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A First Snapshot View into the Belowground Ectomycorrhizal Composition of Pine-Oak Forest in Thailand

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Abstract

Belowground of Ectomycorrhizal (EcM) composition in pine-oak forest at Phu Khieo Wildlife Sanctuary was studied. One hundred and nine samples of EcM roots observed were classified into 59 morphotypes according to mantle colour, branching pattern and host species. The internal transcribed spacer (ITS) sequences of 43 samples were achieved in molecular identification. The total EcM species were identified into 29 species belonged to 10 genera and 10 families. Russulaceae (52%) and Thelephoraceae (21%) were predominant. Moreover other EcM fungal families such as Amanitaceae, Boletaceae, Ceratobasidiaceae, Inocybaceae, Sclerodermataceae, Sebacinaceae, Tremellaceae and *Cenococcum geophilum* were also present. Although from many studies, *C. geophilum* was recorded as dominant species and widely distributed in Pine forest, there was rarely found in this survey. Several species of *Lactarius*, *Russula* and *Tomentella* were noted as unidentified species. The present study provided the first snapshot view insight into the EcM community of pine-oak forests. However, it is necessary to sampling extensively both spatial and temporal scales to improve our understanding of the belowground EcM composition in the tropical forests.

Keywords Ectomycorrhizal fungi, Pinaceae, Fagaceae, pine-oak forest, ITS, Tropical

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 then enhancing host tolerance of stressful situations (Smith & Read 1997). The fine roots of many higher plant families such as Pinaceae, Betulaceae, Fabaceae, Dipterocarpaceae, Fagaceae and Myrtaceae are associated with EcM fungi (Brundrett 2009). Most EcM fungi belong to Phylum Basidiomycota and Ascomycota (Rinaldi et al. 2008), in 162 genera (Tedersoo et al. 2010). The number of plant and fungal species involved in the relationship is approximately 6,000 and 20,000-25,000 species,

respectively (Rinaldi et al. 2008). Thus EcM fungi play essential role in seedlings establishment and tree growth promotion.

Pinaceae and Fagaceae are two of most important forest trees in term of ecologically and economically in tropical regions. Pinaceae is also considered as the oldest plant family that is associated with EcM fungi (Hibbett & Matheny 2009). Mycodiversity and community of EcM fungi in these tropical ecosystems are poorly documented. The most important EcM families observed from fruit body are Amanitaceae, Boletaceae, and Russulaceae, although Thelephoraceae also become numerically important when root tips are examined (Brearley 2012). Recent studies focused in dipterocarp forests using molecular techniques have revealed that member of the Thelephoraceae are the most common ectomycorrhizal fungi followed by Russulaceae and Sclerodermataceae (Sirikantaramas et al. 2003, Yuwa-Amornpitak et al. 2006, Phosri et al. 2012).

Phu Khieo Wildlife Sanctuary located in Chaiyaphum Province. It contributes over one third to the total area of the forest complex in the northeastern where described as the richness resource of biodiversity. There are different types of forests including pine-oak forests, where scant document of EcM fungal diversity and community. Therefore, we selected this forest to study EcM composition for a better understanding of EcM community by combining EcM morphotypes and molecular identification. We hypothesized it is likely that EcM fungal community in pine-oak forests has an affinity to other Dipterocarp forests in Thailand and/or Pine forests in temperate regions.

Experimental and Methods

Sampling sites

EcM root tips were collected in Pine-Oak forest, Phu Khieo Wildlife Sanctuary, Chaiyaphum Province during November to December 2011. The study site was performed in a 50 m × 50 m square plot. Individual samples were collected from 5-10 cm depth of the root system for 20 host plants. The distance of each plant was at least 8 m apart. Two soil samples in opposite directions, 1-1.5 m from the trunk, were collected. Then, all samples were kept in clean plastic bags and labeled site and date of collection.

Morphological observation

EcM roots were cleaned from soil and rinsed three times with tap water. EcM roots were then carefully classified according to their distinct morphotypes such as mantle colour, shapes and branching pattern under a stereomicroscope. EcM roots were hand-sectioned and also observed for mantle structure under DIC Nomarski microscope with high magnification (Olympus BX51TF) and photographed. The representative of cleaned EcM root tips were placed in CTAB buffer and kept at -20°C for molecular study until use.

Molecular identification

DNA extraction of EcM root tips was performed by using CTAB method modified from Attitalla (2011). The ITS regions and 5.8S rDNA were amplified using primers ITSOF-T (5'- ACTTGGTCATTTAGAGGAAGT -3') as forward, and LB-W (5'-

CTTTTCATCTTTCCCTCACGG -3') or ITS4 (5'-TCCTCCGCTTATTGATATGC -3') as reverse (White et al. 1990, Tedersoo et al. 2010, Phosri et al. 2012). The PCR reaction contained 10-100 ng/μl of DNA template, 10 mM of dNTP, 0.5 μM of each primer, 1.5 unit of Phire® Hot Start II DNA Polymerase (Finnzyme) and 1x Phire® PCR buffer containing 1.5 mM MgCl₂, in a total volume of 20 μl. The samples were run in DNA Thermocycler (Eppendorf, Germany) according to the following cycling parameters: initial denaturation for 5 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 1 % agarose gel under UV-light and purified using GEL/PCR Purification Mini Kit (Favorgen). All purified fragments were sequenced and manually edited using BioEdit program. Then, all fungal sequences were identified by running MRGABLAST and BLASTn queries against the International Sequence Database (INSD) and UNITE databases (Abarenkov et al. 2010). In case of unreadable sequences, the purified PCR fragments were cloned using TA cloning kit (RBC Bioscience Corp., Taiwan) before sequenced. The percentage values of species frequently found were calculated from number of identified species per total samples and multiply by 100.

Results and Discussion

A total 109 EcM root tip samples were collected from pine-oak forest. Pinaceae and Fagaceae were recorded as dominant species and commonly found in this survey. Nine different plant species potentially associated with EcM fungi. Most of them belong to *Pinus kesiya* Royle ex Gordon (Pinaceae), *Lithocarpus ceriferus* A. Camus (Fagaceae) and *Castanopsis argentea* (Blume) A. DC. (Fagaceae), respectively (Fig. 1a). The morphological features of EcM root tips examined were divided into 59 different morphotypes based on mantle colours, shapes and branching, (Fig 2). Three types of branching, monopodial pyramidal, dichotomous and irregular, were recognized. In this study, noteworthy, we found more than one morphotypes associated with one species of plant roots (Table 1).

The ITS regions were successfully amplified using primers ITSOF-T/LB-W or ITSOF-T/ITS4 for 65 samples from total 109 samples (59.6%), but only 43 samples were successfully sequenced (66%). The BLAST results were showed in Table1. EcM fungal roots were then identified into 29 and 10 taxa at the species and genus levels, respectively. All EcM fungal members belonged to Amanitaceae (4%), Boletaceae (4%), Ceratobasidiaceae (4%), Inocybaceae (3%), Russulaceae (52%), Sclerodermataceae (3%), Sebacinaceae (3%), Thelephoraceae (21%), Tremellaceae (3%) and *Cenococcum geophilum* (3%) (Fig 1b). The species frequently found were Russulaceae (52%) followed by Thelephoraceae (21%), respectively. This is in agreement with several reports from both tropical and temperate forests (Obase et al. 2011). Substantial dominance of members of the *Russula-Lactarius* and *Tomentella-Thelephora* and the lack of many temperate genera are considered as characteristics of the tropical ecosystems (Phosri et al. 2012). The low diversity of Boletaceae and present of *Cenococcum* were observed. In this study, in the

pine-oak forest site was an indicative of temperate affinities (Tedesoo & Nara 2010, Tedesoo et al. 2010).

By using 97% sequence similarity threshold over the entire ITS region, only 11 samples (25.6%) were identified into species level with high percentage of similarity (97-99%) (Table 1). Totally 32 samples were identified into genus level because of low percentage of similarity (less than 97%) or close to unidentified species in databases. *Lactarius*, *Russula* and *Tomentella* species were found to be a common colonizer in plant roots. Two taxa of *Russula* and *Tomentella* were associated with six different plant species and *Lactarius* spp. was associated with three different host plants (Table 1). EcM associations were highly diverse in *Lithocarpus truncatus* Rehd. & Wils. (Fagaceae) and *Castanopsis argentea* (Blume) A. DC. (Fagaceae) in term of both morphotypes and molecular identification. They contained at least 8 defined EcM fungal species associated to their roots (Table 1). Our study exhibited high diversity of ectomycorrhizal association in pine-oak forest and provide the first snapshot insight into belowground EcM composition in pine-oak forest in Thailand.

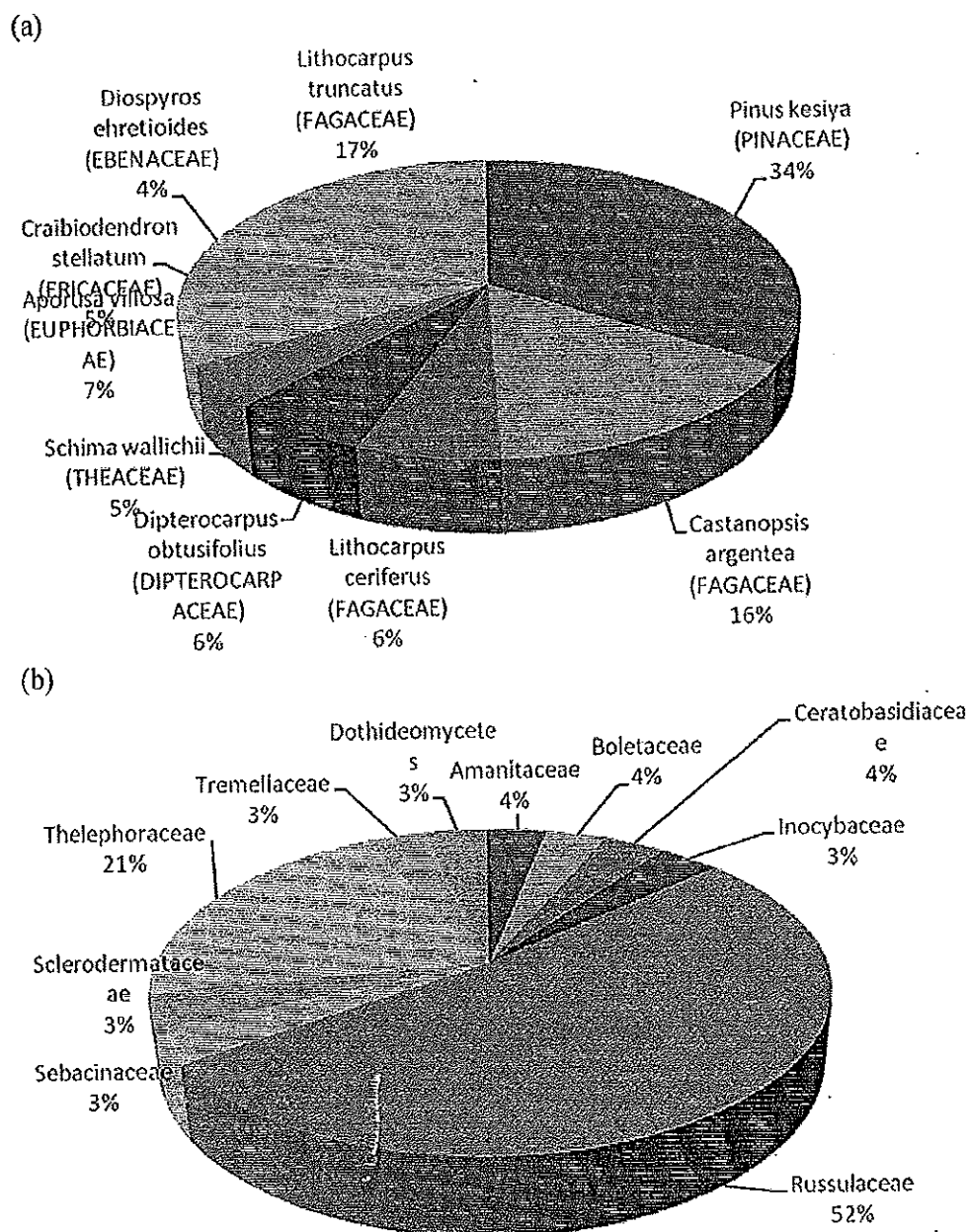


Fig.1 Percentage of potential tree host for EcM association (a), and EcM composition in pine-oak forest in Phu Khieo Wildlife Sanctuary (b).

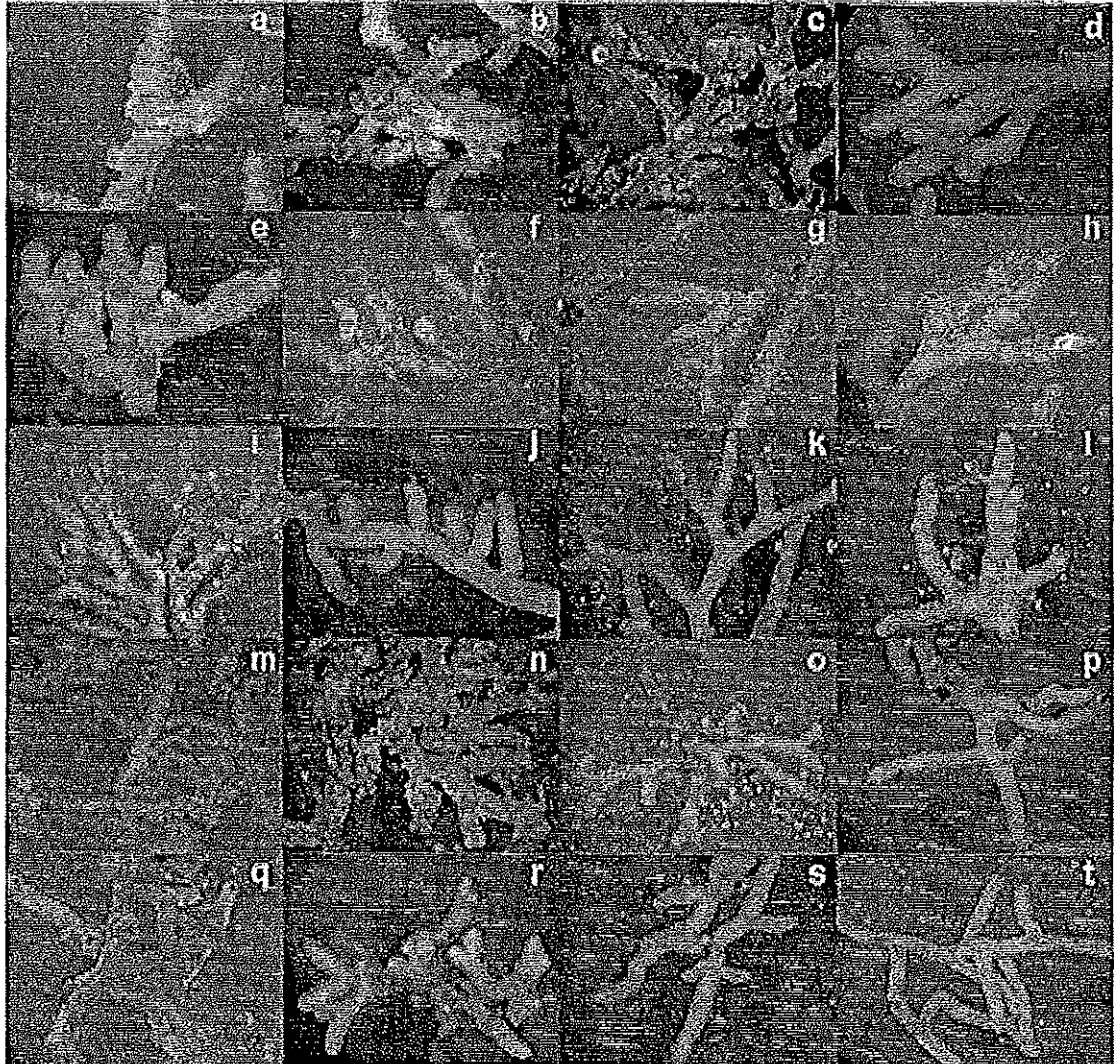


Fig.2 Plan views of ectomycorrhizas: (a) *Ceratobasidium* sp., (b) *Tomentella* sp. 4, (c) *Lactarius friabilis*, (d) *Scleroderma sinnamariense*, (e) *Russula* sp. 6, (f) *Cenococcum geophilum*, (g) *Russula* sp. 4, (h) *Tomentella* sp. 5, (i) *Ceratobasidium* sp., (j) *Boletus* sp., (k) *Lactarius* sp. 2, (l) *Russula* sp. 5, (m) *Sebacina* sp., (n) *Russula* sp. 1, (o) *Russula* sp. 1, (p) *Amanita fuliginea*, (q) *Russula* sp. 3, (r) *Inocybe* sp., (s) *Lactarius* sp. 5 and (t) *Scleroderma sinnamariense*

Table 1 Molecular identification of EcM root tips based on ITS sequences analysis

Fungal family	EcM root taxon	BLAST result (Accession No.)	Similarity (%)	No. of isolates	Host Plant*
Amanitaceae	<i>Amanita fuliginea</i>	<i>Amanita fuliginea</i> (FJ176717)	99%	2	DO
Boletaceae	<i>Boletus</i> sp.	<i>Boletus</i> sp. EMF7 (JF273510)	93%	1	LT
Ceratobasidiaceae	<i>Ceratobasidium</i> sp.	<i>Ceratobasidium</i> sp. RGOLD2 (GQ175300)	93%	2	PK, L
Inocybaceae	<i>Inocybe</i> sp.	<i>Inocybe</i> sp. DED8061 (GQ893021)	98%	2	T PK
Russulaceae	<i>Lactarius friabilis</i>	<i>Lactarius friabilis</i> (EF141553)	98%	1	PK
	<i>Lactarius hatsudake</i>	<i>Lactarius hatsudake</i> isolate sp159 (EF685098)	99%	3	PK
	<i>Lactarius</i> sp. 1	<i>Lactarius</i> sp. 1H2011 (HQ168369)	97%	2	LT
	<i>Lactarius</i> sp. 2	<i>Lactarius fulvissimus</i> Estonia (UDB011533)	93%	1	CA
	<i>Lactarius</i> sp. 3	<i>Lactarius</i> sp. EMF29 (JF273531)	99%	1	CA
	<i>Lactarius</i> sp. 4	<i>Lactarius zonarius</i> Estonia (UDB011468)	94%	1	CA
	<i>Lactarius</i> sp. 5	<i>Lactarius scrobiculatus</i> (EF530942)	95%	1	CA
	<i>Lactarius</i> sp. 6	Uncultured <i>Lactarius</i> (JF519291)	95%	2	CA
	<i>Russula aeruginosa</i>	<i>Russula aeruginosa</i> (HQ604837)	98%	1	LT
	<i>Russula</i> sp. 1	<i>Russula</i> sp. FH CCES19F4SY (DQ778002)	97%	2	LC
	<i>Russula</i> sp. 2	<i>Russula</i> sp. ECM9 (GQ900533)	94%	3	LT, CA
	<i>Russula</i> sp. 3	<i>Russula cascadiensis</i> voucher UBC F19691 (HM240541)	90%	1	PK
	<i>Russula</i> sp. 4	<i>Russula</i> sp. R40 (AF350063)	87%	1	PK
	<i>Russula</i> sp. 5	uncultured <i>Russula</i> (HQ021946)	95%	1	AV
	<i>Russula</i> sp. 6	uncultured <i>Russula</i> (HQ021944)	95%	1	SW
Sclerodermataceae	<i>Scleroderma sinnamariense</i>	<i>Scleroderma sinnamariense</i> (FM213364)	99%	2	LT, AV, SW
Sebacinaceae	<i>Sebacina</i> sp.	Uncultured <i>Sebacina</i> (HQ154314)	92-96%	2	CA
Thelephoraceae	<i>Tomentella</i> sp. 1	uncultured <i>Tomentella</i> (FR852183)	98%	1	LC
	<i>Tomentella</i> sp. 2	Uncultured <i>Tomentella</i> (FR852204)	92%	1	CA
	<i>Tomentella</i> sp. 3	uncultured <i>Tomentella</i> (EF218838)	94%	1	CS
	<i>Tomentella</i> sp. 4	Thelephoraceae sp. EMF48 (JF273548)	94%	2	PK
	<i>Tomentella</i> sp. 5	Uncultured Thelephoraceae (JF519275)	96%	1	LT
	<i>Tomentella</i> sp. 6	uncultured Thelephoraceae (AJ893316)	97%	2	CS
Tremellaceae	<i>Cryptococcus podzolicus</i>	<i>Cryptococcus podzolicus</i> (FN428938)	99%	1	DO
Dothideomycetes	<i>Cenococcum geophilum</i>	<i>Cenococcum geophilum</i> (AB089816)	98%	1	SW

* PK = *Pinus kesiya* Royle ex Gordon., LC = *Lithocarpus ceriferus* A. Camus, LT = *Lithocarpus truncatus* Rehd. & Wils., CA = *Castanopsis argentea* (Blume) A., DC., DO = *Dipterocarpus obtusifolius* Teijsm. ex Miq., AV = *Aporosa villosa* Lindl. Baill., SW = *Schima wallichii* Choisy, CS = *Craibiodendron stellatum* W.W. Smith

Conclusions

Although small sampling area, our study provides the first snapshot view into the EcM composition of pine-oak forest in Thailand. The EcM fungal composition in pine-oak forest is comparable to other forest ecosystems and contained an element of EcM affinities to temperate regions where member of the *Russula-Lactarius* and *Tomentella-Thelophora* were predominant and the occurrence of *Cenococcum* with low composition in *Boletales*. A single tree may be also host for many EcM species as our discovery in this study. It is still need to improve our understanding of belowground EcM community and structure where tropical data are scant by extensively sampling.

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