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DNA Barcoding of *Indomysis annandalei*: Unravelling the Complexity of Molecular Systematics of Shrimps

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Abstract: ***The field of DNA barcoding is transforming conservation efforts by providing a deeper understanding of the complexities of the natural world. This innovative technique allows us to identify known species, uncover hidden variations, and even discover new species. By bridging molecular techniques and traditional taxonomy, DNA barcoding offers a practical and cost-effective way to study and protect biodiversity in unexplored regions. It acknowledges that an organism's place in the hierarchy of life is not fixed, which means that scientists can refine classifications using evidence from various fields. By promoting collaboration and integration with other disciplines, DNA barcoding provides valuable insights into the dynamic nature of our world. This powerful tool is continually advancing our understanding of nature and making significant contributions to conservation efforts.The current findings regarding the distribution of Indomysis annandalei populating the Konkan Region in Maharashtra extended the knowledge on the presence and density of these benthic invertebrates based on molecular identification by DNA barcoding using COI gene sequencing, and complementary meta-data regarding their habitats (substrate type and river depth), thereby adding to the ecological profile of this fauna. The accuracy of species identification by this method was highlighted in the cases of several specimens belonging to the same species of mysids clustered together in monophyletic groups. Moreover, this study contributed to the first mysid barcode dataset.***

Keywords: DNA Barcode, *Indomysis annandalei*, Molecular Systematics, opossum shrimp, phylogenomics

1. Introduction

Phytogeography is a research field that studies the geographic distribution of genealogical lineages within and among species. This field has revolutionized molecular diversity studies and is also known as molecular taxonomy. To conduct phylogenetic analysis and interpret lineage distributions, experts draw on a wide range of fields, such as molecular genetics, population genetics, ethology, demography, evolutionary biology, paleontology, geology, and historical geography.

DNA Barcoding, which advocates the use of mitochondrial genes such as COI, has gained popularity for identifying species globally. Classic phylogenetics relies on physical or morphological features, while modern phylogeny uses information from genetic material. In this context, Internal Transcribed Spacers (ITS) have been useful for studying closely related species and for phylogeographic research.

Marine biology research has extensively examined the spatial distribution of allelic frequencies in natural populations, with a particular focus on the life cycle and mode of reproduction of marine species. As most marine species have a dispersal phase in a boundary-less environment, this approach has proven to be useful. The genetic structuring and gene flow between geographically separated populations depend on the dispersal potential of the species, which is influenced by its mode of reproduction. The genetic divergence among populations of species with planktonic larvae and a continuous habitat is typically low, while species without a pelagic dispersal stage exhibit higher genetic divergence. However, studies have highlighted cases where long-life pelagic larvae do not result in broad dispersal, which is an interesting area for further research.

It is important to consider factors other than dispersal ability when studying population differentiation. A variety of elements may contribute to this phenomenon, such as behavioral mechanisms that limit dispersal, selective processes, local adaptations, complex oceanographic currents, habitat discontinuities, and historical barriers to gene flow. Over the past decade, numerous studies have attempted to understand the relationship between intrinsic (biological, ecological, physiological, or behavioral) and extrinsic (physical, geological, and environmental) factors that influence population structuring. These factors are interconnected and together play a role in shaping population differentiation.

The topic of this study is mysids, which are macro-zooplankton belonging to the Crustacea class. Mysids are crustaceans that look like shrimp and are commonly referred to as "opossum shrimp" because mature females have a brood pouch or marsupium. They can be found in all regions of the ocean, even at depths of up to 7210m. Mysids are highly adaptable and are therefore able to invade new areas easily (Ketelaars et al., 1999). There are many brackish water species, a few freshwater species, and some that have adapted to living in caves and wells. Some mysids live symbiotically with animals like sea anemones, sponges, and hermit crabs (Clarke, 1955; Tattersall, 1962 & 1967; Bowman,1973; Vannini et al.,1993; Price and Head,2004).

Some species of mysids burrow into sediment, while others live just above it or migrate between bottom and surface waters. A few are strictly pelagic, and some live in shallow water in the littoral zone (Mauchline,1980). Although mysids are found throughout the marine environment, the greatest concentration is in coastal regions. Coastal and epipelagic forms are small, but deeper species are large (Mauchline,1980; Brusca and Brusca,2003). Lepidomysidae and Stigiomysidae species inhabit caves and wells, and the Petalophthalmidae species are mainly distributed in deep waters. Unlike Mysidae, these species have no statocyst in the uropod, and the marsupium consists of seven pairs of lamellae. The family Mysidae is the most extensive group, with seven sub-families: Boreomysinae, Thalassomysinae, Siriellinae, Rhopalophthalminae, Gastrobaccinae, Mysinae, and Mysidellinae. Indomysis annandalei was selected for DNA barcoding studies based on its geographical distribution, ecological significance, and specific habitat requirements.

The present study is focusedonthe spatial distribution of mysid *Indomysis annandalei* which is commonly called "***Opossum shrimp"***. In this study, we also describe the synthesis of the *Indomysis annandalei* DNA barcode.

2. Material and Methods

a) Distribution of *Indomysis annandalei*

Indomysis annandalei is a species that can survive in a variety of saltwater and temperature conditions and is typically found in the macro-zooplanktonic communities of the salt-pan reservoirs, creeks, and coastal marine ecosystem of Mumbai (Tattersall and Tattersall, 1951). This species is known for its omnivorous diet and feeds on macrophytes and detritus. It is an important prey for demersal and pelagic fish and plays a crucial role in the salt-pan ecosystems. The female Indomysis annandalei produces several broods throughout the year, with more than three generations annually. The population of this species is highest during the post-monsoon season (Deshmukh, 1995). Indomysis annandalei forms an important part of the food chain in the brackish water ecosystem.

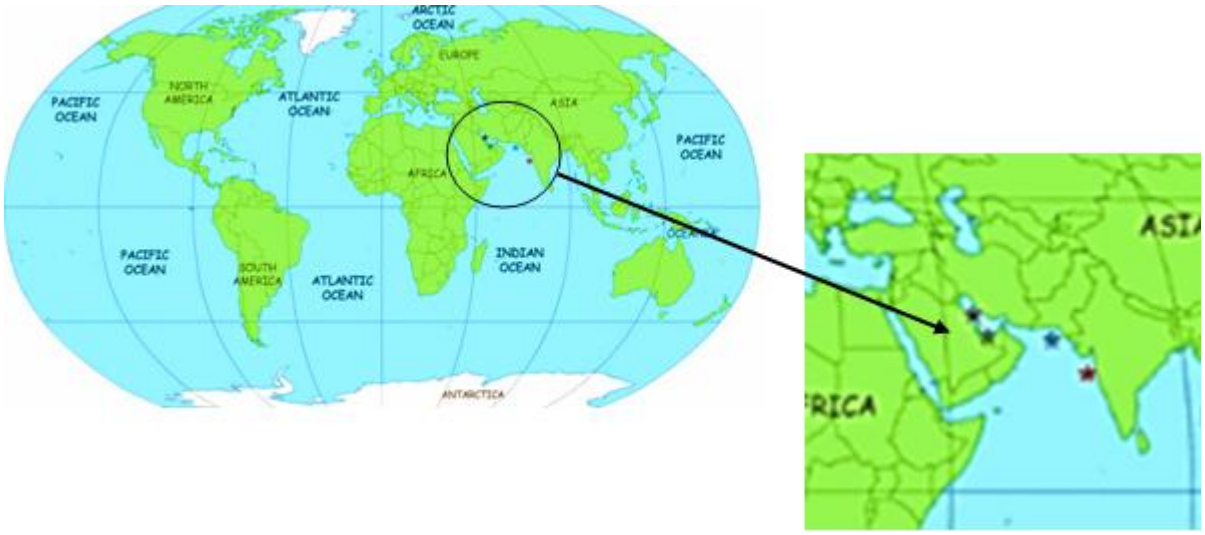


Figure 1: Distribution pattern of Indomysis annandalei.*Mumbai *Pakistan * Saudi Arabia * Arabian Gulf*

b) Morphology of *Indomysis annandalei*: Opossum Shrimps

Indomysis annandalei is an extremely euryhaline, eurythermal species, and abundant in creeks and shallow coastal waters around Mumbai at salinities from 4ppt - 114ppt, whereas temperature tolerance observed was from 10°C to 38°C (Deshmukh, 1995). The occurrence of *Indomysis annandalei* is mainly tropical, until now it has not been reported in latitudes higher than **26°4' N**. Tattersall (1914), was the first to report and describe this species from Panvel Creek near Mumbai, India (**19°N**). Thereafter, Deshmukh (1989), reported this species in the reservoirs of saltpans at Mulund, Mumbai harboring Thane creek. Kazmi and Tirmizi (1995), also recorded the occurrence of *Indomysis annandalei* within Pakistan waters (25° N**)**. Murano (1998), recorded it from Tarut Bay, Saudi Arabia (**26°4' N**); the most recent record is from Tubi Bay, Bahrain, Arabian Gulf (**26°N**) by Grabe *et al*., (2000) (Fig.2).

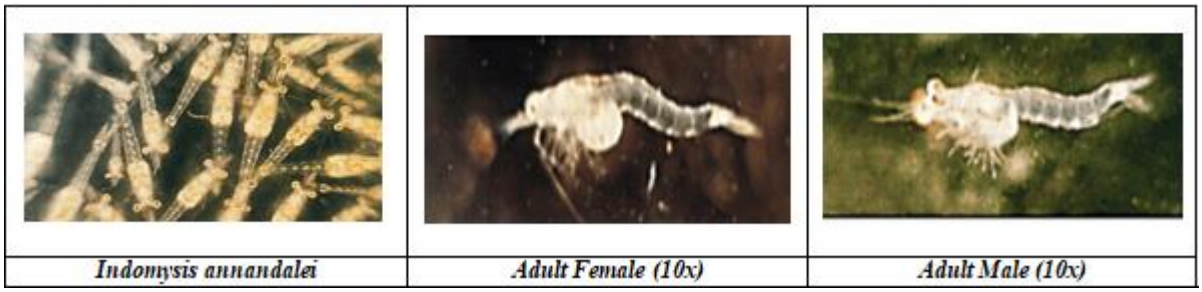
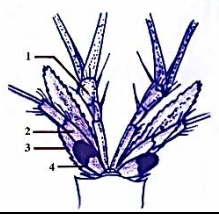
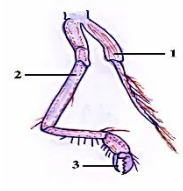
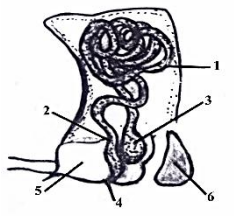


Figure 2: Morphology of *Indomysis annandalei*: Opossum Shrimps

	
a. anterior body end of mysids showing 1.Antennule 2. Antena 3. Antenal scale 4. Eye stalk	**b. Seventh Thoracopod in mysid where 1. Exopod 2. Endopod 3. Chelate dactylus 4. Non-chelate dactylus**
	
c. Figure 1. Urinary Tract 2. Terminal duct 3. End sac 4. External opening 5. Antennal peduncle 6. mandible	**d. Antennal gland in mysid.**

Indomysis annandalei ranges between 7mm and 7.5mm in length with a dull cream color moderately slender but robust body. Eyes well developed with short eye stalks. It has well-developed eyes with long and tubular eye stalks. The major organ involved in maintaining the salt concentration equilibrium and adapting these mysid species to their respective environment is the excretory organ-Antennal Gland. Anatomical differences within the antennal glands of the two selected species are distinctly observed. In the case of *Indomysis annandalei*, the antennal glands consist of a much-convoluted urinary duct, which leads to an end sac that lies in a posterior bulge at the base of the antenna. A terminal duct that widens just behind the external aperture is situated on the second segment of the antennal peduncle.

c) Sample preparation of *Indomysis annandalei*

Adult Mysid species *Indomysis annandalei* (Habitat salinity-36%) and *Indomysis annandalei* (Habitat salinity-114%) were sampled from shore waters of Back Bay, Mumbai where the salinity would reach the maximum of 35ppt.These animals were frozen at -80 °C till further investigation.

d) DNA Barcoding of *Indomysis annandalei*

The sample was extracted by lysing mysid tissue in 1X PBS. This lysed tissue was then centrifuged and the supernatant of respective samples was collected and used for identifying extracted proteins. The extracted protein sample was mixed with gel loading buffer (comprised of glycerol, SDS PAGE Buffer, and bromophenol blue: as tracking dye). All the proteins were loaded onto a 12% Polyacrylamide Gel and the gel was run as per standard protocol. The gel was transferred to a tray containing water and washed thoroughly. The gel was then uniformly stained using 20ml of Coomassie brilliant blue (1%)for 30-45 minutes on a laboratory rocker. The bands were used for identifying proteins using in-gel tryptic digestion followed by mass spectrometric analysis.

e) DNA isolation

Whole genomic DNA was isolated for most samples using the protocol of Miller et al. (1988), but for some degraded samples,DNA easy (Qiagen, Valencia, CA) was used following instructions of the manufacturer, and eluted in 50–200 ml of AE buffer. Extracted DNA was checked by 0.8% agarose gel electrophoresis with ethidium bromide incorporated in 1 × TBE buffer. The concentration of isolated DNA was diluted to a final concentration of 100 ng/ml using a UV spectrophotometer.

f) Amplification and sequencing

The barcode sequence of the COI gene was PCR amplified using the primers (50-TCA ACC AAC CAC AAA GAC ATT GGC AC-30) and Fish R1 (50-TAG ACT TCT GGG TGG CCA AAGAAT CA-30) (Ward et al., 2005) in 25 ml reactions containing 1 × assay buffer (100 mM Tris, 500 mM KCl, 0.1% gelatin, pH 9.0) with 1.5 mM MgCl2 (Genei, Bangalore, India), 5 pmoles of each primer, 200 mM of each dNTP (Genei, Bangalore, India), 1.5 U Taq DNA polymerase, and 20 ng of template DNA. Thermal conditions consisted of initial preheat at 95 ◦C for 3 min, denaturation at 94 ◦C for 30 s, annealing at 50 ◦C for 30 s, extension at 72 ◦C for 35 s, repeated for 29 cycles, followed by a final extension for 3 min at 72 ◦C.PCR products were visualized in a 1.2% agarose gel. Samples with intense bands were selected for sequencing. Sequencing reactions used a BigDye Terminator V.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). All samples were sequenced bidirectionally using an ABI 3730 capillary sequencer following the protocol of the manufacturer.

g) Sequence analysis

RAPD Primer was labeled with FAM which gives a blue colour in genescan analysis. The fluorescently labeled PCR products were run on a Genetic Analyzer to obtain RAPD Peak Data files. The data generated was analyzed to obtain the Binary output. The Binary output was used to create the Specific Barcode.

3. Results and Discussion

Identifying organisms has grown in importance as we monitor the biological effects of global climate change and attempt to preserve species diversity in the face of accelerating habitat destruction. We know very little about the diversity of plants and animals, let alone microbes living in many unique ecosystems on the Earth. Less than two

million of the estimated 5-50 million plant and animal species have been identified. Scientists agree that the yearly rate of extinction has increased from about one species per million to 100-1,000 per million. This means that thousands of plants and animals are lost each year. Most of these have not yet been identified.

a) Genomic DNA Isolated from Mysid Samples (RAPD Agarose Gel):

Genomic DNA was isolated from mysid samples and when loaded on 1% agarose gel, prominent bands appeared on the gel (Fig.1.). These strands were then excised and RAPD PCR was performed. The resultant PCR products were then scanned for their purity on agarose gel with the marker ladders distinguishing the fragment length of the generated PCR product. This showed that five distinct bands developed in the lane where *I. annandalei* RAPD-PCR product was loaded i.e., Lane L1 (DNA ladder) where arrow markings point 100 bp, 500 bp & 1000 bp fragment size); ***Lane I: RAPD Profile of Indomysis sp***. Lane L2: 500 bp DNA ladder (Arrow markings denote 500 bp, 1000 bp & 1500 bp fragment size).

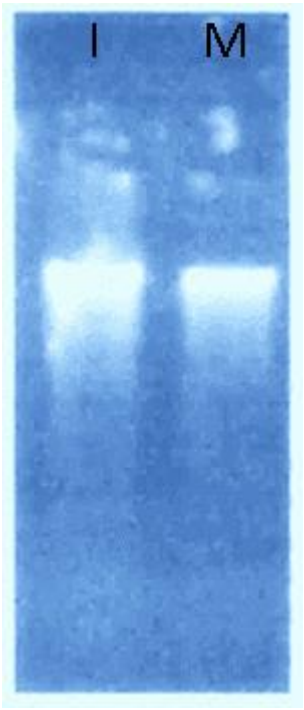


Figure 1: Lane I: DNA isolated from mysid *Indomysis annandalei* Lane M: Marker

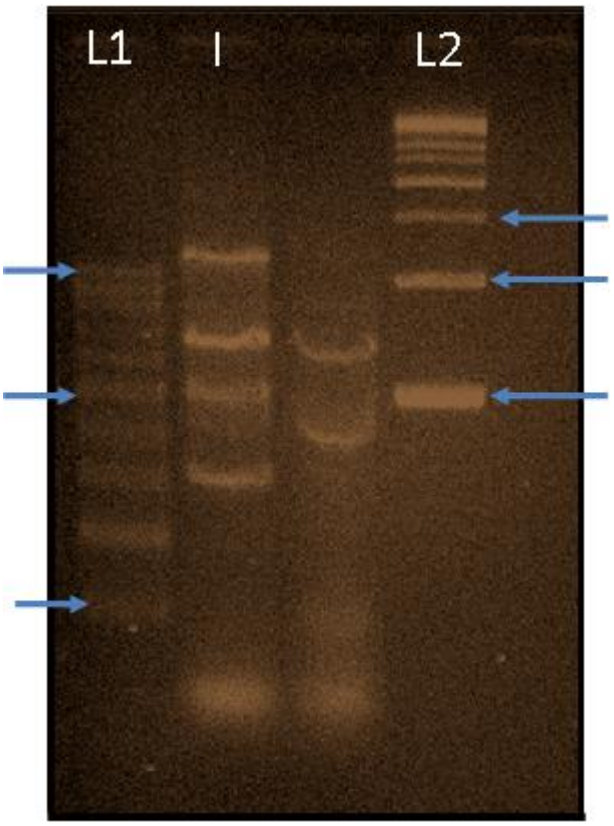


Figure 2: Lane L1: 100 bp DNA ladder (Arrow markings points 100 bp, 500 bp & 1000 bp fragment size); **Lane I:** RAPD Profile of Indomysis sp. **Lane L2**: 500 bp DNA ladder (Arrow markings denote 500 bp, 1000 bp & 1500 bp fragment size)

b) 100 bp Ladder Used for DNA Barcoding

The RAPD peak data thus obtained by running the respective samples on ABI 3130 Genetic Analyzer generated a binary output. This binary output was then ultimately converted into a code for each sample. This code generated the barcode of each respective sample.Thus, the application of DNA barcoding to help unravel the complexity of the dynamics in the natural world.DNA barcoding adds a fast, objective, and repeatable approach to this enormous task that can shift the enterprise into a higher gear.

Barcodes can document and confirm known species while uncovering lots of hidden variations, some of which may lead to the description of new species. A standardized library of barcodes will enable more people to identify species whether abundant or rare, native, or invasive engendering appreciation of biodiversity locally and globally.

Barcoding is a perfect collaboration between molecular techniques and traditional taxonomy, and a practical, cost-effective way to both study this under-explored region and protect its biodiversity. There are two separate tasks to which DNA barcodes are currently being applied. The first is the use of DNA data to distinguish between species (equivalent to species identification or species diagnosis) and the second is the use of DNA data to discover new species (equivalent to species delimitation, species description).

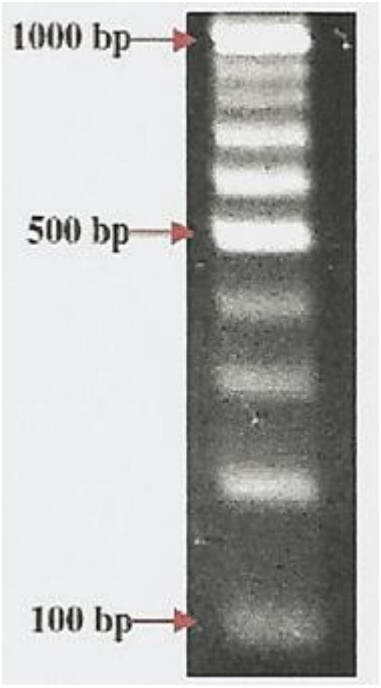


Figure 3:100 bp ladder contains 10 DNA fragments of size 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp and 1kb

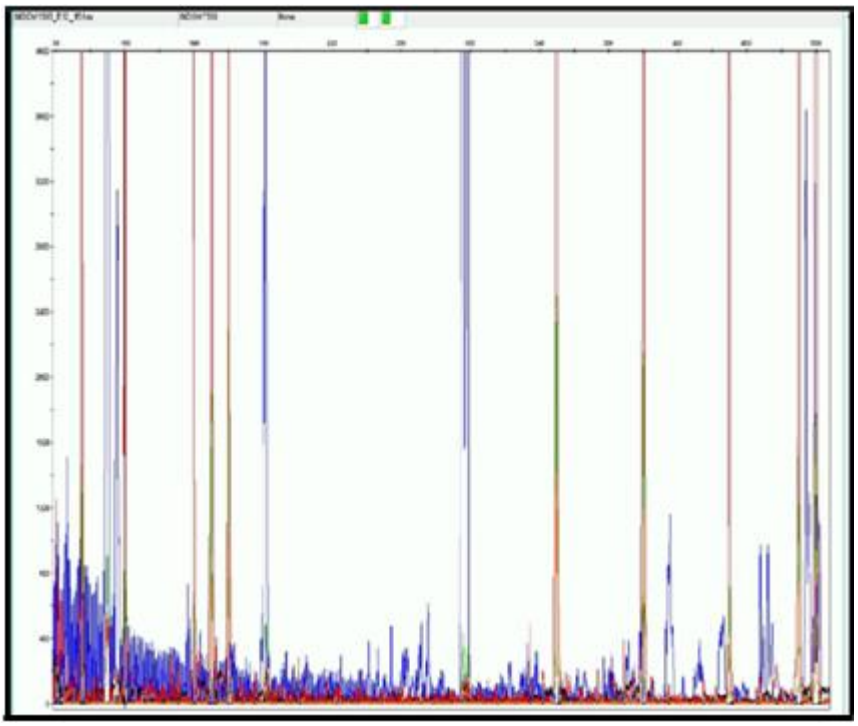


Figure 4: RAPD Peak data of*Indomysis annandalei.*

Figure 5: DNA Barcode of *Indomysis annandalei*
Binary Output: 111011111001111

These two activities differ in the types and amount of data required. biodiversity assessment remains today mainly based on morphological characters. Since morphology is complex and non-neutral, it may lead to under- or over-

estimation of species diversity. Today's technology for sequencing DNA and barcoding has paved the way to molecular taxonomy at a more objective level. For highly diversified crustaceans, sequencing of two mitochondria1 genes, COI and 16 S rRNA has been found useful forthe correlation between taxonomic ranks and molecular divergence. DNA - "barcoding requires defining for each taxonomic group a set of molecular synapomorphies that can be used as taxonomic tags. Cryptic species are common in crustaceans. Crustaceans are also particularly abundant in extreme habitats which tendto morphological convergences leading to biodiversity underestimation. For these reasons, crustaceans constitute a group for which DNA taxonomy could be highly valuable.

4. Conclusion

DNA barcodes obtained in this study for *Indomysis annandalei*define the morphological and anatomical characters playing an important role in the taxonomical classification of the same. Like these characters marked differences among the selected species, their RAPD-PCR profile on the agarose gel showed significant differences. This not only confirmed that the species belong to differentgenera but also, made it easy to identify them in the future with the respective barcodes generated. Barcodes generatedarespecies-specific so there is no confusion of overlapping characters. Moreover, these barcodes generated are universal thus adding up the information in the database available to all.

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Conflict of Interest
None

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