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Diversity of sterile fungi along altitudinal gradients of Mt. Makiling Forest Reserve, Philippines

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Abstract

Mt. Makiling Forest Reserve (MFR), a well-known forest in the Philippines, functions as an instruction, research and training laboratory for the advancement of scientific and technical knowledge on the protection, conservation and development of forests and other natural resources. However, data are scarce on the diversity of fungi in MFR as previous studies relied on characteristics of spores to enumerate fungal population. Sterile fungi have been ignored or discarded. In this study, 37 and 22 sterile fungi were isolated during the wet season and dry season, respectively, from the leaf litter of MFR. The sequences of the internal transcribed spacer (ITS) regions of the ribosomal RNA were used to determine the diversity of sterile fungi along 100 meters above sea level (masl) to 1000 masl altitudinal gradients of the forest. Based on the similarity of sequences at the 97% criterion, the fungal isolates were classified into 28 molecular operational taxonomic units (MOTUs). However, fungal diversity and species richness seemed to decrease with increasing altitude during dry and wet season. While analysis of a bigger fungal population is warranted, the results of this study suggest that fungal communities in MFR are not significantly affected by vegetation and abiotic factors such as temperature and precipitation.

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Introduction

Mount Makiling, located 65 km southeast of Manila, Philippines, covers a total area of 5,907 ha and is considered as a relatively well conserved small mountain ecosystem within a highly diversified and urbanizing environment. Eighty percent of the whole Mt. Makiling is a reservation area set aside primarily as an instruction, research, and training laboratory, and includes the Mt. Makiling Forest Reserve (MFR) (Sargento *et al.*, 1997). MFR, one of the most popular forests in the Philippines, is about 42.44 km² and about 4, 244 ha (Combalicer *et al.,* 2010). It has an annual mean temperature of 27°C, and Macolod clay-loam type soil derived from volcanic tuff and ash. The soil pH ranges from 4.3 to 6.5 (Combalicer *et al.*, 2010; Sargento *et al.*, 1997). The topography of MFR is generally rugged with elevation ranging from 40 to 1100 meters above sea level (masl). MFR is home to a large number of biological species, several of which are considered threatened by the International Union for the Conservation of Nature. On October 2013, ASEAN declares MFR as 33rd ASEAN Heritage Park.

Plant litter represents a major source of organic carbon in forest soils. Fungi are considered the key players in litter decomposition because they can produce a wide range of extracellular enzymes that can efficiently attack the recalcitrant lignocellulose matrix in plants (Osono, 2007). Fungi present in decomposing forest litter are believed to be among the most diverse mycological collection (Zak and Visser, 1996). Previous studies on fungal communities of leaf litter of MFR included identification using morphological features, which depends heavily on characteristics of spores and spore-bearing structures (unpublished data). However, few fungal species do not sporulate in natural habitat and when cultured in the laboratory, and are referred to as sterile fungi. "Sterile fungi" is considered a form group, not a taxonomic division, and is used as a matter of convenience (Hall, 1987; Shivanna *et al.*, 1994; Vinod *et al.*, 2012). Fungi that fail to sporulate *in vitro* are difficult, if not impossible, to identify, hence, have usually been avoided and neglected (Currah and Tsuneda, 1993), and there is no specific species concept that is applicable to them (Vinnere, 2004). Therefore, past studies on biodiversity might have underestimated the fungal flora in leaf litter of MFR.

In this study, the diversity of sterile fungi from leaf litter of MFR was determined based on analysis of the sequences of the internal transcribed spacer (ITS) regions of the ribosomal RNA (rRNA). The ITS regions (ITS 1 and ITS 2) are non-coding but are involved in rRNA processing by forming specific secondary structures needed for recognition of cleavage sites and by providing binding sites for nucleolar proteins and RNAs during ribosome maturation (Hausner and Wang, 2005). However, the ITS regions vary in size and sequence that have been useful in determining inter- and intraspecies variability in fungal isolates (Iwen *et al.*, 2002). The ITS region is now the preferred DNA barcoding marker both for the identification of single taxa and mixed environmental templates ('environmental DNA barcoding') (Bellemain *et al.*, 2010). It has recently been proposed as the official primary barcoding marker for fungi (Schoch *et al.*, 2012).

Materials and methods

Sample collection

Leaf litter samples were collected from 100 masl to 1000 masl altitudinal gradients of MFR on November 2010 and May 2011 to represent wet and dry seasons, respectively. Litter samples included any above-ground leaves. Samples were collected every 100 masl, starting from 100 masl up to 1000 masl altitudinal gradients of MFR; hence, there were 10 sample collection sites. Each specific collection site was 20 m away from the main road or hiking trail. Within each site, three samplings were performed. Three areas, 10 m away from each other, were measured and marked. In each of the three areas, litter samples were collected in 10 points that were 1 m apart in a zigzag manner. Leaf litter samples from the 10 points of each of the three sampling sites were put together in a pail then mixed using a trowel. Consequently, three bags of leaf litter were obtained from each collection site or altitudinal gradient. Samples were placed in autoclavable plastic bags,

sealed and labeled properly.

Isolation and purification of filamentous fungi

Fifty grams of leaf litter samples were first homogenized with 450 ml of 1% peptone using a sterile blender and then serially diluted using 1% peptone diluent. Each dilution was plated onto Potato Dextrose Agar (PDA) supplemented with streptomycin (100 ppm). Cultures were incubated at 30°C for 5-7 days. After incubation, filamentous colonies were picked from the plates and transferred on PDA that were also incubated at 30°C for 5-7 days. Successive transfers to PDA were performed until colonies had been purified as determined by cultural characteristics.

Collection of sterile fungal isolates

Slide culture technique was used for the observation of reproductive structures of fungal isolates. The slide culture setup that used PDA for agar block was incubated up to one month at room temperature (~30°C) and observed periodically under the microscope until sporulation had occurred. Those isolates that failed to produce spores after four weeks of incubation were arbitrarily declared as sterile fungi and collected for this study.

Preparation of isolates for DNA extraction

Hyphae were picked from a fresh culture of each isolate with the aid of dissecting microscope, and then inoculated in Potato Dextrose Broth (PDB) containing streptomycin (100 ppm). Liquid cultures were incubated on a shaker at 125 rpm at room temperature for 3-7 days. After incubation, the spherical-shaped mycelia were collected from the liquid medium by filtration using sterile filter paper.

Genomic DNA extraction

Genomic DNA was extracted from the isolates using the ZR Fungal/Bacterial DNA Kit™ (Zymo Research, California, USA) by following the manufacturer's instructions.

Polymerase Chain Reaction

Universal primers, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5' TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990), were used to amplify the ITS regions. Amplification was performed in a 50 µl reaction containing 25 µl 2X Taq Master Mix (Vivantis Technologies, California, USA), 1.5 mM $MgCl_2$, 0.3 µM of each primer, and 3 µl of template DNA. The thermal cycling program was run on MyCycler™ Thermal Cycler (Bio-Rad Laboratories, Inc., California, USA) as follows: 3 minutes initial denaturation at 95°C, followed by 35 cycles of 40 seconds denaturation at 94°C, 50 seconds primer annealing at 52°C, 1 minute extension at 72°C, and a final 10 minutes extension at 72°C (Lacap *et al.*, 2003).

DNA Sequencing

The PCR products were sent to Macrogen, Inc., (Seoul, South Korea) for purification and sequencing. Both strands of the DNA molecule were sequenced using the universal primers ITS1 and ITS4.

Sequence analysis

Forward and reverse sequences from Macrogen, Inc. of each isolate were aligned using ClustalW2 (Larkin *et al.*, 2007) to obtain overlapping sequences into consensus sequence. Overhang sequences and ambiguous nucleotides were excluded using ChromasPro software (ChromasPro ver. 1.3). The sequence of each isolate was used as query sequence to search for the most similar reference sequences in GenBank using Basic Local Alignment Tool (BLAST) program (Altschul *et al.*, 1990). To reveal the general taxonomic placement of the unknown fungal isolates, reference sequences with 97-100% similarity were considered for multiple sequence alignment in ClustalW in MEGA 6 software. Test for the best-fit substitution model was performed to select for the most suitable model for phylogenetic tree analysis (Posada and Crandall, 2001). Thus, maximum likelihood was used to infer phylogenetic tree using Kimura 2-parameter with gamma distribution as the suitable substitution model. Genetic distance was considered to determine the molecular operational

taxonomic unit (MOTU) of the sequences. The MOTUs were group of sequences that were most similar to each other; i.e, for which the genetic distance was the smallest (Van de Peer, 2009). The sequences with similar smallest genetic distance values were clustered together in one clade and formed one MOTU. The robustness of the resulting tree was evaluated by bootstrap analyses based on 1,000 reiterations.

Diversity correlation analysis

The Shannon index was used to estimate diversity (Hill *et al.*, 2003). The indices were calculated using the function diversity of the vegan package in R software version 3.1.1. Species richness was also measured using the same software. Analysis of variance was performed to determine the correlations between fungal diversity (Shannon diversity) and each variable category (i.e., season and altitude), and the correlation between species richness and each variable category (i.e., season and altitude).

Results and discussion

The fungal isolates used in this study were declared as sterile fungi when they failed to sporulate after incubation for one month at ~30°C. Some isolates exhibited similar cultural characteristics but this information was not sufficient to declare that these isolates belonged to the same taxonomic group. Classifying isolates into morphotypes was not employed in this study since segregation of sterile fungi into morphological units based on cultural similarities does not truly reflect species phylogeny (Lacap *et al.*, 2003). Some sterile fungal isolates may be induced to sporulate by nutritional control and photocontrol (Dahlberg and Van Etten, 1982). However, these strategies require intensive experimentation with media and environmental conditions, show variable success and are time-consuming, thus, impractical for large scale survey.

Fungal composition along altitudinal gradients

The full-length ITS1-5.8S-ITS2 sequences were used to construct the phylogenetic tree. The shortest region analyzed was 363 bp; the longest was 640 bp. The 59 sterile fungi were grouped into 28 molecular operational taxonomic units (MOTUs) (Fig. 1). In terms of the number of isolates, *Schizophyllum commune* (15%) and *Diaporthe* sp. (14%) were the most common. The other MOTUs included only 1 to 4 isolates. Only members of Ascomycota and Basidiomycota had been isolated, with majority of the isolates belonging to Ascomycota (34 isolates, 19 MOTUs). The isolates included species often been reported as plant pathogens, non-pathogenic endophytes or saprobes. A few isolates identified can actually produce asexual spores (*e.g. Trichoderma harzianum*, *Penicillium chrysogenum*, *Aspergillus terreus*) but specific nutritional and environmental requirements for sporulation were apparently not provided under cultivation conditions used in this study.

Table 1. Relationships of altitude to Shannon diversity and species richness during dry and wet seasons.

Season	Shannon Diversity Index		Species Richness	
	r	p-value	r	p-value
Dry	-0.217	0.640	-0.200	0.667
Wet	-0.210	0.652	-0.492	0.150
Both	-0.210	0.645	-0.369	0.415

Influence of altitude and season on fungal composition

Fungal diversity and species richness along altitudinal gradients of MFR follow similar patterns during wet and dry seasons (Fig. 2). For both seasons, weak inverse linear relationship was found between diversity and altitude, and between species richness and altitude (Table 1), indicating that as altitude increases, fungal diversity and species richness decrease. It is surprising to find weak correlation only

as the vegetation of MFR is variable along altitudinal gradient, consisting mainly of grasses at ≤200 masl, Dipterocarp forest at 201-600 masl, mid-mountain forest at 601-900 masl, and mossy forest at 901-1130 masl (Sargento *et al.*, 1998). Other studies had shown that plant distribution or plant species is strongly correlated with fungal diversity (Burke *et al.*, 2009; Fabre, 1996).

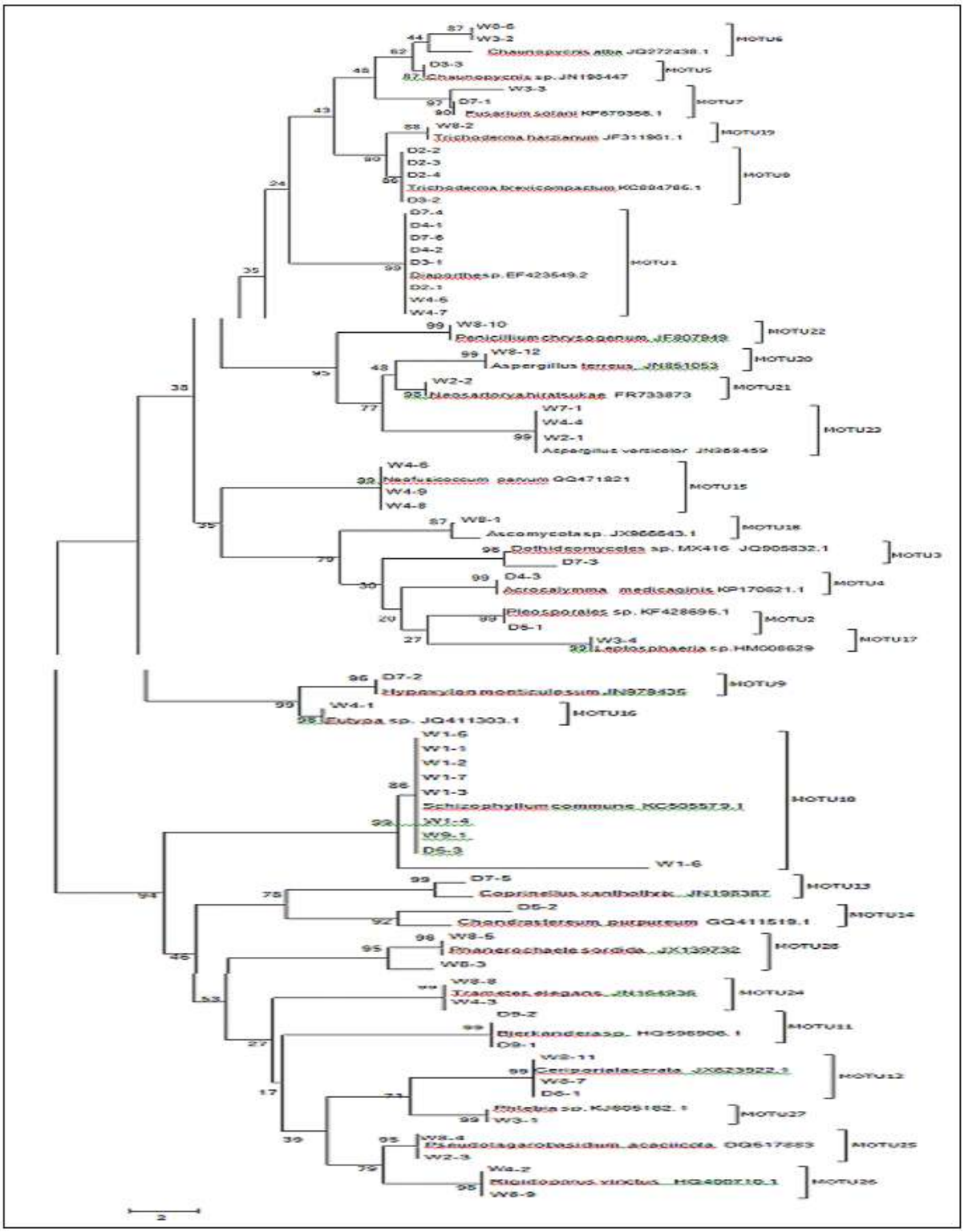

Fig. 1. Maximum likelihood phylogeny inferred from rRNA ITS sequences of 28 fungal molecular taxonomic units (MOTUs) isolated from altitudinal gradients of Mt. Makiling Forest Reserve.

Fungal diversity were unexpectedly equal for dry and wet season (p-value = 0.299). Species richness were also equal for dry and wet season (p-value = 0.554) despite differences in temperature and precipitation. The samples for the dry season were collected in May when the hottest temperature at MFR often registers, occurring during the months of April to July, with a mean annual maximum of 30.4°C. The collection in November that represented the wet season was near the coldest months, December to February, with a mean annual minimum of 23.8°C. The total rainfall on November 2010 and on May 2011 were 159.8 mm and 143.2 mm, respectively. In contrast to our study, high statistical correlation was found between fungal communities and temperature or precipitation (McGuire *et al*., 2012; Shi *et al*., 2014; Zachow *et al*., 2009).

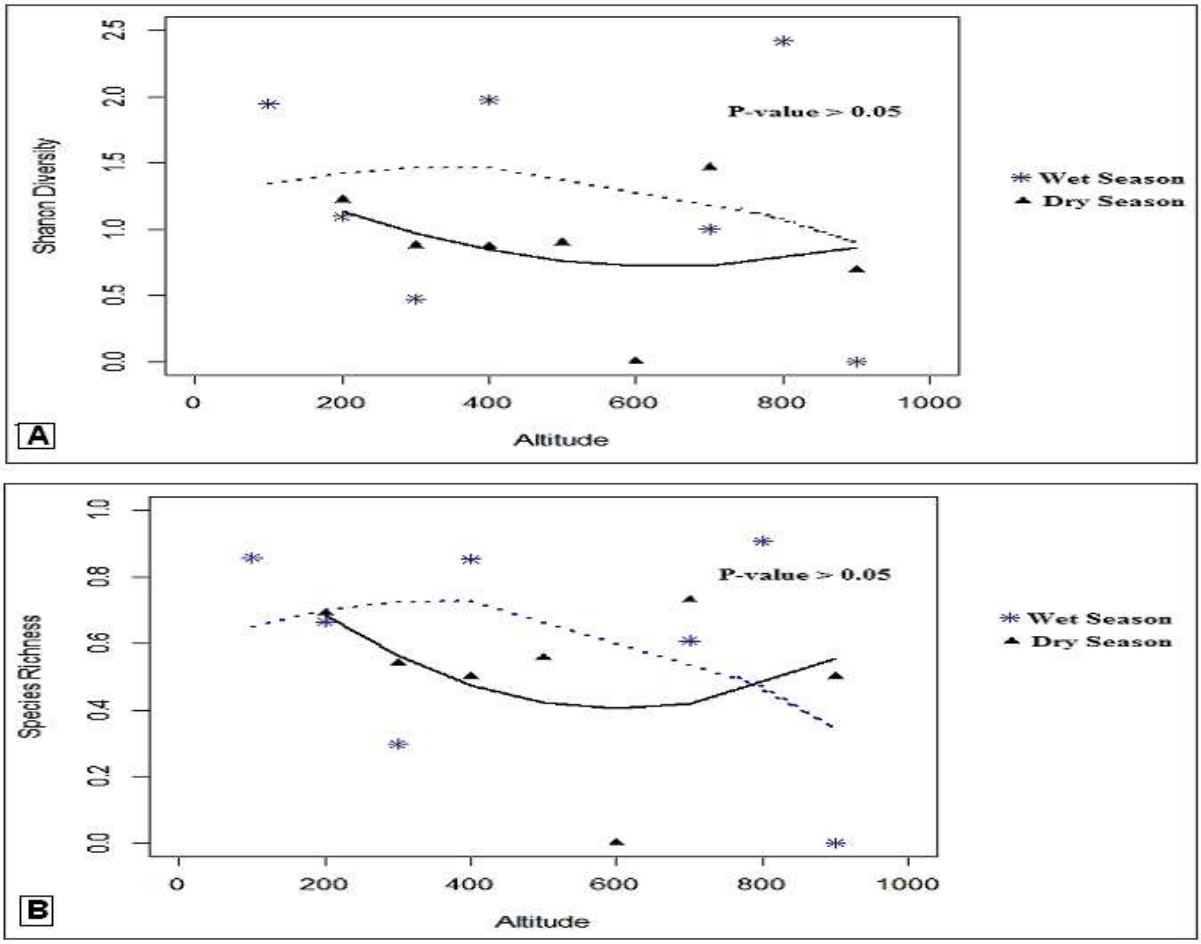

Fig. 2. Fungal diversity (A) and species richness (B) correlated with altitudinal gradients during wet (green) and dry (blue) seasons.

It is likely that the low number of isolates analyzed in this study resulted in weak correlation. This study was limited to the culturable, non-sporulating fungi. We thus recommend 454 pyrosequencing of environmental samples, and correlation analysis with biotic (such as plant species) and abiotic factors (such as temperature and precipitation), to elucidate specific interactions of these players in MFR.

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