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Taxonomic and Phylogenetic study of *Termitomyces entolomoides* in western Assam

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Abstract- *Termitomyces* genus is paleotropical in nature and is known for its obligate symbiotic association with termites belonging to *Macrotermitinae*. The *Termitomyces* sample was collected during the monsoon and post monsoon period (April-October). The basidiocarp was oven dried and used for the macro-morphological and micro-morphological studies. Identification of fungi using morphological characters is very complicated and error prone. Furthermore, internal transcribed spacer (ITS) region 1, 5.8S rRNA gene and ITS 2 region was amplified using ITS 1 and ITS 4 primer pairs. The amplicon of ~600 bps was sequenced and subjected to Phylogenetic analysis in MEGA X sowftware package. Newly obtained sequence and 19 sequences of *Termitomyces* from NCBI GenBank were aligned using MAFFT. Phylogenetic tree was constructed using Maximum Likelihood method with bootstrap values of ≤ 70 %. The morphological characters and molecular data indicate the studied sample as *Termitomyces entolomoides*. Phylogenetic relationship of the studied species with 19 Asian *Termitomyces* species obtained from NCBI GenBank is established and is clearly monophyletic.

Keywords*—Termitomyces, identification, ITS region, molecular phylogeny.*

I. INTRODUCTION

The paleotropical genus *Termitomyces* of Lyophyllaceae family lives in obligate symbiotic relationships with termites belonging to the subfamily *Macrotermitinae* (Isoptera). The termites provide the ambient micro climatic condition suitable for the growth and propagation of the fungi and the later provide enzymatic supplement to aid digestion of the divergent termite food [1]. The fungal combs are made up of partially digested plant material to support the growth of *Termitomyces* and to prevent the comb's colonization by competitive fungi [2]. The detailed study of the genus *Termitomyces* was first described from Central Africa in 1977 and India in 1979 [3]. Several other taxa of *Termitomyces* have been documented and described by many authors including Heim 1951, 1952, 1958, 1977; Otieno 1964; Pegler 1977; Natarajan 1979; Piearce 1987; Van der Vanhaecke 1994; Turnbull and Watling 1999; Mossebo 2000, 2002; Mossebo *et. al.* 2002, 2006, 2009, 2011; Wei *et. al.* 2004; Tang *et. al.* 2005, 2006 [4,5]. *Termitomyces* is one of the most popular edible mushrooms of North Eastern Region of India, highly preferred by the ethnic tribal group viz. Bodo, Rabha, Garo, Kochari, Lalung etc.

Approximately 30 *Termitomyces* species are known [6] all together they covers a wide range of morphological range, includes *Termitomyces titanicus* with a cap diameter of 1 m making it the largest fruiting body of gilled mushroom and *Termitomyces microcarpus* which rarely exceeds 2 cm in diameter. The specific characteristics of *Termitonyces* mushrooms are the pinkinsh spores, long subterranean pseudorhiza and termite association. Most of the species have the 'perforatorium', a knob like structure also called as umbo, which play an important role during penetration of the soil [7].

Identification and classification of *Termitomyces* species based on morphological and physiological characteristics is difficult. Often, the morphological characteristics do not distinguish different species of fungus [2]. Therefore, molecular techniques are required to identify and classify the species to a better extent. Ribosomal DNA (rDNA) studies provide a means for analyzing phylogenetic relationships over a wide range of taxonomic levels [8]. The internal transcribed spacer (ITS) region and intergenic spacer (IGS) region of the nuclear DNA repeat units evolve fastest and may vary among the species within a genus or among populations [9]. The ITS region of rRNA consists of 18S,

ITS1, 5.8S, ITS2 and 28S genes. ITS1 is located between 18S and 5.8S rRNA genes, whereas ITS2 is located between 5.8S and 28S rRNA genes [9]. As the ITS region shows more divergence than their flanking regions they are routinely used to distinguish related species and to infer phylogenetic relationships among populations [2].

Occurrence of 19 species of *Termitomyces* has been recorded in six states of the Western Ghats and on the west coast of India [4]. Literature cited reveals occurrence of 8 species of *Termitomyces* from the North East India [10,11,12,13,14]. The present study confirms the occurrence of *T. entolomoides* in this zone, its morphological study and phylogenetic relationship with other species of *Termitomyces* of Asian origin.

The rest of the paper is organized as follows, Section I contains the Introduction of the genus *Termitomyces*, related Taxonomic and molecular work. Section II contains the methodology used to carry out the present study i.e., sampling, morphological studies and molecular phylogeny study. Section III describes the results and discussions with hand drawings, images of microscopic plates and phylogenetic tree. Section IV contains the Conclusion of the study with future scopes.

II. MATERIALS AND METHODS

Sample Collection and Taxonomic Study

The sample was collected from Kokrajhar District of western Assam during monsoon and post monsoon period. The sample was oven dried at 40^{o} C and used for the subsequent study. Different parts of basidiome viz. cutis, gills, stipe, pileus were studied under the microscope. The sections were manually made by hands using razor blade. Macro and micro morphological characters were studied for the collected *Termitomyces* species. Habitat notes, general characters of pileus, lamellae, stipe were recorded along with field photographs (Canon 600D and 800D, Canon Macro lens 100 mm with image stabiliser and Ultrasonic motor and CMOS sensor). Methuen Handbook of color by Kornerup and Wanscher [15] was referred for color code. Microscopic study was carried out using 5% KOH, Congo red and Phloxin in biological microscope Olympus CX43, eyepiece attached with drawing tube. Measurement of basidiospores were based on 20 readings and the same was done with basidia, cheilocystidia and pleurocystidia (10 readings), at 1000x magnification. Sizes were shown as MIN_L - M_L- MAX_L x MIN_W - M_W - MAX_W where, MIN_L = measurement for minimum length, MAX_L = measurement for maximum length, M_L = arithmetic mean of length and MIN_W = measurement for minimum width, MAX_W = measurement for maximum width, M_W = arithmetic mean of width. For basidiospores, Q value was given (Q means Quotient = Lenght/Width) where, Q = mean quotient value. The measurement of the hyphae were shown as MIN_w - MAX_w and were based on 10 readings.

DNA isolation and PCR

DNA isolation was done by CTAB/ Chloroform-Isoamyl alcohol method [16]. ITS region was amplified using ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') primers [9]. The extracted DNA was used for PCR (Polymerase Chain Reaction) amplification in thermal cycler (Applied Biosystems), which was performed in 25 µl reaction volume containing 120 ng of genomic DNA, 10X PCR buffer, 10 picomole of each primer, 2.5 mM each dNTPs and 1 U of Taq DNA polymerase (Invitrogen), nuclease free water was added to make up the final reaction volume. Cycling conditions were – initial denaturation at 94^{o}C for 7 minutes, denaturation at 94^{o}C for 30 seconds, annealing at 55^{o}C for 2 minutes, extension at 72^{o}C for 2 minutes, final extension at 72^{o}C for 10 minutes and hold at 4^{o}C. The quality of PCR reaction was examined using 2% Agarose gel, bands were visualised in UV-base Gel Documentation Unit (Life Technologies) after staining with Ethidium Bromide.

DNA Sequencing and Alignment

The PCR product was subjected to Sanger sequencing using DNA Sequencer model ABI3730XL-15104-028 (Applied Biosystems) with Sequence Scanner Software 2 v2.0. The newly obtained sequence and sequences obtained from GenBank (Table 1) were aligned and optimized in MAFFT version 7 (https://mafft.cbrc.jp).

Model testing and Phylogenetic Analysis

To estimate the best fit model for the present dataset, likelihood scores and BIC calculations were calculated in JModeltest [17]. Phylogenetic analyses were performed in MEGA X software package [18]. The phylogenetic tree was constructed by Maximum Likelihood method using Tamura-Nei model with discrete Gamma distribution rate of variation. To examine the support of interior branch and for the validity of the analysis Bootstrap tests of 1000 replications were conducted. Codon positions included 1st+ 2nd + 3rd + non-coding. The evolutionary distances were computed using Maximum Composite Likelihood method. Branches with support value lower than 70 % were collapsed; those above 70% were supported.

Table 1

Sl no	Name of Species	Country of origin	GenBank Accession no.
1	*Lyophyllum semitale*	Sweden	HM572552
2	*Lyophyllum decastes*	Sweden	HM572548
3	*Termtomyces fuliginosus*	Thailand	LC068788
4	*Termitomyces eurrhizus*	China	KJ620056
5	*Termitomyces eurrhizus*	India	KY243929
6	*Termitomyces microcarpus*	India	MH542622
7	*Termitomyces microcarpus*	Thailand	HM230661
8	*Termitomyces microcarpus*	Sri Lanka	KP780436
9	*Termitomyces heimii*	India	JQ928938
10	*Termitomyces clypeatus*	Thailand	HQ702552
11	*Termitomyces clypeatus*	Thailand	FJ147329
12	*Termitomyces intermedius*	China	MF488973
13	*Termitomyces intermedius*	China	MF488972
14	*Termitomyces radicatus*	Thailand	LC068787
15	*Termitomyces radicatus*	Thailand	HM230660
16	*Termitomyces bulborhizus*	Thailand	HM230663
17	*Termitomyces heimii*	Malaysia	EU443836
18	*Termitomyces aurantiacus*	Malaysia	GU594650
19	*Termitomyces aurantiacus*	China	JQ228252

III. RESULTS AND DISCUSSION

Taxonomic study

Termitomyces entolomoides **R. Heim,*Termites et Chapignons* (Paris): 52 (1977)** [Index Fungorum accessed on 17th September 2018]

Habitat: on soil, scattered, pleasant odour, edible

Description: *Pileus* 5 cm, initialy conical, later expanding to plano convex with a prominent obtuse conical perforatorium, surface brown (5E4) at the centre, fading towards the edge to orange grey (5B2), moist, smooth, bluish tints at the centre surrounding the perforatorium, margin wavy, smooth and rimose. *Lamellae* free, close ~21/cm, consistency brittle, surface white (1B1) to pale pink, smooth edge. *Stipe* 5-6 x 1.07-2 cm above soil, surface pastel grey (1C1), central, fusiform, solid, fibrous. *Pseudorhiza* 5- 10 cm long, surface yellowish grey (4B3), tapered downwards. *Annulus* absent. *Spore deposit* pale pink. *Basidiospores* 3.7-4.83-5.69 x 2.5-3.5-4.9 µm, Q = 1.4, ellipsoidal, hyaline, thick walled. *Basidia* 18.93-21.94-24.38 x 5.71-6.74-7.6 µm, clavate, sub hyaline, tetrasporate. *Pleurocystidia* 18.87-23.71-46.07 x 8.44-12.63-33.10 µm, scattered, thin walled, sub hyaline. *Cheilocystidia* 14.71-26.39-34.02 x 9.33-12.16-16.9 µm, clavate to fusiform, sub hyaline, thin walled. *Hymenophoral trama* regular, sub hyaline, composed of 4-10.25 µm wide hyphae, septate, presence of oleiloferous hyphae 4.21-5.75 µm wide. *Pileipellis* surface consist of repent and radial parallel hyphae, 2.6-4.7 µm wide, septate, thin walled, sub hyaline, no clamp connections. [Fig. 1 & 2].

Samples examined: INDIA, Department of Biotechnology, Bodoland University, Kokrajhar, Assam, May 2016, (BUMR07). Collected from Sal forest near Magurmari (26°40’74”N, 90°23’E).

T. entolomoides R. Heim is easily recognized by the medium sized dark colour pileus with bluish tints and swollen stipe at the base. It is the second most abundant mushroom found in the western Assam. In India, it has been reported from Goa [4] and Kerela [19,20].

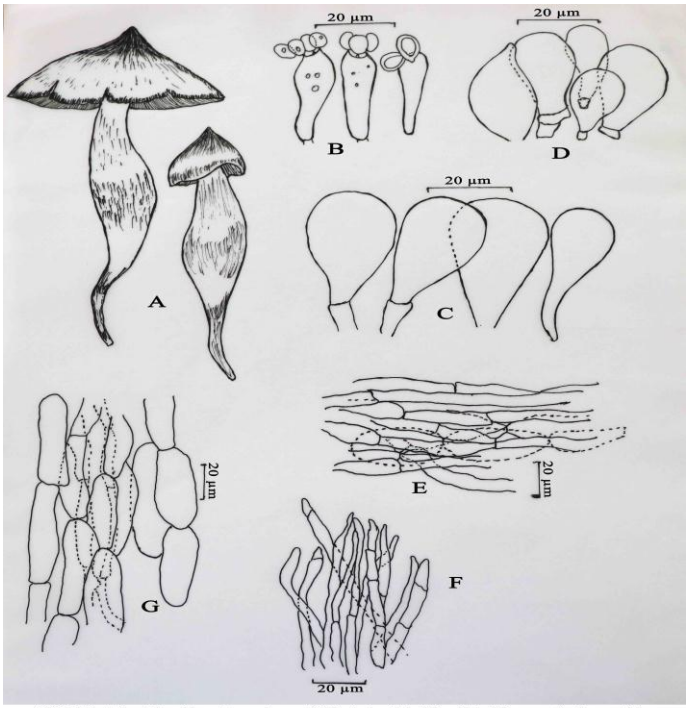

FIGURE 1: A. T. entolomoides mature and young fruiting body , B. basidia with basidiospores, C. pleurocystidia, D. cheilocystidia, E. hymenophoral trama hyphae, F. pileipellis hyphae, G. stipitipellis hyphae.

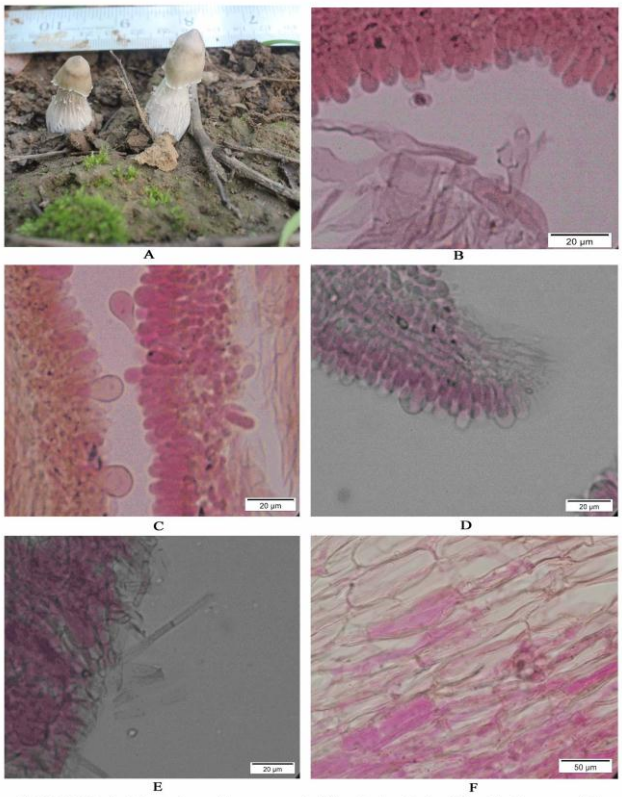

FIGURE 2: A. T. entolomoides young fruiting body, B. basidia, C. pleurocystidia, D. cheilocystidia, E. pileipellis hyphae, F. stipitipellis hyphae.

Phylogenetic Analyses

The PCR product of BUMR07 results in ~600 bps in length. NCBI Blast confirms the sample as *Termitomyces* genus. ITS region sequences of *Termitomyces entolomoides* is not available in GenBank to confirm the species. The phylogentic analysis of the newly obtained sequence of BUMR07 and 19 NCBI downloaded sequence were clustered to form separate clades (Fig 3). BUMR07 is not clustered with any of the *Termitomyces* species, thus forming a separate clade G with 71% Bootstrap (BS) value. The GenBank based Asiatic *Termitomyces* sequences formed different clusters in separate clades A-F (Fig 3). Clade A with *Termitomyces fuliginosus* and *Termitomyces eurhizus* with 99% BS value. Clade B consists of *Termitomyces microcarpus* with 96 % and 79% BS. Similarly, Clade C,D,E and F are separately clustered with *Termitomyces clypeatus* (74%), *Termitomyces intermedius* (100%), *Termitomyces radicatus* (99) and *Termitomyces auranticus* (100%). *Lyophyllum semitale* and *Lyophyllum decastes* forming the outgroup. All the clustering are fully supported by more than 70% of BS value.

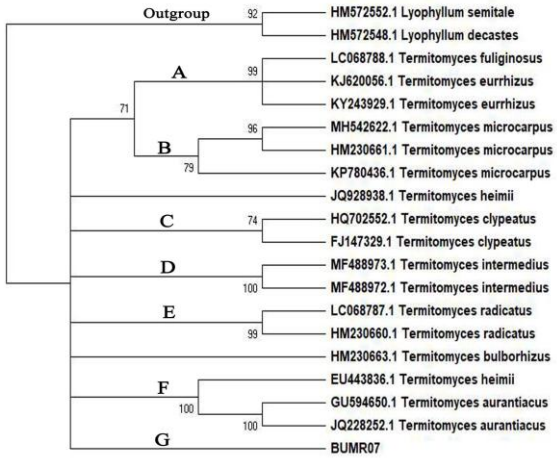

Figure 3. Molecular Phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 70% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. 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. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.9081)). The analysis involved 20 nucleotide sequences. There were a total of 2233 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

The phylogenetic analyses showed the monophyletic tree from the pure Asiatic *Termitomyces* samples that originated from the same clade (Fig 3). Siddiquee *et al.*, 2015 [2] reported the monophyly of pure Malaysian *Termitomyces* isolates and mixture of Asian and African *Termitomyces.* Frøslev *et al*., 2003 [6] revealed monophyly of Termitophilic fungi from separate and combined analyses of nLSU, mtSSU-rDNA and ITS data with BS value 99-100%. Several studies used the ITS barcode with limited number of species or unidentified *Termitomyces* strains [21,22,23]. The above discussion suggested that the combined morphology and phylogeny characters support the identification of BUMR07 as *Termitomyces entolomoides*.

IV. CONCLUSION

From the above study, it can be concluded that the *Termitomyces* species of Asian origin shares a common ancestor and are monophyletic. It is strongly suggested that the combined data of morphology and phylogeny characters are informative and useful equally for identification of a *Termitomyces* at a species level. The study promises potential to explore future possibility of cultivation technology in *Termitomyces*.

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