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A Study on the Diversity and Habitat Specificity of Macrofungi of Assam, India

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ABSTRACT

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Studies on the taxonomy and diversity of macrofungi have gained significance during the recent years, as many macrofungi are facing the risk of extinction because of habitat destruction. We report, here a systematic and detailed study on the diversity and distribution of wild mushrooms in the state of Assam, India, based on phenotypic and molecular characteristics. A total 44 samples were collected from various locations of Assam in different seasons during 2013-2015. Out of the total 44 samples, about 18 macrofungi species occurred during summer season, 9 species were recorded during rainy, 13 species were frequently found during autumn, 5 species in winter and 8 species were prominent during spring. The soil was found as major habitat for 18 collected species, while 10 species were collected from the tree/hardwood tree. This study indicated that the diversity and distribution of macrofungi are dependent on the plant community and the environmental conditions. Molecular characterization based on rDNA -ITS- ((Internal transcribed spacer) sequences revealed that the 44 samples belonged to 16 macrofungal families. Out of the 44 samples, 23 samples were reported to be edible and amongst the rest 21 non-edible strains, 5 strains have medicinal properties, 6 strains are poisonous, two has industrial application and the rest have not been fully explored.

Introduction

Fungi play a significant role in industry, agriculture, medicine, food industry, textiles, and bioremediation (Danielson *et al.*, 1989; Manzi *et al.*, 1999; Krzywinski *et al.*, 2009; Changet *al.*, 2004). Fungi can be broadly classified into three different forms viz., yeast, moulds and macrofungi or mushrooms. Macrofungi are large, conspicuous spore-bearing structures and have fleshy, tough umbrella like sporophores that bear holobasidia on the surface of gills or lamellae that hang down from the cap belonging to basidiomycetes and ascomycetes (few under

Zygomycota) (Alexopoulos *et al.*, 2000). Macrofungi are found in varied types of habitats, depending on the composition of tree species and other substrates. The distribution of macrofungal species is low in hot and dry seasons while they are abundant in spring and autumn due to favourable climate and abundance of flora during that time (Pilz *et al.*, 2001). Similar to yeast and moulds, macrofungi also possess immense biological and economic impact since they serve as food, medicine, biocontrol agents and produce of bioactive compounds such as anti-oxidant,

anti-diabetic, hypocholesterolemic, anti-tumor, anti-cancer, immunomodulatory, anti-allergic, nephroprotective, and anti-microbial agents (Patel *et al.*, 2012). Macrofungi are used in the bioremediation of industrial waste and in the accumulation of heavy metals from the environment (Tuli *et al.*, 2013). There are about 2166 worldwide recognized edible species that include about 470 species possessing medicinal properties. Furthermore, macrofungi are useful indicators of health or age of an ecosystem (Boa *et al.*, 2004).

Assam, situated in the Northeast of India is a constituent unit of the Eastern Himalayan Biodiversity Region; one of the two biodiversity "Hot Spots" in the country. The climatic conditions along with varied physical features in Assam, makes it rich in diversity of ecological habitats such as forests, grasslands, wetlands, which harbour and sustain wide range of floral and faunal species. Assam has a sub-tropical monsoon climate with an average rainfall of around 1,500 mm per year. The day-time temperature in summer, the rainy season, rises to around 35°C and in winter cools to 25°C with a night time minimum of around 10°C which is favorable for the growth of most of the macrofungal species (Das *et al.*, 2005). Although, macrofungi are good resources for agro-industrial, medicinal (Veena *et al.*, 2012) and commercial purposes, very few studies have reported the macrofungal diversity of Northeast India (Khaund and Joshi, 2014). Several varieties of macrofungi are being consumed by the local tribes of Assam which are harvested from wild habitats but no efforts have been made to cultivate these macrofungi for commercial production. Moreover, there are no scientific reports related to characteristics of the wild edible macrofungi which are slowly disappearing due to habitat loss. Because of these reasons, the farmers and entrepreneurs of Assam have not been able to exploit its agro-economic

potential. Therefore, it is crucial to document the diversity, distribution and abundance of these macrofungi in Assam. Such study will not only pave the way towards domestication and commercialization of the wild species for economic benefits but will also help in establishing a strong basis for molecular taxonomy. In this study we aimed to collect macrofungi from different habitats of Assam followed by their taxonomic evaluation using phenotypic and molecular characteristics.

Materials and Methods

Collection of macrofungi

Systematic and periodical survey of different forests, tea gardens and other habitats were undertaken during November, 2013 to April, 2015. For this study, the opportunistic sampling of macrofungi protocol, was followed (Mueller *et al.*, 2004). The sampling method involved walking throughout the entire study region and collecting the macrofungi covering up to 80% of the area. Sampling of the ground-dwelling macrofungi involved careful extraction of the fruit body from the soil and leaf litter, ensuring that the basal surface was intact. The tree-dwelling macrofungi were sampled by cutting off the basidiomata from the trees without disturbing the spore surfaces present below. Different species exhibit different fruiting phenologies, which vary from month to month and at different altitudes and regions.

The survey and collection of mushroom was done from different locations of Assam viz. Nalbari, Barpeta, Jorhat, Sivasagar, Karbi Anglong, Kamrup, Golaghat, Goalpara, Tezpur, Tinsukia, Rangia and Nagaon under different agro-climatic conditions, during the year 2013-2015. The macrofungal samples were collected in different seasons of the year; rainy season (July and August), winter (December and January), spring (March and

April) and summer (May and June) during 2013-15. Soft macrofungi were collected carefully by using forceps/free hand, while the macrofungi growing on wood were collected along with small part of wood. All collected samples were carefully wrapped in aluminum foil, placed in an air-tight zip-lock bag and labeled with the collection number, location, date, and other data. The photograph of the mushroom was taken in their natural habitat.

Morphological characterization

Morphological characterization of the macrofungal strains were carried out based on the cap size, cap structure, colour of the fruiting body, dimensions of stipe, gill shape and gill colour as described earlier (Kumar *et al.*, 2015). Further, the fresh sporocarps of mushroom species were cultured to obtain vegetative mycelium from the tissue (small pieces cut from section between the pileus and stipe) using Potato Dextrose Agar (PDA) media and incubated at $25 \pm 2^\circ\text{C}$.

Molecular characterization and phylogenetic analysis of the macrofungal strains

For Molecular characterization of the 44 macrofungal isolates were performed by sequencing the rDNA internal transcribed spacer (ITS) region. For DNA extraction, individual strains were cultured on PDA at $25 \pm 2^\circ\text{C}$ for 7 days to obtain mycelia. The genomic DNA was extracted from the pure mycelia using the protocol of Singh *et al.*, (2003). Amplification of the ITS (Internal transcribed spacer) region of the nuclear ribosomal repeat unit was done using universal primer set ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Thatoi *et al.*, 2014). PCR reaction mixture was prepared as per TaKaRa Ex Taq® DNA

polymerase protocol (Takara, Japan) with 20 pmols of each primer, 2U Taq DNA polymerase and 50 ng genomic DNA in a final volume of 50 μL and amplification was performed using a thermal cycler (Applied Biosystems, USA). The PCR program was as follows: initial denaturing step of 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 49°C for 1 min and elongation at 72°C for 1 min; a final extension step at 72°C for 7 min. The amplified products were sequenced as per the BigDye Terminator sequencing protocol in an ABI 377 automated DNA Sequencer (Applied Biosystems, USA). The ITS rDNA sequence reads obtained after sequencing were assembled into contig using CodonCode Aligner (CodonCode Corporation, USA). Sequence similarity tool BLAST was employed to find the similarity of the sequences with known 16S rDNA sequences in the GenBank database and the sequences were deposited in GenBank of NCBI to obtain the accession numbers. Phylogenetic analysis of the isolates was carried out based on ITS-rDNA sequences in MEGA6 software (Tamura *et al.*, 2013). A phylogenetic tree was constructed using the ITS-rDNA sequences of the macrofungal strains along with other similar or related macrofungal ITS-rDNA sequences retrieved from the NCBI GenBank. The sequences were aligned with Clustal W using default parameters and a neighbor-joining tree was constructed using MEGA6 software (<http://www.megasoftware.net/>) with Kimura-2 parameter correction and 1000-step bootstrap.

Results and Discussion

Sample collection and morphological characterization

A total of 44 macrofungal samples were collected from different places of Assam, viz., Nalbari, Barpeta, Jorhat, Sivasagar,

KarbiAnglong, Kamrup, Golaghat, Goalpara, Tezpur, Tinsukia, Rangia and Nagaon (Fig. 1) during the study period. The details of the sample collection sites are given in Table 1.

Several workers have studied the diversity of macrofungi from different parts of India namely Kashmir (Sheikh *et al.*, 2014), Garhwal (Vishwakarma *et al.*, 2012), Tamil Nadu (Mani *et al.*, 2009). However very less number of reports appeared from north eastern part of India and only a few attempts have been initiated to document macrofungi from this area. An attempt was made by Gogoi and Sarma to find out some edible macrofungi of Dhemaji district of Assam (Gogoi *et al.*, 2012).

The morphological taxonomic system of classification given by Ainsworth (1963) and modified by Webster (2007) was used for characterization of the macrofungal samples collected from different locations (Table 2). Macroscopic features of the carpophores pertaining to pileus, lamellae, stipe, etc. which play an important role in the taxonomy of macrofungi have been discussed. The photographs of the collected macrofungal samples are shown in Figure 2(a) and 2(b). The fruiting bodies of macrofungi were found to be distinctly different from each other. Some species that grew on wood had caps that attach directly to the wood (e.g. *Pycnoporus*, *Ganoderma*). In some cases, the cap was semi-circular and attached by the straight edge while in others the stalk (also called the stipe, or stem) may be central and support the cap in the middle and in *Pleurotus*, the stalk was off-central or lateral. The morphological data of the collected samples are detailed in Table 2. Similar studies were carried out by Ram *et al.*, (2010) where several edible fleshy fungi were collected from the wilds in Eastern Uttar Pradesh during the rainy season on dead and decaying plant or animal remains. Kalita *et*

al., (2016) documented wild edible macrofungi from Shyrwat and Upper Shillong Reserve Forests of Meghalaya, Northeast India where a total of 22 macrofungi were collected during the rainy season (July to September) 2014, and identified on the basis of macroscopic and microscopic characteristics. The diversity of wild macrofungi in Nagaland, India was reported by Toshinungla *et al.*, (2016) where a total of 87 species of wild macrofungi were collected and identified and out of the collected macrofungi, 37 species were identified as edible, 21 species medicinal, 5 poisonous and 37 inedible/unclassified. Surveys were conducted in Narpuh Reserve Forest, Meghalaya, India to unveil the macrofungal diversity and identified a variety of macrofungi on the basis of macro- and microscopic characteristics.

Seasonal occurrence of the macrofungal species

Climatic and edaphic conditions prevailing in Assam favoured the occurrence of diverse macrofungi. Our study revealed that 18 species occurred during the summer season, 9 species were recorded during rainy, 13 species were frequently found during autumn, 5 species during winter and 8 species grew mostly in the spring. Comparison of the seasonal occurrence is depicted in Figure 3. Some species were observed to overlap two seasons for e.g. *Pleurotus sapidus*, *Chlorophyllum molybdites* were found both in rainy and autumn season; *Physisporinus vitreus* in both autumn and winter season; *Bolbitius titubans* and *Auricularia sp.* grew both during summer and rainy season. Moisture is one of the major factors influencing the fruiting of macrofungi. During the rainy seasons, the availability of adequate moisture favor the growth of the macrofungi. Species diversity was higher in the rainy seasons compared to the early dry seasons, as

there is adequate moisture available during the rainy seasons. While the macrofungal species collected in the early dry season was predominated by the Polyporales, since there is a decrease in rainfall and relative humidity along with increase in temperature and sunshine, most of the fleshy macrofungi cannot withstand these conditions.

Singh and Prasad (2003) recorded a number of mushroom spp. during 1997-98 and 1998-1999. The occurrence of species was found to be greater in September during 1997-98 and in August in 1998-99, while July and October exhibited a lesser number of macrofungi. However, in another investigation, July month of each year (2002-05) showed a greater number of macrofungal species. A varying number of macrofungi of different species in different months may be due to low relative humidity, low temperature range, very little and no rainfall.

It was observed that the diversity of the macrofungal species depend on the habitat (Fig. 4). The highest numbers of samples were from Golaghat followed by Nagaon and Goalpara districts of Assam. The soil was found to be the best source of diverse macrofungal species followed by tree and deadwood. A variety of habitats *i.e.* soil, tree, wood, litter, lawn, etc. supports growth for macrofungi and soil was found as major habitat for 18 collected species, while 10 species were collected from the tree/hardwood tree. The variation observed in occurrence of mushroom species in various habitats may be due to their particular mode of nutrition; the macrofungi growing on the soil are symbiotic, on rotting and dead wood are saprophytic and on trees are parasitic. Cosmopolitan distribution of macrofungi have also been reported (Suryanarayanan *et al.*, 2004), certain species of mushroom are associated with particular kinds of trees and plants (Hawksworth, 2001).

Table.1 GPS data for the macrofungal sample collection sites

Sites	Latitude	Longitude	Elevation(m)
Mariani	26°39'26" N	94°18'55" E	118
Goalpara	26° 10' 12.8028" N	90° 37' 35.8212" E	44
Jorhat	26° 45' 27N	94° 12' 11E	111
Diphu	25°50'36"N	93°25'52"E	2000
KarbiAnglong	26°35' N	93°50' E	186
Sibsagar	26° 59' 3N	94° 38' 16E	95
Kamrup	26° 26' 57N	91° 36' 49E	38
Golaghat	26° 30' 42N	93° 57' 34E	90
Barpeta	26° 19' 22N	91° 0' 23E	27
Tezpur	26° 37' 60N	92° 47' 60E	47
Nagaon	26° 21' 0N	92° 40' 0E	54
Tinsukia	27°29'30.63"N	95°20'55.30"E	125
Rangia	26°26'28.61"N	91°37'7.11"E	53

Table.2 Morphological data of the collected macrofungal samples

Sl. No	Samples	Site of Collection	Habitat	Fruiting Body/Colour	Stipe	Pileus/Gills
1.	M3	Mariani	Tree	Sessile, corky. Scarlet red to flame orange	Absent. A small, single attachment point	5 cm in length. Pores on the underside
2.	Assam	Mariani	Hardwood tree	Flesh is white, firm, and varies in thickness. White to grey or tan to dark-brown	Absent. Cap laterally attached	Fan or oyster-shaped (11 cm). Decurrent with white to cream colour gills
3.	T1	Goalpara	Hardwood tree	Woody brackets. Cream to dark-brown	Absent	Fan-like or hoof-like form honeycomb gills
4.	O	Titabor, Jorhat	Rice straw	Flesh is white, firm, and varies in thickness	Absent. Cap laterally attached	Fan or oyster-shaped
5.	Sap	Diphu	Rice straw	Flesh is white, firm, and varies in thickness	Absent. Cap laterally attached	Fan or oyster-shaped (9 cm). Decurrent
6.	CN	AAU, Jorhat	Soil	Flesh is white, spongy, and varies in thickness	Absent	Conical (8.5 cm). White to cream in colour
7.	H10	AAU, Jorhat	Soil	Fleshy and varies in thickness. Brown	Present (7cm)	Depressed (4 cm). Extended outwards (brown in colour)
8.	K	Karbi Anglong	Dead wood	Tough with a well-developed central stalk. Flesh fibrous. White to pale brown	Tapering to base and a persistent ring is present on stipe (4 cm x 7mm)	Depressed (7 cm). Lamellate, (white to cream in colour)
9.	S1	Goalpara	Tree	Flesh is white, firm, and varies in thickness	Tapering to base.	Fan or oyster-shaped (6 cm) Decurrent white to cream in colour
10.	AM	Sibsagar	Soil	Flesh is cream, and varies in thickness. Grey to pale brown	A persistent ring is present on stipe (7cm x 20 mm)	Flat scales (4.3 cm) serrated, white to cream in colour
11.	HN	Kamrup	Hardwood tree	Corky, hard, woody-textured. dark-brown	Absent	Fan-like or hoof-like form. Decurrent (grey to brown in colour)
12.	N1	Diphu	Soil	Brown spongy well-developed central stalk. Flesh fibrous.	Absent	Radicating, fleshy (4.6 cm)
13.	V	AAU, Jorhat	Hardwood tree	Corky, hard, woody-textured. dark-brown	Absent	Kidney shaped (12 cm). Decurrent (brown in colour)

14.	N2	Rangia	Soil	White with shaggy scales	A loose ring around the stipe of 8 cm length	Gills change rapidly from white to pink, then to black.
15.	P2	Golaghat	Soil	Short brown blunt elevated lines on the lower surface of the cap and well developed with central stalk	Radicating, fleshy (4.6cm)	Depressed (1.3 cm). Decurrent (brown in colour)
16.	T2	Barpeta	Tree	Blunt elevated lines on the lower surface of the cap without central stalk	Absent	Depressed (4.3 cm). Free (cream in colour)
17.	H1	Tezpur	Tree, Wood	Shiny, dry and scaly extremely tough with a well-developed central stalk. Whitish to buff with cinnamon-brown scales.	9 cm x 6 mm, with a slight ring near the apex).	6 cm wide, lamellate gills on lower surface, decurrent, edge sterile, denticulate
18.	C1	AAU, Jorhat	Soil	Flesh is cream in colour, and varies in thickness. whitish -brown	Absent	3 cm wide, top-shaped. white to cream in colour
19.	G2	Nagaon	Soil	Elevated lines on the lower surface of the cap without central stalk. Yellowish white	4 cm x 3 mm, with a slight ring near the apex)	Umbonate. Free (cream in colour)
20.	H2	Golaghat	Soil	Blunt elevated lines on the lower surface of the cap with central stalk. whitish	Fleshy (5.6cm)	Depressed (1.3 cm) brown in colour
21.	H4	Jorhat	Soil	Blunt elevated lines on the lower surface of the cap without central stalk	Radicating, fleshy (2.3cm)	Umbonate. Free (cream in colour)
22.	H7	Golaghat	Soil	Blunt elevated lines on the lower surface of the cap with central stalk	Tapering to base and a persistent ring	Flat (9.3 cm). (Greyish in colour)
23.	H6	Nagaon	Soil	Brownish elevated lines on the lower surface of the cap without central stalk	Tapering to base and a persistent ring is present on stipe	Umbonate. Free (cream in colour)
24.	AP2	Barpeta	Soil	a brick-red colouration in the center	Light yellow (4.5–7 cm in diameter)	Crowded, yellowish

25.	AP3	Golaghat	Dead tree	Basidiocarps, 5 mm thick, waxy and soft when fresh, hard and cartilaginous when dry	5 mm thick, greyish pore surface	Pore surface white to bluish white and translucent
26.	AP5	Barpeta	rotting wood	Cream or light covered in a fine white bloom convex, 4-12cm	Centrally depressed attached to the substrate via an eccentric stem	White; up to 3.5 cm long and 2.5 cm diameter
27.	AP6	Sibsagar	Wood	Oyster shell-shaped to fan-shaped and colour ranges from ivory white to pinkish buff to orange-grey	Absent	Decurrent, running a short ways down the stipe-10 mm broad, white to cream in colour
28.	AP7	Sibsagar	Soil	Pale fawn tan or pale brownish towards the centre	Stem 13 cm x 3-10 mm, centric to excentric	Pileus 1.5-8 cm wide, densely decurrent gills
29.	AP8	Tinsukia	Soil	Brownish or greyish cap measures 17 cm in diameter and is covered with coarse darker brown scale	Pale grey or brown stem (8 cm) and (3.1 in) high and 3 cm (1.2 in) wide	The underside bears soft, pale grey 'teeth' rather than gills
30.	AP9	Nagaon	Wood	Fibrous-corky, usually 3-10 cm in diameter, attached directly to the substrate	Absent	Soft tomentose, semi-circular to elongate Pore surface pale orange
31.	AP11	Tinsukia	Bark of Tree	Gelatinous, orange-yellow fruit body of the fungus, which can grow up to 4.5 cm diameter	Absent	Irregular shape, and usually breaks through the bark of dead branches
32.	AP12	Tinsukia	Lawn	White or pale yellowish developing pressed-down fibers or scales	7 cm tall, the parallel stem with a small double ringed bulb at its base	White and finely scaly, the cap
33.	AP13	Goalpara	Tree	Basidiocarps consist of numerous rosette-like flattened fan-shaped pileus	Absent	Cap surface is pale tan to dull chestnut brown with finely fibrillose tiny scales (squamules)
34.	AP14	Goalpara	Soil	Cap is 1.5 - 5 cm, and grows from egg-shaped when young to broadly	3-10 cm tall and 2-4 mm wide, whitish-yellow colour	Free from the stem and fragile. Whitish or pale yellowish to rusty

				convex		cinnamon
35.	AP15	Golaghat	Forest	Smaller yellow-to-lilac-capped bonnet mushroom	White or pale pink, smooth with longitudinal fibres, with no stem ring	Pink and crowded, the broad gills are deeply sinuate
36.	AP16	Golaghat	Dead wood	White, thin and tough scaly with brown to golden brown scales and fibrils	Central or slightly off-center; 2.5 cm long; 3 mm wide; brown to yellowish brown; scaly to hairy	Running down the stem; whitish
37.	AP17	Dergaon	Tree	Colour of the cap is purple to pinkish brown which is densely haired	3 cm long; 1 cm wide; tough; off-centre densely hairy	Running down the stem; crowded; purplish. Gills decurrent
38.	AP1	Titabar	Dead log	Yellow to brown and has scales	The stalk is thick and short, 5 cm long	Gills decurrent
39.	AP19	Kamrup	Soil	Orange in colour and has a calfskin-like texture	Overlapping clumps, and 4 cm thick	Wide, shaped like a fan and attached direct to the trunk of a tree
40.	AP20	Kamrup	Soil	Brown hygrophanous cap (striate when fresh), the disc usually dingy yellowish-brown	5 cm tall, 2.5 mm thick, slender, thin, fragile	Gills adnexed, moderately broad
41.	AP21	Jorhat	Litter	Yellow to brown with orange caps	8 cm long and 2 cm in diameter, cylindrical; smooth, with fine longitudinal fibres	Adnate; crowded; yellow
42.	AP2	Golaghat	Tree	Flesh is white, firm, and varies in thickness. White to grey or tan to dark-brown	14 cm, convex, Short and offset from the centre of the cap	Gills: Decurrent
43.	AP24	Dergaon	Dead log	Reminiscent of a floppy ear, cup-shaped and has a tough, gelatinous, elastic texture	Attached to the substrate laterally with very short stalk	Gelatinous gills
44.	AP25	Nagaon	Lawn	Brown or beige to straw colour	6 cm tall x 2 mm thick, equal, hollow, straw colour and annulus absent	Gills are adnate or adnexed, grey to purple-brown with whitish

Table.3 Nucleotide nblast results of the isolates

Sl.No	Samples	Sequencing Result	Accession No.
1.	M3	<i>Pycnoporus coccineus</i>	KJ862075
2.	Assam	<i>Pleurotus sapidus</i>	KJ862064
3.	T1	<i>Ganoderma lucidum</i>	KJ862063
4.	O	<i>Pleurotus sapidus</i>	KJ862076
5.	Sap	<i>Pleurotus ostreatus</i>	KM609393
6.	CN	<i>Lycoperdon pyriforme</i>	KM609394
7.	H10	<i>Lactarius friabilis</i>	KM609395
8.	K	<i>Lentinus sajorcaju</i>	KJ862073
9.	S1	<i>Pleurotus floridanus</i>	KJ862077
10.	AM	<i>Chlorophyllum molybdites</i>	KM609396
11.	HN	<i>Ganoderma sp</i>	KM609397
12.	N1	<i>Lentinus sp</i>	KM609398
13.	V	<i>Ganoderma applanatum</i>	KM609399
14.	N2	<i>Coprinus spp.</i>	KM609400
15.	P2	<i>Agrocybe pediades</i>	KM609401
16.	T2	<i>Ganoderma sp</i>	KM609402
17.	H1	<i>Lentinus critinus</i>	KM609403
18.	C1	<i>Hymenopellis megalospora</i>	KM609404
19.	G2	<i>Leucoagaricus leucothites</i>	KM609405
20.	H2	<i>Agaricus campestris</i>	KM609406
21.	H4	<i>Macrolepiota rhacodes</i>	KM609407
22.	H7	<i>Macrolepiota procera</i>	KM609408
23.	H6	<i>Coprinus disseminatus</i>	KM609409
24.	AP2	<i>Hypholomas ublateritium</i>	KR824074
25.	AP3	<i>Physisporinus vitreus</i>	KR824075
26.	AP5	<i>Pleurotus cornucopiae</i>	KR824077
27.	AP6	<i>Pleurotus populinus</i>	KR824078
28.	AP7	<i>Panus lecomtei</i>	KR824079
29.	AP8	<i>Sarcodon imbricatus</i>	KR824080
30.	AP9	<i>Fomitopsis streiformis</i>	KR824082
31.	AP11	<i>Dacrymyces sp</i>	KR824083
32.	AP12	<i>Agaricus arvensis</i>	KR824084
33.	AP13	<i>Meripilus giganteus</i>	KR824085
34.	AP14	<i>Bolbitius titubans</i>	KR824086
35.	AP15	<i>Mycena rosea</i>	KR824087
36.	AP16	<i>Polyporus arcularius</i>	KR824088
37.	AP17	<i>Lentinus strigosus</i>	KR824089
38.	AP1	<i>Polyporus squamosus</i>	KR824090
39.	AP19	<i>Laetiporus sulphurous</i>	KR824091
40.	AP20	<i>Psathyrella corrugis</i>	KR824092
41.	AP21	<i>Gymnopilus dilepis</i>	KR824093
42.	AP2	<i>Pleurotus pulmonarius</i>	KR824093
43.	AP24	<i>Auricularia sp</i>	KR824096
44.	AP25	<i>Psilocybe mexicana</i>	KR824097

Table.4 Classification study of macrofungi from different locations of Assam

ORDER	FAMILY	GENUS	SPECIES	USE
Polyporales	Polyporaceae	<i>Pycnoporus</i>	<i>coccineus</i>	Industrial
		<i>Lentinus</i>	<i>sajorcaju</i>	Edible
		<i>Lentinus</i>	<i>sp.</i>	Edible
		<i>Lentinus</i>	<i>critinus</i>	Edible
		<i>Lentinus</i>	<i>strigosus</i>	Edible
		<i>Laetiporus</i>	<i>sulphurous</i>	Edible
		<i>Panus</i>	<i>lecomtei</i>	Edible
		<i>Polyporus</i>	<i>squamosus</i>	Medicinal
		<i>Polyporus</i>	<i>arcularius</i>	Edible
	Meripilaceae	<i>Physisporinus</i>	<i>vitreus</i>	Industrial
		<i>Meripilus</i>	<i>giganteus</i>	Edible
	Fomitopsidaceae	<i>Fomitopsis</i>	<i>ostreiformis</i>	Non-Edible
	Ganodermataceae	<i>Ganoderma</i>	<i>sp.</i>	Non-Edible
		<i>Ganoderma</i>	<i>applanatum</i>	Medicinal
		<i>Ganoderma</i>	<i>lucidum</i>	Medicinal
		<i>Ganoderma</i>	<i>sp.</i>	Non-Edible
Agaricales	Pleurotaceae	<i>Pleurotus</i>	<i>Sapidus</i>	Edible
		<i>Pleurotus</i>	<i>Sapidus</i>	Edible
		<i>Pleurotus</i>	<i>ostreatus</i>	Edible
		<i>Pleurotus</i>	<i>floridanus</i>	Edible
		<i>Pleurotus</i>	<i>cornucopiae</i>	Edible
		<i>Pleurotus</i>	<i>pulmonarius</i>	Edible
		<i>Pleurotus</i>	<i>populinus</i>	Edible
	Agaricaceae	<i>Lycoperdon</i>	<i>pyriforme</i>	Edible
		<i>Coprinus</i>	<i>sp.</i>	Non-Edible
		<i>Agaricus</i>	<i>arvensis</i>	Edible
		<i>Macrolepiota</i>	<i>rhacodes</i>	Non-Edible*
		<i>Macrolepiota</i>	<i>procera</i>	Non-Edible*
		<i>Chlorophyllum</i>	<i>molybdites</i>	Non-Edible*
		<i>Coprinus</i>	<i>disseminatus</i>	Non-Edible*
		<i>Leucoagaricus</i>	<i>leucothites</i>	Non-Edible
		<i>Agaricus</i>	<i>arvensis</i>	Edible
		<i>Agaricus</i>	<i>campestris</i>	Edible
	Bolbitiaceae	<i>Bolbitius</i>	<i>titubans</i>	Non-Edible
	Mycenaceae	<i>Mycena</i>	<i>roseae</i>	Non-Edible
	Strophariaceae	<i>Agrocybe</i>	<i>pediades</i>	Non-Edible*
		<i>Psilocybe</i>	<i>mexicana</i>	Non-Edible*
		<i>Hypholoma</i>	<i>sublaterium</i>	Non-edible
	Physalacriaceae	<i>Hymenopellis</i>	<i>megalospora</i>	Non-Edible
	Cortinariaceae	<i>Gymnopilus</i>	<i>dilepis</i>	Non-Edible
	Psathyrellaceae	<i>Psathyrella</i>	<i>corrugis</i>	Non-Edible
	Russulales	Russulaceae	<i>Lactarius</i>	<i>friabilis</i>
Thelephorales	Bankeraceae	<i>Sarcodon</i>	<i>imbricatus</i>	Edible
Dacrymycetales	Dacrymycetaceae	<i>Dacrymyces</i>	<i>sp.</i>	Non-Edible
Auriculariales	Auriculariaceae	<i>Auricularia</i>	<i>sp.</i>	Medicinal

*Poisonous macrofungi (Thatoiet *al.*, 2004)

Fig.1 a) Map of India highlighting Assam b) Satellite image of Assam with different districts of Assam c) Satellite image of Assam showing different sites of collection of macrofungi

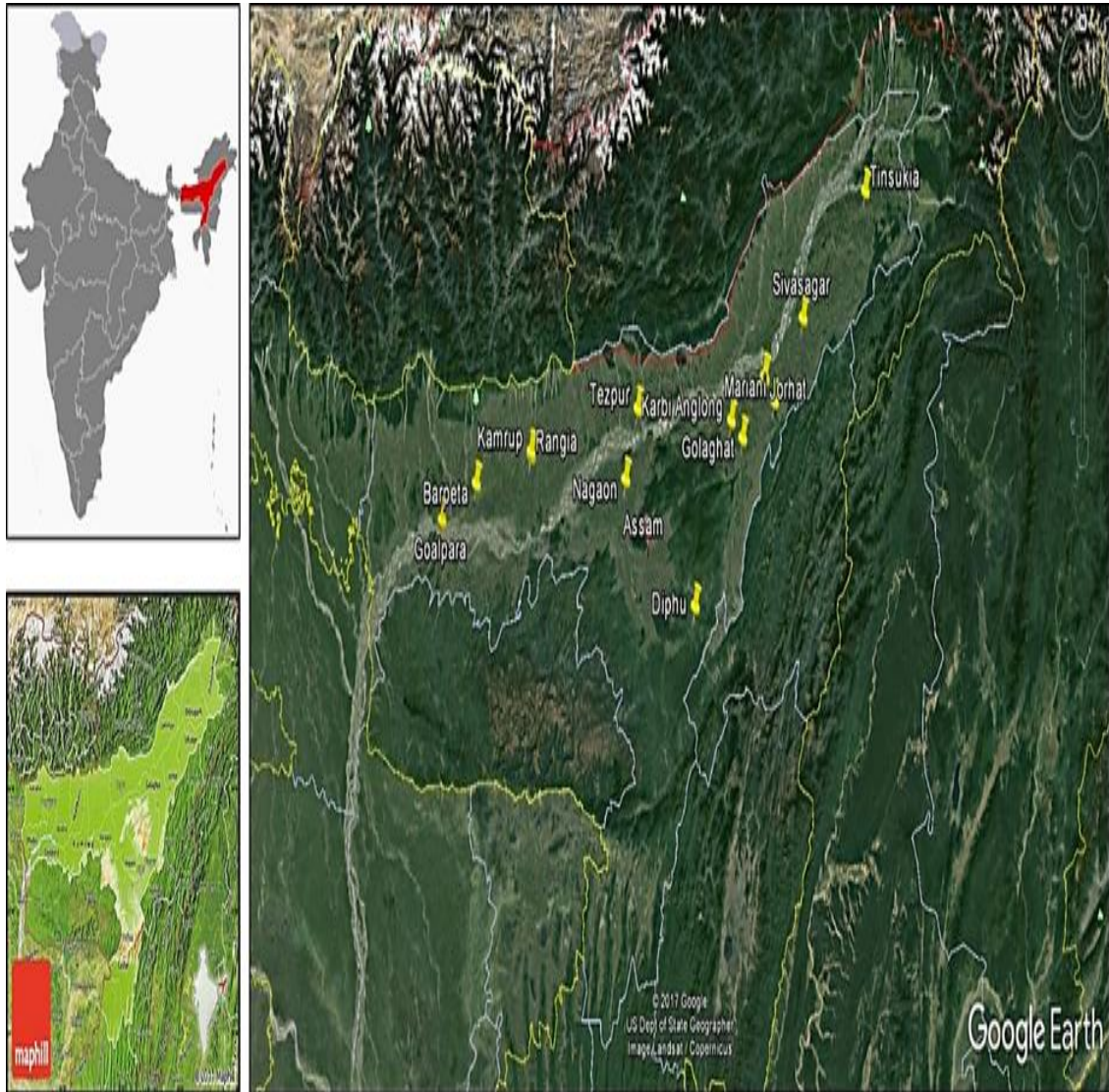


Fig.2 (a) Fruiting bodies of the collected samples. 1= M3, 2=Assam, 3 =T1, 4=O, 5= Sap, 6= CN,7=H10, 8= K, 9=S1,10= AM,11= HN,12= N1,13= V, 14= N2,15= P2,16= T2,17= H1,18= C1,19= G2, 20=H2, 21=H4, 22= H7, 23= H6, 24=AP2

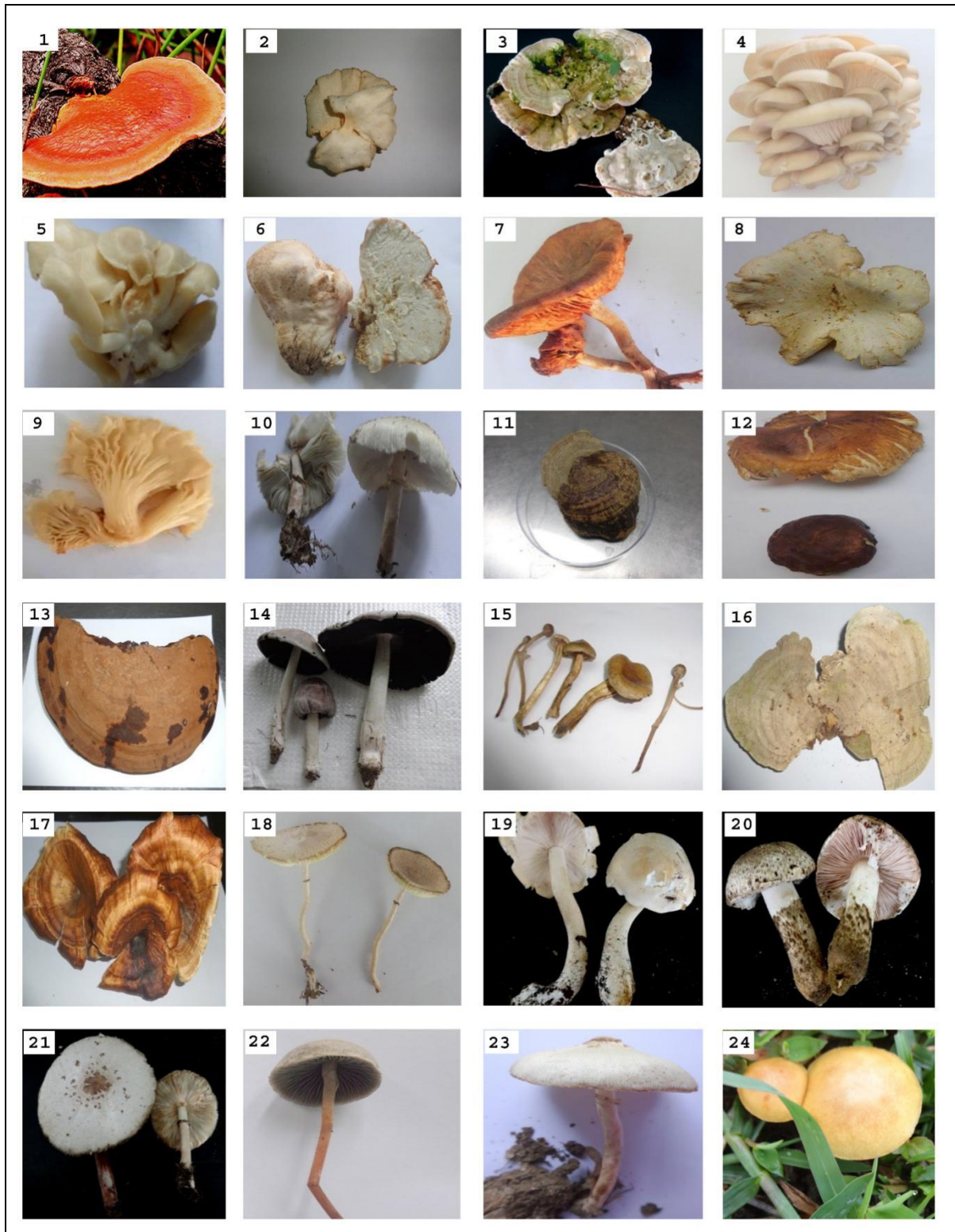


Fig.2 (b) Fruiting bodies of the collected samples. 25= AP3, 26= AP5, 27= AP6, 28= AP7, 29= AP8, 30= AP9, 31= AP11, 32= AP12, 33= AP13, 34=AP14, 35= AP15, 36= AP16, 37= AP17, 38= AP1, 39= AP19, 40= AP20, 41= AP21, 42= AP2 43= AP24, 44=AP25)

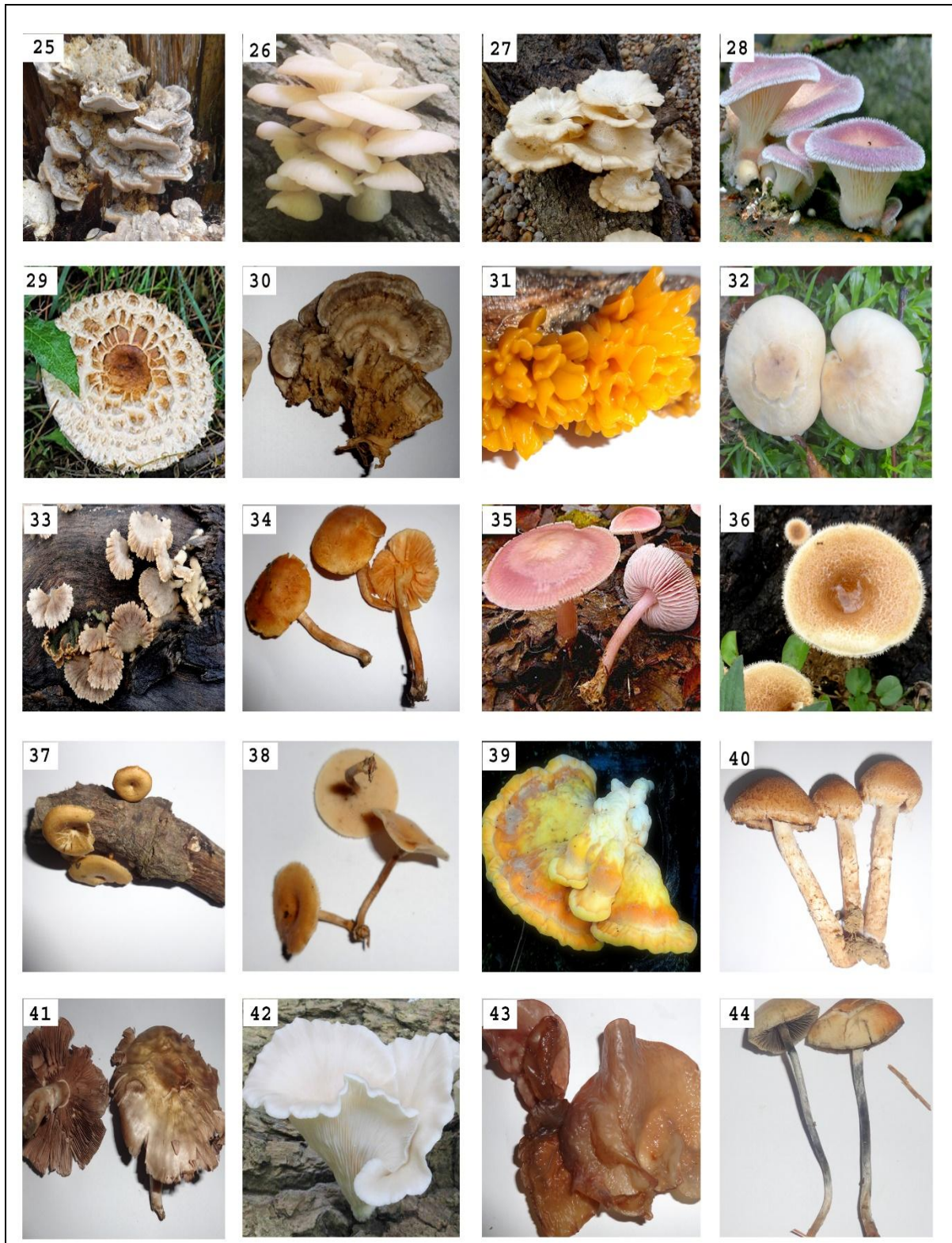


Fig.3 Seasonal distribution of the macrofungal species. Rainy season (July and August), winter (December and January), spring (March and April) and summer (May and June) during 2013-15

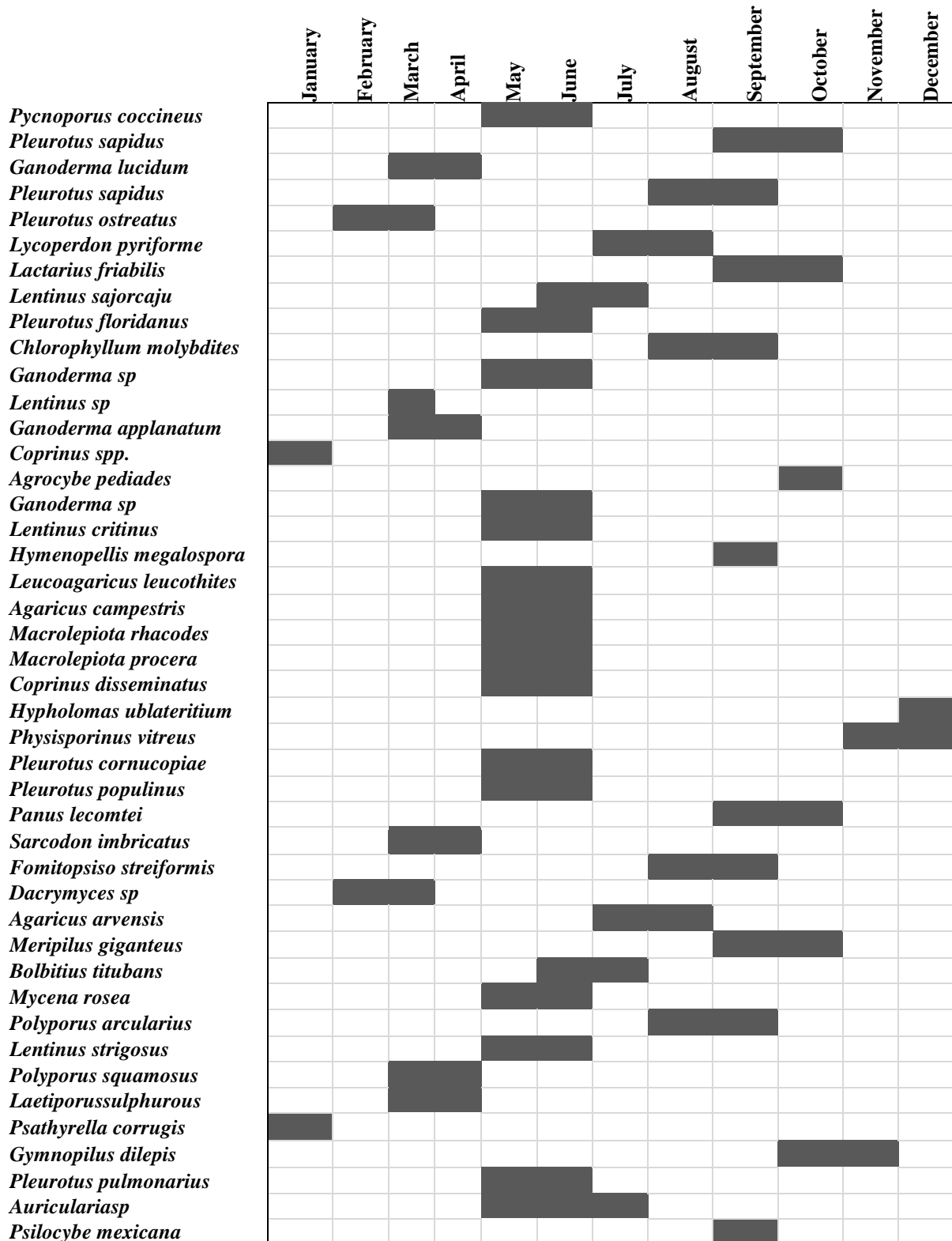


Fig.4 CIRCOS illustration of the samples depicting the relationship between the v macrofungi with that of the site of collection and habitat

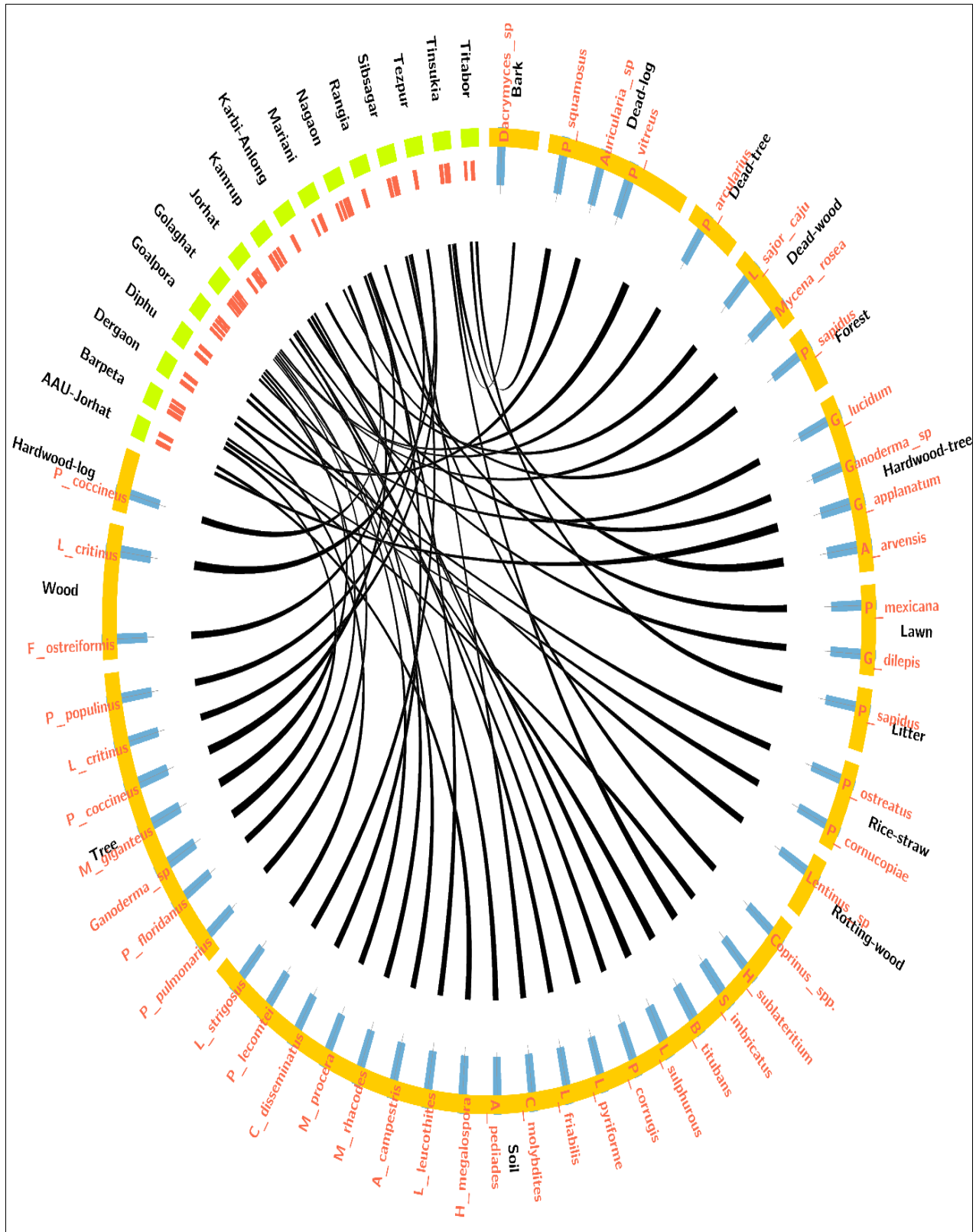


Fig.5 Evolutionary relationships of macrofungal species (NJ Tree Method)

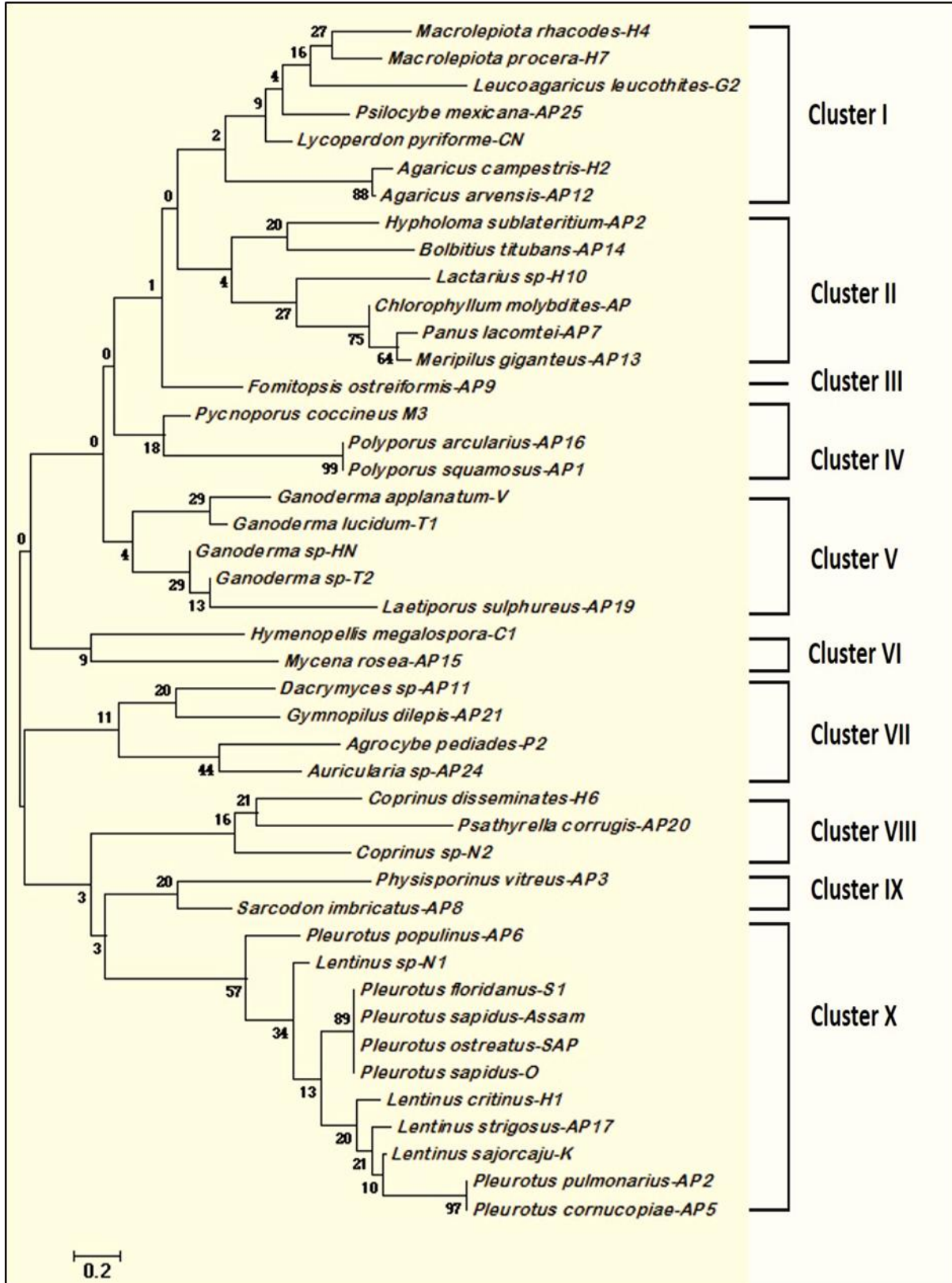
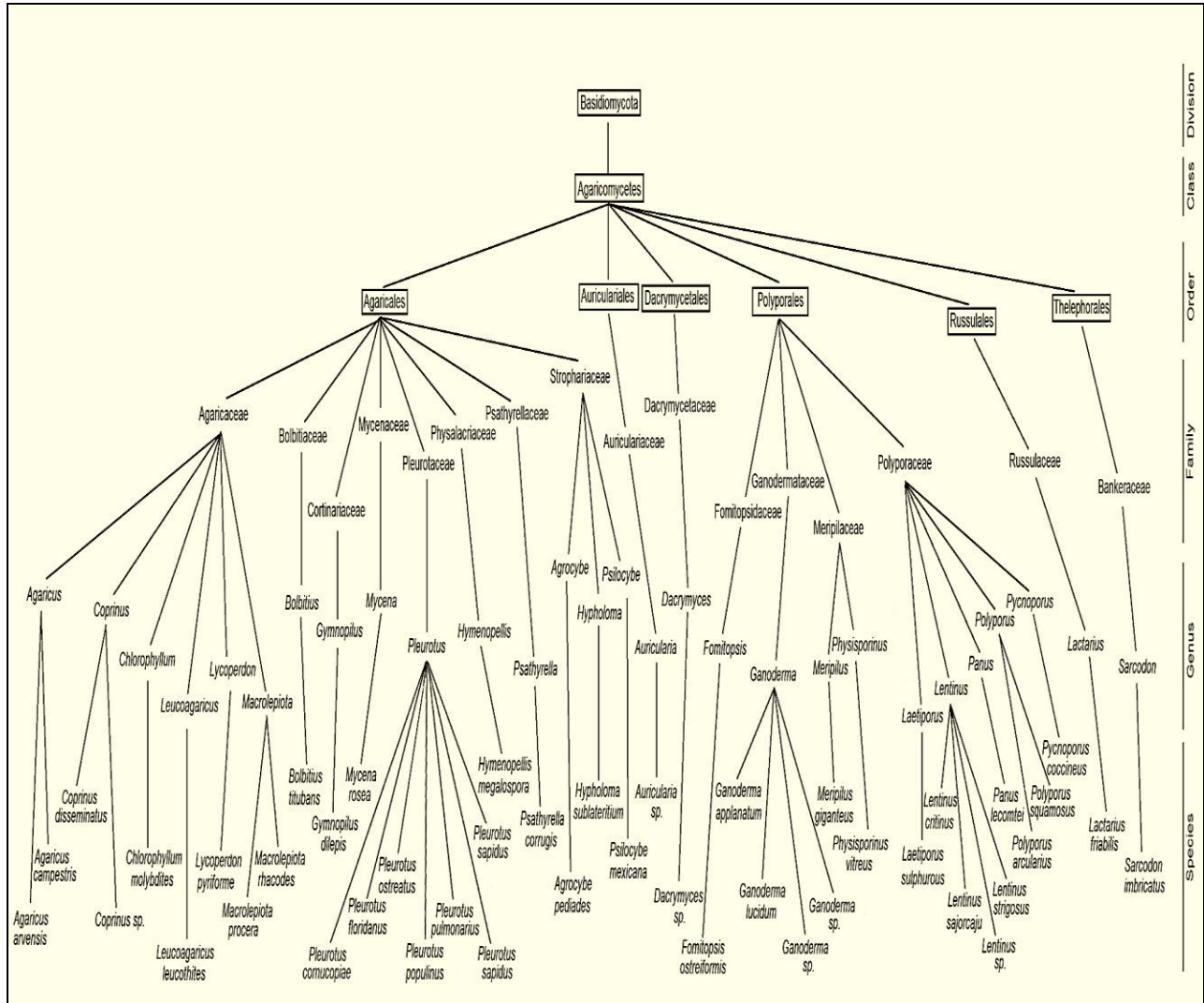


Fig.6 Scientific classification of the identified strains based on molecular data



Molecular characterization of the macrofungal strains

The genomic DNA was subjected to PCR-amplification for the ITS region of the rDNA using ITS1 and ITS4 primers. ITS regions of fungal ribosomal RNA are highly variable sequences, which can be useful markers for determining fungal species. The approximate size of the amplified PCR products (~750bp) was purified and sequenced using ABI 377 automated DNA Sequencer (Applied Biosystems, USA) and resulting contigs were analyzed with CodonCode Aligner version

4.1 (CodonCode Corporation, USA). The consensus sequence obtained for each strain was compared with the ITS rDNA sequences of fungi available in NCBI through a BLAST search and the sequence data has been submitted to GenBank, NCBI and the accession numbers were obtained (Table 3). The sequences were aligned with the help of Codoncode Aligner and analysed for homology using nBLAST (NCBI) to obtain possible identities (Altschul *et al.*, 1997). The BLAST results displayed 97-99% homology with the 44 isolates under study (Table 3). The comparison of ITS gene from different

strain and different species indicates that there exists high species-specific homogeneity.

Many molecular markers have been applied in phylogenetic studies in order to decipher the relationships among species of Basidiomycetes (Shtaer *et al.*, 2005; Shnyreva and Shtaer, 2006). However, internal transcribed spacer (ITS) I and II the two non-coding polymorphic regions, located between highly conserved regions of 18S, 5.8S and 28S rRNA genes (Agarwal *et al.*, 2015), have been widely employed to assess the phylogenetic relationships of mushroom species (Choi *et al.*, 2007). In India, Singh *et al.*, (2006) first reported molecular characterization of mushroom using ITS primers specific to macrofungi and identified 18 mushroom germplasms based on ITS sequence polymorphism. Seven of these isolates were identified as *Podaxis pistillaris*, six belonged to *Phellorinia herculean*, four were identified as belonging to *Phellinus igniarius* and only one was identified as *Gymnopilus subearlei*. A recent report by Rajaratnam *et al.*, (2012) indicated successful identification of wild macrofungi based on ITS (ITS1 and ITS4) sequence. Their study revealed that the wild mushroom belonged to Agaricomycetes which was a new report of a mushroom in India. Some of the wild edible macrofungi have also been reported from Manipur and Arunachal Pradesh of North East India (Sing *et al.*, 2002). Few strains of Basidiomycetes have also been reported from Assam (Baruah *et al.*, 1971).

Verma *et al.*, (1987) described fleshy fungal flora from Manipur and Meghalaya belonging to the family *Auriculariaceae*, *Clavariaceae*, *Cantharellaceae*, *Tricholomataceae*, *Pluteaceae*, *Paxillaceae*, *Cortinariaceae*, *Cycoperdaceae*, and *Sclerodermataceae* of Basidiomycotina and *Halvellaceae* of Ascomycotina. Again, Verma *et al.*, (1995) carried out a macrofungal survey of the North

East Hill region of India and confirmed ninety-five species of higher fungi, among these, 85 species were new records. Recently, diversity of wild edible macrofungi from Khasi hills of Meghalaya, India have been reported and a total of 11 different species were identified based on spore morphology (Khaund and Joshi, 2013). Three new species of *Russula* (*Russulasharmae*, *R. dubdiana* and *R. sikkimensis*) from Sikkim (India) have been reported by Das *et al.*, (2013).

Phylogenetic analysis

Phylogenetic analysis was carried out by comparing the query sequences against a non-redundant database for comparative analysis of ITS partial sequences. The phylogenetic tree generated through the Neighbor-Joining (NJ) method was well supported by the bootstrap values within their nodes. The phylogenetic analysis using partial ITS rRNA gene sequences revealed that the isolate showed closeness with each other. However, further such studies involving more number of isolates (taxa) can better depict their phylogenetic position at a community level. Phylogenetic analysis using the Neighbour-Joining tree of the identified strains is shown in Figure 5. The evolutionary history was inferred using the Neighbor-Joining method and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Efronet *al.*, 1996). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All the 44 macrofungal isolates were grouped into ten clusters (Fig. 5), Cluster I comprised of 7 macrofungal isolates (*Macrolepiota rhacodes*, *Macrolepiota procera*, *Lycoperdon pyriforme*, *Leucoagaricus leucothites*, *Psilocybe Mexicana*, *Agaricus arvensis*, *Agaricus campestris*), Cluster II comprised of 6 macrofungal isolates (*Hypholomas*

ublateritium, *Bolbitius titubans*, *Lactarius sp.*, *Chlorophyllum molybdites*, *Panuslacomtei*, *meripiliusgigantius*), Cluster III comprised of only 1 macrofungal isolate (*Fomitopsis streiformis*), Cluster IV comprised of 3 macrofungal isolates (*Pycnoporus coccineus*, *Polyporus arcularius*, *Polyporus squamosus*), Cluster V comprised of 5 macrofungal isolates (*Ganoderma applanatum*, *Ganoderma lucidum*, *Ganoderma sp.*, *Laetiporus sulphurous*, *Ganoderma sp.*) Cluster VI comprised of 2 macrofungal isolates (*Hymenopellis megalospora*, *Mycena rosea*), Cluster VII comprised of 4 macrofungal isolates (*Dacrymyces sp.*, *Gymnopilus dilepis*, *Agrocybe pediades*, *Auriculariasp.*), Cluster VIII comprised of 3 macrofungal isolates (*Coprinus disseminates*, *Psathyrellacorrugis*, *Coprinus sp.*), Cluster IX comprised of 2 macrofungal isolates (*Physisporinuscitreus*, *Sarcodon imbricatus*) and Cluster X comprised of 11 macrofungal isolates (*Pleurotus populinus*, *Pleurotus floridanus*, *Pleurotus sapidus*, *Pleurotus ostreatus*, *Pleurotus sapidus*, *pulmonarius*, *Pleurotuscornucupaie*, *Lentinus sp.*, *Lentinus critinus*, *Lentinus strigosus*, *Lentinus sajorcaju*).

Classification of the macrofungal species

Classification of the macrofungal species (Table 4) revealed that all the 44 strains belonged to the division Basidiomycota. The macrofungal species can be distributed into 16 families *viz.* Polyporaceae and Agaricaceae (10 species each), Pleurotaceae (6 species), Ganodermataceae (4 species), Meripilaceae and Strophariaceae (2 species each), Fomitopsidaceae, Russulaceae, Bolbitiaceae, Mycenaceae, Physalacriaceae, Cortinariaceae, Psathyrellaceae, Bankeraceae, Dacrymycetaceae and Auriculariaceae (1 species). The order Agaricales consisting of 23 species was found to be dominant followed by Polyporales with 17 species in this region.

It was observed that, among the 44 samples, 23 strains were edible and among the 21 non-edible strains, 4 strains have medicinal properties, 6 strains are poisonous, one has industrial application and the rest are not fully explored. Singer (1986) recognized 230 genera and 5658 species spread over 17 families in order Agaricales. Previous reports worldwide indicated the edibility and medicinal properties of these species and a complete list of all macrofungal species and countries can be retrieved from www.wildusefulfungi.org (Schwarze *et al.*, 2011).

Scientific classification of the identified strains based on their molecular data has been depicted in Figure 6. The Basidiomycota contains about 30,000 described species, which is 37% of the described species of true fungi (Kirk *et al.*, 2001). Zhao *et al.*, (2011) provide an overview of recent work on understanding phylogenetic relationships within the Agaricales, a major group within the Agaricomycotina that is the largest clade of mushroom-forming fungi (Matheny *et al.*, 2006).

A timely research regarding isolation, identification and characterization of the existing mushroom flora is essential due to over exploitation and habitat destruction. Thus, our study was an attempt to survey; collect valuable wild macrofungi and their identification based on phenotypic and molecular characteristics, to know the mycotreasure available in this region that is yet to be fully explored. In this study we observed that 23 strains were edible and the rest 21 were non-edible. Among the non-edible strains, 4 strains have medicinal properties, 6 strains are poisonous, one has industrial application and the rest are not fully explored. Further studies on this less explored may provide valuable information as macrofungi have a great potential for the

production of useful bioactive metabolites and drugs. The list of macrofungi in this study provides the baseline information needed for the identification based on their morphological and molecular characteristics.

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Conflict of interest

The authors declare no conflict of interest.

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