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MOLECULAR IDENTIFICATION AND GERMPLASM CONSERVATION OF WILD MUSHROOMS FROM MAYANTOC, TARLAC, PHILIPPINES

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Abstract– Tarlac is a landlocked province located in Central Luzon, Philippines. It has natural forests containing natural vegetation which offers favorable surroundings for the mushrooms to grow. In this study, a collection of macrofungi was conducted, followed by molecular identification. The genomic DNA was extracted through CTAB method, and amplified the ITS region using Internal Transcribe Spacer (ITS) gene marker. The amplified products were sequenced and identified using BLAST NCBI analysis. There are 25 mushroom samples that were successfully identified and with high similarity with those of other related species retrieved from GenBank. The samples were classified into 6 orders, 12 families, 15 genera, and 25 species from Basidiomycota and Ascomycota which separated into two branches in the phylogeny tree. The tree also shows that the most species collected were from the order Polyporales with 11 species grouped in the first clade followed by Agaricales with 8 mushroom species. A portion of the fruiting body with 15% glycerol and all the mycelial samples were transferred in cryovials and placed in a cryofreezer for preservation.

INTRODUCTION

Asia, including the Philippines has a relatively high number of macrofungi (De Leon *et al.,* 2013). They are morphologically and phylogenetically diverse and known to produce several bioactive molecules (Raja *et al.,* 2017). They can be healthy and nutritious food as well and are consumed worldwide. Wild mushrooms are seasonally and traditionally collected in the forest and or any areas with lignin cellulosic substrates and consumed by farmers and mushroom hunters (Rahi and Malik, 2016). Nowadays, a great variety of mushrooms has been utilized traditionally in many different cultures for the maintenance of health (Yaseen *et al.,* 2016), and also as an important food product for their crucial role in human health, nutrition, and disease control (Abatenh *et al.,* 2018). Because of the advancement of technology, numerous discoveries have been made elucidating the nutritional (high in fibers, proteins, vitamins; low in fats, cholesterols, sodium) and medicinal (anti-oxidative, anti-hypertensive, neuritogenesis) properties of mushrooms, all of which are highly beneficial (Samsudin *et al.,* 2019). But in some regions, people used to grow mushrooms to satisfy their local needs.

Before, the identification of mushrooms is based on macroscopic or morphological observation of each part of the fruiting body, such as the pileus, stipe, lamella, and volva, on an empirical basis (Carvalho *et al.*, 2014) which is sometimes confusing and led to the misidentification of the species.

Therefore, the modern molecular technique is indeed necessary to reduce the challenges of inconspicuous nature, inconsistent morphology, and indiscrimination among fungal species often associated with the traditional method of nomenclature (Blackwell *et al.*, 2006; Nilsson *et al.*, 2011).

This study moreover, is in line with the United Nations' Sustainable Development agenda by 2030 which comprises sustainable consumption and production, food security and nutrition. Mushrooms are relevant in achieving sustainable development for food security and they produce a broad range of biochemicals, secondary metabolites and enzymes. Mushrooms have gained importance in recent years because of their potential use in various areas of research like biotechnology, nutraceuticals and pharmacology.

This study aims to collect mushrooms and identify them molecularly as it provides more accurate methods for identification than the few characteristics afforded by traditional morphological features. Moreover, to preserve collected mushrooms and DNA for future use.

MATERIALS AND METHODS

Collection of mushroom samples

Samples were collected within forest reserve of Sitio Calao, located at the municipality of Mayantoc, Tarlac, Philippines (Figure 1). The municipality of Mayantoc is situated at approximately 15° 37' North and 120° 23' East, on the island of Luzon. Elevation at these coordinates is estimated at 59.2 meters or 194.3 feet above sea level. These natural forests contained natural vegetation which provides favorable surroundings for the mushrooms to grow. Collections were conducted during the August of 2019, when the mushrooms are highly present. The samples were collected from their respective substrate, initially identified based on their morphological characteristics such as color, pileus, gills, and other distinguished observations, placed in labeled paper bags, and immediately brought to the Biotechnology and Analytical Laboratory at College of Science, Central Luzon State University, Philippines.

Fig. 1. The Map of the Philippines expanding Mayantoc, Tarlac as the collection site.

In the laboratory, the surface of the fruiting body was gently cleaned to remove attached material such as soil and leaves and then preceded to DNA extraction. Some parts of the fruiting body were taken and plated on potato dextrose agar (PDA) to rescue the mycelia for cryopreservation. About 5x5mm of rescued mycelia was obtained using a scalpel and then also subjected to DNA extraction.

Genomic DNA extraction

Total genomic DNA was extracted using the Cetyltrimethylammonium Bromide (CTAB) method, following Murray and Thompson (1980) protocol with minor modifications. About 3.0g of fruiting bodies were homogenized using sterilized mortar and pestle. To each sample, 500µL of preheated CTAB buffer to 65 °C was added and incubated at 65 °C in a dry bath for 45 min and 50µL SDS was added and mixed with vortex every 5 min. A ratio of 24:1 of chloroform-isoamyl alcohol was added to each sample after incubation and then mixed using a vortex. The mixtures were centrifuged at 10000 rpm for 30 min. The supernatant was transferred to a new 1.5 mL tube.

To precipitate DNA, 500µl of ice-cold isopropanol was added to the samples and incubated at -20 °C overnight. The DNA was pelleted by centrifugation for 10 min at 10000 rpm and washed twice with 400µl 70% ethanol. The pellets were air-dried for 30 min and then suspended or dissolved in 100µl of low-salt TE (10mM Tris, 0.1mM EDTA [pH 8]) buffer. A 2µl DNA mixed with 3µl loading dye was loaded into 1% agarose gel then a run-in gel electrophoresis system (AccurismyGel™ Mini Agarose Gel Electrophoresis System) was carried out at 100V for 30 min for DNA quality checking using the gel documentation system (UVITEC Gel Documentation Systems).

PCR amplification and sequence analysis

The DNA isolated from fruiting bodies was diluted at 1:100 using sterile distilled water. The rDNA ITS region was amplified using ITS1F (5'-CTTGGT CATTTAGAGGAAGTAA-3') (Gardes and Bruns, 1993) and ITS4R (5'-TCCTCCGCTTATTGATATGC-

3') (White et al., 1990) primers. A total of 25µl PCR reaction mixture containing 2.5µl of 10x PCR Buffer, 1µl of 10mM dNTPs, 1.5µl of 25mM $MgCl_2$, 1µl each primer, 0.1µl of Taq DNA polymerase (KAPA Taq), 1µl of diluted DNA and 16.9µl of sdH2O was performed in Thermal Cycler (Bio-Rad T100™).

The PCR profile was as described by Muruke *et al.* (2002), with slight modification where 35 cycles were initiated with the following PCR profile temperature profile: denaturation of 95 °C for 3 min; 95 °C for 30 sec; primer annealing of 51C for 30 sec; primer extension of 72 °C for 1 min; and final extension of 72 °C for 10 min.

The PCR products were analyzed by agarose gel electrophoresis and the amplified products were DNA quantified using the Spectrophotometer (Thermo Scientific) then sent to 1st BASE Laboratory, Malaysia for PCR clean-up and DNA sequencing. The chromatogram results were analyzed using BioEdit software (Hall, 2011) and assessed in the GenBank database using NCBI-BLAST (Altschul *et al.,* 1990) for identities. Default search parameters on the standard nucleotide BLAST was used. Related species sequences were downloaded and used for phylogenetic analysis.

Phylogenetic Analysis

Sequences were analyzed in MEGA version X (Kumar *et al.,* 2018) and aligned using the MUSCLE multiple alignment program. The evolutionary history was inferred using the Neighbor-Joining method (Saitou *et al.,* 1987) with a Bootstrap analysis of 100 replication rounds. Evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004).

Cryopreservation

About 3.0g of fresh fruiting bodies were placed in cryovials with 15% glycerol. Mycelial stock cultures of collected mushrooms were maintained by regular sub-culturing. Mycelia were then transferred to cryovials containing 15% glycerol as cryoprotectant. Samples were first placed at -20 °C and then stored at -80 °C cryofreezer.

RESULTS AND DISCUSSION

The collection was done during the rainy season when numerous kinds of basidiomycetes grow

Table 1. List of identified macrofungi from Mayantoc, Tarlac

Code	Scientific name	%Identity	GenBank Accession #	Elevation and Coordinates		
				Elevation	Latitude	Longitude
MT02	*Stereopsis radicans*	91.64	KC203487.1	110m	15°36′10.7″	120°19′45.1″
MT03	*Gerronema* sp.	91.22	KR155081.1	154m	15°36′10.5″	120°19′45.0″
MT04	*Scytinopogon* sp.	98.00	MN580122.1	129m	15°36′16.8″	120°19′43.5″
MT05	*Trametes* sp.	100.00	KC848260.1	138m	15°36′06.9″	120°19′43.1″
MT06	*Ganoderma australe*	99.67	LC084717.1	142m	15°36′06.9″	120°19′42.2″
MT10	*Marasmiellus palmivorus*	99.70	JQ653438.1	156m	15°36′04.3″	120°19′36.2″
MT11	*Marasmiellus* sp.	97.51	MN483261.1	173m	15°36′04.1″	120°19′35.3″
MT14	*Auricularia cornea*	100.00	MN156315.1	155m	15°36′04.2″	120°19′36.1″
MT16	*Hygrocybe lucida*	95.16	NR163753.1	157m	15°36′01.6″	120°19′29.7″
MT17	*Hygrocybe firma*	90.31	GU222283.1	159m	15°36′01.6″	120°19′29.7″
MT19	*Trogia infundibuliformis*	99.24	JQ031775.1	170m	15°36′01.9″	120°19′28.3″
MT21	*Physisporinus* sp.	99.65	KY131868.1	175m	15°36′02.2″	120°19′27.5″
MT23	*Trametes hirsuta*	94.07	KX056103.1	173m	15°36′04.8″	120°19′28.5″
MT24	*Haddowia longipes*	99.65	MK345424.1	172m	15°36′05.3″	120°19′26.5″
MT25	*Trametes elegans*	100.00	LC176779.1	195m	15°36′06.0″	120°19′25.6″
MT27	*Ramariopsis laeticolor*	95.67	KM248913.1	166m	15°36′03.5″	120°19′22.6″
MT28	*Trametes cubensis*	100.00	MN068933.1	151m	15°36′01.6″	120°19′19.2″
MT29	*Favolus tenuiculus*	100.00	KU189775.1	298m	15°36′04.8″	120°19′17.8″
MT32	*Marasmiellus palmivorus*	99.56	JQ653438.1	135m	15°36′01.7″	120°19′17.1″
MT33	*Xylaria* sp.	99.82	MK333998.1	135m	15°36′01.7″	120°19′17.1″
MT34	*Efibula* sp.	93.00	KP135018.1	137m	15°36′01.3″	120°19′17.1″
MT36	*Favolus america*	99.51	KP013020.1	204m	15°35′59.1″	120°19′13.0″
MT37	*Auricularia nigricans*	99.59	MT252555.1	202m	15°35′55.3″	120°19′09.4″
MT38	*Xylaria schweinitzii*	99.40	KP133464.1	202m	15°35′55.3″	120°19′09.5″
MT39	*Ganoderma* sp.	99.26	KP027536.1	202m	15°35′55.3″	120°19′09.4″

abundantly. A total of 40 samples were collected. However, PCR amplification and sequencing were successfully only for 25 macrofungi collected. Samples for identification were obtained from the fruiting body of collected macrofungi and an ITS marker was used. This primer has been used for species-level classification for a variety of organisms including fungi (O'Brien *et al.* 2005; Tan *et al.* 2010) and proven to have the highest resolving power for discriminating closely related species with a high PCR and sequencing success rate across a broad range of Fungi (Schoch *et al.*, 2012). Identities were based on the maximum percent similarity of each sample inferred from GenBank as well as the comparison of GenBank sequences to the actual photos and morphological characteristics of the samples. The list of species identity, percent identity, accession numbers, and geographical coordinates were provided in Table 1. Specimens were arranged sequentially based on their sample code. In addition, the photographs of the collected macrofungal samples are shown in Figure 2.

Fig. 2. Collected and molecularly identified macrofungi from Mayantoc, Tarlac, Philippines. A. *Stereopsis radicans* (MT02); B. *Gerronema* sp. (MT03); C. *Scytinopogon* sp. (MT04); D. *Trametes* sp. (MT05); E. *Ganoderma australe* (MT06); F. *Marasmiellus palmivorus* (MT10); G. *Marasmiellus* sp. (MT11); H. *Auricularia cornea* (MT14); I. *Hygrocybe lucida* (MT16); J. *Hygrocybe firma* (MT17); K. *Trogia infundibuliformis* (MT19); L. *Physisporinus* sp. (MT21); M. *Tramete hirsuta* (MT23); N. *Haddowia longipes* (MT24); O. *Trametes elegans* (MT25); P. *Ramariopsis laeticolor* (MT27); Q. *Trametes cubensis* (MT28); R. *Favolus tenuiculus* (MT29); S. *Marasmiellus palmivorus* (MT32); T. *Xylaria sp.* (MT33); U. *Efibula* sp. (MT34); V. *Favolus america* (MT36); W. *Auricularia nigricans* (MT37); X. *Xylaria schweinitzii* (MT38); and Y. *Ganoderma* sp. (MT39).

Based on the molecular and phylogenetic analysis, the 25 samples were classified into 6 orders, 12 families and 15 and genera. A total of 18 mushrooms were identified up to species level, while 8 could only be identified up to the genus level only. The phylogenetic tree includes 50 nucleotide sequences that was divided into two major branches. The first branch belonged to Phylum Basidiomycota while the second branch is Ascomycota.

Polyporaceae formed into a monophyletic group in the first branch that are usually known to be tougher fungi or the bracket fungi. They are consumed as food and tea (i.e., *Ganoderma* sp. and *Trametes* sp.) (Kozarski *et al.*, 2012) and also find widespread application as medicinal mushroom (Wasser, 2002). The Polyporaceae is further subdivided in two lineages representing genera of Favolus and Ganoderma including *Haddowia longipes* and the four genera of Trametes. The *E. tuberculata* and *Physisporinus* sp. are not considered tough fungi so that they separated from the family Polyporaceae. Physisporinus species are white-rot wood-decaying fungi that grow on a wide variety of gymnosperm and angiosperm trees (Dai, 2012; Yamashita *et al.*, 2020), and also characterized by effused reflexed and soft to juicy basidiocarps (Wu *et al*., 2017).

The second monophyletic group composed of family Tricholomataceae, Omphalomataceae and Marasmiaceae from the order Agaricales, also known as gilled mushrooms (Moncalvo *et al.*, 2002) or euagarics, which contains some of the most familiar types of mushrooms. The *Trogia infundibuliformis* and *Gerronema* sp. merged in one group because they both characterized small and having an umbilicate (Norvell *et al.*, 1994; Latha, *et al.*, 2018) or deeply infundibuliform pileus (Senthilarasu, 2014), while three Marasmiellus fused in another group under the Family Marasmiaceae. Marasmiellus species are known to have small basidiocarps and has the potential to produce ligninolytic enzymes, mainly laccases, for degradation of lignocellulosic biomass, especially lignin (Tagger *et al.*, 1998; Klonowska *et al.*, 2001; Gramss *et al.*, 2005; Schneider *et al.*, 2018). A clade comprising Hygrocybe species which are often brightly colored and have waxy to slimy caps, white spores, and smooth, ringless stems recovered as sister to all other species of Agaricales.

Another clade composed of two Auriculariaceae or the jelly fungi which also considered as edible

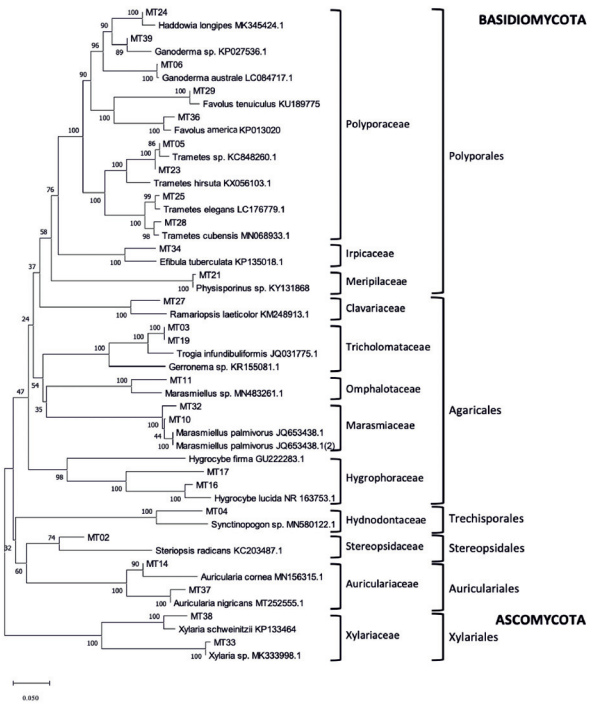

Fig. 3. Phylogenetic tree showing relationship of 25 macrofungi with closely related taxa based on neighbor-joining approach. Bootstrap values obtained with 100 repetitions are indicated as percentage at all branches. The scale bar represents 0.050 substitution per site.

mushroom; and the two coral-like mushroom that belonged to family Hydnodontaceae and Stereopsidaceae. The outgroup of the tree falls to *Xylaria schweinitzii* and *Xylaria* sp.. They belonged to Xylariaceae, a large and well-known ascomycete family, usually wood inhabitants, while some occur in dung, litter, soil, or even insect nests (Edwards *et al.*, 2003).

CONCLUSION

Molecular approach has been introduced as a convenient, rapid, and accurate, and requires only a small amount of sample. For over a decade, mycologists and other fungal researchers has been determined that ITS regions work well for identifying mushroom species and was used in the identification as it was found to be with highest probability of correct identifications especially on fungi samples. Accurate identification of mushroom is the key in utilizing them for the benefit of humans. Although the forest reserve of Mayantocis a small, nevertheless, there are diverse wild edible mushrooms that can be collected. Without proper

understanding and information about wild mushroom species, locals will continuously use them based on their traditional knowledge while overlook some that they believe to be poisonous and with no potential benefits. Thus, this study provided the list of collected macrofungi that may serve them as a guide and provides additional information for further studies and future scientific researches.

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