MOLECULAR PHYLOGENETIC IDENTIFICATION OF WOOD INHABITING FUNGI ISOLATED FROM DAMPA TIGER RESERVE FOREST

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ABSTRACT. In this study, we investigated the taxonomic identities and phylogenetic relationships of fungal species isolated from Dampa Tiger Reserve Forest using a combination of morphological and molecular approaches. Twenty two fungal isolates were selected for molecular phylogenetic analysis using nuclear ribosomal DNA sequences, including both the internal transcribed spacers (ITS1 and ITS2) and the 5.8S gene region. The 22 species were identified to the species level based on fungal sequences with known identities in GenBank.

Keywords: Internal transcribed spacer, Basidiomycetes, Phylogenetic analysis, Molecular analysis

I. Introduction

Fungal species are important components of biodiversity in tropical forests, where they are major contributors to the maintenance of the earth's ecosystem, biosphere and biogeochemical cycle (Satish et al., 2007; Panda et al., 2010). Fungi have beneficial roles in nutrient cycling, agriculture, biofertilizers, antibiotics, food and biotechnological industries (Hawksworth 1991; Hawksworth and Colwell 1992; Lodge 1997; Pointing and Hyde 2001; Manoharachary et al., 2005). The objective of this study was to characterized the fungal species of the protected forest of Dampa Tiger Reserve in Mizoram.

The site occupies an area of 500 sq. km. and lies in west Mizoram in northeastern India, along the border between India and Bangladesh. The hills and forests in this 'Land of the highlanders' are considered by biologists to be "biogeographic highways" connecting India to Malayan and Chinese regions. The site receives rainfall, ranging from 2,000 mm. to 2,500 mm. The minimum temperature in the reserve is 3.5˚C and the maximum temperature is 35˚C. The climate throughout the year at the Dampa Tiger Reserve is pleasant and warm and during the months November and December the area receives moderately chilly climate at higher altitudes.

Fungal taxonomy is traditionally based on comparative morphological features (Lodg et al., 1996; Sette et al., 2006; Crous et al., 2007; Zhang et al., 2008). However, special caution should be taken when closely related or morphologically similar fungi are identified, because the morphological characteristics of some fungi are medium-dependent and cultural conditions can substantially affect vegetative and sexual compatibility (Zhang et al., 2006; Hyde and Soytong, 2007). Furthermore, the conventional methods cannot be applied for identifying fungal isolates that fail to sporulate in culture, which are categorized as mycelia sterilia (Lacap et al., 2003). Various optimization of growth conditions have been used to promote sporulation of these fungi, such as different culture media, potato dextrose agar (PDA), malt extract agar (MEA), corn meal agar (CMA), potato carrot agar (PCA), and water agar (WA), as well as the inclusion of host tissues in plate cultures (Guo et al., 2000). Nevertheless, a large number of fungi still do not sporulate in culture media.

In contrast, molecular techniques exhibit high sensitivity and specificity for identifying microorganisms and can be used for classifying microbial strains at diverse hierarchical taxonomic levels (Sette et al., 2006). Ribosomal genes and spacers regions within the fungal genome have proven good candidates for amplification via PCR because they are comprised of highly conserved tracts with heterogeneous regions in between (Grades et al., 1991). The conserved tracts are ideal for universal primer design that can allow for the amplification of the separating heterogeneous regions. Most molecular fungal species identification relies on the amplification and sequencing of the internal transcribed spacer (ITS) region of the fungal genome, which is highly variable among species or even populations of the same species (Hibbett, 1992; Horton and Bruns, 2001). This region lies between the 18S small subunit(SSU) and the 28S large subunit (LSU) ribosomal RNA (rRNA) genes and contains two non coding spacer regions (ITS-A and ITS-B) separated by the 5.8S rRNA gene (Kendrick, 2000). In fungi, the ITS region is typically 650900 bp in size, including the 5.8S gene and is usually amplified by the universal primer pair ITS 1 and ITS 4 designed by White et al., (1990). Once the region is amplified, it can be sequenced and that sequence can be compared to those of known species.

II. Materials and Methods
II.1 Study site: Dampa Tiger Reserve

Dampa Tiger Reserve is located in the western part of Mizoram bordering Bangladesh and within Mamit District of Mizoram. Dampa Sanctuary has an area of 500 sq km and is the largest protected area in Mizoram. Tropic of cancer passes through the sanctuary. The distance from Aizawl at W.Phaileng is 127 km to the west. The reserve forest falls under Tropical evergreen forest in the lower part and Tropical semi-evergreen forest in the higher elevation. The common trees are *Michelia champaca* L., *Terminalia myriocarpa* Van Heurck & Müll. Arg. *T.chebula* Retz., *Diptero carpus* C.F.Gaertn .spp., and so on. On the south-eastern side, the village of Lallen, Saithah, Phuldungsei, Pukzing are situated on roadsides and the villages of Terei, Damparengpui, Tuipuibari are on the north and West Hnahva and Andermalik of the south and W.Phaileng on the east.
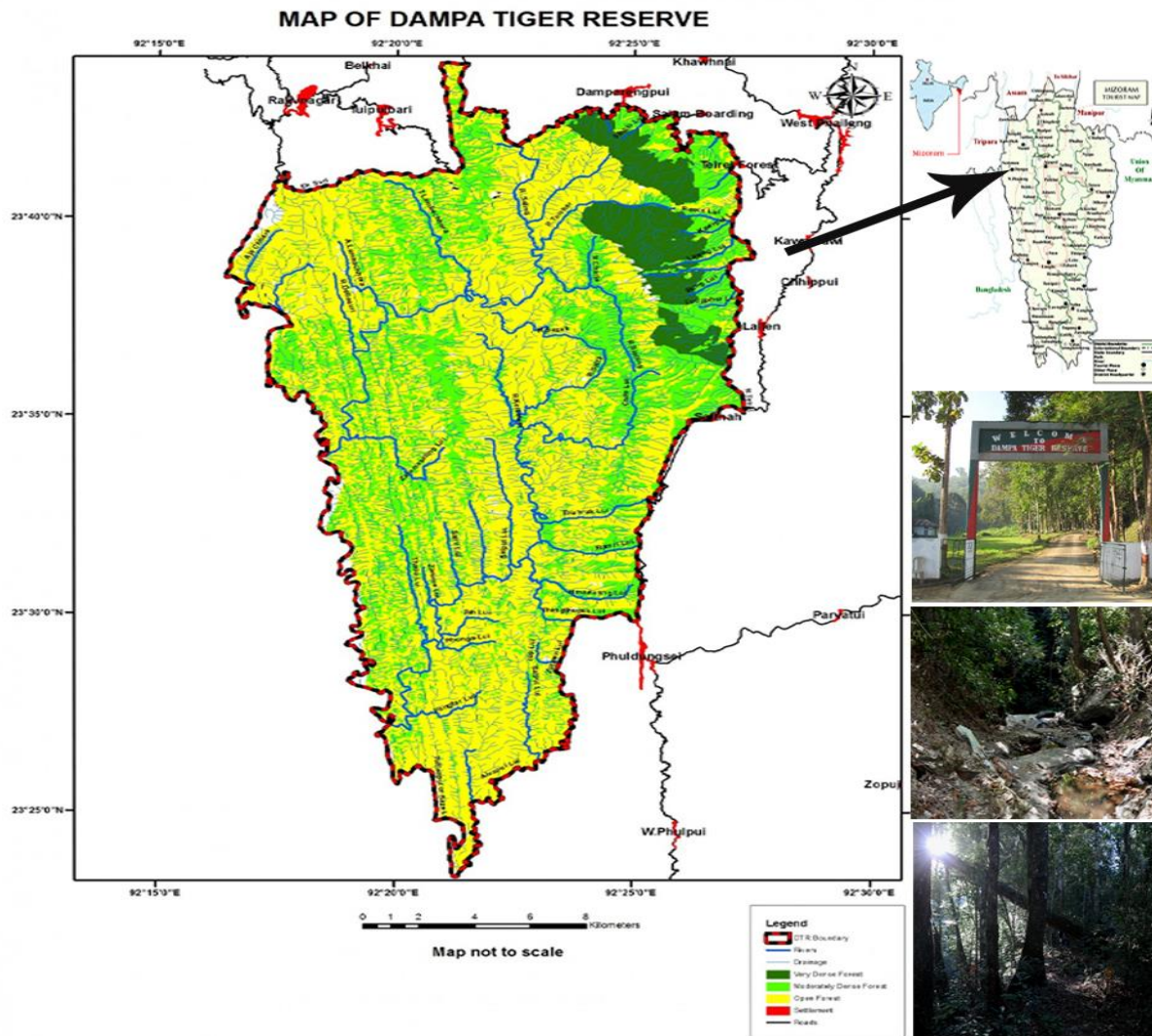
Fig1: Map of Dampa Tiger Reserve

II.2 *Collection and Preservation of Specimens*

The samples were collected or isolated from its substrates or host (dried wood/branches) with the help of knife or other sharp materials and sometimes simply plucked with bare hand (in case of soft samples). Samples collected were kept in air-tight container or plastics bags which are labeled after collection. Photograph of each sample collected were taken in the field and in the laboratory with measuring scales (Prasher, 2015).

The specimens are preserved by air drying and deep freezing. Voucher numbers are given to the specimens and stored in the Department of Environmental Science, Mizoram University.

II.3 *Identification of specimens*

The collected specimens were identified according to standard macroscopic and microscopic characteristics through consultation with appropriate literature (Gilbertson & Ryvarden, 1986; Núñez & Ryvarden, 2000).

For microscopic study, thin sections of dried specimens are taken with the help of a sharp razor blade and were mounted in 3% KOH solution and stained in 2% aqueous phloxine. Sections are mounted in Lactophenol or 60% lactic acid + cotton blue. Spore print of the collected specimens were taken by cutting off the cap and placing it in a piece of white paper (Surcek, 1988).

II.4 *Molecular analysis*

DNA was extracted from fruting bodies using a CTAB extraction. A small amount of tissue from the inside of the fruiting body was added to a sterile 1.7 ml microcentrifuge tube with glass beads and 500 µL of CTAB lysis buffer. The centrifuge tube was then vortexed for a minute homogenize lyse fungal cells. For drier and harder fruiting bodies, the tissue was vortexed for 2 minutes to allow for a better homogenization of the tissue. The tubes were then briefly centrifuged to move the larger tissue segments to the bottom of the tube and then the supernatant was transferred to another tube. The new microcentrifuge tube was then placed into a 65°C hot water bath to further lyse the cells. After 20 minutes, the tubes were removed and 500 µL of chloroform were added to the tube, mixed and then centrifuged at 13,000 rpms for 5 minutes. The top layer of the supernatant was then transferred to a new microcentrifuge tube. The amount of liquid transferred was then measured and two thirds of that amount was added of isopropanol stored in a minus 20C freezer. The tube was then incubated for 5 minutes at room temperature before being centrifuged for 7 minutes at 15,000 rpm. The supernatant was removed and 500 µL of 70% ethanol was added. The tubes were centrifuged again at 15,000 rpm for 3 minutes and the supernatant was removed. The tubes were left open in a hood to allow the last of the ethanol to evaporate before the DNA pellet was re-suspended in 100 µL of sterile water.

PCR reactions were setup in 0.2 ml centrifuge tubes that contained 12.5 µl GoTaq Green Mastermix (Promega, Madison, WI),

9.5μl nuclease free water , 1μl forward primer 5μM), 1μl reverse primer (5μM) and 1μl of fungal DNA template for a total reaction volume of 25.5μl. PCR was performed using primers ITS1-F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') ITS4-B (5'-CAG GAG ACT TGT ACA CGG TCC AG-3') (White et al., 1990) with the following parameters; 94ºC for 5 minutes, followed by 35 cycles of 94ºC for 1 minute, 52ºC for 1 minute and 72ºC for 1 minute with a final extension step of 72ºC. PCR amplicons were verified by electrophoresis on a 1% agarose gel with SYBR green and visualized on a Gel Documentaion System. Sequencing was performed using both primers by using Sanger sequencing using a DNA sequencer. Consensus sequences for contigs were trimmed and aligned using Bioedit sequence alignment editor. Sequences were then compared to those in GenBank database using the BLASTn (Altschul et al., 1990) search tool for similarities and were submitted to Genbank.

II.5 *Phylogenetic analysis*

Phylogenetic analysis was conducted based on the ITS gene data using both maximum likelihood (ML) approaches. ML searches were carried out using MEGA 7. All characters were equally weighted. Alignment gaps were treated as missing data. NJ trees were constructed based on the total character differences and bootstrap values were calculated from 1,000 replications.

III.Results

A total of 22 species were identified and submitted to the NCBI Genbank. Morphological identification of the 22 fungal isolates from Dampa Tiger Reserve Forest was first carried out according to characteristics of the spores, and reproductive structures if discernible (Barnett and Hunter, 1998). Based on these features, the 22 specimen could be classified into different morphological taxa (Table 3). Using the traditional morphological techniques, only some of the fungal isolates could be identified to the genus level. Among them *Lentinus badius* and *Schizophyllum commune* were common. The rest were identified to the genus level using molecular techniques.

Molecular identification and phylogenetic analysis of representative fungi

In addition to the morphological characterization, molecular analyses were carried out to confirm the identification of 22 representative fungal isolates from the Dampa Tiger Reserve Forest. The ITS1- 5.8S- ITS2 sequences of these isolates were compared to 61 corresponding sequences of reference fungal taxa in the database (Table 1).

Table 1: List of Species, Voucher No. and GenBank Accession No. used for the analysis.

Sn	Species	GenBank Accession Nos.
1	*Favolus acervatus*	KP012981
2	*Favolus acervatus*	KM385427
3	*Favolus acervatus*	MG981026
4	*Polyporus philippinensis*	KX548977
5	*Polyporus philippinensis*	KX548976
6	*Polyporus philippinensis*	KX548978
7	*Abundisporus fuscopurpureus*	KC455255
8	*Abundisporus fuscopurpureus*	KC455254
9	*Abundisporus fuscopurpureus*	JN048771
10	*Pycnoporus coccineus*	KP255840
11	*Pycnoporus coccineus*	KP255839
12	*Leiotrametes flavida*	MF774416
13	*Leiotrametes flavida*	KC569130
14	*Leiotrametes flavida*	KC569132
15	*Lenzites warnieri*	KP794599
16	*Lenzites warnieri*	AY855916
17	*Lenzites warnieri*	GU731567
18	*Microporus vernicipes*	MH221090
19	*Microporus vernicipes*	KU863046
20	*Microporus vernicipes*	KU863045
21	*Coriolopsis aspera*	KP012720
22	*Coriolopsis aspera*	KP012718
23	*Coriolopsis aspera*	KR131760
24	*Hexagonia tenuis*	KC414233
25	*Hexagonia tenuis*	KU194308
26	*Hexagonia tenuis*	JX559277
27	*Lentinus badius*	KP283481
28	*Lentinus badius*	KP283480
29	*Lentinus badius*	GU207275
30	*Filoboletus manipularis*	KF746998
31	*Filoboletus manipularis*	KF746993

| 32 | *Filoboletus manipularis* | KF746991 |
33	*Schizophyllum commune*	MH307932
34	*Schizophyllum commune*	KX958030
34	*Schizophyllum commune*	KR706163
35	*Campanella sp.*	EF175518
36	*Campanella sp.*	EF175519
37	*Campanella sp.*	EF175520
38	*Campanella buettneri*	MF075136
39	*Marasmius palmivorus*	MF100969
40	*Marasmius palmivorus*	MF100967
41	*Marasmius palmivorus*	JQ653438
42	*Marasmius sp.*	JQ586347
42	*Fomitopsis cajanderi*	KC595915
44	*Fomitopsis dochmia*	DQ491401
45	*Coprinopsis clastophylla*	KY654717
46	*Coprinopsis aff. clastophylla*	KP012971
47	*Coprinopsis cinerea*	JN943128
48	*Coprinopsis cinerea*	NR148066
49	*Elmerina cladophora*	JQ764638
50	*Elmerina cladophora*	KX963793
51	*Ceriporiopsis semisupina*	KU509525
52	*Ceriporiopsis semisupina*	NR158302
53	*Ceriporiopsis semisupina*	MH114630
54	*Nigroporus vinosus*	AB811861
56	*Nigroporus vinosus*	MH114824
57	*Panus conchatus*	KR818817
58	*Panus conchatus*	KM282285
59	*Spongipellis delectans*	HQ728301
60	*Spongipellis delectans*	HQ728300
61	*Spongipellis delectans*	KP135301
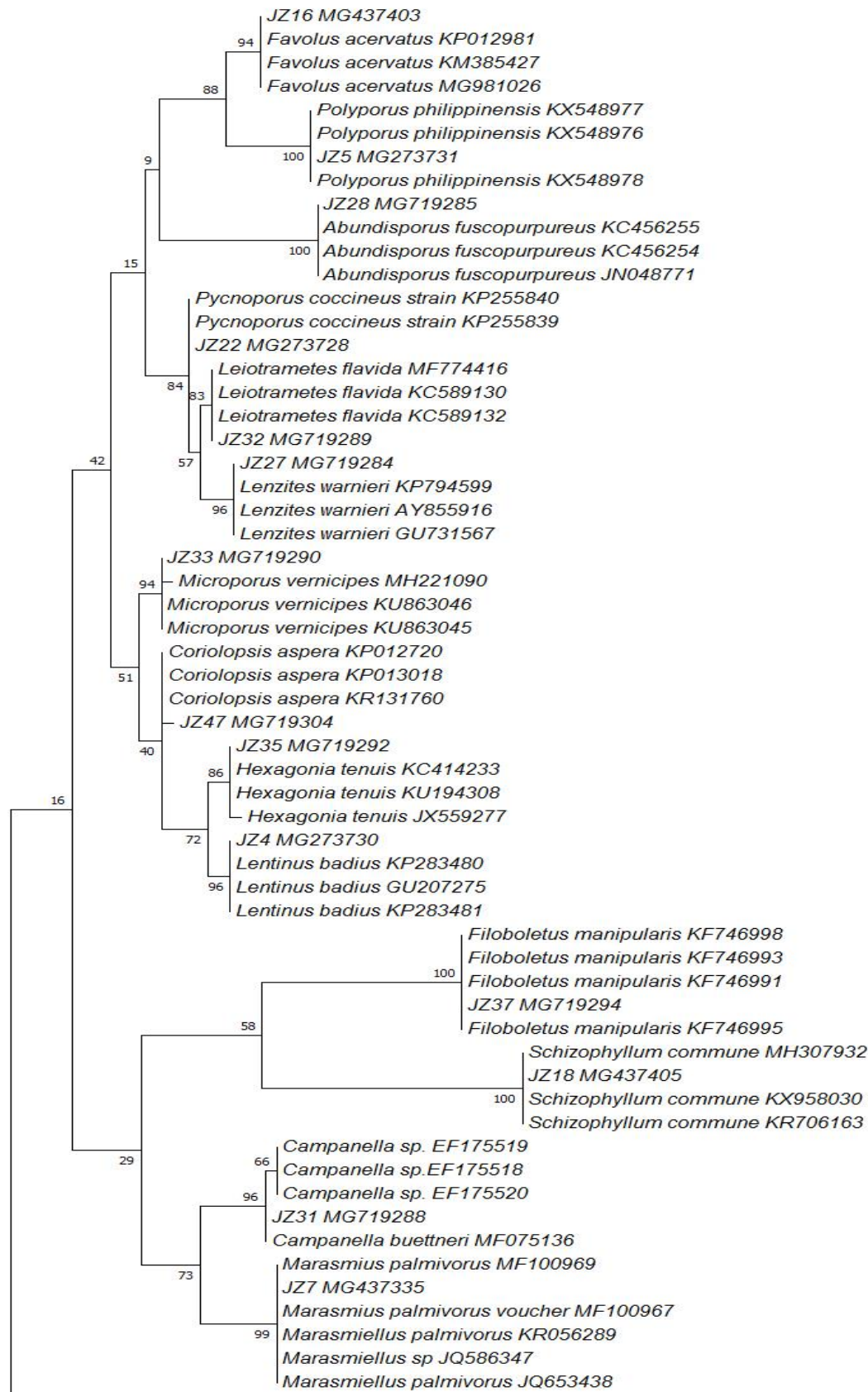
Figure 2. Tree 1- Molecular Phylogenetic analysis by Maximum Likelihood method

The analysis involved 83 nucleotide sequences (Figure 1 and Figure 2). In the maximum Likelihood tree generated, the specimens of the different species from Dampa Tiger Reserve forest clustered with their related species with high support value.
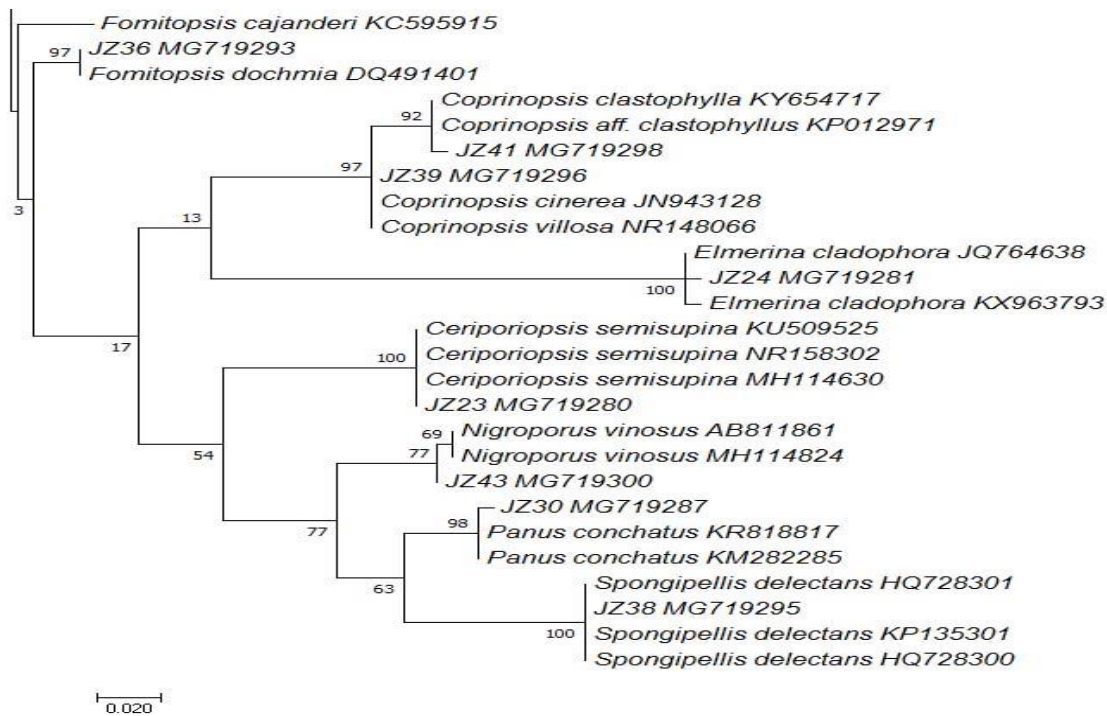
Figure 3. Tree 2- Molecular Phylogenetic analysis by Maximum Likelihood method

Table 2: Voucher No, Species Name and Genbank sequences of Fungal specimens collected from Dampa Tiger Reserve Forest.

Sl.No	Voucher No.	Morphological identification	NCBI ACCESSION NO.	Max identity %
1	JZ16	*Favolus acervatus*	MG437403	98
2	JZ5	*Polyporus philippinensis*	MG273731	100
3	JZ28	*Abundisporus fuscopurpureus*	MG719285	97
4	JZ22	*Pycnoporus coccineus*	MG273728	98
5	JZ32	*Leiotrametes flavida*	MG719289	98
6	JZ27	*Lenzites warnieri*	MG719284	99
7	JZ33	*Microporus vernicipes*	MG719290	98
8	JZ47	*Coriolopsis aspera*	MG719304	97
9	JZ35	*Hexagonia tenuis*	MG719292	98
10	JZ4	*Lentinus badius*	MG273730	98
11	JZ37	*Filoboletus manipularis*	MG719294	99
12	JZ18	*Schizophyllum commune*	MG437405	97
13	JZ31	*Campanella sp.*	MG719288	98
14	JZ7	*Marasmiellus palmivorus*	MG437335	98
15	JZ36	*Fomitopsis dochmia*	MG719293	99
16	JZ41	*Coprinopsis clastophylla*	MG719298	98
17	JZ39	*Coprinopsis cinerea*	MG719296	97
18	JZ24	*Elmerina cladophora*	MG437338	98
19	JZ23	*Ceriporiopsis semisupina*	MG437402	99
20	JZ43	*Nigroporus vinosus*	MG719300	98
21	JZ30	*Panus conchatus*	MG719287	99
22	JZ38	*Spongipellis delectans*	MG719295	99

Table 3: Species identified and their Taxonimical position.

Basidiomycetes	
Polyporaceae	*Favolus acervatus, Polyporus philippinensis, Lentinus badius, Pycnoporus coccineus, Lenzites warnieri, Hexagonia tenuis, Microporus vernicipes, Panus conchatus, Leiotrametes flavida, Leiotrametes lactinea, Abundisporus fuscopurpureus, Spongipellis delectans, Nigroporus vinosus, Coriolopsis aspera*
Meruliaceae	*Ceriporiopsis semisupina*
Marasmiaceae	*Campanella sp., Marasmiellus palmivorus*
Fomitopsidaceae	*Fomitopsis dochmia*
Psathyrellaceae	*Coprinopsis clastophylla, Coprinopsis clastophylla, Coprinopsis cinerea*
Aporpiacaea	*Elmerina cladophora*
Schizophyllaceae	*Schizophyllum commune*
Mycenaceae	*Filoboletus manipularis*

As shown in **"Table 3"** all the isolates from Dampa Tiger Reserve Forest can be classified as Polyporaceae, Meruliaceae, Marasmiaceae, Fomitopsidaceae, Psathyrellaceae, Aporpiacaea, Schizophyllaceae and Mycenaceae.
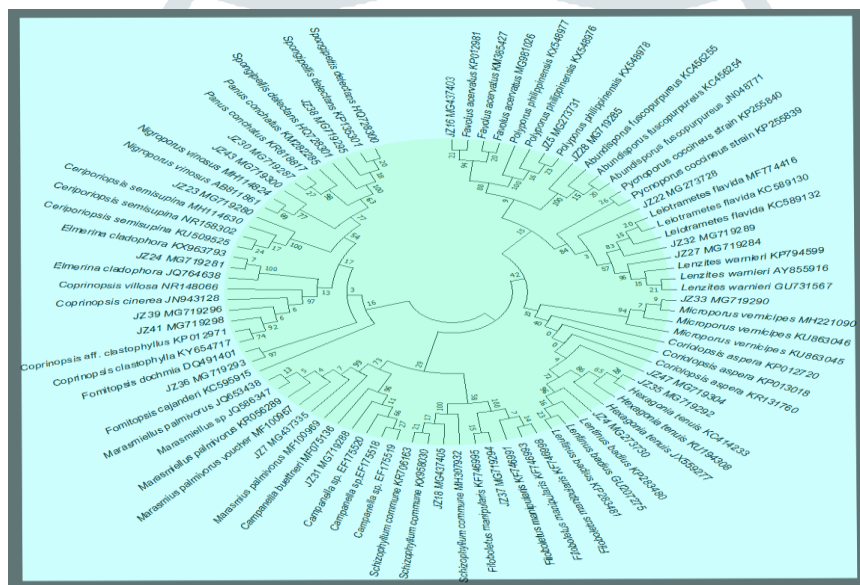

Figure 4. Molecular Phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. 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 Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 83 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 212 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

IV. Discussion

Diversity of fungi in this study

In this study, 22 fungal isolates were obtained from Dampa Tiger Reserve. For fungal identification, we relied on a combination of traditional and molecular methods. All the twenty two representative morphological isolates were further identified with molecular phylogenetic analysis of ITS1- 5.8S- ITS2 sequences. Generally, there was a good agreement between morphological and ITS-sequence based approaches.

The use of molecular techniques in identifying Fungal isolates

Molecular techniques have been successfully used for identifying fungi in recent studies (Promputtha et al., 2005; Sette et al., 2006; Tedersoo et al., 2006; Morakotkarn et al., 2007). Culture-independent DNA methods, such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), and ITS sequencing, have been developed for the investigation of complex microbial communities. Our study also shows that molecular identification based on ITS sequences can be used to complement or verify morphological identification of unknown fungi. Some fungal isolates could be identified to the species level, and others to the level of genus or family. Based on DNA analysis, problems associated with taxonomic identification of mycelia sterilia could be solved. However, molecular analysis alone also has limitations. It cannot overcome the problem of over-isolating fast growing fungal species at the expense of slow growing taxa, nor isolating species that will not grow in culture (Duong et al., 2006; Hyde and Soytong 2007). The use of ITS sequences also has limitations in phylogenetic analysis. Because the noncoding ITS sequence is fast evolving with many variable characters, it is usually difficult to achieve a perfect sequence alignment at high taxonomic levels. Moreover, it has been shown that 20-30% of sequences downloaded from GenBank for comparative analysis may not be accurate in their identification (Nilsson et al., 2006; Hyde and

Soytong, 2007). Further studies using different gene sequences can be conducted to resolve this type of difficulties in the phylogenetic analysis of fungi.

V. Acknowledgements

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