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Ethno-mycological Survey and Molecular Identification of Mushrooms in the Shimoga Region of the Western Ghats of Karnataka (India)

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Shimoga region of Western Ghats is one of the richest biodiversity hotspots in India, which receives an average rainfall of 2869 mm and provides congenial atmosphere for mushrooms. In the present study eleven mushrooms were collected from Siddapura, Theertha halli and Agumbe forests of Shimoga district during monsoon (June-September 2013). The samples were labeled as WGM-1 to WGM-11 (Western Ghats Mushroom). These mushrooms were identified by ITS (Internal Transcribed Spacer) region sequence homology available at National Centre for Biotechnological Information (NCBI) Gen Bank as *Lentinus squrossulus* (WGM-1), *Pleurotus salmoneostramenius* (WGM-2), *Termitomyces* sp.-1 (WGM-3), *Termitomyces* sp.-2 (WGM-4), *Leucoagaricus purpureolilacinus* (WGM-5), *Tricholosporum porphyrophyllum* (WGM-6), *Agrocybe pediades* (WGM-7), *Leucocoprinus birnbaumii* (WGM-8), *Podoscypha petalodes* (WGM-9), *Xylaria* sp. (WGM-10) and *Antrodia serialis* (WGM-11). Among the eleven species, *L. squrossulus, P. salmoneostramenius, Termitomyces* sp.1, *Termitomyces* sp.2, were edible mushrooms. This study unravels the abundance of the mushroom species in parts of Shimoga region of Western Ghats area.

Keywords: Western Ghats, Mushroom, ITS region, Molecular identification.

Ethno mycology refers to the relationship between Man and fungi. Human association with mushroom fungi is from the time immemorial as wild mushrooms are picked up and eaten by man kind since antiquity. Thousands of years ago, fructifications of higher fungi have been used as a source of food due to their attractive flavor and taste (Rai, 1997). Mushrooms are efficient degraders of lingo-celluloses; hence they play a vital role in biodegradation. Some mushrooms have pharmaceutical value such as antimicrobial, anticancer, antioxidants etc., (Chadha and Sharma, 1995).

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Mushrooms grow during rainy season when climatic conditions are cool and humid. Diversity of mushroom species varies according to the ecosystem and their habitat. Shimoga region is in the heart of Western Ghats, which is one of the hot-spots of biodiversity in India. This district comes under south-eastern transitional zone and receives an average annual rainfall of 2869 mm (Bhat, *et al*., 2012) making an ideal habitat for blooming variety of mushrooms. Defining the number and kinds of fungi on earth has been a point of discussion and several studies have focused on enumerating the world fungal diversity (Crous *et al*., 2006). Only a fraction of total fungal wealth has been subjected to scientific scrutiny and mycologists continue to unravel the unexplored and hidden wealth. One third of fungal diversity of the globe exists in India and of this

only 50 % are characterized so far (Manoharachary *et al.*, 2005).

Mushrooms are ephemeral and disappear within a day. Therefore, documentation of mushrooms needs constant survey during appropriate season. Mushrooms can be identified based on their morphological and molecular characters. The Phenotypic characters include the shape, size, texture, colour and odour of the fruiting body. Molecular tools such as 18S rRNA/ITS (Internal transcribed spacer) region can be used to identify mushrooms at any stage (Rajarathnam and Thiagarajan., 2012). Several wild fungi were documented elsewhere and identified using ITS sequence (Oyetayo., 2012). In this study, we report molecular characterization of 11 mushrooms documented from Shimoga region of Western Ghats.

MATERIALS AND METHODS

Collection and documentation of mushrooms

Field survey was made to document the wild mushrooms in forest area of Shimoga district (Shimoga, Siddapura, Agumbe and Theertha halli) of Karnataka from June to September 2013. The survey was carried out with the help of information provided by tribal communities like Adivasis, Halakki vokkals and Siddis in the locality during the visits as they were familiar with mushroom types and season of their appearance. The mushroom samples were collected in paper bags and field notes like date, weather condition, abundance, habitat and phenotypic characters were recorded.

Molecular characterization

Genomic DNA Isolation

Total genomic DNA from cap tissue was extracted using CTAB method (Sambrook et al., 1989). The DNA obtained was stored in Tris-EDTA (10:1) buffer at -20°C. The DNA concentration was measured using nano drop (Eppendorff) and then PCR amplification was carried out in 40 µl reaction mixture containing 4.0µl of 10 X PCR Taq. Buffer, 4.0 µl of 10 mM dNTP's mix, 2.0 µl of ITS primers (ITS1-5'TCCGTAGGTGAACCTGCGG3'and ITS4-5' TCCTCCGCTTATTGATATGC 3', 0.6 µl of Taq. DNA polymerase, 2.0 µl of Template DNA (~50 ng) and 27.4 µl of sterile distilled water.

PCR amplification and elution

The PCR reaction was carried out in a Thermal Cycler (Applied Biosystems). Programmed as initial denaturation at 96°C for 3min, 40 cycles of denaturation of 94°C for 1 min, annealing at 60°C for 30 sec and extension at 72°C for 1 min and final extension at 72°C for 10 min. The amplified products were separated by agarose gel electrophoresis. The gel was visualized under UV light and documented using Alpha Innotech Gel documentation unit. The amplified product was eluted using Gene JET™ Gel Extraction Kit (Thermo Scientific) following manufacturer protocol. The eluted product was cloned into pTZ57R/T cloning vector using Ins T/A clone PCR product cloning kit [MBI, Fermentas Life Sciences, USA (#K1214)] after determining the appropriate vector: insert ratios (Sambrook *et al.*, 1989). The ligation reaction was performed in a 10µl reaction volume at 16°C overnight. The ligated product was transformed in to *E. coli* (DH5á) cells using heat shock method (Sambrook *et al.*, 1989) and plated on Luria Berton (LB) agar medium containing antibiotic (ampicillin, 100 µg/ml). The recombinant clones were initially screened by blue white selection, followed by colony PCR using M13 forward and reverse primers (Sambrook *et al.*, 1989). The transformed colony was multiplied in LB broth containing 100µl ampicillin for overnight and the recombinant plasmid was isolated using GenElute™HP Plasmid MiniPrep Kit (Sigma, USA) following the manufactures protocol. The isolated plasmid was sequenced at Sci Genome Labs Private Ltd. Kerala, INDIA using M13 forward and reverses primers.

Sequence analysis and homology search

Sequence results were analysed with Vec Screen online software from NCBI for removing the vector contamination. Forward and reverse primer sequences were checked against each other by generating the reverse complement of the "reverse" sequence using Fast PCR Professional (Experimental test version 5. 0. 83) and aligning it with the "forward" sequence with the help of CLUSTAL W Multiple Sequence Alignment Programme using the online software SDSC Biology Workbench (San Diego Supercomputer Center). The full length gene homology search was performed with blast programme of National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al*., 1990).

RESULTS AND DISCUSSION

Mushrooms have been used as food and medicine by the ancient Egyptian, Greek, Roman and Chinese civilizations. The diversity of fungi and their natural beauty occupy prime place in the biological world and India has been a cradle for these species. To understand the occurrence, abundance, locality or habitat and edibility of the mushrooms, traditional knowledge of the tribal folks was very much essential, therefore, we sought villager's knowledge and accompanied them during the survey for collection of mushrooms. Field information of the mushroom species was recorded during collection (Table 1). Then the samples were labeled as WGM-1, WGM-2, WGM-3, WGM-4, WGM-5, WGM-6, WGM-7, WGM-8, WGM-9, WGM-10 and WGM-11. The habitats were varied from soil to tree stump and leaf litter as it is a versatility of the forest ecosystem which provides diversified niche for different types of mushrooms under same umbrella. Srivastava *et al.* (2011) collected mushrooms belonging to four species of *Termitomyces* namely, *Termitomyces heimii, Termitomyces clypeatus, Termitomyces mammiformis and Termitomyces microcarpus* from Gorakhpur forest division and characterized by different morphological traits such as shape of stipe, pileus, margin of fruit body, colour of fruit body, gills, flesh, annulus, pseudorrhiza and spore print. Dwivedi *et al*., (2012) reported 52 mushroom species from Amarkantak Biosphere Reserve of Madhya Pradesh. Those mushrooms belonged to different genera out of which only 14 mushroom

Table 1. Field characters of mushrooms collected from Western Ghats region of Karnataka

S. No	Mushroom collected	Date of collection	Place of collection	Vernacular name	Habitat
1	WGM-1	22/09/2013	Siddapura, shimoga district.	Mara anabe	Tree
2	WGM -2	18/09/2013	Theerthahalli Shimoga district.	Henne anabe	Branch of tree
3	WGM -3	17/09/2013	Siddapura shimogha district	Beru anabe	Soil
4	WGM -4	22/09/2013	Agumbe forest of shimogha district	Huthada anabe	Termite mound
5	WGM -5	22/10/2013	Siddapura, of shimogha district	Thuppada anabe	Soil
6	WGM -6	22/10/2013	Agumbe forest of shimogha district	Nayee anabe	Humus
7	WGM -7	17/08/2013	Siddapura shimogha district	Chikka anabe	Soil
8	WGM -8	19/08/2013	Agumbeforest, shimogha district	Hucha anabe	Soil
9	WGM -9	10/10/2013	Siddapura, shimogha district	Mara anabe	Black soil
10	WGM -10	28/09/2013	Theerthahalli shimogha district.	Kaddi anabe	Humus soil
11	WGM -11	26/08/2013	Agumbe forest shimogha district	Mara anabe	Decay wood

WGM-Western Ghat Mushroom

Table 2. List of Mushroom species identified by ITS region sequence and their DNA amplicon size

S. No	Mushroom SpeciesDesignation	Size of amplified DNA(bp)	Mushroom species identified	Blast search homology (%)
1	WGM-1	684	*Lentinus squrossulus*	97
2	WGM-2	737	*Pleurotus salmoneostramenius*	99
3	WGM-3	735	*Termitomyces sp.*	99
4	WGM-4	773	*Termitomyces sp.*	99
5	WGM-5	716	*Leucoagaricus purpureolilacinus*	94
6	WGM-6	719	*Trycholosporum porpyrophyllum*	87
7	WGM-7	729	*Agrocybe pediades*	99
8	WGM-8	764	*Leucocoprinous bimbaumii*	99
9	WGM-9	693	*Podoscypha petalodes*	99
10	WGM-10	455	*Xylaria sp.*	89
11	WBG-11	735	*Antrodia serialis*	95

WGM-Western Ghat Mushroom

GTCAGAGGTGAAATTCTTGGATTTACTCAAGACCAACTACTCGCGAAAGCATTTGCCAAGGA
TGTTTTCATTAATCAAGAACGAAGGTTAGGGGATCGAAAACGATCAGATACCGTTGTAGTCT
TAACAGTAAACTATGCCGACTAGGGATCGGGCAAACTCAAACATGATGTGTTGCTCGGCACC
TTACGAGAAATCAAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAA
GGAATTGACGGAAGGGCACCACCAGGTGTGGAGCCTGCGGCTTAATTTGACTCAACACGGG
GAAACTCACCAGGTCCAGACATAACTAGGATTGACAGATTGATAGCTCTTTCATGATTTTAT
GGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGATAACG
AACGAGACCTTAACCTGCTAAATAGCCAGGCCGGCTTTGGCTGGTCGCCGGCTTCTTAGAGG
GACTGTTGGCGTCTAGCTGACGGAAGTTTGAGGCAATAACAGGTCTGTGATGCCCTTAGATG
TTCTGGGCCGCACGCGCGCTACACTGACAGAGCCAGCGAGTTTTTTTCCTTGGCCGGAAGGT
CTGGGTAATCTTGTGAAACTCTGTCGTGCTGGGGATAGAGCATTGCAATTATTGCTCTTCAAC
GAGGAATACCTAGTAAGCGTGAGTCATCAGCTCGCGTTGATTACGTCCCTGCCCTT

Fig. 1. Full length sequence and homology search of *Pleurotus salmoneostramenius* (The above figure is representative of molecular identification)

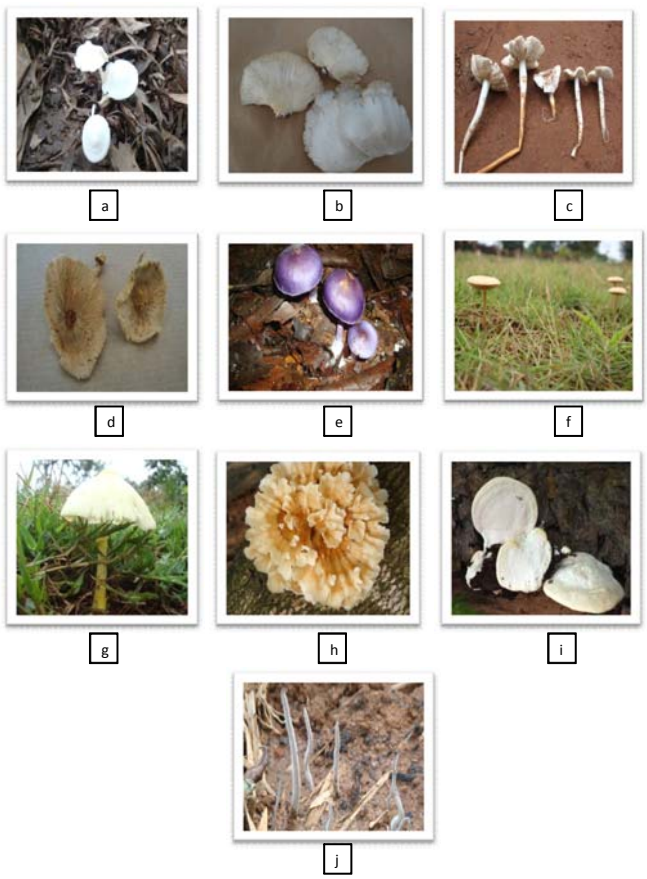

Fig. 2. Mushrooms collected from Shimoga regions of Karnataka. a) *L.squrossulus*, b) *P. salmoneostramenius*, c) *Termitomyces sp.*, d) *Termitomyces sp.* e) *L. purpureolilacinus*, f) *T. porphyrophyllum*, g) *Agrocybe pediades*, h) *Leucocoprinous bimbaumii*, i) *Podoscypha petalodes*, j) *Antrodia serialis*, k) *Xylaria sp*

samples were identified up to species level.

Molecular tools provide more accurate methods for identification of both prokaryotes and eukaryotes. The eleven mushrooms were identified up to species level by using ITS region sequence. PCR amplification of genomic DNA of the 11 mushrooms in this study yielded amplified product sizes varying from 455 bp to 773 bp (Table, 2) which were corresponding to almost full length gene sequence of ITS. The sequence homology of the11 species ranged from 83-99% (Table, 2) when aligned with the sequences present in NCBI Gen Bank. WGM-1 has 98% homology with *Lentinus squrossulus,* WGM-2 has 99 % homology with *Pleurotus salmoneostramenius* (Fig.1). WGM-3 has 99% homology with *Termitomyces* sp.WGM-4 showed 97% homology with *Termitomyces* sp. WGM-5 showed 90% homology with *Leucoagaricus purpureolilacinus,* WGM-6 with 83% homology with *Triholosporum porphyrophyllum*, WGM-7 with 99% homology for *Agrocybe pediades*, WGM-8 with 99% homology for *Leucocoprinus birnbaumii,* WGM-9 showed 99% homology with *Pedoscypha petalodes*, WGM-10 had 89% homology with *Xylaria* sp. and WGM-11 had 99% homology with *Antrodia serialis.*

The ITS region/ 18S rRNA gene sequence are the most widely used techniques in molecular phylogenetics of mushroom as these sequences are conserved irrespective of life history and evolution (Rajaratnam and Thiagarajan, 2012). An edible mushroom from the Theertha halli forest area of Western Ghats of Shivamoga district of Karnataka was identified using ITS region of ribosomal DNA sequences as *Termitomyces* sp. (Earanna *et al.,* 2013). Our study documented the abundance of the 11 mushroom flora from the Western ghats region (Shimoga) of Karnataka.

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