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"Exploring Mushroom Diversity in Bhopal Through Its Region Molecular Identification"

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ABSTRACT:
This study presents a systematic methodology for the molecular characterization of wild mushrooms collected from the Bhopal region. A part of three years, mushroom biodiversity was documented, and DNA was extracted, preserved, and amplified using internal transcribed spacer (ITS) region primers (ITS1 and ITS4). The protocol includes cryopreservation in liquid nitrogen, DNA extraction with a Tris-EDTA-SDS lysis buffer, and purification using chloroform:isoamyl alcohol and ethanol precipitation. Amplification of the ITS region was performed to validate the quality and presence of fungal DNA, enabling further molecular identification and phylogenetic analysis. This methodological framework contributes significantly to the genetic cataloging of mushroom species in Central India.

KEY WORDS:
Mushroom, Biodiversity, Bhopal, DNA, PCR, ITS Region, Fungal Taxonomy, Molecular, Phylogenetic, ETC.

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I. LITERATURE OF PAPER:

DOMINIQUE STRAUSS, SOUMYA GHOSH, ZURIKA MURRAY, MARIEKA GRYZENHOUT: *Genomic DNA Extraction from Minimal Amount of Dried Mushroom Samples*(4 September 2021): This study outlines an optimized protocol for extracting high-quality genomic DNA from very small quantities of dried mushroom tissue. The method is designed to be efficient and reliable, particularly for mycological research where only limited or preserved material is available.

II. INTRODUCTION:

This article provides an overview of the methodology used in my research, which forms part of my PhD work. Mushrooms, the reproductive structures (fruiting bodies) of macrofungi, are a vital group within the fungal kingdom. They contribute significantly to terrestrial ecosystems by participating in nutrient recycling, decomposing organic matter, and forming symbiotic associations with plants through mycorrhizal networks. Their ecological relevance extends to sustaining forest ecosystems and improving soil health.[1] Mushrooms inhabit a broad spectrum of ecological niches, including dense forests, grasslands, decaying wood, and cultivated soils. They display extensive morphological and genetic diversity, which, while offering rich potential for scientific exploration, also presents considerable challenges for accurate classification and identification. These challenges necessitate systematic documentation supported by advanced analytical tools.[2] Beyond their ecological roles, mushrooms are increasingly recognized for their nutritional and therapeutic value. Edible mushrooms serve as an excellent source of proteins, essential vitamins, minerals, and other bioactive compounds. Concurrently, medicinal mushrooms have long-standing applications in traditional medicine for their immunomodulatory, antioxidant, and anticancer properties.[8] Despite their biological and practical importance, mushroom biodiversity—particularly in underexplored regions such as Central India—remains insufficiently studied.[7] This lack of comprehensive documentation impedes both ecological understanding and conservation efforts. Therefore, it is essential to adopt integrative approaches combining ecological surveys with molecular characterization techniques.

MOLECULAR APPROACHES IN MUSHROOM BIODIVERSITY STUDIES:

Molecular biology has revolutionized fungal taxonomy, particularly through DNA barcoding—a precise method for identifying organisms at the species level using short genetic markers. For fungi, the Internal Transcribed Spacer (ITS) region of ribosomal RNA genes is widely accepted as the standard barcode due to its high interspecies variability and robust amplification success.[23] The ITS region, located between the 18S and 28S rRNA genes, provides sufficient variability to distinguish even closely related fungal species. This makes it a powerful tool for resolving taxonomic ambiguities and facilitating the discovery of novel or cryptic species.[30] ITS-based DNA barcoding thus supports both species-level identification and broader phylogenetic studies.

III. GEOGRAPHIC MUSHROOM COLLECTION SITES BHOPAL REGION:

The temporal and spatial aspects of mushroom collection are fundamental to understanding the dynamics of fungal biodiversity in a given region. This study involves the systematic collection of mushroom specimens from three distinct locations within the Bhopal region, Madhya Pradesh, India, with sampling conducted on two different dates in September 2024.

3.1: 12TH SEPTEMBER 2024:

Two collection sites were sampled on this date, allowing comparison of fungal diversity across different microhabitats within the same temporal window.[SELF]

- MP Police Line Quarters, Idgah Hills (Latitude: 23.271878°, Longitude: 77.392403°) was sampled at 3:27 PM. This semi-urban site presents a mixed environment of natural vegetation and human activity, creating diverse niches that support a range of fungal species adapted to variable ecological conditions.

FIGURE 1:(A) MP POLICE LINE QUARTERS, IDGAH HILLS, BHOPAL, MADHYA PRADESH

- Sarvdharm Colony, Kolar Road (Latitude: 23.191133°, Longitude: 77.418149°) was sampled later the same day at 5:04 PM. This peri-urban area includes residential and vegetated patches, potentially favoring fungi that thrive in fragmented habitats and altered landscapes.

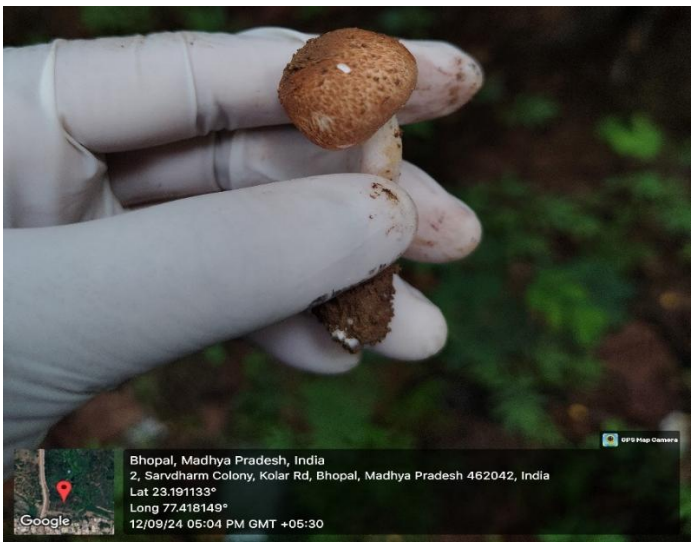

FIGURE 1:(B) SARVDHARM COLONY, KOLAR ROAD, BHOPAL, MADHYA PRADESH

6.2: 20TH SEPTEMBER 2024:

- Ekant Park, Char Imli (Latitude: 23.21494°, Longitude: 77.420792°) was sampled at 4:47 PM. This urban green space offers a relatively undisturbed environment with rich plant diversity, providing an ideal microhabitat for saprophytic and symbiotic fungi involved in organic matter recycling.

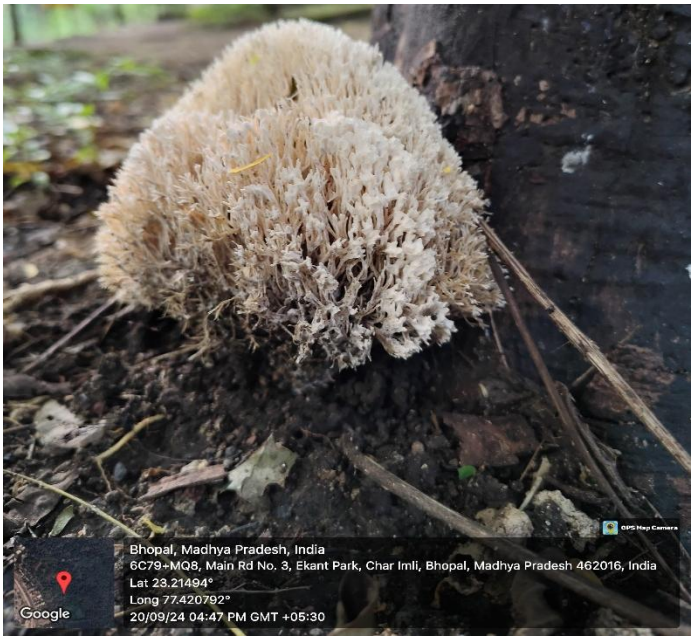

FIGURE 1:(C)EKANT PARK, CHAR IMLI, BHOPAL, MADHYA PRADESH

IV. MATERIALS AND METHODS:

4.1.STUDY AREA AND SAMPLE COLLECTION:

For three consecutive monsoon seasons, from June to November, wild mushroom specimens were collected from forested and semi-urban ecological niches in and around Bhopal, Madhya Pradesh. The collection period spanned from mid-June (onset of the monsoon) to late October or early November, coinciding with peak mushroom emergence on decaying plant material such as teak (Tectona grandis) and tade (Cassia tora). Samples were gently brushed to remove debris, sealed in sterile zip-lock bags, and transported under cold conditions to the laboratory.[4]

FIGURE 2: SAMPLE COLLECTION

4.2.CRYOPRESERVATION AND SAMPLE PROCESSING:

Upon arrival, fresh mushroom tissues were immediately immersed in liquid nitrogen to arrest cellular degradation. Samples were then pulverized in the frozen state to form a fine powder. This powder was stored at –20°C in sterile zip-lock bags until further processing.[5]

4.3.DNA EXTRACTION PROTOCOL:

DNA extraction is a fundamental procedure in molecular biology used to isolate genomic material from cells. The process relies on chemical reagents that break open cells, inactivate nucleases, and separate DNA from proteins and other contaminants. Tris buffer (100 mM) is used to maintain a stable pH during the extraction process, protecting DNA from degradation. EDTA (10 mM) acts as a chelating agent by binding divalent metal ions such as Mg^{2+} and Ca^{2+}, which are essential for nuclease activity, thereby inhibiting enzymes that could

degrade DNA.[23] SDS (2%), an anionic detergent, disrupts the lipid bilayer of cell membranes and denatures proteins, facilitating cell lysis and release of nucleic acids. Chloroform:Isoamyl alcohol (24:1) is added to promote phase separation. Chloroform helps in removing proteins and lipids, while isoamyl alcohol minimizes foaming and stabilizes the interphase during centrifugation. 100% ethanol is used to precipitate DNA. In the presence of salts, DNA becomes insoluble in ethanol and forms a visible pellet upon centrifugation. This purified DNA can then be further used for downstream molecular applications such as PCR, sequencing, or cloning.[9][23] The process of DNA extraction involves several steps that require specific reagents, each serving a vital role in isolating high-quality genomic DNA. The following reagents were used in the protocol.

TABLE 1: REAGENTS USED FOR DNA EXTRACTION WITH THEIR CONCENTRATIONS AND FUNCTIONS:

S.NO	REAGENT	CONCENTRATION / RATIO	PURPOSE / FUNCTION
1	Tris	100 mM	Buffering agent to maintain pH during DNA extraction
2	EDTA	10 mM	Chelates divalent ions (e.g., Mg^{2+}) to inhibit nucleases
	SDS	2%	Detergent used to lyse cells and denature proteins
3	Chloroform: Isoamyl alcohol	24:1 (v/v)	Separates DNA from proteins and other cellular debris
4	Ethanol	100%	Precipitates DNA from the aqueous solution

V. PROCEDURE:

5.1.DNA EXTRACTION PROCESS FROM MUSHROOM SAMPLES:

5.1.1.DRIED MUSHROOM SAMPLE (0.04 G):

A small amount of finely powdered mushroom tissue provides a sufficient source of fungal genomic DNA for extraction. Drying reduces enzymatic activity and microbial contamination, preserving nucleic acids.[6]

FIGURE 3: DRIED MUSHROOM SAMPLE

5.1.2.FRESHLY PREPARED TRIS-EDTA-SDS LYSIS BUFFER:

This buffer facilitates cell lysis. Tris maintains pH stability, EDTA chelates divalent cations to inhibit nucleases, and SDS disrupts cell membranes and denatures proteins.

FIGURE 4: LYSIS BUFFER

5.1.3.MIXING SAMPLE AND LYSIS BUFFER (VORTEX MIXING):

Proper homogenization ensures that the lysis buffer comes into full contact with the sample, improving cell disruption and DNA release.

FIGURE 5: SAMPLE AND LYSIS BUFFER (VORTEX MIXING)

5.1.4.INCUBATION AT 65°C FOR 30 MINUTES:

Heat treatment enhances the efficiency of cell lysis and helps denature proteins and other macromolecules that may interfere with DNA extraction.[10][27]

5.1.5.CENTRIFUGATION AT 13,000 RPM FOR 5 MINUTES AT 4°C:

High-speed centrifugation separates cellular debris from the soluble components, leaving DNA in the supernatant. Low temperature helps preserve DNA stability.[19]

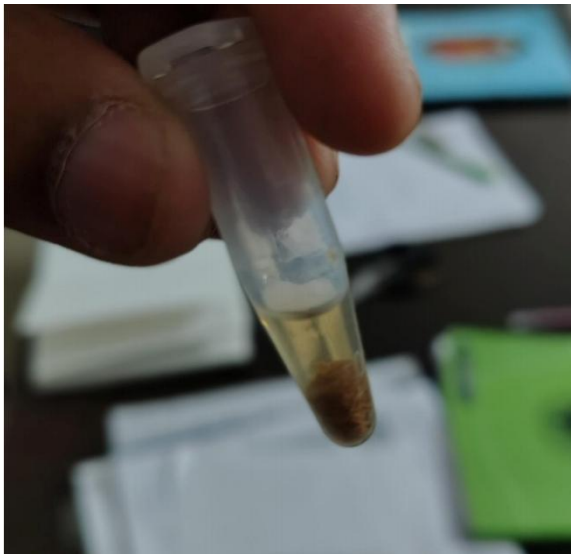

FIGURE 6: AFTER CENTRIFUGATION

5.1.6.COLLECTION OF SUPERNATANT:
The clear supernatant contains the released genomic DNA and is carefully transferred to avoid contamination from the pellet.

5.1.7.CHLOROFORM:ISOAMYL ALCOHOL (24:1) TREATMENT:
This organic solvent mix removes proteins and lipids. Chloroform denatures proteins, while isoamyl alcohol reduces foaming during mixing.[16]

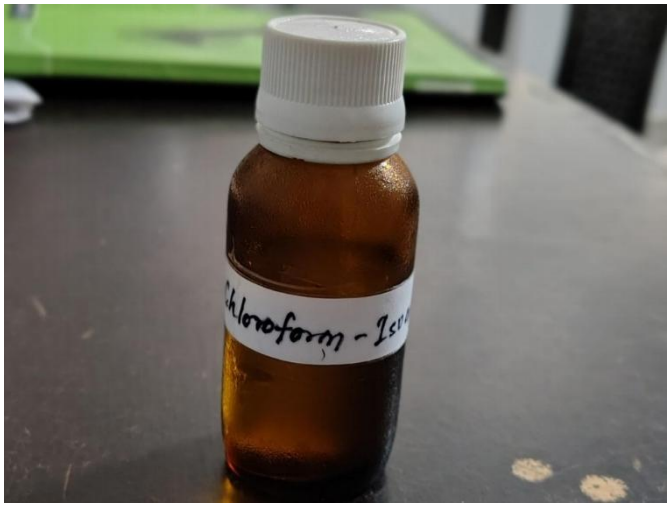

FIGURE 7: LIPID CHLOROFORM

5.1.8.SECOND CENTRIFUGATION AND PHASE SEPARATION:
Centrifugation leads to clear separation between the aqueous phase (DNA) and the organic phase (debris and proteins). Only the upper aqueous layer is retained.

FIGURE 8: AQUEOUS PHASE (DNA) AND THE ORGANIC PHASE (DEBRIS AND PROTEINS)

5.1.9.REPEAT EXTRACTION WITH CHLOROFORM:ISOAMYL ALCOHOL:
Repeating this step ensures the removal of any residual contaminants, enhancing DNA purity.

5.1.10.DNA PRECIPITATION WITH CHILLED 100% ETHANOL:
Cold ethanol causes the DNA to precipitate by reducing its solubility in the aqueous environment. This step is crucial for visualization and recovery of DNA.

FIGURE 9: DNA PRECIPITATION WITH CHILLED 100% ETHANOL

5.1.11.CENTRIFUGATION AT 15,000 RPM FOR 30 MINUTES AT 4°C:
Extended centrifugation helps form a compact DNA pellet at the bottom of the tube. The cold temperature further protects DNA from degradation.

5.1.12.AIR-DRYING AND RESUSPENSION OF DNA PELLET:
Removing residual ethanol is essential to avoid PCR inhibition. The pellet is resuspended in 40 µL of sterile, pre-warmed nuclease-free water to dissolve the DNA for further use.

5.1.13.AGAROSE GEL ELECTROPHORESIS WITH 100 KB LADDER:
The extracted DNA is visualized using gel electrophoresis. Comparing the sample bands against a 100 kb molecular ladder helps confirm the presence and approximate size of the DNA fragments, validating extraction quality.[23]

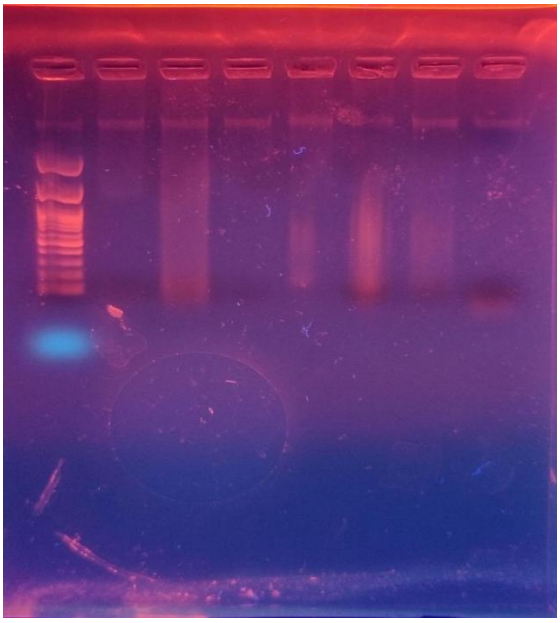

FIGURE 10: AGAROSE GEL ELECTROPHORESIS(100 KB LADDER)

5.2.DNA QUALITY ASSESSMENT:

The integrity of the extracted DNA was confirmed by electrophoresis on a 1% agarose gel stained with ethidium bromide. Sharp and distinct bands indicated successful extraction and the absence of shearing or contamination.

5.3.PCR AMPLIFICATION OF ITS REGION:

Polymerase Chain Reaction (PCR) is a powerful molecular biology technique used to amplify specific DNA sequences. In this study, the internal transcribed spacer (ITS) region of fungal ribosomal RNA genes was targeted for amplification due to its high variability and reliability in fungal species identification.[23] The primers used for amplification were **ITS1** and **ITS4**, which flank the ITS region. The ITS1 primer (5'-TCCGTAGGTGAACCTGCGG-3') is 19 base pairs long and serves as the forward primer, while ITS4 (5'-TCCTCCGCTTATTGATATGC-3') is 20 base pairs in length and functions as the reverse primer. These primers are widely used in fungal barcoding and enable accurate amplification of the target region across a broad range of species.[25]

TABLE 2: PCR WAS CONDUCTED TO AMPLIFY THE ITS REGION ENCOMPASSING ITS1, 5.8S RDNA, AND ITS2 USING THE FOLLOWING UNIVERSAL PRIMERS:

S.NO	PRIMER NAME	SEQUENCE (5' → 3')	LENGTH (BP)
1	ITS1	5'-TCCGTAGGTGAACCTGCGG-3'	19 bp
2	ITS4	5'-TCCTCCGCTTATTGATATGC-3'	20 bp

A standard 25 µL PCR reaction was prepared, consisting of 1 µL of template DNA (approximately 100–200 ng), 1.25 µL each of ITS1 and ITS4 primers (10 µM concentration), and 12.5 µL of OneTag® 2X Master Mix, which contains the necessary components for amplification, including DNA polymerase, dNTPs, and buffer system. The volume was adjusted to 25 µL using nuclease-free water to ensure optimal reaction conditions.[29] This PCR setup provides a consistent and efficient method for amplifying the ITS region, enabling downstream applications such as sequencing, species identification, and phylogenetic analysis.

TABLE 3: PCR REACTION MIXTURE (TOTAL VOLUME: 25 µL):

S.NO	Component	Volume	Final Concentration / Notes
1	Template DNA	1 µL	~100–200 ng
2	ITS1 Primer (10 µM)	1.25 µL	Forward primer
3	ITS4 Primer (10 µM)	1.25 µL	Reverse primer
4	OneTag® 2X Master Mix	12.5 µL	Contains DNA polymerase, dNTPs, buffer
5	Nuclease-free water	Up to 25 µL	Adjusts final volume

Amplicons were visualized by electrophoresis on a 1% agarose gel. The expected product size (~500–700 bp) confirmed successful amplification of the ITS region.

VI. RESULTS AND DISCUSSION:

The described protocol successfully yielded high-quality DNA from field-collected mushroom samples. The liquid nitrogen freezing method, followed by buffer-based lysis and chloroform extraction, minimized degradation and contamination. Repeated organic extractions ensured the removal of PCR inhibitors typical of fungal tissues. Amplification of the ITS region provided sharp and specific bands, indicating intact fungal DNA suitable for sequencing and downstream phylogenetic studies.

VII. CONCLUSION:

This study successfully establishes a reliable, reproducible, and efficient protocol for the molecular identification of wild mushrooms using Internal Transcribed Spacer (ITS) region analysis. The methodology presented here demonstrates the effective use of minimal amounts of dried mushroom tissue for DNA extraction, amplification, and sequencing—an approach that is especially valuable in regions where fresh samples are limited or access to advanced laboratory resources is constrained. The ITS region, being highly variable among fungal species, proved to be a powerful marker for accurate species-level identification. The research highlights the significance of documenting fungal biodiversity in ecologically rich yet underexplored regions like Bhopal, Madhya Pradesh. By focusing on molecular tools rather than solely on morphological characteristics, this study overcomes the limitations posed by convergent phenotypic traits, seasonal availability, and the expertise required for classical taxonomic identification. The resulting genetic data not only contributes to a deeper understanding of local mycobiota but also supports broader mycological research efforts, including phylogenetic studies, species discovery, and biogeographical mapping. The successful implementation of this protocol sets the foundation for future fungal barcoding initiatives and biodiversity monitoring programs in India and other biodiversity-rich developing regions. It aligns with global efforts to build comprehensive fungal DNA databases such as UNITE and GenBank, ultimately facilitating the integration of molecular taxonomy into mainstream fungal research. This study emphasizes the role of molecular identification in conservation planning and sustainable utilization of fungal resources. Understanding the diversity and distribution of mushrooms at the molecular level aids in recognizing endemic, rare, or endangered species, and can inform policies aimed at habitat protection and sustainable harvesting.

VIII. ABBREVIATIONS TABLE:

TABLE 4: LIST OF ABBREVIATIONS USED IN THE STUDY:

S. NO.	ABBREVIATION	FULL FORM	S. NO.	ABBREVIATION	FULL FORM
1	**ITS**	Internal Transcribed Spacer	**11**	**HOD**	Head of Department
2	**PCR**	Polymerase Chain Reaction	**12**	**°C**	Degree Celsius
3	**rRNA**	Ribosomal Ribonucleic Acid	**13**	**v/v**	Volume by Volume
4	**SDS**	Sodium Dodecyl Sulfate	**14**	**µL**	Microlitre
5	**EDTA**	Ethylenediaminetetraacetic Acid	**15**	**mg**	Milligram
6	**Tris**	Tris(hydroxymethyl)aminomethane	**16**	**M.P.**	Madhya Pradesh
7	**DNA**	Deoxyribonucleic Acid	**17**	**UV**	Ultraviolet
8	**kb**	Kilobase	**18**	**DNA Ladder**	A set of known DNA fragments for size comparison in gel electrophoresis
9	**bp**	Base Pair	**19**	**RPM**	Revolutions Per Minute
10	**dNTPs**	Deoxynucleotide Triphosphates	**20**	**Ph.D.**	Doctor of Philosophy

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