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Research Article

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The first report on the molecular identification of Termitomyces of Central Luzon, Philippines

Renato G. Reyes*, Jesusa Q. Undan, Rich Milton R. Dulay, Sofronio P. Kalaw and Jerwin R. Undan

Center for Tropical Mushroom Research and Development, Department of Biological Sciences, College of Arts and Sciences, Central Luzon State University, Science City of Munoz, Nueva Ecija, Philippines

****Email for correspondence:** renato.reyes@clsu.edu.ph*

Telephone Number: +63-44-4560107

ABSTRACT

DNA samples from the fruiting bodies of Termitomyces which were previously collected from their natural habitats (termite mounds) in Central Luzon, Philippines were extracted following the standard protocol on the isolation, amplification and sequencing of DNA fragments. The rDNA-ITS regions of the samples were PCR amplified using ITS3R and ITS4D primer and subsequently sequenced and analyzed using BLAST and compared with the existing NCBI data. Two species of Termitomyces namely T. bulborhizus and T. clypeatus were identified. The rDNA-ITS sequences of the different samples yielded lower percentage value i.e. 92% for T. bulborhizus and 87% for T. clypeatus when compared to other NCBI reported - Termitomyces.

***Keywords:** mushroom identification, termite-associated mushroom, Termitomyces*

INTRODUCTION

Termitomyces is a genus of termite-associated mushroom under Family Tricholomataceae of the Kingdom Fungi [1]. Its fruiting bodies which are being consumed by Filipinos in the rural areas of the country are usually collected from the wild during rainy season. This mushroom is considered as nutraceutical due to the health benefits that it provides in addition to its nutrient content. Previous reports confirmed that different species of *Termitomyces* exhibit biofunctionalities such as antioxidant [[1] [2], anti-inflammatory [3] [4] and hypocholesterolemic [5] activities. This nutraceutical mushroom remains to be in the wilderness due to the unavailability of mushroom production technology. Most of the studies conducted on the taxonomy of *Termitomyces* in the Philippines were only based on its morphological characterization which oftentimes led to the misidentification of the unknown mycological collection. Efforts on the taxonomic accounting of the unknown species of *Termitomyces* in the Philippines which was based on the visual observation of the morphology of the fruiting bodies have been exerted by local researchers. *Termitomyces striatus*, *T. robustus* and *T. clypeatus* were reported in Southern and Northern Luzon, Philippines [6] [7] [8]. To date, there is no in-depth investigation in the Philippines on the molecular identification of this genus. Thus, our research team initiated this investigation to provide an accurate taxonomic accounting of the unknown species of *Termitomyces* based on their molecular profile.

MATERIALS AND METHODS

Source of specimen

Fruiting bodies were collected from the active termite mounds in the middle of rainy season (August-September) in 5 different sampling sites in Central Luzon.

Extraction of DNA

The mushroom fruiting bodies were ground in liquid nitrogen and transferred into a 2 mL sterile tube. A 750 µl of pre-warmed 2X CTAB buffer (20g CTAB dissolved in 860 ml sterile double distilled water, 81.82g NaCl, 100 ml 1M Tris pH 8.0, 40 ml 0.5M EDTA pH 8.1) and 50 µl of 20% SDS were added to the samples and mix using vortex and incubated at 65°C for 30 min using dry bath (Labnet D1200-230V Accublock Digital dry bath). After 1 hour of incubation and cooling, 750 µl of chloroform was added to the samples and these were mixed thoroughly and carefully using a vortex. The tubes containing the suspensions were centrifuged for 30 minutes at 10,000 rpm (Heraeus; Pico17) and the upper layer was transferred into a new 1.5 ml sterile tube. Subsequently, 600 µl of ice-cold isopropanol was added, incubated overnight at -20°C, mixed gently then centrifuged for 10 minutes at 10,000 rpm. After centrifugation, the isopropanol was decanted and the pellet was washed by 500µl of 70% ethanol. The samples will be subjected in the centrifuge for 10,000 rpm for 3 minutes. The ethanol was decanted and the formed pellets were subjected to air-drying until the alcohol was completely removed from the pellet. The pellet was dissolved using TE buffer with RNAse. One (1) uL of DNA stained with 1 µL of gel red (Biotium) was evaluated and checked by running in 1% agarose gel (prepared in 1X TAE buffer) using gel electrophoresis system (Enduro Gel XL) at 100V for 30 minutes and analyzed under gel photo documentation system (Labnet GDS-1302 Enduro Imaging System) The concentration and purity of the DNA samples were determined using Fluorometer (E6150 Quantus, Promega). Finally, the total genomic DNA of each sample was diluted to 50 ng/µl and stored at -20°C.

PCR amplification

Amplification of specific fragment of samples was performed using a pair of universal primer: ITS3R (5′-ATCGATGAAGAACACAG -3′) and ITS4D (5′- TCCTCCGCTTATTGATAGC -3′). DNA amplification was performed by PCR (2720 Thermal Cycler) with the following parameters: an initial cycle of 5 min at 94 °C; 35 cycles of 30 s at 94 °C, 45 s at 56 °C, 30 s at 72 °C; and final extension of 7 min at 72 °C. Reactions were carried out in 0.2 mL PCR tubes of 50 µL containing 33.96 µL of distilled water, 1 µL of forward primer (10 µM), 1 µL of reverse primer (10 µM), 5 µL of dNTP's (25 mM), 5 ng (4µL) of DNA template, 5 µL 10X buffer and .04 µL of Taq polymerase. One 1 µL of amplification products and the 1 kb DNA ladder stained with 1 µL gel red (Biotium) and ran for 30 minutes at 100 V on 1.0 % agarose gel (prepared in 1X TAE) was analyzed under gel photo documentation system (Labnet GDS-1302 Enduro Imaging System).

Sequence analysis

Once the expected size of amplified fragments have been confirmed, the PCR products were sent to 1st Base Laboratory, Malaysia for PCR purification and sequencing. The chromatogram results were evaluated using 4peaks (NUCLEOBYTES.COM) software.

BLAST and Phylogenetic analysis**.** The nrDNA ITS sequence of the mushroom species was used for BLAST (Basic Local Alignment Search Tool) analysis. The DNA sequences were aligned using the ClustalW program version 2.0.3 provided in the Phylogeny.fr software [9] using the default parameters. Phylogenetic analysis was performed using Phylogeney.fr and tree rendering using the programs BioNJ (neighbour-joining) and TreeDyne 198, respectively.

RESULT AND DISCUSSION

Two species of *Termitomyces* (Fig. 1) namely *T. bulborhizus* and *T. clypeatus* were identified. The molecular identity of the collected samples based on the rDNA-ITS (Ribosomal DNA Internal Transcribed Spacers) region is shown in Table 1. Based on the results, the rDNA-ITS sequences of the different samples gave low % value compared to other *Termitomyces* with available sequences from NCBI.

T. clypeatus was reported in Southern and Central Luzon by previous researchers [1] [6] [7] [8] who based their identification on the morphological features of their collection. *T. bulborhizus* was not mentioned in the said reports, however 2 unidentified species of *Termitomyces* collected in the provinces of Central Luzon namely, Pampanga, Tarlac and Zambales were emphasized by De Leon et al [6] in addition to *T. clypeatus, T. striatus* and *T. robustus*. In this paper most of the samples examined which were obtained from different geographical areas in Central Luzon are *T. bulborhizus.*

Table 1. Molecular identity of the collected samples based on the rDNA-ITS (Ribosomal DNA Internal Transcribed Spacers) region.

Code	Species	% Identity	GenBankAcc. N0.
Sample_01	*Termitomyces_bulborhizus*	92%	HM230663.1
Sample_02	*Termitomyces_bulborhizus*	92%	HM230663.1
Sample_03	*Termitomyces_bulborhizus*	92%	HM230663.1
Sample_04	*Termitomyces clypeatus*	87%	HQ702552.1
Sample_03	*Termitomyces_bulborhizus*	93%	HM230663.1

Phylogenetic analysis

The phylogenetic analysis was done using Phylogeny Fr. Based on the phylogenetic tree (Fig. 2), samples 01, 02, 03 and 05 are *T. bulborhizus* (HM230663.1). On the other hand, Sample 5 was identified as *T. clypeatus* (HQ702552.1) and found clustered with *T. fulginosus* (LC068788.1) and *T. eurrhizuz* (KJ620056.1).

Fig. 1. Fruiting bodies of *Termitomyces bulborhizus* (upper photo) *and T. clypeatus* (lower photo) growing in their natural habitat

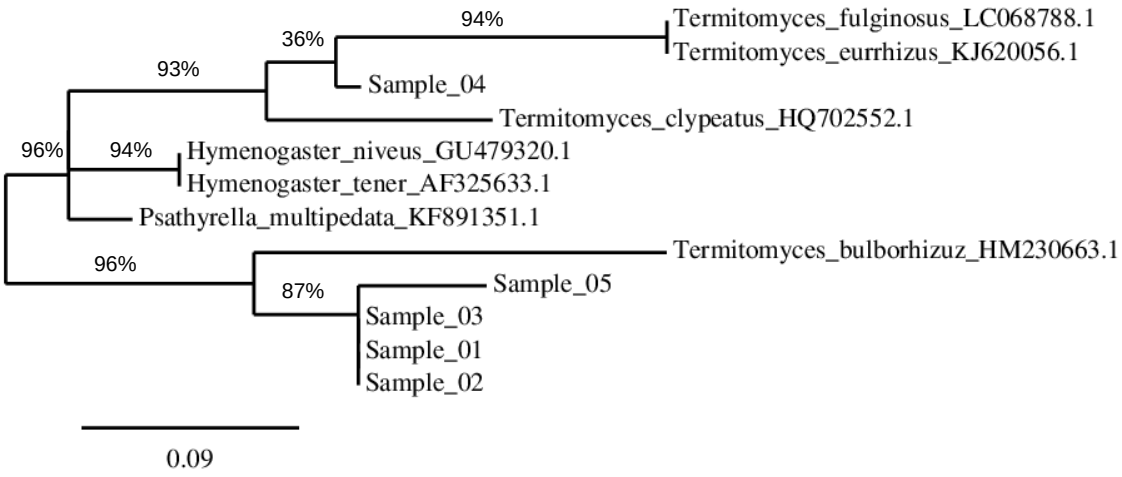

Fig. 2. Cluster analysis of the collected samples of *Termitomyces*

CONCLUSION

This is the first report on the molecular identification of *Termitomyces* species in Central Luzon, Philippines. Two species of *Termitomyces* namely *T. bulborhizus* and *T. clypeatus* were molecularly analyzed and defined.

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