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Above- and Below-Ground Ectomycorrhizal Diversity in a Pine-Oak Forest in Northeastern Thailand †

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Abstract

A diversity of ectomycorrhizal (ECM) fungi in a pine-oak forest in the PhuKhieo Wildlife Sanctuary (PKWS) in remote northeastern Thailand, was investigated both above-ground (fruit-bodies) and below-ground (root tips). The total number of fungi studied were 27 and 109 samples, respectively. Due to a high variation of mushroom morphotypes and limitations in identification of ECM roots, nucleotide sequences of ITS and 5.8S nrDNA were determined and analysed. The results revealed a high diversity of ECM fungi, especially in the below-ground samples. ECM mushrooms belonged to 6 families whereas root tip samples belonged to 10 families. Russulaceae was a dominant and frequently found fungal family in our study. Other families such as Amanitaceae, Boletaceae and Sclerodermataceae were also identified from both above-ground and below-ground samples. In addition, two families, Astraeaceae and Gomphaceae were only noted above-ground, whereas Ceratobasidiaceae, Inocybaceae, Sebacinaceae, Tremellaceae and Thelephoraceae were only detected below-ground. Our study provide the first molecular characterizations of an ECM diversity in pine-oak forest in Thailand.

Keywords: Ectomycorrhizal fungi, pine-oak forest, ITS, above-ground, below-ground

1. Introduction

Ectomycorrhizal (ECM) associations are symbiotic, mutualistic relationships between soil fungi and fine plant roots. They are common in temperate and tropical forests and play a significant role in plant establishment by providing nutrient and water uptake in host plants and by enhancing host tolerance of stressful situations [1]. The fine roots of many higher plant families such as Pinaceae, Betulaceae, Fabaceae, Dipterocarpaceae, Fagaceae and Myrtaceae are associated with ECM fungi [2]. Most ECM fungi belong to Phyla Basidiomycota and Ascomycota [3], and represent 162 genera [4]. The number of plant and fungal species involved in ECM relationships is approximately 6,000 and 20,000-25,000 species, respectively [3]. Thus ECM fungi play an essential role in seedling establishment and tree growth promotion.

Pinaceae and Fagaceae are two of the most ecologically and economically important forest trees in tropical regions. Mycodiversity and community of ECM fungi in these tropical ecosystems are relatively poorly documented. The most important ECM families observed from fruiting bodies are Amanitaceae, Boletaceae, and Russulaceae, although Thelephoraceae also become numerically important when root tips are examined [5]. Recent studies focused on dipterocarp forests using molecular techniques have revealed that members of the Thelephoraceae are the most common ectomycorrhizal fungi followed by Russulaceae and Sclerodermataceae [6-8].

PhuKhieo Wildlife Sanctuary is located in Chaiyaphum Province. It contributes over one third of the total area of the forest complex in the northeastern region and is a rich resource of biodiversity. The sanctuary encompasses different types of forests including pine-oak forests that have not been investigated for ECM fungal diversity and community. Therefore, we selected this forest to study ECM diversity to better understand of ECM diversity by combining a survey of above-ground and below-ground together with molecular identification. We hypothesized it is likely that ECM fungal diversity in pine-oak forests would be similar to other Dipterocarp forest in Thailand and/or Pine forest in temperate regions.

2. MATERIALS AND METHODS

The study site was performed in 100 m^2 plot in a pine-oak forest at PhuKhieo Wildlife Sanctuary, Chaiyaphum Province, Thailand during November to December 2011. Ectomycorrhizal fungi both from fruit-bodies and root tips were collected. The fruit-bodies found in this site were collected in paper bags or plastic boxes. The ectomycorrhizal roots were collected from a 5-10 cm depth from the root systems from 20 host plants. The distance between each plant was at least 8 m. Two soil samples in opposite directions, 1-1.5 m from the trunk, were collected. Then, all samples containing root tips were kept in plastic bags and labeled by site and date of collection.

2.1 Morphological Observation

Collected fruit-bodies were observed within 24 h for their morphological characteristics such as colour, size and shape of stalks, gills, caps, rings, etc. Spore prints were then made and studied for microscopic characteristics [9-12]. The inner tissues of each collection were then cut and placed in CTAB buffer before preservation at -20°C for molecular studies. The specimens were then oven dried at 40°C for overnight and kept in desiccators until dry and were deposited at the Faculty of Science Herbarium, Srinakharinwirot University, Bangkok.

ECM roots were cleaned to remove soil debris and rinsed several times with tap water. They were then carefully classified according to their distinct morphotypes such as mantle colour, shapes, surface texture and branching pattern under a stereomicroscope [13]. ECM roots were hand-sectioned and also observed for mantle structure using a DIC Nomarski-equipped microscope under high magnification (Olympus BX51TF) and photographed. Representative samples of cleaned ECM root tips were placed in CTAB buffer and kept at -20°C for molecular studies.

2.2 Molecular Identification

DNA extraction of mushrooms and ECM root tips was performed by using a CTAB method modified from Attitalla (2011)

[14]. The ITS regions and 5.8S rDNA were amplified using primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) as forward and ITS4 (5′-TCCTCCGC TTATTGATATGC-3′) as reverse for mushrooms, while primers ITSOF-T (5′-ACTTGGTCATTTAGAGGAAGT-3′) as forward, and LB-W (5′-CTTTTCATC TTTCCCTCACGG-3′) or ITS4 (5′-TCCT CCGCTTATTGATATGC-3′) as reverse were used for ECM roots [4, 8, 15]. The PCR reaction contained 10-100 ng/ml of DNA template, 10 mM of dNTP, 0.5 mM of each primer, 1.5 unit of Phire® Hot Start II DNA Polymerase (Finnzyme) and 1x Phire® PCR buffer containing 1.5 mM $MgCl_2$, in a total volume of 20 ml. The samples were run in a DNA Thermocycler (Eppendorf, Germany) according to the following cycling parameters: initial denaturation for 4 min at 98°C, followed by 40 cycles of denaturation for 5 sec at 98°C, annealing for 5 sec at 56°C and extension for 20 sec at 72°C. The cycling was ended by an extension phase for 1 min at 72°C. PCR products were examined on a 1% agarose gel under UV-light and purified using the GEL/PCR Purification Mini Kit (Favorgen). All purified fragments were sequenced using the same primers at the 1st BASE Laboratories Sdn Bhd (Malaysia) and manually edited using the BioEdit program. Then, all fungal sequences were identified by running BLASTn against the International Nucleotide Sequence Databases (INDS) and UNITE databases [16]. In the case of unreadable sequences, the purified PCR fragments were cloned using TA cloning kit (RBC Bioscience Corp., Taiwan) before being sequenced.

3. RESULTS AND DISCUSSION

Twenty-seven samples of fruit-bodies and 109 samples of ectomycorrhizal root tips were collected from the site of pine-oak forest. Ectomycorrhizal fruit-bodies obtained were identified as belonging to 6 families and 9 genera i.e. Amanitaceae (1 *Amanita*), Astraeaceae (1 *Astraeus*), Boletaceae (4 *Tylopilus*, 1 *Boletellus*), Gomphaceae (6 *Ramaria*, 4 *Gomphus*, 1 *Ramariopsis*), Russulaceae (6 *Russula*) and Sclerodermataceae (3 *Scleroderma*) (Figure 1). The dominant ECM families were the Gomphaceae, Russulaceae and Boletaceae (Figure 2). Among these collections, only twelve samples were selected to sequence because they were very difficult to distinguish in species level. The BLAST results showed in Table 1. There was only four samples (*Amanita fuliginea* PKWS11-11, *Ramaria largentii* PKWS11-18 and *Scleroderma sinnamariense* PKWS11-12, PKWS11-16) revealed clear identification based on ITS sequences, whereas nine samples revealed less than 97% similarity against known species from the GenBank database. Then, they remained unidentified at species level. This result indicated that there was a high diversity among the fruit-bodies studied. Some of them might be new species.

Ectomycorrhizal root tips were divided into 23 different morphotypes based on mantle colours, shapes and branching (Figure 1). Three types of branching, monopodial pyramidal, dichotomous and irregular, were recognised. It is noteworthy, that we found more than one morphotype associated with one species of plant roots. From a total 109 ECM root samples, 65 (59.6%) samples were successfully amplified, but only 43 samples were successfully sequenced. The BLAST results are shown in Table 1. ECM fungal roots were divided into 10 families and 11 genera i.e. Amanitaceae (1 *Amanita*), Boletaceae (1 *Boletus*), Ceratobasidiaceae (1 *Ceratobasidium*), Inocybaceae (1 *Inocybe*), Russulaceae (8 *Lactarius*, 7 *Russula*), Sclerodermataceae (1 *Scleroderma*), Sebacinaceae (1 *Sebacina*), Thelephoraceae (6 *Tomentella*), Tremellaceae (1 *Cryptococcus*) and *Cenococcum geophilum* (Figure 2). The most frequent fungal taxa found were Russulaceae (51.7%) followed by Thelephoraceae (20.7%). This is in agreement with several reports from both tropical and temperate forests [17]. Substantial dominance by members of the *Russula-Lactarius* and *Tomentella-Thelephora* lineages are considered as characteristics of both tropical and temperate ecosystems [7, 18]. The low diversity of Boletaceae and the presence of *Cenococcum* in the pine-oak forest site in this study are indicative of their temperate affinities [4, 19]. By using a 97% sequence similarity threshold over the entire ITS region, only 11 samples (25.6%) were identified to the species level with high % similarity (97-99%) (Table 1). Totally, 32 samples were identified to the genus level because of low % similarity (less than 97%) or closeness to unidentified species in databases. *Lactarius*, *Russula* and *Tomentella* species were found to be common colonizers in plant roots in our study.

Due to a species number of ECM roots obtained was higher than a number of ECM root morphotypes (Table 1). It could be implied that one morphotype may include several fungal species. In addition, the sequence results of below-ground revealed four fungal families of Amanitaceae, Boletaceae, Russulaceae and Sclerodermataceae related to fruit-bodies from above-ground (Figure 2). Two families of Astraeaceae and Gomphaceae were only found above-ground, whereas Ceratobasidiaceae, Inocybaceae, Sebacinaceae, Tremellaceae and Thelephoraceae were only detected below-ground. From this study, eleven genera of ECM fungi were discovered in the soil and three genera correlated with fruit-bodies. Therefore, approximately 17.6% (3 genera from 17 genera in total) of the ECM fungi observed were of genera represented above-ground from this study. Even though some genera of *Gomphus* and *Ramaria* were common found above-ground, there were not found below-ground. In contrast, the genus *Lactarius* was frequently detected below-ground, but no fruit-bodies was found above-ground. However, several ECM fungal species have never been found producing fruit-bodies [19]. Further the absence of fruit-bodies and short-term superficial search efforts have resulted in the discrepancy view of above- and below- ground ECM diversity [20]. Also a number of abiotic (light, moisture, litter, organic matter) and biotic (tree root age, tree species) environmental factors which occur at the forest, may plays important roles in the dynamic changes of some ECM fungal species observed. New mycorrhizae are continually formed as roots proliferate, and other mycorrhizae die, and new species can come to dominate [21].

Pinaceae and Fagaceae are recorded as the dominant plant species and are commonly found in the area surveyed. Nine different plant species were potentially associated with ECM fungi. There were *Pinus kesiya* Royle ex Gordon. (Pinaceae), *Lithocarpus ceriferus* A. Camus (Fagaceae), *Lithocarpus truncatus* Rehd. & Wils. (Fagaceae), *Castanopsis argentea* (Blume) A. DC. (Fagaceae), *Dipterocarpus obtusifolius* Teijsm.ex Miq. (Dipterocarpaceae), *Aporusa villosa* Lindl. Baill. (Euphorbiaceae), *Schima wallichii* choisy (Theaceae), *Craibiodendron stellatum* W.W. Smith (Ericaceae). The most common ECM associations were with *P. kesiya*, *L. ceriferus* and *C. argentea*. Two taxa, *Russula* and *Tomentella*, were associated with six different plant species and *Lactarius* was associated with three different host plants. ECM associations were highly diverse in *L. truncatus* and *C. argentea* in term of both morphotypes and molecular identification. They host at least eight defined ECM fungal species within their roots. Bahram et al. [22] estimated that a single European aspen tree individual may host at least 200 ECM species. Our study identified a high diversity of ectomycorrhizal associations and provided a brief insight into their plant relationships in a pine-oak forest in Thailand.

4. CONCLUSIONS

Although a small sampling area was used, our study provides significant information of the ECM diversity in a remote pine-oak forest in Northeastern Thailand. We found that the ECM fungal diversity in the pine-oak forest is comparable to other forest ecosystems. It contained an element of ECM fungi present in temperate regions including the *Russula-Lactarius* and *Tomentella-Thelophora* groups, *Cenococum* and a low incidence of the *Boletales*. A single tree may also host as many ECM species as recovered from our study. These information was important for understanding the mycorrhizal diversity both from above- and below-grounds because of their influence on forest dynamics.

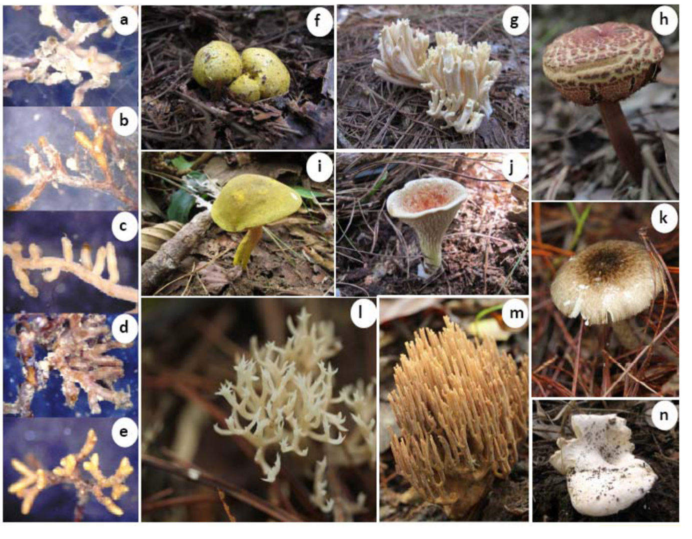

Figure 1. Ectomycorrhizal fungi (a) *Tomentella* sp. 4 RPK12-1A2, (b) *Russula* sp. 4 RPK12-3B2, (c) *Boletus* sp. RPK12-6B1, (d) *Russula* sp. 1 RPK12-10A2, (e) *Inocybe* sp. RPK12-14A1, (f) *Scleroderma sinnamariense* PKWS12-03, (g) *Ramaria largentii* PKWS12-18, (h) *Boletellus* sp. PKWS12-23, (i) *Tylopilus* sp. PKWS12-12, (j) *Gomphus* sp. PKWS12-01, (k) *Amanita fuliginea* PKWS12-11, (l) *Ramaria* sp. 1 PKWS12-24, (m) *Ramaria* sp. 2 PKWS12-25, (n) *Russula* sp. PKWS12-09.

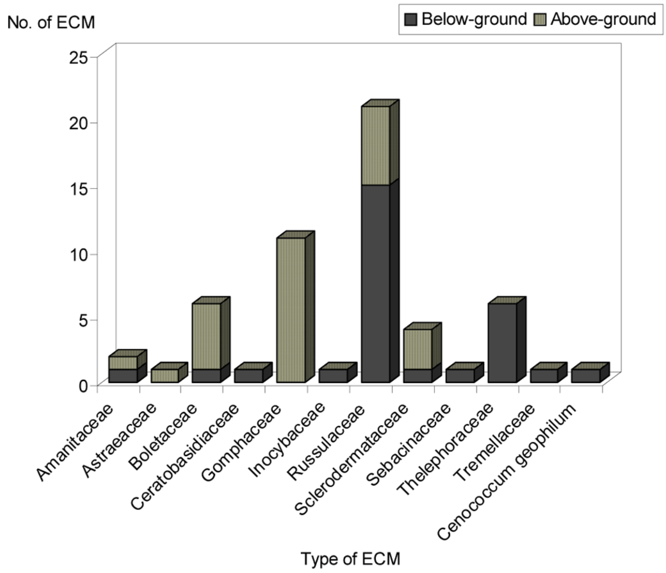

Figure 2. Numbers of fungal families and species from above-ground and below-ground found in pine-oak forest.

Table 1. Molecular identification of fungal samples from above-ground and below-ground based on ITS sequences analysis.

Fungal family	Mushroom taxon	ECM root taxon	BLAST result (Accession No.)	Similarity(%)	Representative vouchers
Amanitaceae	Amanita fuliginea	-	*Amanita fuliginea* (FJ176717)	99%	PKBP12-11
		Amanita fuliginea	*Amanita fuliginea* (FJ176717)	99%	RPK12-11A1, RPK12-11A2
Astraeaceae	*Astraeus* sp.	-	*Astraeus hygrometricus* (FJ536664)	92%	PKBP12-08
Boletaceae	*Boletellus* sp.	-	*Boletellus* sp. (AM412293)	83%	PKBP12-23
	Tylopilus sp.	-	*Tylopilus* sp. 2 CG-2012 (HE814223)	97%	PKBP12-12
		Boletus sp.	*Boletus* sp. EMF7 (JF273510)	93%	RPK12-6B1
Ceratobasidiaceae		*Ceratobasidium* sp.	*Ceratobasidium* sp. RGOLD2 (GQ175300)	93%	RPK12-4A1
Gomphaceae	*Gomphus* sp.	-	*Gomphus bonarii* (EU846244)	89%	PKBP12-01, PKBP12-07, PKBP12-20
	Ramaria largentii	-	*Ramaria largentii* OSC:143964 (KC346862)	99%	PKBP12-18
	Ramaria sp. 1	-	*Ramaria* sp. OSC 111765 (DQ365621)	89%	PKBP12-24
	Ramaria sp. 2	-	*Ramaria gelatiniaurantia* var. *violeitingens* (EU697261)	85%	PKBP12-25

Table 1. Continued.

Fungal family	Mushroom taxon	ECM root taxon	BLAST result (Accession No.)	Similarity(%)	Representative vouchers
	Ramaria sp.3	-	*Ramaria rubribrunnescens* (EU697262)	93%	PKBP12-02
Inocybaceae		*Inocybe* sp.	*Inocybe* sp. DED8061 (GQ893021)	98%	RPK12-14A1, RPK12-14A2
Russulaceae	*Russula* sp.	-	*Russula cascadensis* (FJ845426)	86%	PKBP12-09, PKBP12-10
		Lactarius friabilis	*Lactarius friabilis* (EF141553)	98%	RPK12-1A3
		Lactarius hatsudake	*Lactarius hatsudake* isolate sp159 (EF685098)	99%	RPK12-3B1, RPK12-3B2, RPK12-3B3
		Lactarius sp. 1	*Lactarius* sp. IH2011 (HQ168369)	97%	RPK12-6A1, RPK12-6A2
		Lactarius sp. 2	*Lactarius fulvissimus* | Estonia (UDB011533)	93%	RPK12-7A1
		Lactarius sp. 3	*Lactarius* sp. EMF29 (JF273531)	99%	RPK12-9B1
		Lactarius sp. 4	*Lactarius zonarius* | Estonia (UDB011468)	94%	RPK12-16B1
Sclerodermataceae	Scleroderma sinnamariense	*Lactarius* sp. 5	*Lactarius scrobiculatus* (EF530942)	95%	RPK12-16B2
		Lactarius sp. 6	Uncultured *Lactarius* (JF519291)	95%	RPK12-7A2, RPK12-7A3
		Russula aeruginea	*Russula aeruginea* (HQ604837)	98%	RPK12-6A3
		Russula sp. 1	*Russula* sp. FH CCES19F4SY (DQ778002)	97%	RPK12-10A1, RPK12-10A2, RPK12-3B1
		Russula sp. 2	*Russula* sp. ECM9 (GQ900533)	94%	RPK12-7A1, RPK12-7A2, RPK12-5A1
		Russula sp. 3	*Russula cascadensis* voucher UBC F19691 (HM240541)	90%	RPK12-13B1
		Russula sp. 4	*Russula* sp. R40 (AF350063)	87%	RPK12-3B2
		Russula sp. 5	uncultured *Russula* (HQ021946)	95%	RPK12-8A1
		Russula sp. 6	uncultured *Russula* (HQ021944)	95%	RPK12-2A1
		-	*Scleroderma sinnamariense* (FM213364)	97%	PKBP12-03, PKBP12-05, PKBP12-08, PKBP12-16
		Scleroderma sinnamariense	*Scleroderma sinnamariense* (FM213364)	99%	RPK12-1A1, RPK12-17A1
Sebacinaceae		*Sebacina* sp.	Uncultured *Sebacina* (HQ154314)	92-96%	RPK12-9A1, RPK12-9A2
Thelephoraceae		*Tomentella* sp. 1	uncultured *Tomentella* (FR852183)	98%	RPK12-6A4

Table 1. Continued.

Fungal family	Mushroom taxon	ECM root taxon	BLAST result (Accession No.)	Similarity(%)	Representative vouchers
		Tomentella sp. 2	Uncultured *Tomentella* (FR852204)	92%	RPK12-7A4
		Tomentella sp. 3	uncultured *Tomentella* (EF218838)	94%	RPK12-15A1
		Tomentella sp. 4	Thelephoraceae sp. EMF48 (JF273548)	94%	RPK12-1A2, RPK12-1A3
		Tomentella sp. 5	Uncultured Thelephoraceae (JF519275)	96%	RPK12-12A1
		Tomentella sp. 6	uncultured Thelephoraceae (AJ893316)	97%	RPK12-15B1, RPK12-15B2
Tremellaceae		Cryptococcus podzolicus	*Cryptococcus podzolicus* (FN428938)	99%	RPK12-11A3
Dothideomycetes		*Cenococcum geophilum*	*Cenococcum geophilum* (AB089816)	98%	RPK12-2B1

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