Molecular identification and phylogeny of some wild microscopic fungi from selected areas of Jaen, Nueva Ecija, Philippines

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ABSTRACT
Background: The rDNA-ITS (Ribosomal DNA Internal Transcribed Spacers) region of the genomic DNA of the collected wild macroscopic fungi from selected areas in Jaen, Nueva Ecija, Philippines was amplified and used for nucleotide sequence homology analysis using the Basic Local Alignment Search Tool (Nucleotide BLAST). Using the rDNA-ITS sequences of the collected macrofungi, the 7 wild macroscopic were identified. There are 3 macrofungi identified as Lentinus swartzii (GU207276.1) with 100% identity, 1 species was identified as Lentinus squarrosulus (KT207470.1) with 99% identity, 1 species was identified as Panaeolus foenisecii (KR867660.1) with 100%, 1 species was identified as Coprinellus aureogranulatus (GQ249274.1) with 100% identity and 1 species was identified as Schizophyllum commune (KR706163.1) with 100 % identity. All of the collected species were identified up to species level. The molecular identification strategy in this study was proven accurate for identifying mushroom. This study in the molecular identification of collected species was the first report in the Philippines and the sequences may provide additional information in the molecular taxonomy of the mushroom and thus important in its domestication and characterization as reference for further research and exploration of its benefits.

KEYWORDS: rDNA-ITS, BLAST, macroscopic fungi, taxonomy

INTRODUCTION

Macrofungi have been part of the fungal diversity for around 300 million years and they are known to be among the largest of group of fungi that attracted the attention of naturalists before microscopes were invented [1].

Fungi or also known as "mushroom" is defined as “macrofungus” with a distinctive fruiting body, it can be hypogeous or epigeous, large enough to be seen by the naked eye and usually pick up by hands. Most macrofungus or mushroom species are under the Basidiomycota and Ascomycota, the two phyla under the Kingdom Fungi [2]. Mushrooms are important source of food and utilize to gain income in both developing and
developed countries. Furthermore, mushrooms are great recyclers, decomposers and bioremediators; and therefore play a significant role in the ecosystem. With these, mushrooms which happened to be in the wilderness, growing on fallen logs, decomposing piles of straws, lawns, meadows and gardens were now intentionally cultivated for consumption [3].

Mushrooms can be roughly divided into four categories: (1) edible mushrooms (2) medicinal mushrooms, (3) poisonous mushrooms, and (4) those in a miscellaneous category, which includes a large number of mushrooms whose properties remain less well defined. Certainly, this approach of classifying mushrooms is not absolute. Studies on identification and characterization of more mushrooms under the miscellaneous category must be given attention for research for their possible benefits to mankind.

Ecologically, mushrooms can be classified into three groups: the saprophytes, the parasites and the symbiotic species (which includes Mycorrhiza sp.). There are only a few parasitic mushrooms that are known while most of the cultivated gourmet mushrooms are saprophytic fungi. These species have a symbiotic relationship with some vegetation, particularly trees, i.e. there is a relationship of mutual need.

Moreover, indigenous community are utilizing mushroom for the treatment of different type of diseases and also as an aphrodisiac and tonic. Different types of edible mushrooms are cultivated on large scale for commercial use and many more species of mushrooms that grow in the wild, which has much nutritional and medicinal value. The Philippines as a tropical country has very rich mycological resources and Filipinos are known to be mushroom eaters. Unfortunately these mycological resources are not yet known and fully utilized [4].

The study on the molecular identification of wild mushrooms was an important activity to identify those unknown mycological resources that the Philippines has. Through the advent of molecular marker technology, it is now possible to determine fungi based on their molecular data. The molecular markers, PCR (Polymerase Chain Reaction) and non-PCR based are widely used for mushroom identification and characterization [5]. Recently, The Internal Transcribed Spacer (ITS) of nuclear DNA has been proposed as the official bar coding marker for molecular identification of fungi [6].

The ITS regions of fungal ribosomal DNA (rDNA) are highly variable sequences of great importance in distinguishing fungal species. The objective of this study was to identify the wild macroscopic fungi found in selected areas of Jaen Nueva Ecija, Philippines using the amplified rDNA-ITS region collected mushroom. The morphology of the mushroom was also examined for verification and confirmation. The study also determined the phylogenetic relationships of the different mushrooms collected.

MATERIALS AND METHODS

Mushroom collection:
The mushrooms were collected at three different areas in the municipality of Jaen Nueva Ecija. The areas were Barangay Dampulan, Barangay Langla and Barangay Putlod, Jaen, Nueva Ecija, Philippines. Fruiting bodies of wild mushroom were collected during the summer season (April-May) of 2015. The samples were either carefully handpicked or with the use of knife. The global coordinates of the sampling sites were recorded with the use of Global Positioning System (GPS). Specimens were place in a paper bag with tag of location and other important information. The samples were stored at the at the Molecular Biology and Biotechnology Laboratory, Department of Biological Sciences, College of Arts and Sciences, Central Luzon State University, Science City Munoz, Nueva Ecija. The samples were cleaned using fine brush, documented with picture and standard measurements were taken. The specimens were preserved with 95% ethanol and coded until use for DNA extraction.

DNA Extraction:
Approximately 10g of the fruiting body preferably from the gills was collected and placed in 2ml tubes for genomic DNA extraction. Tissue homogenization was carried-out by grinding specimens in liquid nitrogen using mortar and pestle. Total DNA was extracted using the cetyltrimethyl ammonium bromide (CTAB) method based on Murray and Thompson (1980) [7] with minor modification. Pre-warmed 750μl 2X CTAB buffer and 50 μl of 20% sodium dodecyl sulfate (SDS) were added to the homogenized specimens. The mixture was thoroughly mixed in a vortex shaker and incubated in a water bath at 65°C for 3 1 hour, after that 750μl of chloroform was added and thoroughly mixed using vortex. Tubes containing specimens were centrifuged for 30 min at 10,000 rpm.

The aqueous phase was transferred into a new 1.5ml tube, and 600 μl of cold isopropanol were added and incubated at -20°C overnight. Specimens were centrifuged for 10 min at 10,000 rpm, and then decant the isopropanol. The DNA pellet was washed with 70% ethanol and centrifuged twice for 3 min at 10,000rpm. After draining the ethanol and air-drying, the pellet was dissolved in 50μl TE buffer and incubated at room temperature for 2 to 3 h until the pellets dissolve completely. To check the DNA quality, 1 μl of the DNA mixed with 1μl loading dye was loaded into 1% agarose gel containing 1 μl of gel red (GelRed™ Nucleic acid,
Biotium). Electrophoresis was carried out at 100 V for 30 minutes using Enduro™ Gel XL, Labnet International Inc. The gel was viewed in Enduro™ GDS the for gel imaging. Quantification of DNA was done using Quantus™ Fluorometer (Promega). The genomic DNA was diluted 1:100 by means of sterilized distilled water. A volume of 1μl of diluted DNA was used for PCR analysis.

**PCR amplification, sequencing and phylogeny analysis:**

The polymerase chain reaction was performed using the following component: 2.5μl of 10X PCR buffer, 1.5μl of MgCl₂ (25mM), 1.25 μl of dNTP mix (25mM each), 1.13μl of forward primer [5'-GTAAGTGCAGATTCGAAATAACTGGAATGGGA-3' (10pm/μl)], 1.13μl of reverse primer [5'-AGGGCTTGGGATCTCATTCAGGTCTATCACCTC-3' (10pm/μl)], .09 μl of Taq polymerase and 15.4μl of DNase- and RNase-free water, and 2.0μl of DNA. The PCR was performed in a DNA Thermal Cycler (Applied Biosystem® 2720) programmed as follows: 5 min at 94°C (initial denaturation) followed by 35 cycles of 30 sec at 94°C (denaturation), 30 sec at 56°C (annealing), 45 s at 72°C (extension), and 1 cycle of 10 min at 72°C (final extension). The final step was held at 10°C. The PCR products were stored at 4°C. PCR products (1 μL) was loaded in the gel with loading dye and gel red along with 1kb DNA ladder (KAPA™ Universal Ladder). The gel was run at 100 volts for 30 minutes and the amplified products were visualized in the UV trans-illuminator and photographed in Gel Doc system (Enduro™ GDS Gel Documentation System). The PCR samples were quantified using Fluorometer (Promega) and were sent to 1stBASE Laboratory at Malaysia for PCR clean up and sequencing.

After sequencing, the sequences were viewed using 4peaks (nucleobytes.com) and was used for homology analysis using blastn a default parameter for nucleotide sequence homology from Basic Local Alignment Search Tool (BLAST). Phylogenetic analysis was performed using advance mode of Phylogeny fr. [8] using MUSCLE for sequence multiple alignment, PhyML for the construction of phylogeny tree and TreeDyn for tree rendering.

**Results:**

**Molecular identification of macrofungi:**

There were seven wild "mushrooms" collected in the three areas. Two were collected at Dampulan (Lat N 15° 20' 28" Long N 120° 54' 14"), two were collected from Langla (Lat N 15° 19' 26" Long N 120° 55' 04") and three from Putlod (Lat N 15° 25' 5" Long N 120° 53' 39") (Table 1).

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Collection area</th>
<th>Latitude</th>
<th>Longitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. SP1</td>
<td>Dampulan</td>
<td>N 15° 20' 28&quot;</td>
<td>N 120° 54' 14&quot;</td>
</tr>
<tr>
<td>2. SP2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. SP3</td>
<td>Langla</td>
<td>N 15° 19' 26&quot;</td>
<td>N 120° 55' 04&quot;</td>
</tr>
<tr>
<td>4. SP4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. SP5</td>
<td>Putlod</td>
<td>N 15° 25' 05&quot;</td>
<td>N 120° 53' 39&quot;</td>
</tr>
<tr>
<td>6. SP6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. SP7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The different samples were collected from varying habitat and environment as presented in Table 2. The fruiting body of SP1 was found in weedy backyard on a compost area, the SP2 and SP3 were found in weedy backyard on a decayed branch of tree, the SP4 was found in highland and composted area. The SP5 were found in small groove decaying wood. The SP6 were collected in wide level highland. The partial nucleotide sequence of the 7 samples were obtained and analyzed for homology analysis and sample identity query. All of the samples were identified up to species level. The sample code SP1, SP4 and SP5 were all identified as Lentinus squarrosulus with 99% sequence similarity and wth a GenBank Accession number GU207276.1, respectively. The SP2 was identified as Schizophyllum commune with 100% identity (KR706163.1). Lastly, SP7 was identified as Panaeolina foenisecii with 100% identity (KR867660.1) (Table3).

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Species identification</th>
<th>Maximum identity</th>
<th>GenBank Accession Number</th>
<th>Taxon</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. SP1</td>
<td>Lentinus swartzii</td>
<td>99%</td>
<td>GU207276.1</td>
<td>Fungi, Basidiomycota, Agaricomycetes, Polyporaceae, Lentinus, L. swartzii</td>
</tr>
<tr>
<td>2. SP2</td>
<td>Schizophyllum commune</td>
<td>100%</td>
<td>KR706163.1</td>
<td>Fungi, Basidiomycota, Agaricomycetes, Agaricales,</td>
</tr>
</tbody>
</table>
Phylogenetic analysis:

The phylogenetic tree was derived from 23 nucleotide sequences closely related from the samples used in this study using ITS partial sequences (Figure 1). The phylogenetic tree was composed of four main branches that roughly correspond to four groups or clades. The first group consisted of SP2 [Schizophyllum commune (KR706163.1)] together with Auricularia polytrichia (FJ617293.1) with a percentage bootstrap support value of 100%. These species belongs to division Basidiomycota and both were commonly found at fallen branch or logs. Both species are sessile type of mushroom. The second group consists of the SP7 [Panaeolus foenisecii (KR867660.1)], together with other closely related species belong to Panaeolus species, the P. fimicola (JF908514.1), P. cyanescens (EU834287.1) and P. retirugis (FJ478119.1) with a bootstrap support value of 99%. This group of species usually has a smooth solid cup and black gills underneath. The third group formed the cluster of species belong to Coprinellus species (100% bootstrap support value) where in the SP3 [Corinellus aureogranulatus (GQ249274.1)] belong. The other Coprinellus species in this group are; C. micaceus (JX160060.1), C. radians (JN943117.1), and C. xanthrothrix (JN159578.1). Most of the Coprinellus species were commonly known as sink cup mushrooms because they dissolve into black ink in maturity. The SP1, SP4, SP5 [L. swartzii (GU207276.1)] and SP6 [L. squarrosulus (KR183767.1)] belong to the fourth group composed of Lentinus species with percentage support value of 99%. Lentinus species have white color with brownish shade and become yellowish when mature and most of them had funnel shaped cup and produced white spore print.

Discussion:

The Schizophyllum commune Fr. like the L. squarrosulus and L. swartzii are edible mushrooms and also known to have medicinal properties. The molecular data for Schizophyllum commune was still limited and was just reported recently on oititis externa in human [9] other than its benefits, S. commune was a known pathogen. The molecular data of Panaeolus foenisecii (Pers.) J. Schroet was first reported in Sri Lanka, collected from elephant dung in dry zone forest reserves [10]. Panaeolus species was known as umbrella mushrooms because of their umbrella cup like structures. Most of them have grayish black gills and the cups quickly crumble when handled.

The differences between them can be distinguished by their specific details in their structures and appearance. The C. aureogranulatus was first reported as Coprinus aureogranulatus [11] and the taxon was further characterized and verified using molecular approach [12]. This species was found at Papua New Guinea in 1996 [13]. C. aureogranulatus is a species of mushroom in the Psathyrellaceae family and was later transferred to Coprinellus in 2001 [12]. The Lentinus Fr. is belongs to genus of decaying-wood Agaricomycetes, the species found to have tough basidiocarps, with hyaline spores and decurrent lamellae. Previous studies on the molecular taxonomy of this species still limited and still the application of the generic name Lentinus has been controversial [14][15][16][17]. Due to different classification scheme, its was the molecular data that have been strongly proposed and with the support of phylogenetic analysis in order to settle nomenclature issues surrounding its taxonomic classification. The species of Lentinus species play an important role in the environment and locally found in diverse ecosystem, from boreal to tropical regions [18][19]. The identification of mushroom in the Philippines using molecular data using ITS region of Schizophyllum commune, Coprinus aureogranulatus, Panaeolus foenisecii, Lentinus swartzii and Lentinus squarrosulus are important information for its taxonomy, distribution and phylogeny studies.
Fig. 1: Constructed phylogeny tree based on PhyML method using the partial rDNA-ITS sequence of the species and other closely related species available from NCBI.

**Conclusion:**

The study collected and characterized 7 samples from three areas in Jaen, Nueva Ecija, Philippines. The molecular approach using the rDNA-ITS region firmly verify the different macrofungi in the selected areas. The samples were separated into four distinct clades with other closely related species in the Basidiomycota. The complex diversity of macrofungi in nature possesses difficulty in terms of its taxonomic identity using its morphology alone. With the advent of advance molecular tools and technique, specimens can now be verify and identify properly and may overpass the limitation of using classical taxonomic classification. Thus, the molecular identification are very important to elucidate the proper identity of the species exist in our environment and therefore must fully utilized to identify other species of macrofungi in the different areas in the Philippines.

**REFERENCES**


