Identification and Molecular Analysis of Novel Mushroom Varieties of MDI Using ITS Sequencing and Phylogenetic Tools

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Abstract

This study aimed to molecularly characterize and identify newly introduced mushroom varieties preserved at the Mushroom Development Institute (MDI), Bangladesh. Five varieties—*Pleurotus ostreatus* (BPO), *Pholiota adiposa* (PA), *Flammulina filiformis* (Fv-C), *Psilocybe cubensis* (PC), and *Hypsizygus marmoreus* (HT-C)—were selected for genetic analysis. Genomic DNA was extracted and amplified using ITS4 and ITS5 primers targeting the internal transcribed spacer (ITS) region of nuclear rDNA. PCR products ranged from 602 to 672 bp and were sequenced using the Sanger method. BLASTn analysis confirmed species identity, with sequence similarities ranging from 93.99% to 99.85% compared to GenBank references. Evolutionary divergence among the isolates was estimated using the Tamura-Nei model, revealing the lowest divergence (0.024) between PA and HT-C, and the highest (0.941) between PA and PC. A phylogenetic tree constructed via the Maximum Likelihood method clearly grouped isolates into distinct clades with high bootstrap support, validating molecular identification. This ITS-based profiling confirms the genetic distinctiveness of these mushroom varieties and supports their conservation and future breeding potential at MDI.

Keywords: *Pleurotus ostreatus*, *Pholiota adiposa*, *Psilocybe cubensis*, Molecular identification, Mushroom germplasm.

INTRODUCTION

Mushrooms are globally recognized for their nutritional, medicinal, and ecological importance. Among the thousands of known fungal species, several edible and medicinal mushrooms have gained widespread popularity due to their high protein content, low fat, and presence of bioactive compounds (Chang and Wasser, 2017). In particular, genera such as *Pleurotus, Pholiota, Flammulina, Psilocybe*, and *Hypsizygus* are well-studied for their commercial cultivation, therapeutic potential, and genetic diversity (Valverde *et al.*, 2015; Lindequist *et al.*, 2005).

In Bangladesh, mushroom cultivation has seen significant growth over the past two decades, particularly among small-scale farmers and rural entrepreneurs. This expansion is supported by favorable climatic conditions, increased public awareness, and governmental initiatives aimed at improving nutrition and income generation. The Mushroom Development Institute (MDI), the country's principal research and development center for mushrooms under the Department of Agricultural Extension (DAE), plays a crucial role in strain development, extension services, and the dissemination of cultivation technologies. MDI maintains a collection of both local and exotic mushroom germplasm, with a strong focus on species adaptation, yield improvement, and conservation.

Despite the increased popularity of mushroom cultivation in Bangladesh, systematic molecular characterization of the available strains remains limited. Traditional methods of morphological and phenotypic identification are often insufficient for distinguishing between closely related strains or identifying cryptic diversity. Therefore, molecular profiling techniques offer a more reliable and reproducible approach to assess genetic variation, identify novel or elite germplasm, and inform breeding and conservation strategies.

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The Internal Transcribed Spacer (ITS) region of ribosomal DNA has been widely adopted as a universal DNA barcode for fungi due to its high interspecific variability and ease of amplification (Schoch *et al.*, 2012). Phylogenetic analysis based on ITS sequences enables researchers to resolve evolutionary relationships among mushroom strains and supports accurate taxonomic identification. This study aims to conduct a molecular profiling of selected mushrooms varieties preserved at the Mushroom Development Institute (MDI).

MATERIALS AND METHODS

The experiment was conducted at Mushroom Development Institute, Sobhanbag, Savar, Dhaka, Bangladesh in 2024. In this experiment, newly introduced mushroom varieties BPO (*Pleurotus ostreatus*), PA (*Pholiota adiposa*), Fc-V (*Flammulina filiformis*), PC (*Psilocybe cubensis*), and HT-C (*Hypsizygus marmoreus*) were selected and grown at the Mushroom Development Institute's culture house. DNA isolation, fingerprint, and genetic chromatography were examined in the Invent Technology.

Molecular Identification

DNA extraction and purification: Genomic DNA was extracted from the selected mushrooms fruiting bodies using the Maxwell® Blood DNA Extraction Kit (Model AS1010, Promega Corp., Madison, WI, USA) according to the manufacturer's protocol. The extracted DNA was purified using the Wizard® Genomic DNA Purification Kit (A1120, Promega Corp.). DNA concentration and purity were assessed using a NanoDrop 2000c Spectrophotometer (Thermo Scientific), with a final concentration measured at 40.9 ng/µL.

PCR amplification of ITS region: The Internal Transcribed Spacer (ITS) region of the nuclear ribosomal DNA (rDNA) was amplified using the primer pair ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). PCR reactions were carried out in a 25 µL total volume using the GoTaq® Green Master Mix Kit (M7122, Promega Corp.) at a 1× final concentration. Each reaction contained 5 µM of each primer and 25 ng of template DNA. Amplification was performed in a C1000 Touch Thermal Cycler (Bio-Rad®, Germany) using the following thermal profile (Fig. 1).

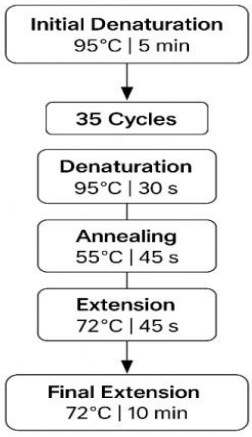

Fig. 1. The flowchart of the PCR protocol performed to amplify the DNA of different mushroom varieties.

Gel electrophoresis and purification: PCR products were separated by electrophoresis on a 2% agarose gel at 80 V for 40 minutes and visualized under UV light using SYBR® Gold stain (Invitrogen, Carlsbad, CA, USA). Amplicons of approximately 700 base pairs were selected and purified using ExoSAP-IT® (N/P 78200, USB Affymetrix, Inc., Cleveland, OH, USA) to remove excess primers and dNTPs.

DNA sequencing: Sequencing was performed using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (P/N 4336917, Applied Biosystems, Foster City, CA, USA) with both ITS5 and ITS4 primers. Sequence reactions were analyzed on an ABI 3130 Genetic Analyzer (Applied Biosystems® HITACHI, Tokyo, Japan). Raw sequences were assembled and edited using SeqMan v8 (DNASTAR®, Madison, WI, USA). The resulting Sanger sequences were processed using Chromas 2.6. software to generate a FASTA file containing the partial sequence. Species identification was performed by comparing the assembled ITS sequences against the GenBank database using BLASTn. The species was determined based on the highest identity match.

Sequence analysis: The FASTA files of all the obtained sequences were analyzed through nucleotide BLAST by submitting the sequences in the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to match with existing sequences in the GenBank and obtained accession numbers. The estimation of evolutionary divergence between sequences was analyzed using the Tamura-Nei model. After that, the evolutionary history was inferred using the Maximum Likelihood method and the Tamura-Nei model (Tamura and Nei, 1993). The bootstrap consensus tree inferred from 1000 replicates was constructed to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with superior log likelihood value. This analysis involved 5 nucleotide sequences. Codon positions included were 1st+ 2nd+ 3rd+ Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Evolutionary analyses were performed in MEGA11 software (Tamura *et. al.,* 2021).

RESULTS AND DISCUSSION

Molecular Identification of Different Mushroom Varieties

A molecular approach was performed and different mushroom varieties were identified at the MDI. A total of five different mushroom varieties were identified which are shown in (Table 1).

Table 1. Identification of newly introduced different mushroom varieties by molecular analysis at Mushroom Development Institute

Sl. No.	Name of the mushroom	Scientific name	Variety name
1.	Shimeji/ Beech mushroom	*Hypsizygus marmoreus*	HT-C
2.	Black pearl oyster mushroom	*Pleurotus ostreatus*	BPO
3.	Magic/ Shroom/ Golden halo mushroom	*Psilocybe cubensis*	PC
4.	Chestnut mushroom	*Pholiota adiposa*	PA
5.	Enoki mushroom	*Flammulina filiformis*	Fv-C

Study on PCR products

The Gel-Doc system visualized PCR products of mushroom isolates generated by ITS4 and ITS5 primers, which resulted in the DNA bands typically ranging between 500-750 base pairs (bp). Variability in amplicon sizes ranging from 602 (isolate Fv-C) to 672 (isolate PC) bp was observed in the GenBank database. The generated DNA bands indicated the successful PCR amplification, which were extremely similar in size to the target band. The size of the amplified DNA fragment was verified using a Bench Top 1 kb DNA ladder, which acts as a size marker. Fig. 2 shows the successful amplification of DNA bands obtained from several mushroom isolates. These confirm the specificity and successful amplification of the desired DNA fragments under the utilized primer and PCR conditions, as demonstrated by the Gel-Doc system.

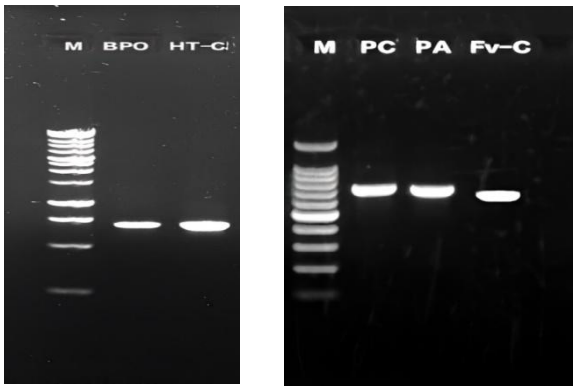

Fig. 2. PCR amplified products of mushroom obtained from the isolates BPO, HT-C, PC, PA, and Fv-C. M: denotes 1kb DNA ladder (Marker).

Analysis of DNA Sequences

The obtained FASTA sequences were analyzed using the BLAST tool on the NCBI website. These sequences were found to match existing nucleotide sequences in the NCBI GenBank database. All the sequences exceeded 94% (93.99-99.85%) homology with their corresponding sequence. Subsequently, unique accession numbers were assigned to the partial sequences for five mushroom isolates (Table 2).

Table 2. Results of the mushroom's gene identification with the BLAST Program

Lab Strain			BLAST Alignment				
Sl. no.	Identified Species	Obtained accession no.	Species	Query coverage	E value	Percent identity	Accession no.
1.	*Pleurotus ostreatus* isolate BPO	PV643226.1	*Pleurotus ostreatus* strain NBRC 33211	99%	0.0	93.99%	AB733144.1
			Pleurotus ostreatus isolate ITCC4006	96%	0.0	94.30%	OP597497.1
2.	*Pholiota adiposa* isolate PA	PV643232.1	*Pholiota adiposa* isolate ET37	100%	0.0	98.64%	MN209721.1
			Pholiota adiposa strain Du 167	99%	0.0	98.35%	OQ927086.1
3.	*Flammulina filiformis* isolate Fv-C	PV643233.1	*Flammulina filiformis* strain JIN2	99%	0.0	97.69%	MH469705.1
			Flammulina filiformis strain F004-Y-A	99%	0.0	97.68%	MT734029.1
4.	*Psilocybe cubensis* isolate PC	PV643234.1	*Psilocybe cubensis* voucher FLAS-F-69275a	100%	0.0	99.70%	OR052554.1
			Psilocybe cubensis strain CBS	99%	0.0	99.85%	HM035075.1
5.	*Hypsizygus marmoreus* isolate HT-C	PV643235.1	*Hypsizygus marmoreus* isolate 3	100%	0.0	98.15%	MN893870.1
			Hypsizygus marmoreus strain LE-BIN 3789	99%	0.0	97.99%	OP980886.1

Estimation of Evolutionary Divergence Between Sequences

Estimates of evolutionary divergence between the ITS sequences of the five mushroom isolates revealed a range of genetic distances, indicating varying degrees of relatedness among the isolates. The lowest divergence (0.024 substitutions/site) was observed between *Pholiota adiposa* (PA) and *Hypsizygus marmoreus* (HT-C), suggesting a close evolutionary relationship. Similarly, *Pleurotus ostreatus* (BPO) and *Flammulina filiformis* (Fv-C) exhibited low divergence (0.034), indicating moderate genetic similarity.

In contrast, the highest divergence (0.941) was found between *Pholiota adiposa* (PA) *and Psilocybe cubensis* (PC), followed by PC and HT-C (0.861), implying substantial evolutionary separation. Notably,

Psilocybe cubensis consistently showed higher divergence values with all other isolates, indicating it is the most genetically distinct among the sampled species (Table 3).

These findings underscore the molecular variability among the examined mushroom strains and support the effectiveness of ITS-based profiling in fungal species discrimination and germplasm differentiation.

Table 3. Pairwise evolutionary distances among the different mushroom isolates based on ITS sequences

	Isolate BPO	Isolate PA	Isolate Fv-C	Isolate PC	Isolate HT-C
Isolate BPO		0.067	0.077	0.034	0.068
Isolate PA	0.756		0.033	0.091	0.024
Isolate Fv-C	0.746	0.336		0.084	0.031
Isolate PC	0.391	0.941	0.818		0.075
Isolate HT-C	0.741	0.234	0.310	0.861	

Here, Values below the diagonal represent base substitutions per site; values above the diagonal represent standard error estimates obtained by 1000 bootstrap replicates under the Tamura-Nei model.

Analysis of Phylogenetic Tree

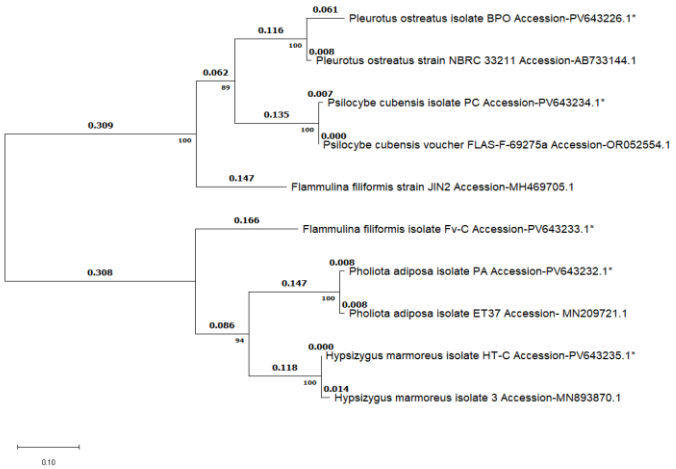

Fig. 3. Phylogenetic tree of the selected mushroom isolates and reference strains, constructed using the Maximum Likelihood method. Bootstrap values from 1,000 replicates are shown. Asterisks (*) indicate newly introduced isolates from the Mushroom Development Institute (MDI). The scale bar represents substitutions per site.

A phylogenetic tree was constructed using ITS region sequences from selected mushroom isolates to evaluate their evolutionary relationships. The analysis included newly characterized strains—marked with an asterisk (*)—alongside reference sequences retrieved from GenBank (Fig. 3). A total of 1,065 aligned nucleotide positions were used in the final dataset. Phylogenetic reconstruction was performed using the Maximum Likelihood method, and the highest log likelihood score was -4413.79, indicating a reliable tree topology.

The phylogenetic tree grouped the mushroom isolates into distinct clades corresponding to their respective genera. The *Pleurotus ostreatus* isolate BPO (PV643226.1*) clustered closely with the reference strain NBRC 33211 (AB733144.1), supported by a strong bootstrap value of 100 and a minimal evolutionary distance of 0.008. Similarly, the *Psilocybe cubensis* isolate PC (PV643234.1*) was found to be nearly identical to its GenBank counterpart (OR052554.1), indicating genetic consistency.

The *Flammulina filiformis* isolate Fv-C (PV643233.1*) formed a distinct clade with its reference strain JIN2 (MH469705.1), while the *Pholiota adiposa* isolate PA (PV643232.1*) clustered tightly with the ET37 strain (MN209721.1), showing minimal divergence (0.008). Notably, the *Hypsizygus marmoreus* isolate

HT-C (PV643235.1*) grouped strongly with isolate 3 (MN893870.1) with a bootstrap value of 100 and zero genetic distance, confirming strain-level similarity.

Overall, the phylogenetic tree clearly distinguished species and validated the molecular identities of the newly introduced isolates at the Mushroom Development Institute. The close alignment with GenBank references supports the accuracy of ITS sequencing for mushroom identification and germplasm conservation.

CONCLUSION

The molecular characterization of five newly introduced mushroom varieties at the Mushroom Development Institute (MDI) confirmed their taxonomic identities and genetic relationships using ITS region sequencing. The study successfully amplified and sequenced the ITS regions, with high sequence similarity to GenBank references, validating species-level identification. Evolutionary divergence and phylogenetic analyses revealed both close and distant genetic relationships among the isolates, highlighting their diversity. These findings underscore the reliability of ITS-based molecular profiling for fungal identification and provide a valuable foundation for germplasm conservation, breeding, and future genetic improvement programs at MDI.

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