively.

According to the work of Zhou et al., (2009), the limited access to taxonomic expertise is an issue for large-scale biodiversity surveys. Their study shows that a 7 comprehensive DNA barcode library built on expertly identified specimens enables fast and accurate species identification. There will be easier ways of analysing bulk environmental samples which will become more widespread and less expensive over time, facilitating ecological and monitoring applications of the barcode library. Continued interaction with the taxonomic community during barcode-based

biodiversity and monitoring studies, involving submitting specimens with novel sequences for determination or revision, will ensure the growth and maintenance of a high-quality database.

Leiognathus equulus also known as common pony fish, is found in river mouths and muddy inshore areas (Allen., 1991) and often in mangrove areas (Kuiter et al., 2001). Adults are coastal inhabitants found on soft bottoms, usually between depths of 10-70 metres. (Allen et al., 2002). They're strictly carnivorous fish feeding on blue-green algae, green algae, diatoms, rotifers, gastropods, nematodes etc. (Lankadhikara et al., 2004). They are deep bodied with short rounded snout and large eyes, strongly arched back, naked head with nuchal spines and protracted mouth (Frosskal., 1775). The gas bladder has the purpose of reflecting bioluminescent light from circumesophageal light organ (Margaret .,1983). They have 8 dorsal spines and 15-16 dorsal soft rays and 3 anal spines 14-15 anal soft rays. The body is black greyish colour with silver belly

Priacanthus prolixus commonly known as elongate bull-eye is found in the Western Indian Ocean region, ranging from Arabian Sea and Gulf of Aden. It belongs to the Priacanthidae family represented by Priacanthus genera which consists of 12 species in the world. *Priacanthus prolixus* is closely related to *Priacanthus hamrur*, *Priacanthus arietinus*, and *Priacanthus meeki*.(Starnes.,1988). They are pelagic species found in outer reef slopes and sometimes schools in oceanic locations. (Froese et al ., 2017). Generally found in 35m to 250m depth (Sommer et al.,2017) feeds mainly on small fish, crustaceans, and other small invertebrates. The elongate bull-eye can grow upto 25cm (Starnes.,1988) and has an elongated and laterally

compressed body, with a big eye on the head, with extremely adherent modified cycloid scales covering the body. Scales in the lateral line series ranging from 74 to 84, and scale rows between the dorsal fin origin and the lateral line ranging from 10 to 11. The body, head and fins are dark orange red in colour. Pectoral fins are reddish yellow; pelvic fins are light dusky to dark, especially distally, with a black spot basally on dorsal surface (Bineesh et al ., 2015)

METHODOLOGY

SPECIMEN COLLECTION

The fishes were collected from Beypore Fishing Harbour, Kozhikode. A total of 21 fish types were selected and labelled for analysis by DNA barcoding. The specimens were stored in the Museum at Kerala University of Fisheries and Ocean Studies, Panangad, Kerala.

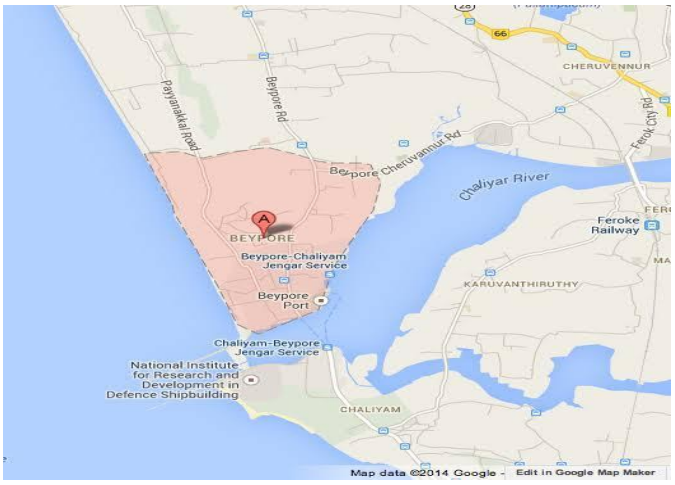

Fig. 1: Map showing Beypore Fishing Harbour, Kozhikode

Fig 2: Fish sample collection

PROCEDURE

PREPARATION OF SAMPLE

The selected mature fish specimens were measured and mounted on a hard surface to observe the characteristic features. Muscle tissue samples were dissected using sterilized tools, preserved in 10% Formalin, properly labelled and stored in the refrigerator.

The protocol for DNA extraction, PCR amplification of CO1 gene, product purification and sequencing follow the study of Lakra et al. (2011).

Fig 3: Preparation of fish sample

DNA ISOLATION

The preserved tissue samples were taken out of the vial and a portion (<25mg) of the flesh was transferred to a centrifuge tube and labelled. 200µL of Buffer ATL (Lysis Buffer) was added to the centrifuge tube followed by 20µL of Proteinase K. The mixture was vortexed to homogenise the contents and incubated in a thermomixer at 56°C for 2 hours until the whole tissue was completely digested. 200µl of Buffer AL (Lysis Buffer) was added to the centrifuge tube and incubated for another 10 minutes at 56°C. After removing the centrifuge tubes from 10 the thermomixer, 200µL of 95% chilled Ethanol was added to it and then incubated at room temperature for 5 minutes. The contents of the centrifuge tube were then transferred into labelled spin columns taken in 2ml collection tubes. The tubes were placed in a balanced configuration and centrifuged for 1 minute at 8000 rpm.

The collection tubes were then replaced with new 2ml tubes. 500µL of Buffer AW1 (Wash Buffer) was added to the centrifuge tube and centrifuged for 3 minutes at 14000 rpm. The process was repeated with Buffer AW2 (Wash Buffer). The collection tubes were replaced with labelled centrifuge microtubes. 50µL of Buffer AE (Elution Buffer) was added to the centrifuge tube (incubated at room temperature for 1 minute) and then centrifuged for 1 minute at 8000 rpm. Another 50µL of Buffer AE was added to the centrifuge tube and incubated at room temperature for 1 minute and centrifuged at 1 minute at 8000 rpm. The 11 Buffer AE elutes the DNA from the spin column membrane into the centrifuge tube. The eluted DNA in the labelled centrifuge microtube was stored at -4°C.

Fig 4: Vortexing of microtubes

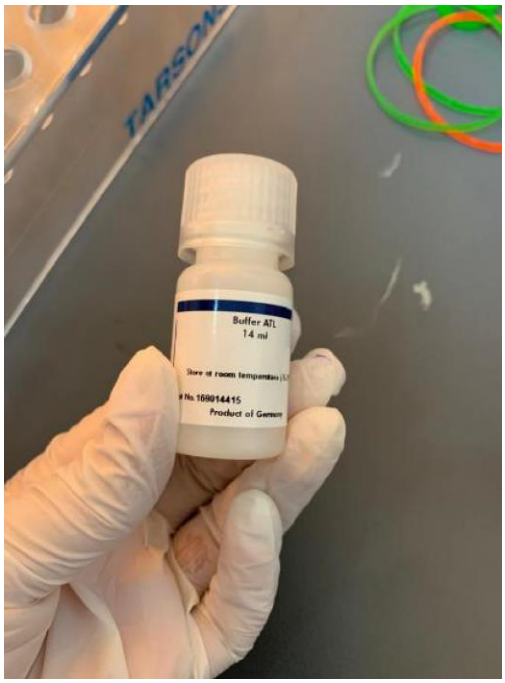

Fig 5: ATL Buffer

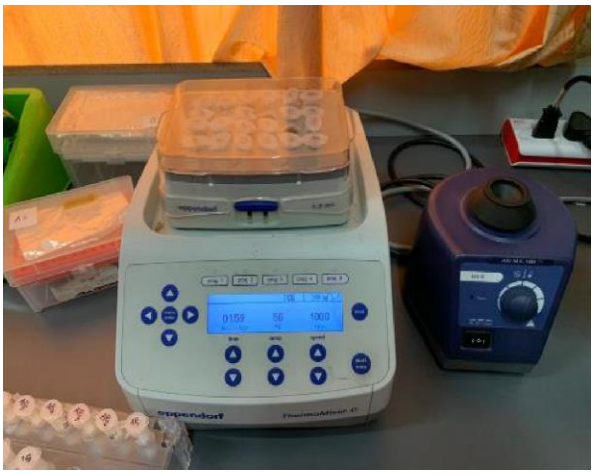

Fig 7: Thermomixer

Fig 8: Centrifugation

PCR

Species-specific variations or polymorphisms in the DNA sequence that are spread randomly over the entire genome and result in characteristic DNA fingerprints have been exploited through use of polymerase chain reaction (PCR) and its variants. (Priyanka et al., 2015). The procedures are followed using INVITROGEN Genomic DNA Mini Kit. 24 µL Master Mix (312.5 µL Emerald Amp GT PCR, 31.25 µL Forward F1 Primer, 31.25 µL Reverse R1 Primer and 225 µL dH2O) was added to new individual vials which were properly labelled. The primer pair LCO1490 (59-GGTCAACAAATCATAAAGATATTGG-39) and HCO2198 (59-TAAACTTCAGGGTGACCAAAAAATCA-39) was subsequently used to amplify a 658 bp fragment of the COI gene. The samples were taken out of storage and added to the Master Mix vials. The next step in the process involved 35 cycles of PCR (involving Denaturation, Annealing and Extension followed by Final Extension) maintained at 4°C. The vortexed vials were kept in the PCR Machine until a temperature of 105°C was attained after which the process started and took around two and a half hours to complete.

- DENATURATION

  Done at 95°C for 5 minutes.
- ANNEALING

  Done at 58°C (but may vary depending on Primers used).
- EXTENSION

  Done at 72°C

Fig 9: Genomic DNA Mini Kit

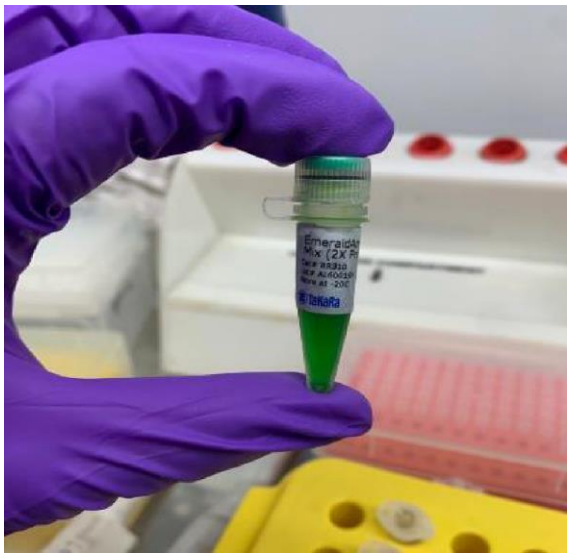

Fig 10: Master Mix

Fig 11: PCR Machine

Fig 12: Progress of PCR

PREPARATION OF GEL

0.4g Agarose Special Powder was added to 100mL Borosil and mixed well. This was transferred to a gel tray with wells/pits.

GEL ELECTROPHORESIS

Anode and Cathode electrodes were placed in a container and TE Buffer was added until the electrodes were completely immersed. The amplified sample mixtures were poured into the wells/pits. The Electrophoresis Machine was set to 90-91 Volts and run for about 20 minutes. The gel is then transferred onto a Bio Rad Imager for analysis of results of DNA imaging on the computer. The isolated DNA samples were given for sequencing.

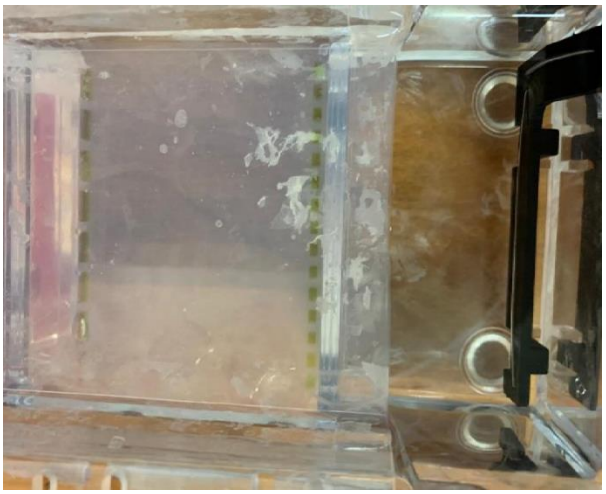

Fig 13: DNA Bands separation after Gel Electrophoresis

Fig 14: X-ray analysis of DNA Bands

PHYLOGENETIC TREE

A phylogenetic tree is made and analyzed using various softwares. In the field of genome analysis, biologists seek to identify important genes or chromosome

regions by comparing phylogenetic trees and analyzing the mutation at which locus might affect phenotypic traits (Ge et al., 2020).

Name of the species	**Database of number of base pairs acquired from ICBN nucleotide sequence of COI gene**
Leiognathus equulus	643 base pairs
*Priacanthus prolixu*s	636 base pairs

These sequences were then aligned using the MUSCLE (Edgar, 2004) sequence algorithm implemented in MEGA 11. From the aligned sequences a phylogenetic tree was constructed by using the Maximum Likelihood (ML) method. The most common way to estimate the reliability of a phylogenetic tree is by the bootstrap method (Hall, 2013).

OBSERVATION AND RESULT

The amplified Cytochrome Oxidase I (COI) sequences were identified using BLAST. The fishes identified based on matches of ≥97% similarity to a published sequence in the NCBI GenBank database were *Leiognathus equulus*, and *Priacanthus prolixus*.

Leiognathus equulus

Fig 15: *Leiognathus equulus*

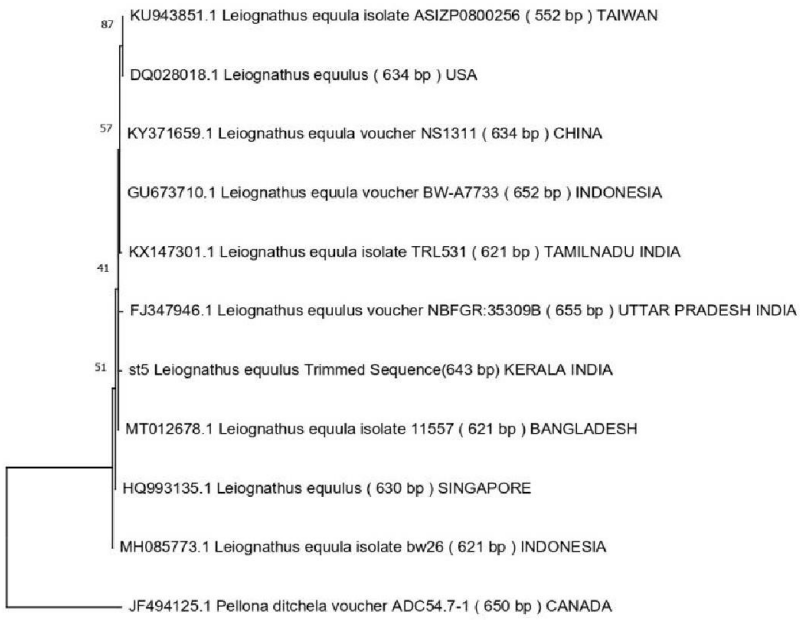

Fig 16: Phylogenetic tree of *Leiognathus equulus*

Outgroup- JF49412

Priacanthus prolixus

Fig 17: *Priacanthus prolixus*

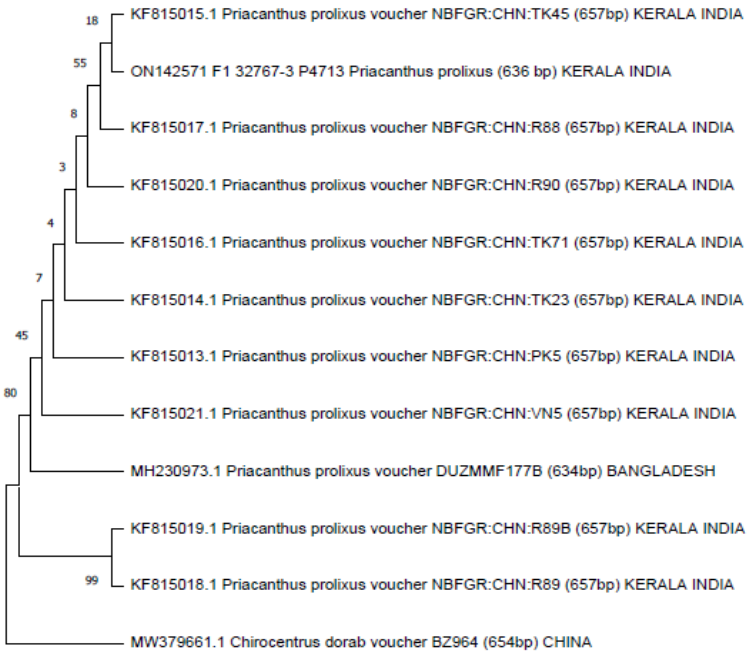

Fig 18: **Phylogenetic tree of *Priacanthus prolixus***

Voucher Specimen- ON142571

Outgroup- MW379661

DISCUSSION

The DNA barcoding method is an effective tool for species identification, particularly with specimens that are damaged, incomplete, unknown, or consisting of several morphologically distinct stages. The objective of DNA barcoding analyses is simple- to assign each unknown sequence to a set of referenced (tagged-specimen) sequences extracted, for instance, from databases like BOLD (Casiraghi., 2010). Many different bioinformatics approaches are available to reach this aim. Although it has many advantages, it also has its limitations. In some cases, related species may present identical sequences making DNA barcodes useless for species discrimination.

Leiognathus equulus

Leiognathus equulus are marine fishes living in brackish waters. They are distributed along the Red Sea, Persian Gulf and East Africa. Body is deep, compressed with a strongly humped back. Found in river mouths and muddy inshore areas often in mangrove areas. Adults move in schools. They feed on polychaetes, small crustaceans, small fishes and worms (Froese et al., 2022).

In the phylogenetic tree of *Leiognathus equulus*, the voucher specimen is named as st5 Kerala India. The native place of *Leiognathus equulus* is Tranqebar, Tamil Nadu, making the region its type locality. The voucher specimen is collected from Beypore Kerala. The type locality voucher is named as KX 147301 and is found in Tami Nadu.

If the difference in Bootstrap value is less than 40, one could say that the similarities between two species are very vague or they belong to two different species. Here an outgroup is chosen to show the significant difference between the two genus of fishes. The outgroup chosen is Pellona ditchela and it has no similarities to the genus Leiognathus. Since the similarities between the type locality voucher and voucher specimen is below 40, it can be said that even though they belong to the same genus, they are dissimilar. The similarities are vague and differences are more prominent in the dendrogram making it a cryptic species of the genus Leiognathus.

Priacanthus prolixus

Priacanthus prolixus are pelagic species found in outer reef slopes or commonly found in schools in oceanic locations . They are distributed along the western Indian ocean ranging from Arabian sea to the Gulf of Aden (Froese et al., 2017).The species is distinguished by an elongated and laterally compressed body. A distinctive feature of the Priacanthidae family is big eyes, 74-84 scales in the lateral line area, and 10-11 scale rows between the lateral line and dorsal fin origin (Bineesh et al., 2019)

In the dendrogram of *Priacanthus prolixus*, the voucher specimen is named as ON142571. It is collected from Beypore Kerala.The native region of *Priacanthus prolixus* is the Vizhinjam Kerala, India, making the region its type locality. The type locality voucher is named KX805015.1. Here the outgroup chosen is *Chirocentrus dorab* and it has no similarities with the voucher specimen. Since the similarities between the voucher specimen and type locality specimen is less than 40. The

differences in the dendrogram suggests that the voucher specimen maybe a cryptic species. But the species has less than 10 samples in the public database, hence the construction of phylogenetic tree is not a reliable technique.

CONCLUSION

DNA barcoding is a very useful tool for identifying unknown specimens. One of the main advantages of this technique is its speed and accuracy. This is particularly important for identifying cryptic species, which may look very similar to one another but have distinct genetic differences. In addition to its applications in species identification and conservation, DNA barcoding of marine fishes can also provide important insights into the evolutionary history of these organisms. By comparing the DNA sequences of different fish species, researchers can reconstruct their evolutionary relationships and better understand the processes that have led to the diversity of marine fishes we see today. Compared to traditional methods such as identifying fish using morphology, which can be misleading and has a higher possibility of error, analysis involving genetic material is significantly better. It is a valuable tool in forensic science, which can provide important information in criminal investigations, identification of human remains, and wildlife forensics. A phylogenetic tree might be used to interpret the results. A single glance reveals the clade or group to which the unknown species belongs.

Despite its many benefits, DNA barcoding of marine fishes also has some limitations. It relies on the availability of high-quality DNA samples, which can be difficult to obtain from some species, particularly those that are rare or have a small body size. For the species *Leiognathus equulus* the availability of sample sequences in public database enabled the identification of the species and the study of the degree of genetic changes in the species. But for *Priacanthus prolixus* there are less

than 10 DNA sequences available in public databases, hence it is challenging to conduct a robust phylogenetic analysis that includes this species. Phylogenetic analysis requires a sufficient number of DNA sequences from different species to compare and identify evolutionary relationships among them, DNA barcoding has been most extensively applied to many taxonomic groups, but the insufficient reference database for the identification of species that belong to taxonomic groups like invertebrates and vertebrates can lead to inaccurate identification of species, so it is important to interpret the results of the analysis with caution and to consider the limitations of the available data.

While phylogenetic studies based on DNA barcoding may be limited by the availability of sequences, they can still provide valuable insights into the evolutionary relationships and taxonomic classification of species. Improving DNA barcoding requires a combination of efforts to expand reference databases, use multiple genetic markers, employ more advanced sequencing technologies, develop standardized protocols, integrate DNA barcoding with other data sources, and incorporate machine learning and artificial intelligence. These initiatives can aid in overcoming DNA barcoding's limitations and enhancing its accuracy and dependability as a tool for species identification.

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