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MOLECULAR IDENTIFICATION AND PHYLOGENETIC ANALYSIS OF WILD MUSHROOMS COLLECTED FROM OKITIPUPA METROPOLIS, SOUTHWEST NIGERIA USING ITS RDNA SEQUENCING AND BLAST ANALYSIS

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Abstract

Morphological identification of fungal species is often laborious, error-prone, and insufficient for accurate classification. This study utilized molecular techniques, specifically internal transcribed spacer (ITS 1 and 4) regions and polymerase chain reaction (PCR), to identify three wild mushroom species collected from Okitipupa Metropolis, Ondo State, Nigeria. BLAST analysis of the amplified sequences against the NCBI database confirmed the species as *Trametes polyzona*, *Pleurotus ostreatus*, and *Pleurotus pulmonarius*. Phylogenetic analysis revealed low genetic relatedness between the isolates and reference strains, suggesting regional diversity or possible genetic variation within species. The results obtained from this study affirms the reliability of ITS-based molecular identification and emphasizes the importance of incorporating molecular tools in fungal taxonomy. The results also lay the groundwork for further exploration of the ecological and commercial potential of wild mushroom species in Nigeria.

Keywords: Molecular identification, ITS region, wild mushrooms, phylogenetic analysis, *Pleurotus* species.

Introduction

Mushrooms are macrofungi that play essential ecological roles in nutrient cycling, symbiotic associations, and organic matter decomposition (Ogwu *et al.*, 2025). They are also valuable for their nutritional, medicinal, and biotechnological potential. The accurate identification and classification of mushrooms are critical for understanding fungal biodiversity, assessing their ecological functions, and ensuring safe consumption and utilization, especially in regions where wild mushroom harvesting is common (Martínez-Rodrigo *et al.*, 2025). Traditional identification methods based on morphological and microscopic characteristics often fall short due to phenotypic plasticity and environmental influences (Cox, 2014). Moreover, many fungal species exhibit convergent morphologies, making accurate taxonomic resolution difficult. These limitations necessitate the application of molecular techniques, which offer precise, reproducible, and reliable tools for fungal taxonomy (Stengel *et al.*, 2025).

The Internal Transcribed Spacer (ITS) region of ribosomal DNA (rDNA) has emerged as the universal barcode for fungi due to its high interspecific variability and ease of amplification. The ITS region, comprising ITS1, 5.8S rRNA, and ITS2, provides sufficient genetic resolution for species-level identification across diverse fungal taxa. ITS-based sequencing, when coupled with database comparison tools such as the Basic Local Alignment Search Tool (BLAST), enables rapid and accurate identification of both cultivated and wild mushrooms (Antil *et al.*, 2023).

In recent years, the integration of molecular data with phylogenetic analysis has transformed fungal systematics by revealing evolutionary relationships and uncovering cryptic species (Ekanayaka *et al.*, 2025). Species evolution: cryptic species and phenotypic noise with a particular focus on fungal systematics. Frontiers in Cellular and Infection Microbiology, 15, 1497085. Such approaches are especially important in under-explored regions like southwestern Nigeria, where mushroom biodiversity remains poorly documented at the molecular level.

This study aims to molecularly identify and analyze the phylogenetic relationships of wild- collected mushroom species from Okitipupa, Ondo State, Nigeria, using ITS rDNA sequencing and BLAST analysis. By combining classical and molecular methods, this work contributes to the growing body of knowledge on indigenous fungal diversity and supports the safe and sustainable use of wild mushrooms

Materials and Methods

Sample Collection and Preliminary Identification

Wild mushroom strains used in this study were collected from various locations across Okitipupa Metropolis, Ondo State, Nigeria. The Global Positioning System (GPS) was used to determine and record the precise coordinates of each collection site (Krenn *et al.*, 2011). Samples were collected aseptically in sterile containers and promptly transported to the Research Laboratory at the Olusegun Agagu University of Science and Technology (OAUSTECH), Okitipupa, where they were cultured on the same day of collection. Initial identification of the mushroom samples was carried out based on morphological features and spore print characteristics. Selected specimens were air-dried under natural light at ambient room temperature (30 ± 2°C) for further analysis.

Preparation of Sterile Work Environment

The laminar flow hood was disinfected thoroughly using 70% ethanol prior to use. All tools, including scalpels and forceps, were flame-sterilized using a Bunsen burner. Additional materials such as Petri dishes and media components were sterilized prior to use to maintain aseptic conditions throughout the procedure.

Preparation of Growth Media

Potato Dextrose Agar (PDA) was prepared following the manufacturer's instructions, 39 g of agar powder was dissolved in 1 liter of distilled water, the media were mixed thoroughly and transferred into heat-resistant flasks or bottles, leaving adequate space for expansion. The media were sterilized in an autoclave at 121°C for 15 minutes and allowed to cool to 45–50°C.

Mycelial Culture and Isolation (Tissue Culture Method)

Healthy and mature fruiting bodies of mushrooms were selected for tissue culturing. Under sterile conditions in a laminar flow hood, the mushrooms were bisected using a sterile scalpel. Tissue samples were aseptically excised from the inner sterile parts, preferably from the stipe or cap, while avoiding the gills to reduce contamination risk.

Each tissue piece was transferred using sterile forceps to the center of a prepared Petri dish containing solidified growth medium. The tissue was gently pressed into the agar to ensure good contact. Plates were labeled with the date and species designation, and then incubated in the dark or under low-light conditions at 25–28°C. After several days, the development of mycelium was observed. A clean, uncontaminated section of mycelium was subcultured onto fresh medium in a new sterile Petri dish. This process was repeated until a pure culture was obtained. The resulting pure cultures were maintained through regular subculturing and preserved in sterile distilled water for long-term storage.

Molecular Methods for DNA Extraction

Genomic DNA was extracted from mushroom fruiting bodies using the Transgen DNA Extraction Kit (Tiangen Biotech Co. Ltd., Beijing, China) according to the manufacturer's instructions, with slight modifications. Approximately 10 mg of finely powdered mushroom tissue was transferred into sterile Eppendorf tubes. A volume of 500 µL of extraction buffer—comprising 100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.7 M NaCl, 1% (w/v) cetyltrimethylammonium bromide (CTAB), and 1% 2-mercaptoethanol—was added to each sample, followed by sonication for 15 minutes.

Samples were then subjected to thermal lysis by heating at 100°C for 5 minutes, immediately followed by incubation on ice for 4 minutes. This cycle of heating and cooling was repeated five times to maximize DNA yield. Purification of the extracted DNA was performed using the Transgen DNA Purification Kit (Tiangen Biotech Co. Ltd., Beijing, China) following the manufacturer's protocol (Abdel-Latif and Osman, 2017).

PCR Amplification of the ITS Region

The Internal Transcribed Spacer (ITS) region of fungal rDNA was amplified using the universal primers ITS1 (5′-TCC GTA GGT GAA CCT GCGG-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′). The polymerase chain reaction (PCR) was performed in a 25 µL total volume, which included 15.5 µL of nuclease-free distilled water, 2.5 µL of 10× TransTaq™ Hifi Buffer I, 2.0 µL of TransTaq™ dNTPs at a concentration of 2.5 mM, 1.0 µL each of ITS1 and ITS4 primers at 0.2 µM concentration, 0.25 µL of TransTaq™ Hifi DNA Polymerase, and 3.0 µL of genomic DNA template.

PCR amplification was performed under the following thermal cycling conditions: initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 1 minute. A final extension was carried out at 72°C for 10 minutes. PCR products were purified using the TIANamp DNA Purification Kit (Tiangen Biotech Co. Ltd., Beijing, China) per the manufacturer's instructions (Abdel-Latif and Osman, 2017).

Sequencing and Analysis of the ITS Region

The purified ITS PCR products were cloned using the pGEM®-T Easy Vector Ligation Kit (Tiangen Biotech Co. Ltd., Beijing, China) and sequenced using the same ITS1 and ITS4 primers. Sequencing was conducted on an ABI 3100 Genetic Analyzer by Tsingke Biotechnology Co. Ltd., Chengdu, China. Raw sequence data were assembled and edited using BioEdit version 7.2.5, and aligned with ClustalW. The edited sequences were subjected to nucleotide similarity searches using the BLAST algorithm on the NCBI database. For phylogenetic analysis, sequence alignments were performed, and a tree was constructed using the Neighbor-Joining method in MEGA version 7.0.21 (Kumar *et al.*, 2015). Evolutionary distances were calculated using the Maximum Composite Likelihood method, and the final

tree was optimized to a total branch length of 4.0691.

Construction of Phylogenetic Tree

The DNA sequences of interest (in FASTA format) were queried against the NCBI BLAST database to retrieve homologous sequences. These sequences were downloaded and combined with the query sequences into a single FASTA file. The merged file was imported into MEGA X software for alignment using either the ClustalW or MUSCLE alignment tool. The aligned sequences were saved and used to construct a phylogenetic tree in MEGA X using the Neighbor-Joining algorithm. The Maximum Composite Likelihood model was employed to estimate evolutionary distances, and bootstrapping with 1000 replicates was performed to assess the robustness of the branching patterns (Suzuki et al., 2015).

Results

All three mushroom samples were successfully identified to the species level using sequences from the GenBank database. Molecular identification based on the ITS region of the rDNA corroborated the morphological identification, confirming the identities of *Pleurotus pulmonarius*, *Termitomyces polyzona*, and *Pleurotus ostreatus* (Table 4.6). BLAST (Basic Local Alignment Search Tool) analysis (Table 7) revealed a high degree of similarity between the sample sequences and those in the NCBI database. *T. polyzona* and *P. ostreatus* both showed 100.0% identity, indicating perfect sequence matches. *P. pulmonarius* exhibited 99.9% similarity. These high percentage identities provide strong confidence in the molecular identification. GenBank accession numbers associated with each sequence support data transparency and enable further validation and reproducibility.

Phylogenetic analysis (Figures 3, 4, and 5) provided insights into the evolutionary relationships among *Pleurotus* species. The tree topology showed well-defined clades, with *P. pulmonarius* and *P. ostreatus* forming separate, strongly supported clusters. Bootstrap values as high as 100 confirmed the reliability of these groupings. The *P. pulmonarius* clade included multiple accessions (GU722282.1, MK346233.1), exhibiting minimal intraspecific variation. Minor sub-clades, supported by bootstrap values of 67 and 78, indicated slight genetic diversity within the species. Similarly, *P. ostreatus* formed a cohesive cluster, including related accessions such as JN126344.1, with 100% bootstrap support. A closely related clade included *P. ostreatus* and *P. cornucopiae* (KY962504.1, FJ379271.1), consistent with their known genetic and phenotypic similarities. These findings reinforce current taxonomic classifications and demonstrate the robustness of ITS-based phylogenetic inference in resolving species-level distinctions within the genus *Pleurotus*.

Table 1: ITS Primer Sequences of mushrooms obtained from the wild

Name of Primer	Target	Sequence (5' to 3')
ITS-1	ITS rDNA sequence	TCCGTAGGTGAACCTGCGG
ITS-4	ITS rDNA sequence	TCCTCCGCTTATTGATATGC

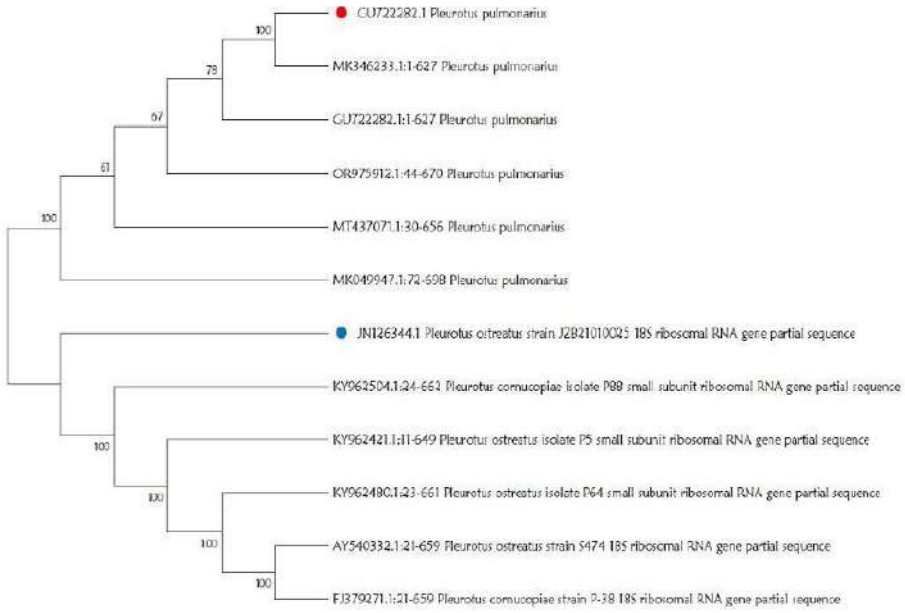

Figure 5: Comparing Phylogenetic trees of *Pleurotus ostreatus* and *Pleurotus pulmonari*

Discussion

The primer sequences used in this study targeted the Internal Transcribed Spacer (ITS) region of ribosomal DNA (rDNA), a genetic marker widely recognized for its high variability and ease of amplification. This makes the ITS region ideal for fungal species identification and phylogenetic analysis (Xing et al., 2024). The primers ITS-1 and ITS-4 were employed for the amplification of the entire ITS region. Specifically, ITS-1 targets the start of the ITS1 region, while ITS-4 targets the end of the ITS2 region, enabling comprehensive coverage of the ITS sequence (Imran and Nafees, 2024). These primers are well-established tools in fungal taxonomy and ecology, frequently used in the identification of diverse wild-collected mushroom species, including edible, medicinal, and toxic varieties (Xing *et al.*, 2024). Sequenced ITS regions were compared to reference databases such as UNITE to ensure accurate species identification. This molecular approach has also been applied in the authentication of commercial mushroom products (Imran and Nafees, 2024).

BLAST (Basic Local Alignment Search Tool) analysis was utilized to compare the obtained sequences against the NCBI database (Bradshaw *et al.*, 2024). This bioinformatics tool identifies the closest matching sequences based on percentage identity (% ID), predicted organism, and associated GenBank accession numbers (Eldem and Balc, 2024). The sample labeled "TP" showed 100% identity to *Trametes polyzona* (OL685335.1), a wood-decay fungus with ecological and medicinal significance (Kijpornyongpan *et al.*, 2022). The sample "PO" matched 100% with *Pleurotus ostreatus* (JN126344.1), a widely cultivated oyster mushroom (Lesa *et al.*, 2022), while sample "PP" displayed 99.9% identity to *Pleurotus pulmonarius* (GU722282.1), another valuable oyster species (Guleria *et al.*, 2024).

The high sequence identity (≥99.9%) provides strong confidence in species-level

Table 1: The BLAST result to the Similarities between the sequence queried and the biological sequence with NCBI database

Name of sample	Percentage ID (%)	Predicted organism	Gen Bank Accession
TP	100.0	*Trametes polyzona*	OL685335.1
PO	100.0	*Pleurotus ostreatus*	JN126344.1
PP	99.9	*Pleurotus pulmonarius*	GU722282.1

TP= *Trametes polyzona*, PO= *Pleurotus ostreatus*, PP=*Pleurotus plumonari*

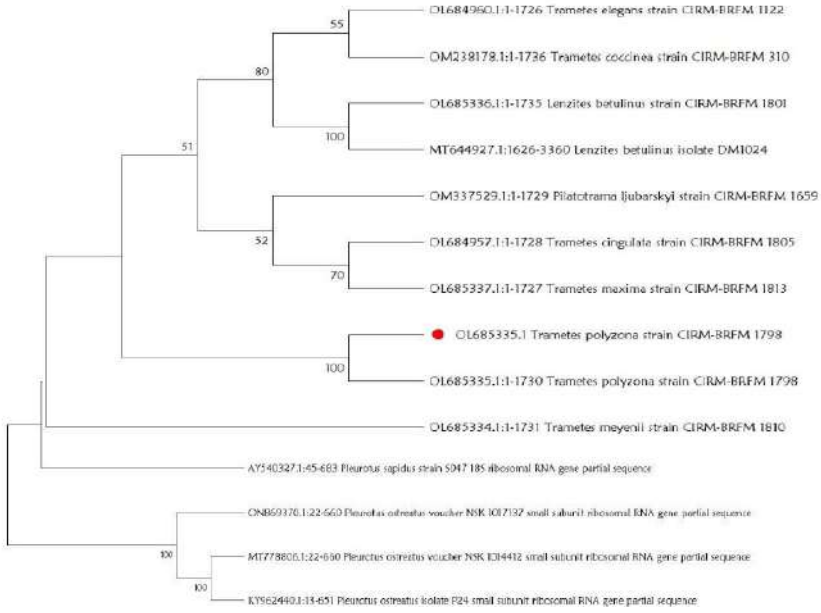

Figure 3: Phylogenetic tree of *Pleurotus ostreatus*

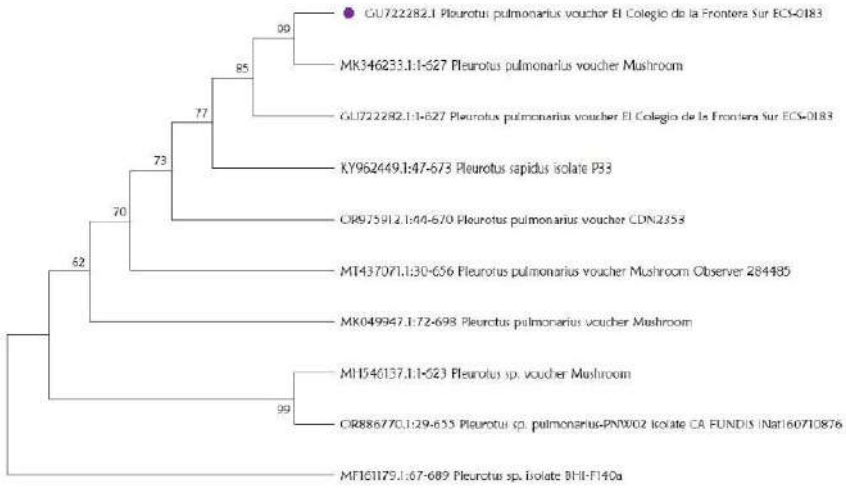

Figure 4: Phylogenetic tree of *Pleurotus pulmonarius*

identification. The combination of ITS sequencing and BLAST analysis offers a reliable and accepted method for identifying wild mushroom species with high accuracy (*Abdi et al.*, 2024). Phylogenetic analysis based on ITS sequences revealed evolutionary relationships among the identified mushroom species. Two entries for *Trametes polyzona*—OL685335.1 and OL685335.1:1-1730— represent full and partial sequences, respectively, and clustered closely with other *Trametes* 16 species, including *T. elegans*, *T. coccinea*, *T. cingulata*, and *T. maxim*a (Olou *et al.*, 2020). The inclusion of multiple strains supports the evaluation of intraspecific genetic variation (Schwartz *et al.*, 2000).

Similarly, several *Pleurotus ostreatus* sequences—JN126344.1, KY962421.1, KY962480.1, and AY540332.1—clustered together with 100% identity, confirming precise identification. The presence of other species such as *P. cornucopiae* and *P. sapidus* within the same phylogenetic tree, albeit with lower identity values, reflects distinct evolutionary lineages within the genus (Gonzalez and Labarère, 2000). Sequences of *P. pulmonarius* (GU722282.1, MK346233.1, OR975912.1) also demonstrated high similarity (85–99%), highlighting intraspecific diversity and supporting the resolution of closely related taxa (Avin *et al.*, 2016).

Conclusion

This study establishes the effectiveness of ITS rDNA sequencing, in combination with BLAST analysis and phylogenetic tools, as a reliable molecular approach for the identification and classification of wild mushroom species. The successful amplification of the ITS region using ITS1 and ITS4 primers enabled accurate species determination and highlighted the utility of DNA barcoding in distinguishing between closely related fungi. Phylogenetic analysis further clarified the evolutionary relationships within the Trametes and Pleurotus genera, revealing intraspecific variation and taxonomic structure, and indicating a low genetic relatedness among local strains. This suggests notable regional diversity and underscores the potential for discovering novel genetic traits in indigenous mushroom populations.

Notably, this research provides the first molecular documentation of *Trametes polyzona*, *Pleurotus ostreatus*, and *Pleurotus pulmonarius* from Okitipupa, Nigeria. The findings contribute valuable data to the regional understanding of fungal biodiversity and phylogeny, reinforcing the importance of molecular identification in ecological studies, species conservation, and the safe utilization of mushrooms for food and medicine. Moreover, the integration of ITS-based sequencing with BLAST database comparison and phylogenetic analysis demonstrates a powerful strategy for resolving taxonomic ambiguities and verifying species identity, which is critical for ecological monitoring, public health, and biotechnological applications.

To build upon this foundation, future studies should broaden molecular surveys across diverse ecological zones in Nigeria to develop a comprehensive national reference database of indigenous mushroom species. The integration of morphological and molecular approaches is also recommended to enhance the accuracy and efficiency of fungal identification. Establishing local ITS sequence libraries would reduce dependence on global databases and enable rapid, region-specific authentication of species. Furthermore, identified mushrooms should be investigated for their pharmacological, nutritional, and biotechnological potential, especially in the context of sustainable agriculture and environmental remediation.

Insights gained from molecular characterization of fungal diversity should also inform conservation strategies aimed at protecting native species from threats such as habitat loss and climate change.

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