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Taxonomic studies and molecular characterisation of *Tricholoma giganteum* and *Calocybe indica* isolates from Bangalore.

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Abstract

The agarics *Tricholoma giganteum* Massee and *Calocybe indica* Purk. & Chandra showed varied morphological and ecological features, as these data along with microscopic studies of basidiocarps were found to be inadequate to delimit them to species level. This led to explore using molecular methods to distinguish the species of *Tricholoma* and *Calocybe* by analysing the ITS region of rDNA from pure culture. The DNA sequences obtained in this study were deposited in the GenBank database. ITS sequence of *Tricholoma giganteum* and *Calocybe indica* were subjected to the BLAST programme to generate the significant alignment and the close matches to the query sequence. ITS sequence isolated from the pure culture of *T.giganteum* showed 99% similarity with *Tricholoma giganteum* (EU051917.1) an isolate from China. Similarly ITS sequence of the pure culture of *Calocybe indica* of our isolate showed 100% similarity with *Calocybe indica* (AY636067). Hence the molecular techniques can be used as a tool to avoid misidentification of fungal isolates along with classical identification methods.

Key words: *Tricholoma giganteum*, *Calocybe indica*, ITS sequence, rDNA, BLAST, Clustal W2.

Introduction

The taxonomic identification of an organism is an essential part of an investigation of a new species or isolate of a known species. Classical identification techniques used in the identification of mushrooms include the macroscopic and microscopic characters of the basidiocarps. However, the age of the basidiocarp and environment have a marked effect on the colour, size and brightness of the fruiting body, and the presence, absence or length of the stipe (Moncalvo *et al,* 2000). With the progress in molecular genetics, these identification techniques are now being replaced by more modern methods that analyse and compare the ribosomal RNA (rRNA) genes from different fungal isolates. Such molecular techniques used for fungal identification to date include PCR coupled with RFLP (Miller *et al.*, 1999), isozyme analysis (Gottlieb *et al.*, 1998), direct sequencing of the rRNA genes (Moncalvo *et al.*, 1995) and rDNA (ITS), Bruns *et al,* (1998). In addition, developments in the field of bioinformatics have enabled the interpretation of this sequence information to yield information about the evolutionary relationships between new and existing fungal species. Molecular genetic markers have been employed for rapid identification of different kinds of mushrooms by several workers. (Lee *et al*, 2006; Yadav *et al*, 2007; Lian *et al,* 2008).

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In the present investigation, the taxonomy of the genera *Tricholoma* and *Calocybe* using ecological, morphological, microscopic and microchemical tests were found to be inadequate to delimit them to species level as these species showed pleomorphism. Hence they were identified by analysing the ITS sequence of rDNA from the pure culture obtained from the basidiocarps.

Materials and methods

Molecular taxonomy

The pure cultures of *Tricholoma* (Fig 01 and 02) and *Calocybe* (Fig 02 and 03) were obtained by placing a portion of context of the pileus aseptically on sterilized Potato Dextrose Agar in petiplates and incubated at 25±1ºC for 6-7 days for initial mycelial growth. The extraction of DNA from the pure cultures was performed by – Cetyl tri methyl ammonium bromide (CTAB) method (Ausubel *et al.*, 1994). Agarose gel electrophoresis was performed in a horizontal submarine apparatus (Genei, Bangalore, India) as outlined by Sambrook and Russell (2001). 10 µl of Gene Ruler 1kb DNA Ladder (Chromous Catalogue No. LAD03) was loaded into one well as a standard molecular weight marker. Electrophoresis was carried out at 60V for 40–60 min. The gel was viewed under

UV transilluminator (352 nm). DNA band obtained was removed from the gel aseptically and Polymerized chain reaction (PCR) was performed in a Thermocycler (PTC-100TM programmable thermal controller, USA) to produce multicopies of a specified DNA using following PCR condition

1. Initial Denaturation 94°C for 5 min.
2. Denaturation 94°C for 30 sec.
3. Annealing 55^{0}C for 30 sec
4. Extenshion 72^{0}C for 1 min
5. Final extenshion 72^{0}C for 15 min
6. Stop at 4°C for 1 h.

Universal primers for fungal genome, ITS 1 - (5'-TCC GTA GGT GAA CCT GCC G-3') forward ITS 4 - (5'- TCC TCC GCT TAT TGA TAT GC-3') reverse primers were used. These primers were obtained from Chromous Biotech Pvt. Ltd. Bangalore, India. ITS region of rDNA was visualised by UV trans-illumination (352 nm) and the expected DNA band was excised from the gel using a sterile scalpel and placed into a 1.5 ml microtube. This DNA was purified using gel extraction kit (Chromous Biotech Pvt. Ltd. Bangalore, India) according to the manufacturer's specifications. The purified PCR product was sequenced at Chromous Biotech Pvt. Ltd. Bangalore, India. Sequences were determined by the chain termination method using an ABI 3130 Genetic Analyzer. Sequencing was done in the forward and reverse direction. The sequence was generated using data analysis software (Seq Analysis_ v 5.2).

Sequence Data Analysis

Sequence alignments provide a powerful way to compare novel sequences with previously characterized genes. Both functional and evolutionary information can be inferred from well designed queries and alignments. Basic Local Alignment Search Tool (BLAST) provides a method for rapid searching of nucleotide and protein database. The rDNA gene sequence was used to carry out BLAST with the data base of NCBI gene bank. Based on maximum identity scores first 10 sequence were selected and aligned using Multiple sequence alignment software program Clustal W2 was used to prepare Cladograms for the analysed species

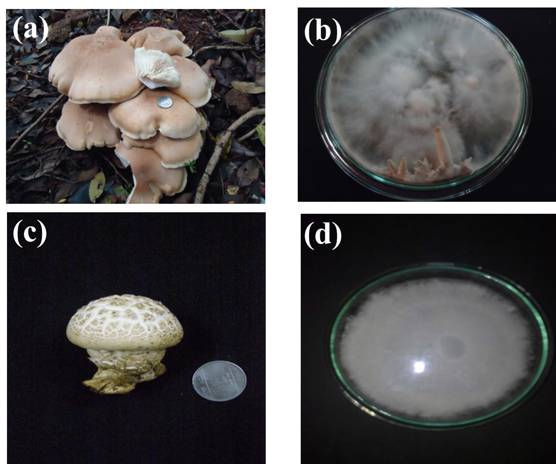

Figure 1: a) Basidiocarps of *Tricholoma giganteum*; b) Pure culture of *T.giganteum*; c) Basidiocarp of *Calocybe indica*; d) Pure culture of *C. indica*

Result and discussion

The genomic DNA was extracted from the pure cultures of *Tricholoma and Calocybe*. The ITS region of *Tricholoma* and *Calocybe* yielded an amplified PCR product of approximately 600 bp respectively (Fig: 05a, 5b).

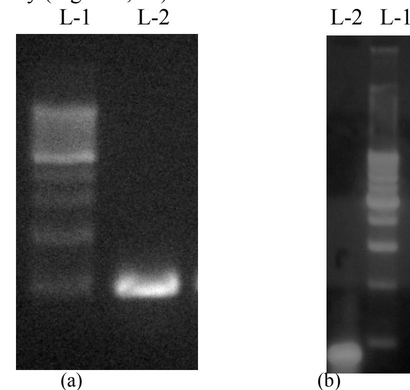

Figure 5: a) Gel image of rDNA amplicon of *Tricholoma giganteum;* L1: Marker DNA Ladder (Chromous Catalogue No. LAD02); L-2: rDNA amplicon; b) Gel image of rDNA amplicon of *Calocybe indica;* L-1: Marker DNA Ladder (Chromous Catalogue No. LAD02); L-2: rDNA amplicon

The sequence data of *Tricholoma* and *Calocybe* exhibited 601 bp and 625 bp respectively (Fig: 6 and 7). The DNA sequences obtained in this study were deposited in the GenBank database with accession numbers JN006792 and JN874408 respectively.

TGATGACTTGGTCAGTTGTTGCTGGCCCTTTCAGAGCATGTGCACG
CTTGGCTATTGTTTCTTAAACCACTTGTGCACCTTTTGTAGACTTTG
GGTAAAGTTTTGAGTCGAGAGTGATCTTGGCCCTTATACTCCAAA
GTCTATGTCTTTTCATATCATTTACTCTATGTATAAGAATGTTTTCT
AAGGCATTTCTTTGATGCCTTTAAATCATATACAACTTTCAACAAC
GGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGA
TAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA
ACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAG
TGTCATGAAATTCTCAACCTTTGTTACTTTTTTGTTTCAAAGTGTTT
GGATGTGGGAGTTGCTGGCTTTTTGTGTTCAAAGAGTCAGCTCTTC
TGAAATACATTAGTGGGACCCATCGTTGATTTAGCCCCTGGTGTGA
TAGTTATCTACGCCGTGGCTTAGCACGATATTGTGTGGTTCAGCTT
TCTAACAGGACAATTAACTGTCTTTGATCATTTGACCTCAAATCAG
GAG

Figure 6: ITS sequence data of *Tricholoma giganteum* showed 601 bp

GACTGCGGAAGATTCTTTATGAAATGAAACTGGTTAAATTGGTTG
CGGGTTTTTTAGGGGATTGTCCACATTGGGTTTTGGTTTTTTAAAC
CCACTTGTGCACCTTTTGTAGACTTTGGAAGAGTTTGAGTCGAGAG
TGATCTTGGCCCTTACACATCCGAGTCTATGTCTTTTTCATATCATT
TTACTCTGTGTATAAGAATGTTTTCTAAGGCATTATTAATGCCTTT
AAATCATATACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCG
ATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAA
TTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTA
TTCCGAGGAGCATGCCTGTTTGAGTGTCATGAAATTCTCAACCTTT
GTTACTTTTTTTGTTTCAAAGAGTCTGGAAGTGGAGGTTGCTGGCT
TTTGTTGAAAGAGTCGGCTCCTCTGAAATACATTAGTGGGACCCAT
CGTTGATTAGCTCCCTGGTGTGATAGTTATCTACGCCGTGGCTCAT
CACGATATTGTGTGGTTCAGCTTTCTAACGAGACAACAACAACTG
TCTTTGATTATTTGACCTCCAAATCAGGAC

Figure 7: ITS sequence data of *Calocybe indica* showed 625 bp

ITS sequence of *Tricholoma* and *Calocybe* were subjected to the BLAST programme to generate the significant alignment and the close matches to the query sequence. ITS sequence isolated from the pure culture of *Tricholoma* showed 99% similarity with *Tricholoma giganteum* (EU051917.1) an isolate from China (Tang 2007), 98% identity with (HM120872.1) *Tricholoma giganteum* isolate CBE (Coimbatore) (Prakasam *et al.,* 2010); The Phylogenetic position confirms that our isolate corresponds to *Tricholoma giganteum*.(Fig: 8)

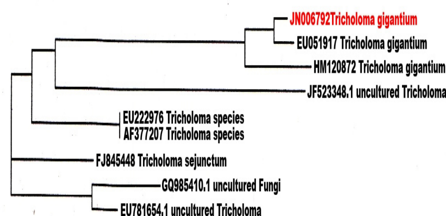

Figure 8: Cladogram of *Tricholoma giganteum* (Clustal W2)

Similarly ITS sequence of the other pure culture of *Calocybe* showed 100% similarity with *Calocybe indica* (AY636067) (Sing *et al.,* 2003). The phylogenetic position confirms that culture corresponds to *Calocybe indica*. (Fig. 9). From this Cladogram it is also confirmed that *Tricholoma giganteum* would have been originated from *Calocybe indica*, which supports (Hofstetter *et al*, 2002) who suggested that agarics with siderophilous granules are of single origin, but would have lost during the course of evolution.

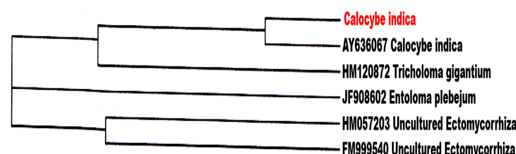

Figure 9: Cladogram of *Calocybe indica* (Clustal W2)

Conclusion

Hence our results suggest that molecular techniques can be effectively used as a tool for precise identification of mushrooms which show high morphological variations.

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