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# Molecular Phylogenetic Relationships and Cultural Conditions of *Calocybe indica*

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*Key words*: *Calocybe indica*, ITS sequence, Phylogenetic relationship, RAPD, Vegetative growth

## Abstract

*Calocybe indica* known as a milky white, indigenous and medicinally important edible mushroom. The cultural conditions, molecular identification, and phylogenetic relationships of selected strains of *Calocybe indica* were examined. The optimal temperature for mycelial development was found to be 30°C, whereas the lowest growth occurred at 15°C. The most favorable growth occurs at a pH of 5. Based on the growth characteristics of various culture media, potato dextrose agar proved to be the most advantageous, while Hennerberg and Czapek Dox media were less suitable. Molecular identification and the phylogenetic relationships of the chosen IUM strains of *C. indica* were examined through their ITS and RAPD analyses. The selected strains of *C. indica* had total sequence lengths of $ITS_1$, $ITS_2$, and 5.8S rDNA ranging from 570 to 620 bp. The sizes of the ITS1 and ITS2 regions vary among strains, with ITS2 showing greater variety than ITS1. All strains contained identical 5.8S rDNA sequences, according to sequencing analysis. Eleven out of the twenty arbitrary 10-base oligonucleotide primers utilized to amplify genomic DNA segments for the chosen *C. indica* strains were effective. RAPD-PCR resulted in distinct multiple products, demonstrating significant variability among the strains assessed. The quantity of amplified bands differed based on the primers utilized or the strains evaluated. The DNA polymorphisms displayed consistent traits in the replicated trials. Thus, it can be concluded that ITS sequencing and RAPD methods were effective in identifying the genetic diversity of the chosen strains of *Calocybe indica*.

## Introduction

*Calocybe indica*, commonly referred to as milky white, is a well-known edible mushroom in Bangladesh (Alam et al. 2008). It is highly valued for its appealing, sturdy and white

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fruiting bodies, along with its distinctive taste and flavor. This mushroom is classified under the class Basidiomycetes, within the Tricholomataceae family of the order Agaricales (Amin et al. 2010, Alam et al. 2022).

Milky white mushroom thrives in temperatures between 25-35°C and requires relative humidity levels of 60-70%, achieving a good biological efficiency of 60-70% under optimal conditions (Alam et al. 2019). Therefore, the environmental conditions in Bangladesh are conducive to the cultivation of milky white mushrooms. Only secondary mycelia are capable of producing fruiting bodies when the proper conditions are met. In the basidia of developed fruiting bodies, the two nuclei merge into one, subsequently undergoing meiosis to generate four haploid nuclei. These four haploid nuclei are then formed into four new basidiospores (Upadhyay et al. 2008, Yoon et al. 2012). The milky white mushroom exhibits morphological diversity in its basidiospores, leading to taxonomic confusion and challenges in defining species boundaries.

Molecular biology has emerged as a crucial tool for characterizing the milky white mushroom. This technique has become increasingly accessible owing to the use and automation of the polymerase chain reaction (PCR). However, molecular methods may be more suitable for confirming the taxonomic classification of mushrooms (Alam et al. 2009). Several molecular genetics methods have been developed for identifying mushrooms, including restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), as well as analyses of small subunit ribosomal DNA (SSU rDNA) and internal transcribed spacer (ITS) sequences. Recent studies in molecular phylogenetics have shown that analyzing the ITS region of genomic DNA is highly effective for examining phylogenetic relationships at more specific taxonomic levels. Comparing ITS sequences is becoming a more popular for both phylogenetic analysis and population differentiation. The ITS region of rDNA is viewed as a variable area among different species and various strains (Park et al. 2004, Ahmmed et al. 2022). Genetic studies on mushroom species have revealed that RAPD techniques outperformed rDNA sequencing methods in differentiating strains within species. RAPD proved especially effective in confirming mushroom strains from diverse hosts across various geographical locations (Lopandic et al. 2005). The milky white mushroom stands out as a promising species for Bangladesh. Nonetheless, the molecular identification and phylogenetic relationships among different strains of this mushroom have yet to be explored. Consequently, this research aims to identify various indigenous strains of *C. indica* through ITS and RAPD analysis and to assess the ideal culture conditions for the vegetative growth of the milky white mushroom.

## Materials and Methods

Nine distinct strains (IUM-4374, IUM-4375, IUM-4376, IUM-4377, IUM-4378, IUM-4379, IUM-4380, IUM-4381, and IUM-4382) of *C. indica* were gathered from various ecological regions across Bangladesh. Incheon University Mushroom (IUM) was assigned an accession number,

and pure cultures were kept in the Culture Collection and DNA Bank of Mushrooms (CCDBM). For phylogenetic comparisons with specific IUM strains, GQ-259881 was used as a control strain. The control strains' sequencing information obtained from the GenBank database maintained by the National Center for Biotechnology Information (NCBI).

To determine the optimal temperature for vegetative growth, nine different strains of *C. indica* were incubated at temperatures of 15, 20, 25, 30 and 35°C. A 5 mm diameter agar plug was taken from cultures that were 10 days old and grown on PDA then placed at the center of each PDA plate. The medium was adjusted to a pH of 5 and incubated for 10 days at the specified temperatures. For the pH testing, the medium was adjusted to pH levels of 4, 5, 6, 7 and 8 using 1 N NaOH or HCl prior to autoclaving and was then incubated for 10 days at 25°C. The mycelial growth measurements were carried out using the methods outlined by Sikder et al. (2019).

A total of nine distinct culture media, including czapek dox, glucose peptone, glucose tryptone, Hamada, Hennerberg, Lilly, mushroom complete, potato dextrose agar, and yeast malt extract, were prepared to examine the mycelial development of the chosen strains of *C. indica*. Prior to autoclaving, the pH of the media was adjusted to 5. The formulations of the culture media and the assessment of mycelial growth were carried out following the procedures outlined by Alam and Rahman (2019).

The genomic DNA was isolated using the method described by Lee and Taylor (1990). A 10-day-old culture of the chosen *C. indica* strains was used to collect fresh mycelium, which was subsequently frozen using liquid nitrogen. After being pulverized in a sterile mortar and pestle, the frozen mycelium was put into a 1.5 ml microcentrifuge tube. The microcentrifuge tube was filled with an equal amount of extraction buffer, which contained 50 mM Tris-HCl (pH 7.5), 50 mM EDTA (pH 8) and 1% sarkosyl. The tube was then incubated for 30 min at 65°C. Following the incubation, an equivalent volume of PCI (25 ml phenol: 24 ml chloroform: 1 ml isoamyl-alcohol) was introduced, vortexed, and centrifuged at 4°C for 10 min at 12,000 rpm. The supernatant was collected in a new 1.5 ml microcentrifuge tube and 1,000 μl of 99.9% alcohol was added before centrifuging at 4°C for 5 min at 12,000 rpm. The supernatant was subsequently discarded and 500 μl of 70% alcohol was added to the precipitated DNA, vortexed and centrifuged again at 4°C for 5 min at 12,000 rpm. After removing the supernatant, the tube was left to air dry until any residual alcohol had evaporated. Finally, 500 μl of sterilized distilled water was added. The DNA concentration was determined using a spectrophotometer (Optizen POP; Mecasys Co. Ltd., Daejeon, Korea) and was stored at -20°C (Cubero et al. 1999).

The ITS region of the rDNA from selected strains of *C. indica* was amplified via PCR with universal primers ITS1 (5′-TCCGTAGGTGAACCTGCG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), which were obtained from Bioneer Corporation, located at 49-3, Munpyeong-dong, Daedeok-gu, Daejeon 306-220, Korea. The amplification reactions were conducted in a final volume of 20 μl, containing 2 μl of 10× PCR buffer, 1.6 μl of dNTPs, 0.5 μl of each primer, 0.2 μl of Taq polymerase

(SolGent Co. Ltd., Korea), 1 µl of genomic DNA, and 14.2 µl of sterilized distilled water. The PCR process was performed using a thermal cycler (Veriti thermal cycler, Applied Biosystems, USA), starting with an initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 52°C, extension for 1 min at 72°C and a final extension for 10 min at 72°C. The resulting amplified PCR products were resolved by electrophoresis on a 1.5% agarose gel in 1 × TAE buffer for 1 hrs at 100V alongside a 1 kb DNA ladder as a size marker and then stained while agitated in an EtBr solution (0.5% µg/ml). The stained gels were visualized and photographed using a UV transilluminator (Kodak Image Station 4000R; Molecular imaging system, Carestream Health Inc., 150 Verona Street, Rochester, NY 14608). The ITS sequences were analyzed using the SolGent sequencing service (SolGent Co. Ltd. Korea) and sequence alignment for phylogenetic analysis was conducted using the Cluster W program (Thompson et al. 1994). The phylogenetic tree was created using the neighbor-joining method through the CLC free Workbench software. Bootstrap analysis was carried out 1,000 times to assess the reliability of the internal branches and the validity of the phylogenetic trees (Saitou and Nei 1987).

Genomic DNA was amplified utilizing the RAPD method (Williams et al. 1990), which involved 20 different arbitrary 10-base oligonucleotide primers (Operon Technologies Inc.). The RAPD-PCR reaction was conducted using a thermal cycler, beginning with an initial denaturation phase lasting 5 min at 94°C, succeeded by 35 cycles that included denaturation for 1 min at 94°C, annealing for 1 min at 36°C, extension for 2 min at 72°C and a concluding extension period of 7 min at 72°C. The RAPD products were subjected to electrophoresis on a 1.4% agarose gel in 1×TAE buffer for 1.15 hrs at 100V, utilizing a 1kb DNA ladder as a size standard, after which they were stained in an EtBr solution (0.5% µg/ml) while being agitated. The stained gels were observed and photographed using a UV transilluminator. The RAPD bands were noted as either present (1) or absent (0), creating a data matrix. The similarity coefficients (S) among isolates across bands for all primers were computed using the formula S= 2Nxy/ (Nx +Ny), where Nx and Ny denote the number of bands common to the two strains (Nei and Li 1979).

A measurement of mycelial growth was made seven days after incubation (dpi). MS Excel, one of the common statistical analysis tools, was used to examine the data produced during the experiment. The MEGA-11 software and the nucleotide BLAST tool were used to evaluate the sequence data of the tested strains.

## Results and Discussion

To ascertain the ideal temperature for mycelial growth, a range of 15 to 35°C was evaluated. The strain IUM-01, 02, 04 and 06 exhibited the highest mycelial growth (87.0 mm) at 30°C. The average mycelial growth peaked at 81.88 mm at 30°C, followed by 76.88, 74.66, 65.43 and 19.37 mm at temperatures of 25, 35, 20 and 15°C, respectively

(Table 1). These results are consistent with Alam et al. (2009), who reported that 30°C is optimal for the mycelial growth of Pleurotus eryngii. Shim et al. (2003) found that the mycelial growth of Paecilomyces fumosoroseus increased with temperature, reaching its most favorable range between 25 to 30°C, while growth declined when temperatures exceeded 30°C.

**Table 1. Effects of temperature on the mycelial growth in different strains of *Calocybe indica*.**

| Strain | Mycelial growth (mm)* | | | | |
|---|---|---|---|---|---|
| | 15°C | 20°C | 25°C | 30°C | 35°C |
| IUM-4374 | 30.7 ± 2.5 | 70.7 ± 2.1 | 85.3 ± 2.9 | 87.0 ± 0.0 | 84.0 ± 0.0 |
| IUM-4375 | 20.3 ± 2.1 | 73.3 ± 2.1 | 79.7 ± 6.4 | 87.0 ± 0.0 | 83.3 ± 1.5 |
| IUM-4376 | 19.3 ± 3.8 | 78.0 ± 3.6 | 82.3 ± 7.5 | 82.7 ± 8.1 | 71.7 ± 2.9 |
| IUM-4377 | 17.7 ± 2.5 | 66.3 ± 3.5 | 79.3 ± 1.5 | 87.0 ± 0.0 | 82.7 ± 2.1 |
| IUM-4378 | 11.7 ± 1.5 | 43.0 ± 3.0 | 63.0 ± 1.0 | 66.3 ± 1.5 | 64.7 ± 2.1 |
| IUM-4379 | 19.0 ± 2.6 | 74.0 ± 3.5 | 87.0 ± 0.0 | 87.0 ± 0.0 | 72.7 ± 2.1 |
| IUM-4380 | 12.0 ± 1.0 | 61.0 ± 6.6 | 66.0 ± 5.3 | 68.3 ± 3.2 | 64.0 ± 0.0 |
| IUM-4381 | 25.0 ± 1.0 | 76.3 ± 6.3 | 81.7 ± 1.5 | 84.7 ± 3.2 | 75.9 ± 1.6 |
| IUM-4382 | 18.7 ± 4.5 | 46.3 ± 0.6 | 67.7 ± 3.8 | 87.0 ± 0.0 | 73.0 ± 2.6 |
| Mean | 19.37 ± 1.96 | 65.43 ± 4.23 | 76.88 ± 2.96 | 81.88 ± 2.80 | 74.66 ± 2.51 |

*Mean of 3 replications, Values expressed as means ± SD.

The ideal pH levels for mycelial growth across nine different strains of *C. indica* were examined within a pH range of 4 to 8. The most substantial growth (76.33 mm) occurred at pH 5, while the least growth (8.08 mm) was noted at pH 8 (Table 2). This indicates a preference for acidic conditions in mushroom growth, a finding that aligns with Hur's (2008) study. He noted that *Phellinus linteus* and *Pleurotus eryngii* thrive under acidic, neutral, or alkaline conditions, with optimal mycelial growth typically found at pH levels of 5-6.

To identify the best culture medium for mycelial growth of *C. indica*, nine different media, including czapek dox, glucose peptone, glucose tryptone, Hamada, Hennerberg, Hoppkins, Lilly, mushroom complete, potato dextrose agar, and yeast-malt extract, were assessed. Findings indicated that PDA was the most suitable growth medium, whereas Hennerberg and czapek dox were the least beneficial for the vegetative growth of *C. indica* (Table 3).

According to Alam et al. (2009), glucose peptone, yeast malt extract, and PDA media were found to be the most effective, while Hennerberg and Hoppkins media were the least conducive for the vegetative growth of *P. eryngii*.

**Table 2. Effects of pH on the mycelial growth in different strains of Calocybe indica.**

| Strain | Mycelial growth (mm)* | | | | |
|---|---|---|---|---|---|
| | pH 4 | pH 5 | pH 6 | pH 7 | pH 8 |
| IUM-4374 | 61.0 ± 1.7 | 74.5 ± 2.5 | 50.5 ± 2.6 | 20.3 ± 1.2 | 18.7 ± 2.7 |
| IUM-4375 | 67.3 ± 1.2 | 85.3 ± 1.5 | 65.0 ± 0.6 | 18.0 ± 1.4 | 15.3 ± 1.2 |
| IUM-4376 | 64.7 ± 3.2 | 84.7 ± 0.6 | 62.7 ± 1.5 | 21.3 ± 1.7 | 18.7 ± 1.7 |
| IUM-4377 | 59.5 ± 0.0 | 80.3 ± 1.5 | 58.0 ± 2.4 | 23.0 ± 3.8 | 19.0 ± 1.0 |
| IUM-4378 | 55.3 ± 2.6 | 72.3 ± 1.2 | 53.7 ± 1.7 | 23.7 ± 1.5 | 14.5 ± 0.6 |
| IUM-4379 | 61.7 ± 1.7 | 76.7 ± 3.2 | 49.3 ± 6.9 | 17.3 ± 2.3 | 12.7 ± 2.5 |
| IUM-4380 | 60.0 ± 0.6 | 63.3 ± 1.2 | 38.7 ± 2.0 | 20.1 ± 2.1 | 15.1 ± 5.4 |
| IUM-4381 | 49.7 ± 1.2 | 64.6 ± 3.2 | 37.5 ± 0.6 | 19.0 ± 1.7 | 16.5 ± 0.6 |
| IUM-4382 | 68.3 ± 1.7 | 85.3 ± 2.4 | 53.7 ± 3.9 | 17.7 ± 2.5 | 14.3 ± 1.5 |
| Mean | 60.83 ± 1.93 | 76.33 ± 2.82 | 52.12 ± 3.16 | 20.04 ± 0.76 | 16.08 ± 0.75 |

*Mean of 3 replications, Values expressed as means ± SD.

**Table 3. Effects of culture media on the mycelial growth in different strains of *Calocybe indica*.**

| Strain | Culture Media | | | | | | |
|---|---|---|---|---|---|---|---|
| | CZA | GLP | HEN | LIL | MUC | PDA | YEM |
| IUM-4374 | 29.6 ± 2.5 | 62.6 ± 2.5 | 31.0 ± 0.6 | 76.6 ± 2.4 | 85.6 ± 2.6 | 85.0 ± 5.6 | 72.6 ± 3.4 |
| IUM-4375 | 30.6 ± 1.5 | 61.3 ± 3.5 | 31.6 ± 1.5 | 82.0 ± 1.8 | 86.3 ± 3.5 | 84.6 ± 3.8 | 77.3 ± 1.5 |
| IUM-4376 | 71.0 ± 5.6 | 70.3 ± 1.5 | 66.0 ± 2.4 | 80.3 ± 4.5 | 75.3 ± 2.8 | 77.6 ± 3.6 | 82.3 ± 6.4 |
| IUM-4377 | 23.6 ± 1.4 | 66.0 ± 1.8 | 16.0 ± 0.6 | 57.3 ± 1.2 | 69.3 ± 1.5 | 86.3 ± 6.2 | 79.0 ± 3.5 |
| IUM-4378 | 69.6 ± 3.7 | 76.3 ± 2.7 | 64.0 ± 3.4 | 76.3 ± 2.2 | 71.6 ± 2.4 | 77.6 ± 2.6 | 86.3 ± 5,4 |
| IUM-4379 | 63.0 ± 2.5 | 68.3 ± 5.2 | 60.3 ± 2.8 | 72.6 ± 1.5 | 71.3 ± 5.2 | 64.3 ± 2.5 | 70.6 ± 3.2 |
| IUM-4380 | 72.0 ± 4.2 | 70.3 ± 4.5 | 66.6 ± 2.5 | 85.0 ± 3.8 | 70.6 ± 2.4 | 79.6 ± 4.5 | 77.3 ± 1.8 |
| IUM-4381 | 71.3 ± 3.7 | 53.3 ± 3.5 | 62.3 ± 1.7 | 73.3 ± 3.6 | 61.6 ± 1.5 | 80.3 ± 5.2 | 83.3 ± 4.5 |
| IUM-4382 | 21.0 ± 0.6 | 56.3 ± 2.2 | 24.0 ± 1.2 | 60.3 ± 2.4 | 59.0 ± 3.2 | 67.6 ± 2.8 | 48.3 ± 1.2 |
| Mean | 50.18 ± 7.69 | 64.96 ± 2.43 | 46.86 ± 6.90 | 73.74 ± 3.12 | 72.28 ± 3.08 | 80.32 ± 1.9 | 75.22 ± 3.75 |

*Mean of 3 replications, Values expressed as means ± SD. CZA: czapek dox, GLP: glucose peptone, HEN: Hennerberg, LIL: Lilly, MUC: mushroom complete, PDA: potato dextrose agar and YEM: yeast-malt extract.

To explore the molecular identification and genetic diversity of selected IUM strains of *C. indica*, the ITS region was amplified with ITS1 and ITS4 primers and subsequently sequenced. The results revealed that the length polymorphism at the sequence level ranged from 570 to 620 bp. The sizes of the ITS1 and ITS2 regions exhibited variability among the strains, ranging from 168 to 204 bp and 178 to 211 bp, respectively. The total G

+ C and A + T contents of the ITS regions varied between 46.1-47.2% and 52.8-53.9%. Sequence analysis indicated that the 5.8S rDNA sequence was consistent (160 bp) across all tested strains of *C. indica* (Table 4). The observed size variation resulted from differences in nucleotide quantity, indicating that these strains can be distinctly identified from one another based on ecological distribution, substitution, and polymorphisms involving insertions or deletions in base positions (Ro et al. 2007).

**Table 4. Nucleotide distribution, ITS1, 5.8S, and ITS2 of rDNA sequences in different strains of *Calocybe indica*.**

| Strains | Nucleotide distribution (bp) | | | | | | Sequence information (bp) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A | C | G | T | G+C | A+T | ITS1 | 5.8S | ITS2 | Length |
| IUM-4374 | 145 | 142 | 149 | 184 | 46.9 | 53.1 | 188 | 160 | 192 | 620 |
| IUM-4375 | 144 | 141 | 149 | 183 | 47.0 | 53.0 | 178 | 160 | 192 | 617 |
| IUM-4376 | 144 | 140 | 148 | 183 | 46.8 | 53.2 | 178 | 160 | 192 | 615 |
| IUM-4377 | 143 | 140 | 148 | 183 | 46.9 | 53.1 | 178 | 160 | 192 | 614 |
| IUM-4378 | 136 | 131 | 132 | 171 | 46.1 | 53.9 | 168 | 160 | 178 | 570 |
| IUM-4379 | 143 | 141 | 144 | 182 | 46.7 | 53.3 | 178 | 160 | 180 | 610 |
| IUM-4380 | 138 | 140 | 140 | 178 | 47.0 | 53.0 | 204 | 160 | 211 | 596 |
| IUM-4381 | 139 | 140 | 142 | 179 | 47.0 | 53.0 | 204 | 160 | 210 | 600 |
| IUM-4382 | 137 | 139 | 142 | 177 | 47.2 | 52.8 | 202 | 160 | 207 | 595 |

The phylogenetic tree created from the nucleotide sequences of the ITS region in selected strains of *C. indica* was developed using the neighbor joining method (Fig. 1). Reciprocal homologies of the ITS region sequences ranged from 98% to 100%. The ITS region is relatively short and can be easily amplified by PCR utilizing universal single primer pairs. The genetic distance displayed a high level of similarity among identical ITS sequences. The greatest difference was noted between IUM-4378 and IUM-4374 strains, while the highest similarity (99.53%) was recorded between IUM-4380 and IUM-4382 strains. The findings from the phylogenetic tree revealed that IUM strains of *C. indica* were very closely related to strains found in the NCBI GenBank. White et al. (1990) mentioned that ITS sequences are largely consistent or exhibit minimal variation within a species, but show variation between species within a genus. The genetic diversity detected among the groups is likely due to effective gene flow and a high degree of genetic compatibility among the tested strains. These conclusions support the findings of Zervakis et al. (2001). The ITS region, being relatively short, can be easily amplified by PCR with the use of universal single primer pairs. The genetic distance demonstrated a high level of similarity among identical ITS sequences. The base sequences within the ITS region of rDNA varied among the strains examined and can be utilized to assess genetic distances and offer insights into phylogenetic studies.

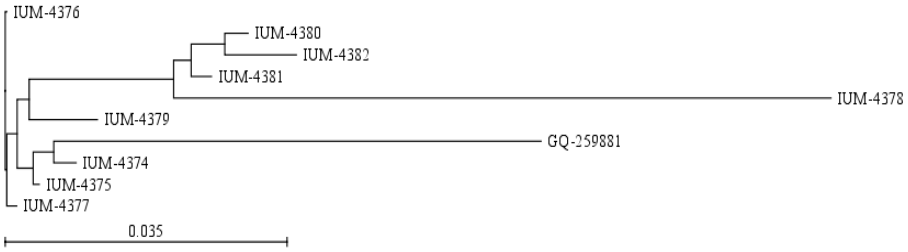


Fig. 1. Phylogenetic tree of ten strains of *Calocybe indica* (nine IUM and one GenBank strain) based on the nucleotide sequences of the ITS regions using neighbor-joining method with 1,000 boot-strapping trails.

Twenty arbitrary 10-base oligonucleotide primers were employed to amplify genomic DNA segments from the selected IUM strains of *C. indica*. Eleven primers (OPA-1, 3, 5, 7, 8, 9, 10, 11, 17, 19 and 20) effectively amplified genomic DNA (Table 5).

These primers produced significant band profiles in the tested strains, making them suitable candidates for strain screening (Figs 2, 3 and 4). RAPD-PCR yielded distinct multiple products that exhibited substantial variability among the tested strains. The quantity of amplified bands varied based on the primers selected or the strains assessed. The size of these polymorphic fragments ranged from 0.1 to 2.3 kb. The DNA polymorphisms displayed consistent properties in replication tests. Thus, by using the same primers for screening DNA polymorphisms, it becomes feasible to differentiate genetically distinct strains of *C. indica*. To enhance the specificity of the polymorphic patterns, a combined dendrogram was generated using RAPD-PCR amplified bands obtained from the ten RAPD primers. Cluster analysis based on banding patterns and the sizes of amplified products revealed two potential groups among the nine strains of *C. indica* (Fig. 5). Among the nine strains examined, 99-100% similarity was found between IUM-4374 and IUM-4377, as well as between IUM-4378 and 4381. These findings align with the research conducted by Alam et al. (2009, 2010).

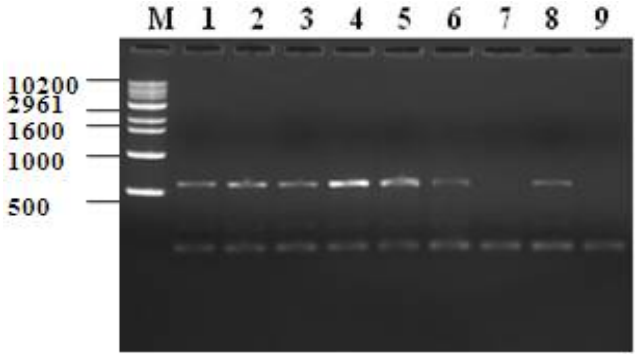


Fig. 2. RAPD profiles in nine different strains of *Calocybe indica* with primer OPA-8. M, molecular size marker (1 kb DNA ladder); lane 1, IUM-4374; lane 2, IUM-4375; lane 3, IUM-4376; lane 4, IUM-4377; lane 5, IUM-4378; lane 6, IUM-4379; lane 7, IUM-4380; lane 8, IUM-4381; lane 9, IUM-4382.

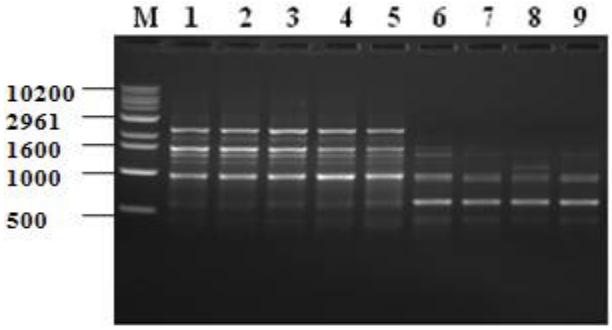


Fig. 3. RAPD profiles in nine different strains of *Calocybe indica* with primer OPA-11. M, molecular size marker (1 kb DNA ladder); lane 1, IUM-4374; lane 2, IUM-4375; lane 3, IUM-4376; lane 4, IUM-4377; lane 5, IUM-4378; lane 6, IUM-4379; lane 7, IUM-4380; lane 8, IUM-4381; lane 9, IUM-4382.

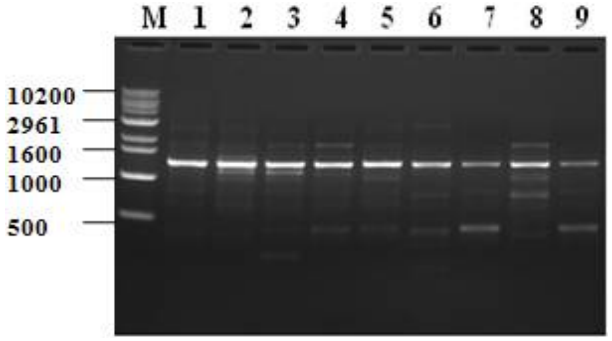


Fig. 4. RAPD profiles in nine different strains of *Calocybe indica* with primer OPA-17. M, molecular size marker (1 kb DNA ladder); lane 1, IUM-4374; lane 2, IUM-4375; lane 3, IUM-4376; lane 4, IUM-4377; lane 5, IUM-4378; lane 6, IUM-4379; lane 7, IUM-4380; lane 8, IUM-4381; lane 9, IUM-4382.

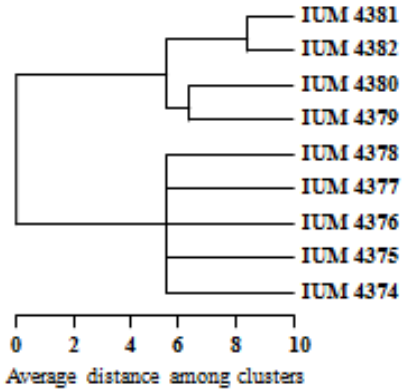


Fig. 5. Dendrogram constructed based on the random amplification of polymorphic DNA markers of *Calocybe indica* strains determined by the average linkage cluster.

**Table 5. DNA bands in different strains of *Calocybe indica* by random amplification of polymorphic DNA assay using 10-base OPA primers.**

| Primers | DNA band (kb) | IUM- Strains | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| OPA-01 | 1.5 | + | + | + | + | + | + | + | + | + |
| | 1.0 | - | - | - | - | - | + | + | - | - |
| OPA-03 | 1.5 | + | + | + | + | + | + | + | + | + |
| | 0.8 | + | + | + | + | + | + | + | + | + |
| | 0.5 | + | + | + | + | + | + | + | + | + |
| OPA-05 | 1.2 | + | + | + | + | + | + | + | + | + |
| | 0.7 | + | + | + | + | + | + | + | + | + |
| | 0.5 | + | + | + | + | + | + | + | + | + |
| OPA-07 | 1.6 | + | + | + | + | + | - | - | - | - |
| | 0.9 | + | + | + | + | + | - | - | - | - |
| | 0.6 | + | + | + | + | + | + | + | + | + |
| OPA-08 | 0.6 | + | + | + | + | + | + | - | + | - |
| | 0.1 | + | + | + | + | + | + | + | + | + |
| OPA-09 | 1.9 | + | + | + | + | + | + | - | + | - |
| | 1.0 | - | + | + | + | + | + | + | + | + |
| | 0.5 | + | + | + | + | + | + | + | + | + |
| OPA-10 | 1.5 | + | + | + | + | + | + | + | + | + |
| | 1.2 | + | + | + | + | + | + | + | + | + |
| | 0.7 | + | + | + | + | + | + | + | + | + |
| OPA-11 | 2.3 | + | + | + | + | + | - | - | - | - |
| | 1.6 | + | + | + | + | + | - | - | - | - |
| | 0.9 | + | + | + | + | + | + | + | + | + |
| | 0.5 | - | - | - | - | - | + | + | + | + |
| OPA-17 | 1.3 | + | + | + | + | + | + | + | + | + |
| | 0.8 | - | - | - | - | - | - | - | + | - |
| | 0.3 | - | - | - | - | - | - | + | - | + |
| OPA-19 | 1.6 | + | + | + | + | + | + | + | + | + |
| | 1.3 | + | + | + | + | + | - | - | - | - |
| | 1.0 | + | + | + | + | + | + | + | + | + |
| OPA-20 | 1.6 | + | + | + | + | + | + | + | + | + |
| | 1.3 | + | + | + | + | + | + | + | + | + |

1,IUM-4374; 2,IUM-4375; 3,IUM-4376; 4,IUM-4377; 5,IUM-4378; 6,IUM-4379; 7,IUM-4380; 8,IUM-4381; 9,IUM-4382, - indicate absence of DNA band, + indicate presence of DNA band.

The results obtained from the RAPD analysis corresponded with those gained from the analysis of ITS region sequences. Selecting strains and creating a molecular map focused on breeding would be a viable approach to enhance the yield and quality of milky white mushrooms. RAPD primers serve as an effective tool for elucidating the genetic relationships among different strains (Alam et al. 2009). The molecular findings

indicated that the strains tested were genetically similar, exhibiting some variations; both RAPD and ITS techniques proved to be effective for identifying the genetic diversity among all tested strains of *C. indica*.

The optimal physicochemical conditions for the mycelial growth of the milky mushroom are outlined; thus, the foundational information derived from this study could benefit database analysis as well as assist farmers and policymakers in establishing protocols for commercial cultivation. Based on the experimental outcomes of molecular characterization, it can be concluded that all IUM strains assessed were identified as *Calocybe indica*, showing genetic similarity with some variations; moreover, RAPD and ITS techniques were well-suited for revealing the genetic diversity.

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