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ORIGINAL ARTICLE

Diversity of fungal endophytes isolated from *Marchantia polymorpha* populations from Baguio City, Philippines

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ABSTRACT
This study was undertaken to isolate, identify and determine the diversity of endophytic fungi from Marchantiapolymorpha. Four hundred fifty thallus fragments from 15 individuals were surface sterilized and plated onto potato dextrose agar. Nineteen morphospecies of fungal endophytes were characterized from the plated segments. Of the eighteen morphospecies, twelve were identified up to the species level by sequence homology using the ITS1/ITS4 primer pair. These were Acremonium alternatum, Colletotrichum boninense, Colletotrichum cymbidiicola, Colletotrichum karstii, Colletotrichum novae-zelandiae, Fusariumoxysporum, Penicillium commune, Penicillium crustosum, Penicillium purpurogenum, Pestalotiopsis disseminata, Pestalotiopsis microspora and Setophoma terrestris. Four isolates were identified to the genus level namely, Aspergillus sp., Pseudozyma sp., Fusarium sp., and Nodulisporium sp. Two remained unidentified due to sequence homology of less than 95%. However, it was enough to designate them to belong to the class Eurotiomycetes and Sordariomycetes. Results showed that diversity of the endophytes of the thallus of M. polymorpha was comparable to many angiosperms investigated to date.
***Keywords**: Endophytic fungi, liverwort, diversity*

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INTRODUCTION

Plants have symbionts that live within their organs. Any organism that are found inside the living tissues of plants are called endophytes. Fungal endophytes are microorganisms that colonize living internal tissues of plants without causing any immediate and apparent negative effects [1]. They have been found in all plant species examined to date. They are ubiquitous and have been found in above ground tissues, and in roots. They have been associated in all lineages in plants including liverworts, mosses, seed-free vascular plants, conifers, and angiosperms [2]. Endophytic fungi may receive nutrition and protection from the host plant while the host may benefit from enhanced competitive abilities and increased resistance to herbivores, diseases, and various abiotic stresses [3]. Accordingly, endophytes likely play an important role in ecological communities [4].

However, the majority of mycological researches on endophytes have been conducted on spermatophytes, especially the domesticated ones [5]. Researches undertaken to characterize the fungal communities in other lineages such as in bryophytes is scarce [6]; more so in the Philippines. This research aims to contribute to the limited information about symbiotic fungi to bryophytes, specifically to *Marchantiapolymorpha* - a thalloid liverwort. The sampled population of this liverwort was found in the campus of the University of the Philippines Baguio campus.

MATERIALS AND METHODS

Plant Collection and Isolation of fungal endophytes

Fungal endophytes were obtained from *Marchantia* polymorphathalli. Fifteen healthy thalli were collected randomly from 15 different plants. Endophytes from the living tissues were isolated according to methods used in Arnold and Lutzoni (2007) with several modifications. The thalli were washed in a running water to remove debris and patted dry. They were surface-sterilized using sequential immersion in 95% ethanol

(1 minute), NaOCl solution (2:1 v/v water; 5 minutes), and 95% ethanol (30 seconds), and washed with sterile water three times and were allowed to dry under sterile conditions. Ten fragments were cut from each of the thalli and plated onto to potato dextrose agar (PDA) containing chloramphenicol. Triplicate plates were prepared for each individual liverwort thallus. The plates were incubated at 30°C for 7-14 days. Emergent hyphae were transferred onto new PDA slants.

Morphological characterization and identification of fungal endophytes

The isolated endophytic fungi were identified using the amplified and sequenced internal transcribed spacer (ITS) region with the ITS1 and ITS4 primer pair using the genomic DNA extracted from the fungal mycelia using the methods of Liu et al. [7]. Amplification of the target gene segment was performed using the EmeraldAmp MAX HS PCR Master Mix following the manufacturer's instructions. Cycling conditions were as follows: 2 min initial denaturation at 94 °C followed by 35 cycles of 1 min denaturation at 94 °C, 30 s primer annealing at 55°C, and 45 s extension at 72°C. Final extension was at 72°C for 5 min. Amplified DNA size was confirmed through agarose gel electrophoresis demonstrating single bands for all reactions at around 550 bp. PCR products were sent to 1st Base in Singapore for sequencing. All sequencing reads were used for the Basic Local Alignment Search Tool (BLAST) searches of the NCBI GenBank database and Mycobank database for provisional identification.

In the study, sequences that presented ≥98% similarity were considered to belong to the same species while sequences that presented ≥95 % similarity were considered to belong to the same genus. However, species was considered unidentified when sequences presented <95 % similarity. Multiple sequence alignment and phylogenetic analyses were performed using Molecular Evolutionary Genetics Analysis version 6 (MEGA 6).

Diversity Analyses

Diversity of cultured fungal endophytes was measured using Shannon-Wiener, Simpsons', Evenness, and Chao-1 indices using Paleontological Statistics (PAST).

RESULTS

Identification and Phylogenetic Analyses

Eighteen morphospecies of fungal endophytes were characterized from the leaves of *M. polymorpha.* Table 1 summarizes the fungal endophyte isolates from this research with their corresponding GenBank accessions. Of the eighteen morphospecies, eighteen were identified through a query search using the sequences from 1st Base Intl to the Genbank and Mycobank databases. The genus and species of the isolates were determined by sequencing the PCR products of each fungal endophytes using the primers ITS1 and ITS4 separately. The identified species were *Acremonium alternatum, Aspergillus* sp., *Colletotrichum boninense, Colletotrichum cymbidiicola, Colletotrichum karstii, Colletotrichum novae-zelandiae, Fusariumoxysporum, Fusarium*sp., *Nodulisporium*sp., *Penicillium commune, Penicillium crustosum, Penicillium purpurogenum, Pestalotiopsisdisseminata, Pestalotiopsismicrospora, Pseudozyma* sp., *Setophoma terrestris,* Unidentified Eurotiomycete, and Unidentified Sordariomycete.

Among the identified species, seventeen were ascomycetes while only one was basidiomycete yeast. The basidiomycete was *Pseudozyma*sp. Of the isolates that were successfully sequenced. Phylogenetic analyses showed three major subclades of Ascomycetes (Figure 1). The Ascomycete clade was monophyletic and included the seventeen gene sequences of fungal endophytes isolated from *M. polymorpha*. The Sordariomycetes (A) included most of the isolates of this research. There was a single representative of the Dothideomycetes being *Setophomaterrestris.* SubcladeC included the Eurotiomycetes representatives of the *Aspergilllus* and *Penicillium* genera. The Basidiomycete clade included the yeast identified as*Pseudozyma*sp. This clade served as out group.

Table 1: The registered accession numbers provided by the GenBank for the different isolates in this study.

Isolate	Accession Number
Acremoniumalternatum	KP714265
Aspergillus sp.	KP714267
Colletotrichumboninense	KP714268
Colletotrichumcymbidiicola	KP714269
Colletotrichumkarstii	KP714270
Colletotrichum novae-zelandiae	KP714271
Fusariumoxysporum	KP714275
Fusarium sp.	KP714276
Nodulisporium sp.	KP714282
Pestalotiopsisdisseminata	KP714292
Pestalotiopsismicrospora	KP714295
Penicillium commune	KP714283

Penicilliumcrustosum	KP714284
Penicilliumpurpurogenum	KP714288
Pseudozyma sp.	KP714299
Setophomaterrestris	KP714301
Unidentified Eurotiomycetes	KP714308
Unidentified Sordariomycetes	KP714310

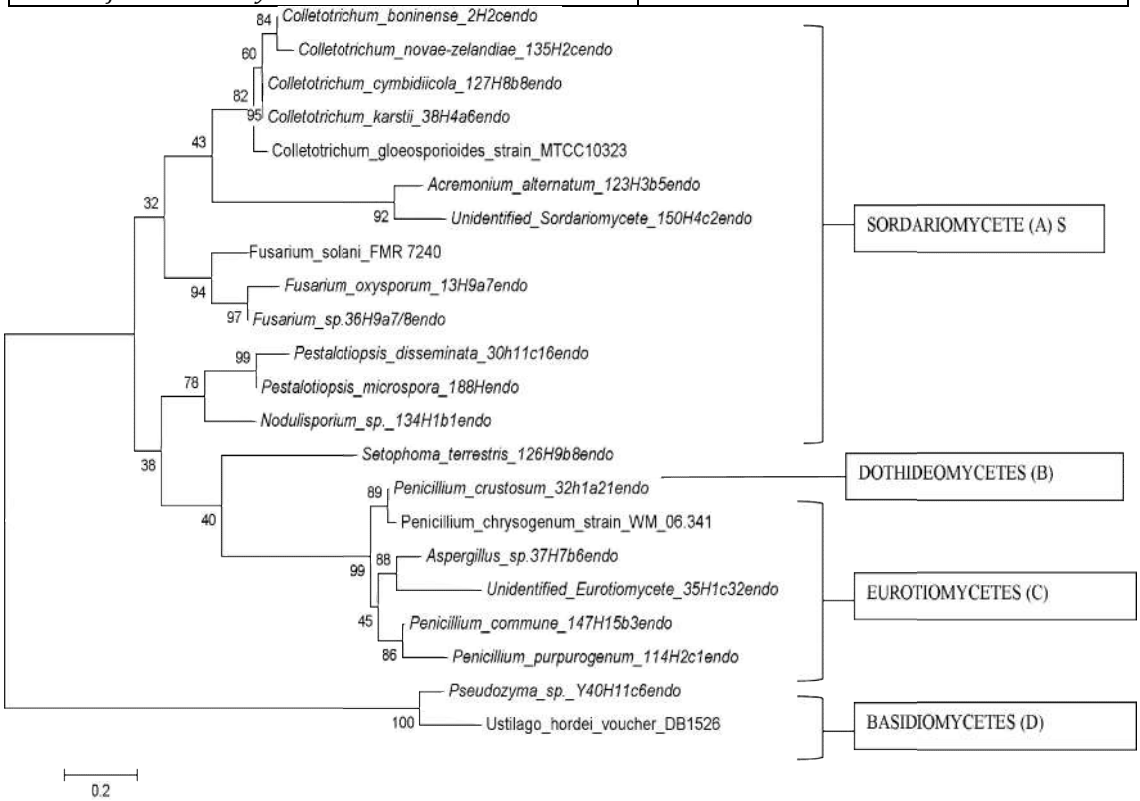

Figure 1. Molecular Phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The tree with the highest log likelihood (-5321.0461) is shown. The percentage of trees in which the associated taxa clustered together is 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 = 0.6265)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 22 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 475 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 .

Diversity Analyses

Four hundred fifty fragments from the living tissues of *M. polymorpha* leaves were collected and sampled for fungal endophytes. The distribution of the isolates showed a log-normal pattern with few common species and many rare species (Figure 2).

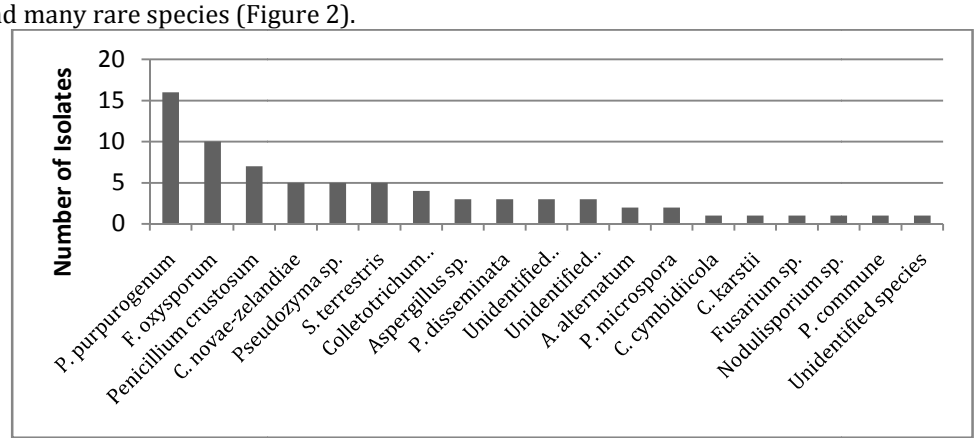

Figure 2.Frequency of endophytic fungi from *M. polymorpha.*

The most frequent species was *P. purpurogenum*, *F. oxysporum*and *P. crustosum.* These species are all filamentous fungi belonging to the Ascomycetes. Diversity analyses show that the total evenness of fungal endophytes was at 0.7072, the Shannon-weiner index was at 2.7089 and the Simspon's index at 0.9196. While the evenness of the endophytes is high, the diversity of fungal endophytes from *M. polymorpha* is low. Chao-1 was used to calculate the estimated number of missing species. The results showed that there is an estimated 27 total species in *M. polymorpha*and that there are up to nine possible species that were not detected in the sampling protocol

The total species accumulation curve (Figure 3) showed that the number of individuals did not reach an asymptote. This supports the inference that the total diversity of fungal endophytes from the *M. polymorpha*thalli was not sampled.

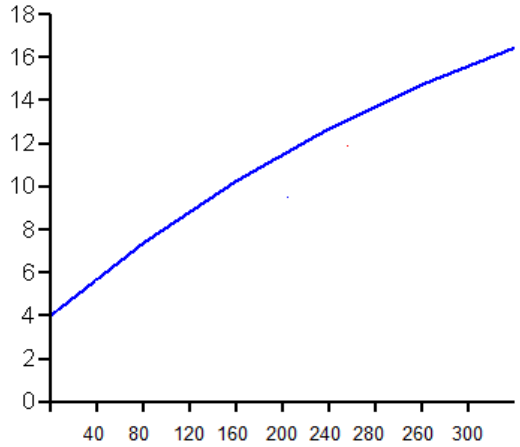

Figure 3.Total Species accumulation curve of fungal endophytes from *M. polymorpha.*

DISCUSSION

Identification and Phylogenetic Analyses

There were 74 total isolates of fungal endophytes collected from the thalli of *M. polymorpha*. They were characterized and reduced to eighteen morphospecies. Morphospecies are morphological groupings that seem to be unreliable method for the identification of fungal endophytes. However, Lacap et al. [8] proved the validity of this method as taxonomic groupings in their study of the ribosomal DNA sequence analysis. Of the sequenced and identified species, fungal endophytes were mainly composed of Ascomycetes and one species belonged to Basidiomycetes. This is probably because the fungal endophytes of *M. polymorpha* belong to Class III non-clavicipitaceous group of endophytes. Non-clavicipitaceous endophytes are highly diverse, representing a polyphyletic assemblage of primarily ascomycetous fungi with diverse and often poorly defined or unknown ecological roles [9].They have been recovered from every major lineage of land plants, and from all terrestrial ecosystems, including both agro-ecosystems and biomes ranging from the tropics to the tundra [10]. Most of them belong to the Ascomycota, with a minority of Basidiomycota [9]. Class 3 endophytes are restricted to above-ground tissues of nonvascular plants, conifers, and woody and herbaceous angiosperms [11,12]. The identified genus of *Aspergillus, Penicillium,* andUnidentified Eurotiomycete belong to Eurotiomycetes; *Acremonium, Colletotrichum, Fusarium, Nodulisporium, Pestalotiopsis,* and Unidentified Sordariomycete belong to Sordariomycete; *Setophoma*belong to Dothideomycetes; and *Pseudozyma* sp. belong to Ustilaginomycetes.

The frequency of occurrence of the different endophytic species was observed to be log-normal, which is common to the majority of living communities such as plants and animals, including microorganisms. This pattern is also common for fungal endophytes as reported by Santamaria and Bayman [13], Gazis and Chaverri [14].

Diversity analyses by Shannon-Wiener, Simpson's indices, and Evenness showed that the diversity of fungal endophytes from liverwort were not high.Possibly, the reason behind this is the relatively simple structure and short lifespan of these organisms. Fungal endophytes had been found to be more diverse in more structurally complex and longer-lived organisms such as woody plants [15].

Chao-1 revealed the number of expected missing species in the total sample is approximately nine. The Chao-1 value for the computed total species count was 27; implying that there are species that were not detected with the sampling protocol. Possibly, more sample segments plated may have detected these. Thisis confirmed by the species accumulation curve, which did not reach asymptote. Asymptotic curves

assume a finite number of species that can be collected from the source. The curve will continue to increase until it reaches plateau, which means that the species all will be collected with more samples. However, the diversity analyses done on the fungal endophytes of *M. polymoprha* in this research are basedsolelyon culture dependent procedures. The diversity was limited to the culturable fungal endophytes that were able to grow on PDA. Little is known about unculturable and slow-growing fungal endophytes. Culture-free methods such as environmental PCR can be used to approximate the total diversity of fungal endophytes in *M. polymoprha*thalli to address the limitations of the current research. Researches in the future regarding possible interactions between these endophytes and their liverwort host are also interesting activities.

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