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The First Report on the Molecular Identification of Wild Macroscopic Fungi *Oudemansiella australis* G. Stev. and G. M. Taylor from La Union, Philippines

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ABSTRACT

Towards the effort of documenting mushroom resources in the Philippines, precise identification using nucleotide base approaches and geo-tagging activities were conducted in this study. Often times, identification of macroscopic fungi or mushroom species was done using morphological methods. Due to the growing potential of mushrooms as food, nutraceutical and or medicinal applications, sources of where these important mushrooms can be found must seriously be taken into consideration. The first report on the molecular identification of macroscopic fungi collected in La Union, Philippines was carried out by amplifying its ITSnrDNA region. Basic Local Alignment Search Tool (BLAST) analysis of the amplified gene region the wild macroscopic fungi has 98.07% identity to *Oudemansiella australis* with accession number AF321473. The morphology of the basidiocarp was also determined for proper documentation. Phylogeny tree was also rendered using the MEGA X software based on the ITS sequence of the mushroom.

Key words : *ITS nrDNA, Oudemansiella australis, Phylogeny, Molecular identification*

Introduction

The diverse mycological resources of the Philippines have promising pharmacological and nutraceutical potentials. These mycological resources are proliferating on different substrates and in different geographical locations across the archipelago (Reyes *et al.,* 2013). The growing interest of researchers in the field paved the way for the continued efforts and research works in the discovery of their potential use that would benefit the society as a whole. Interestingly, as Filipinos are becoming acquainted with the good taste and healthful benefits of consuming mushrooms, mushroom production in the Philippines is progressively improving (Dulay *et al.,* 2015).

It is then very important to search for more potential mushroom species that have promising nutraceutical or medicinal applications. Interestingly, past research in mushrooms expressed its great potential not only in nutrition but in a wide range of pharmacological benefits. Several mushrooms like *Pleurotus* species are very good dietary foods which have been reported to contribute positive effects on body metabolism, where it helps in decreasing the free lipid triglycerides thus preventing arteriosclerosis. Aside from that, it was also re-

ported that many mushrooms have medicinal properties such as antiviral, antitumor, antibiotic, and immuno-modulating activities (Adebayo-Tayo *et al.,* 2011). Likewise, the need to collect, identify, and conserve mushroom species for fungal diversity is imperative because no one knows when and how these valuable life forms might be lost forever (Ram *et al.,* 2010).

The identification of a mushroom species is being done using morphological features of the mushroom under study. Apparently, the use of morphological features in the identification of mushroom species is sometimes inaccurate and limited. When the specimen is damaged or in a young stage of development there is insufficient information making it hard to identify the mushroom sample (Hebert *et al.,* 2003). With the advent of biotechnology nowadays, a more powerful tool in mushroom identification is in practice, i.e., molecular identification with the use of the DNA sequence. In the case of mushrooms, the nuclear ribosomal internal transcribed spacer (ITS) region serves as an appropriate barcode in ascertaining the identity of mushroom species (Schoch *et al.,* 2012; Dentinger *et al.,* 2011). Molecular tools provide more accurate methods for identification using molecular markers and DNA techniques which are quick and reliable in establishing identities of various wild mushrooms (Rajaratnam and Thiagarajan, 2012).

Materials and Methods

Collection of the macroscopic fungi

The fruiting body of the wild macroscopic fungi was taken and the morphological features were described and recorded. Likewise, it was also subjected to spore printing by gently removing the pileus from the stipe using a blade and placing it in black paper and then covered with an opaque container (small bowl) and was set aside for 6 to 8 hours. After which the spore print was labeled and properly stored. The coordinates where the sample collected was recorded.

DNA extraction

Initially, a portion of the collected specimen was washed with distilled water then air dried in an ambient area for 4 to 6 hours. After which, the dried sample was soaked in 500 µl of CTAB (Cetyltrimethylammonium Bromide) (Sigma USA) overnight. Then, the sample was ground using mortar and pestle aseptically in a laminar flow. A volume of 500 µL of the ground sample with CTAB was added with 50 µL of SDS (Sodium Dodecyl Sulfate) (Vetec India) which was subjected to dry bath (Labnet D1200) for 45 min at 70 °C and was mixed occasionally using vortex every 5 min. The sample was cooled briefly prior to the addition of 500 µL of fresh isoamyl-chloroform (1:24) then the tube was mixed gently by flipping it slowly several times.

After which, the sample was centrifuged (Centurion Scientific) at 10,000 rpm for 30 min. A volume of 200 µL from the supernatant liquid was taken and then added with 500 µL of isopropanol then incubated at a temperature freezer (Operon Ultralow Temperature Freezer) -70 °C for 4 hours. After which, the sample was centrifuged at 10,000 rpm for 10 min, the samples were decanted to remove the isopropanol and allow it to evaporate for a few minutes. The sample was further washed with ethanol twice in the same process. Then, the pellet was dissolved in 20 µL TE Buffer and was incubated at room temperature for 3 to 4 hours.

Amplification of the ITS region using PCR and sequencing of amplified products

Using a micropipette, a mixture of 5 µl of DNA and 5 µl loading dye (KAPPA) was loaded into previously made 1% agarose gel. Gel electrophoresis (Enduro gelXL) was done at 50 V for 60 min. The gel was viewed in a gel documentation system (Labnet GDS-1302) for imaging to observe and check the quality of the DNA.

For molecular identification of the collected fungi, ITS 1 (´5-TCCGTAGGTGAACCTGCGG-3´) forward primer and ITS 4 (5´-TCCTCCGCT TATTGA TATGC-3´) reverse primer were used to amplify the nuclear ribosomal ITS region. The final component concentration per 20 µl PCR reaction were: 3 µl of diluted DNA, 2.5 µl 10x PCR Buffer, 0.5 µl DNTP Mix, 1 µl Forward ITS 3D, 1 µl Reverse ITS 4, 15 µl sterile distilled water and 0.2 µl Taq Polymerase (KAPA). The PCR profile that was used to amplify the nrDNAinternal transcribed spacer (ITS) region was set up to 35 cycles with initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 30 sec, annealing at 57 °C for 45 sec, extension at 72 °C for 30 sec and final extension 72 °C for 7 min and final hold at 10 °C.

The amplified PCR products were placed in microtubes, and were sent to 1st BASE DNA se-

quencing Division of Apical Scientific Laboratory at Malaysia for sequencing.

BLAST and phylogenetic analysis

The partial ITS sequence of the mushroom was analyzed using the Basic Local Alignment Search Tool (BLAST) program of the National Center for Biotechnology Information (NCBI) website against the whole Genbank database. The obtained sequence match in the database with the highest similarity percentage was noted with its accession number. MEGAX software (Kumar *et al.*, 2018) was used for multiple sequence alignment, specifically the ClustalW (Thompson *et al.*, 1994) and eventually inferring the phylogeny of the collected wild mushroom using the same software.

Results and Discussion

The wild mushroom was collected near the foot of Mt. Nagdaingan situated in Barangay Luzong Norte, Bangar, La Union, Philippines with coordinates of N 16° 52.328′, E 120° 26.694′ and an elevation of 27 m above sea level (Figure 1).

Morphology of the basidiocarp and spore print

The collected basidiocarp of the wild macrofungus catches one's attention for its size and the unusual clusters they make whenever they sprout. The collected mushroom C was formed in clusters due to the clump connections they make with each other on the base of each stipes. The stipe can grow as much as 16 cm in height with thickness ranging from ~3.0 cm (base) to ~1.8 cm (top) and circumference of ~9.3 cm (base) and ~5.0 cm (top). Though it seems to be thick, it is only solid at the base and quite spongy from the middle to the top, true for the mature basidiocarp. The surface of the stipe is smooth and white to dirty white in color. The pileus, on the other hand, is ~1.3 cm thick with a diameter of ~10.5 cm and circumference of ~32.9 cm. The surface of the pileus is smooth and white to dirty white in color. The pileus is entirely convex even after reaching maturity. The gills (underside of pileus) are also white in color and it is not continuous with the stipe. The mushroom is generally white in color and quite aromatic. The spore print is white in color (Figure 2).

BLAST and phylogenetic analysis

The amplified sequence of the ITS region of the mushroom sample C was aligned using Nucleotide BLAST. Table 1 presents the summary of the BLAST analysis of the macrofungal sample using the nuclear internal transcribed spacer sequence fragment. It is clearly shown in the table that the mushroom sample C with 415-bp of sequence length has the 98.07% similarity with *Oudemansiella australis* with accession number AF321473.

Furthermore, phylogenetic analysis also revealed that by using the ITS region, the sample C was very

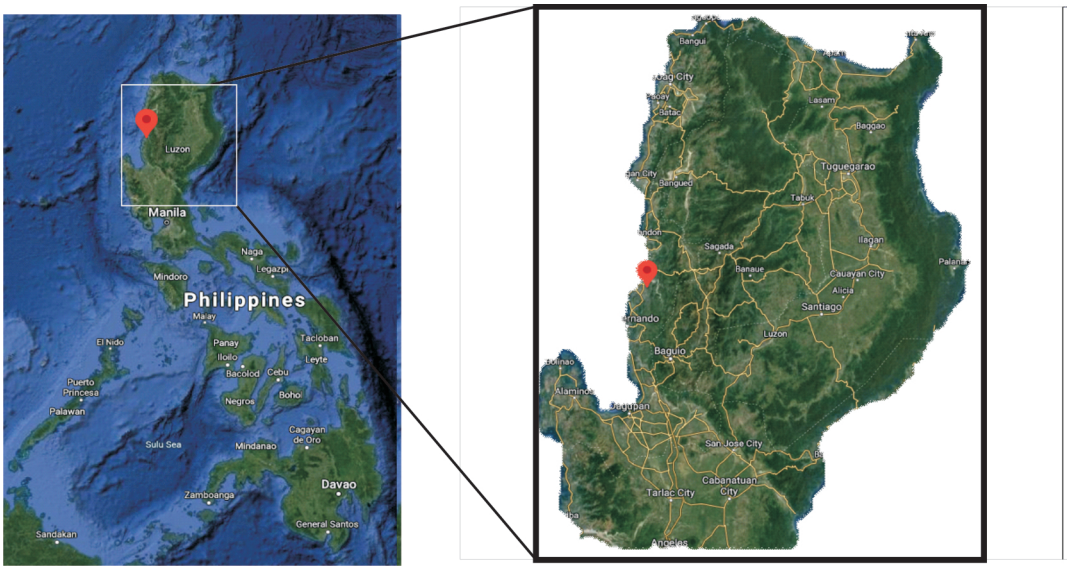

Fig. 1. Map of the Philippines and the Luzon Island where the La Union, Philippines was located on where the sample was collected.

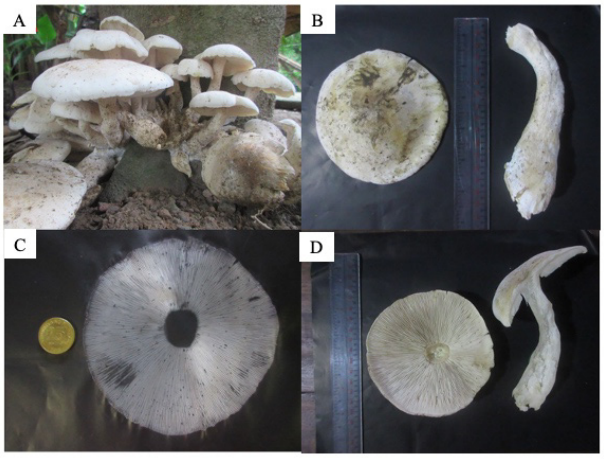

Fig. 2. Wild macroscopic fungi in its natural habitat (A), the pileus and stipe (B), the sporeprint (C), and the pileus showing the gills and the stipe showing its internal structure and the connection with the pileus (D).

much closely related to the *Oudemansiella australis* (Figure 3) and as also with other species under Physalacriaceae family. The genus *Oudemansiella,* where *O. australis* belongs, is claimed by some authors as a broadly circumscribed genus including *Xerula* and *Mucidula.* However, many authors consider *Oudemansiella, Xerula,* and *Mucidula* as separate genera slowly validated by the growing contributions of various authors as they discern and summarize their taxonomic history (Alberti *et al.,* 2020).

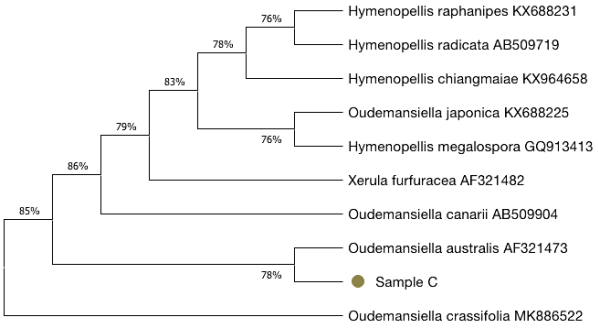

Fig. 3. Phylogenetic relationships of the collected mushroom inferred from ITS nrDNA gene sequences.

With the advancement in molecular studies, Oudemansiella is placed in the family Physalacriaceae of the order Agaricales comprising around 39 currently acceptable species (Alberti *et al.*, 2020; Niego *et al.*, 2021).

Morphological distinctions of *Oudemansiella* include ixotrichodermpileipellis composed of filamentous hyphae often intermixed with chains of inflated cells (Niego *et al.*, 2021), pileus dry to generally viscid, lamellae white to off-white, and stipe, central without veil remnants and lacks persistent annulus (Alberti *et al.*, 2020; Niego *et al.*, 2021). The *Oudemansiella* is distributed from tropical to temperate areas however, Niego *et al.,* (2021) stresses that most of them are found in tropics. Interestingly several research noted that several species of *Oudemansiella* have biofunctional activities and has antibacterial, antifungal, cytotoxic and antioxidant potential (Vahidi and Namjoyan, 2004; Rosa *et al.,* 2005; Hleba *et al.,* 2016; Acharya *et al.,* 2019). Surprisingly, there is no or limited research published on the biofunctional activity of *O. australis* in the Philippines, hence, uncovering its biological activities or functional benefits must be explored.

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Table 1. BLAST analysis summary of the mushroom sample using the nuclear internal transcribed spacer sequence fragment.

Sample code	Sequence Length (bp)	% Identity	Accession No.	Identity	Coordinates
Sample C	415	98.07%	AF321473	*Oudemansiella australis*	N 16° 52.328′, E 120° 26.694′

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