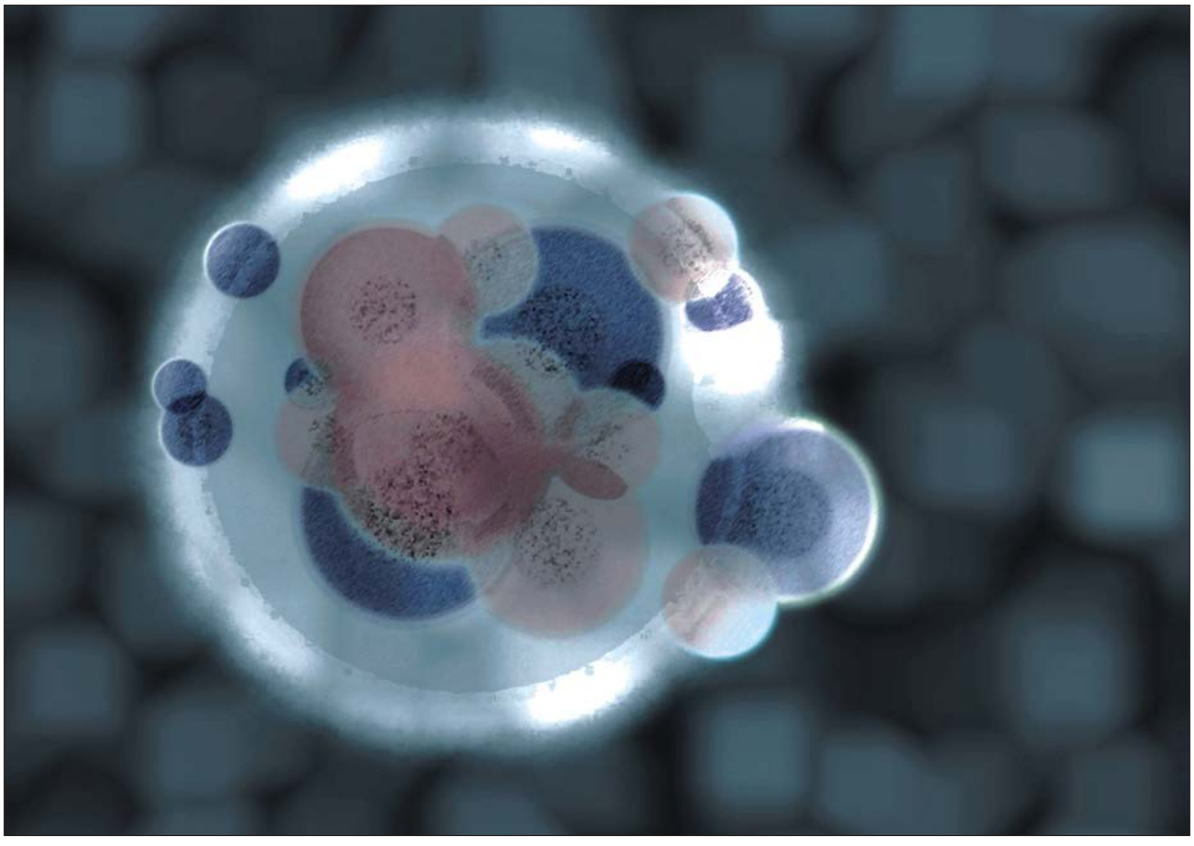

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Diversity of Ectomycorrhizal Fungi on Dipterocarpaceae in Thailand

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Abstract: Mycorrhizae are symbiotic association between specialized soil fungi and plants. Trees of Dipterocarpaceae have long been known for their ability to associate with ectomycorrhizal (ECM) fungi. Apart from modifying the lateral root system of their host, ECM fungi benefit their host plant in many ways, such as: protecting against root pathogens, providing mineral nutrients, increasing drought tolerance and enhancing seeding growth in the nursery. In this study, DNA from ectomycorrhizal roots was successfully amplified from 15 species in 6 genera of regular type. These included *Anisoptera* (1 species), *Cotylelobium* (1 species), *Dipterocarpus* (5 species), *Hopea* (3 species), *Parashorea* (1 species) and *Shorea* (5 species). Molecular techniques were used to identify the ECM. ITS (internal transcribed spacer) fragments located between the 18 S and 28 S rRNA genes in the nuclear genome were amplified and sequenced. The sequences of ITS were compared with sequences in the GenBank. The results revealed that the 33 mycobionts participating in the symbioses in ECM roots were in 8 families including Thelephoraceae (13), Russulaceae (6), Amanitaceae (1), Cortinariaceae (3), Sclerodermataceae (4), Agaricaceae (1), Pisolitaceae (3) and Boletaceae (2). The observed host specialization showed that these fungi could associate with more than one host plant species.

Key words: Dipterocarpaceae, ectomycorrhiza, symbiosis, internal transcribed spacer

INTRODUCTION

Mycorrhizae (fungus-roots) are symbiotic multualistic relationships between soil fungi and fine plant roots. Since the association is multualistic, both organisms benefit. The fungus receives carbohydrates (sugars) and growth factors from the plant, which in turn receives many benefits. Protection against root pathogens, increased efficiency of nutrients and water uptake are a few examples. Most of the ECM hosts are woody type plants in temperate regions. ECM establish with the fine roots of many plants such as pine (*Pinus*), spruce (*Picea*), fir (*Abies*), poplar (*Populus*), willow (*Salix*), beech (*Fagus*), birch (*Betula*) and oak (*Quercus*). The southern Eucalyptus and Northofagus (Southern Beech) are important plants that associate with ectomycorrhizal fungi, as are the Dipterocarpaceae in the Southeast Asia (Smith and Read, 1987). The ECM community and it's diversity in temperate and southern regions are widely studied (Harvey *et al*., 1976, 1979; Sylvia and Jarstfes, 1997; Gorgan *et al*., 2000). Also, in recent years, the ectomycorrhizal fungi on Dipterocarpaceae have received more comprehensive investigations (Smith, 1994; Sim *et al*., 1997).

In order to study the association between ECM and members of Dipterocarpaceae, basidiomes in their rhizoid connections with plant roots were collected and identified. By this method, over 50 different agarics, boleti, earthballs and a species of *Pisolithus* were identified by Watling and Lee (1995). The distribution of ectomycorrhizal Basidiomycetes especially members of the genus *Russula*, was studied by the same survey team. Amanitaceae and Boletaceae were the second and the third most commonly found basidiomes, respectively (Watling *et al*., 2002). Ectomycorrhizal studies by this method are limited, particularly in the case of those ectomycorrhizae that rarely or never produce sporocarp (Gardes and Bruns, 1996). Inspecting the anatomical feature of transverse sections of root tips and viewing distinct morphotypes are other methods for studying

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ectomycorrhizae. Different ectomycorrhizal morphotypes and their description is a key to their identify. Under these procedures, 24 ectomycorrhizal types on seedling and roots of *Shorea leprosular* were identified and placed into 20 Basidiomycotina, 2 Ascomycotina and 2 either members of Ascomycotina or Russulaceae (Lee *et al*., 1997). This method is needed for the well-trained eyes to identify ectomycorrhizae.

Recently, molecular approaches have become alternative methods for rapid identification of fungi at various taxonomic levels. Comparison of mycorrhizal Restriction Fragment-length Polymorphisms (RFLPs) of two similar morphotypes or fungal fruiting body RFLPs helps to identify ectomycorrhizae. However, a RFLP pattern is very limited in terms of the enzyme used, the primer used, fragment size estimation and intraspecific variation (Horton and Bruns, 2001). Direct sequence analysis is an additional practical used to identify ectomycorrhizae. The sequences of the Internal Transcribed Spacer (ITS) regions are studied more for identification to the species level or at least within a species group. Then, only using the sequences from unknown samples, can closely related taxa be retrieved from GenBank or EMBL that contains currently deposited the ITS data (Bruns *et al*., 1998).

The purpose of this study was to examine the diversity of ectomycorrhizae on Dipterocapaceae in Thailand. ITS regions of ECM were amplified and sequenced and phylogenetic analyses and comparison of ITS sequences were performed to identify ECM.

MATERIALS AND METHODS

Study sites description: This study was conducted in 4 sections of Thailand (Fig. 1) during the rainy season of 2002 and 2003 in order to collect ectomycorrhizal fungi on dipterocarp. Thailand located in South-East Asia between latitudes 5° 37′ - 20° 27′ N and longitudes 97° 22′ - 105° 37′ E, with an area of 513,115 km^2. In the past, Dipterocarpaceae species were distributed in all areas of Thailand. Nowadays, however, most dipterocarps are scarily found in the open area except in forests or conservation areas. The types of forests in the North region are dry deciduous to mixed deciduous and large tracts of dry evergreen forest occur intermittently. Natural forests have almost disappeared in the central part of Thailand; most of those remaining are under cultivation. Dry dipterocarp forests are a main feature of the eastern region. Evergreen forest include Malayan species (*D. kerrii*, *D. baudii*, *D. dyeri*) are to be found in South and Peninsular Region of Thailand.

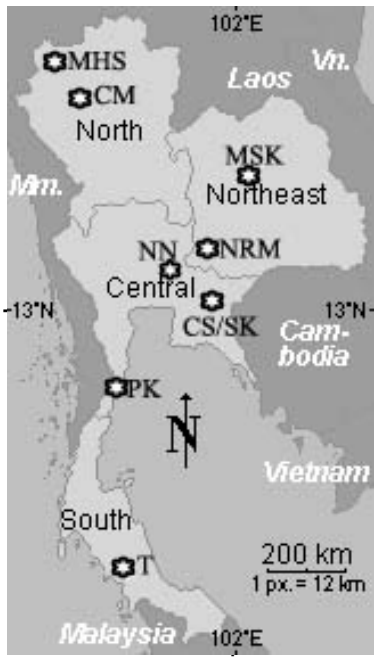

Fig. 1: Sample sited location in 4 parts of Thailand. North region; TPPNP = Tham Pla Phaseau National Park, Maehongson (MHS) province; AMT = Ampher Mae Tang, Chiang Mai (CM) province, North-East region; PKDK = Pa Kok Dong Keng, Mahasarakham (MSK) province, SERS = Sakaerat Environment Research Station, Nakornratchasima (NRM) province, Central region; WSM = Wat Srimuang, Nakhon Nayok (NN) province, CBG = Centennial Botanic Garden, Chachoengsao/Sakaew (CS/SK) province, PKA = Pa Klang Ao Forest park, South region; Prachaub Kiri Khan (PK) province, PBG = Peninsular Botanic Garden (Thung Khai), Trang (T) province

Mycorrhizal sampling: Ectomycorrhizal root tips were removed from 5-10 cm depth of the root system and kept ice-boxed before transfer to the laboratory. ECM roots were cleaned in water to remove soil and sand. Ectomycorrhizae were separated into distinct morphological types after examination of mantle strncture under a compound microscope and stored in absolute ethanol.

DNA extraction, amplification and sequencing: DNA was extracted from ectomycorrhizal root tips using CTAB method (Doyle and Doyle, 1987). TheInternal Transcribed

Spacer (ITS) region of nuclear ribosomal DNA was amplified using the primers ITS1-F and ITS4-B (Gardes and Bruns, 1993). The PCR reaction contained 10 mM each of dATP, dCTP, dGTP, dTTP, 25 ρmol of each primers, 2.5 unit of Taq DNA Polymerase (Promega) and 2 mM $MgCl_2$, in a total volume of 50 μL. After an initial denaturation step at 95°C for 3 min, PCR cycle parameters were set as follows: denaturation of template DNA for 30 sec at 94°C, primer annealing for 30 sec at 50°C, primer extension for 1 min at 72°C. After 30 cycles, a final extension step for 7 min at 72°C was added to allow completion of unfinished strands. PCR-amplified products were purified by GeneClean II kit following the manufacturer specification and ligated to pGEM-T vector (Promaga). Nucleotide sequences of plasmids inserts were determined using the same primers. Cycle sequencing was done by the reaction termination method using flurorescence-labled dideoxyribonucleotides. The sequencing reactions and the processing of the reaction products for electrophoresis were performed following the instructions for the sequencing kit (ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystem Co.). Electrophoresis and data collections were done on a ABI Model 377 DNA Sequencer (Perkin-Elmer Corporation).

Data analysis: The Dipterocarpaceae ectomycorrhizae were identified by comparing their ITS sequences with the sequences a database of GenBank. From Blast search the most similar sequences were aligned with the ITS sequences of ECM using ClustalX version 1.8 in multiple alignment mode. Phylogenetic analyses were constructed by Parsimony method using PAUP program (Swofford, 1993). A bootstrap analysis with 1000 replications were performed to test the significance of the strict consensus of parsimonious trees of nucleotide sequences. The ITS sequence of *Sparassis crispa* was used as the outgroup.

RESULTS

Identification and phylogenetic grouping: Uninfected roots are thin, light to slightly dark brown. The infected roots are modified and developed the root system (Peterson and Farquhar, 1994) to difference morphotypes. In two collecting rainy season (2002, 2003), over 40 ectomycorrhizal morphotypes were observed on Dipterocarp roots. Generally, only one morphotype was found on infected roots. However, in some cases, more than one was found on some roots, especially on the adult trees (Agerer and Rambold, 1998). The ITS region of 33 ectomycorrhizal root tips were successfully amplified

Table 1: The similarity of ECM root tips compared with GenBank data base

Taxa (Accession No.)*	ECM of blast match	(Accession No.)*	Similarity (%)	Dipterocarp species (ECM host)	Study sites**
ECM1 (DQ146392)	ECM root tip 133Ny1.C23.1	(AF476987)	74.00	*H. odorata*	PKA
ECM2 (DQ146366)	*Russula foeten*	(AF418613)	96.55	*D. turbinatus*	AMT
ECM3 (DQ146367)	*Amanita virosa*	(AB015676)	83.97	*D. alatus*	PBG
ECM4 (DQ146368)	*Thelephoraceae* sp. EC117 A52	(AY751562)	91.99	*S. farinosa*	PBG
ECM5 (DQ146369)	*Lactarius fluvissimus*	(AF204679)	90.48	*D. kerrii*	PKA
ECM6 (DQ146393)	*Tomentella* sp. 041	(AJ534912)	89.13	*S. roxburghii*	TPPNP
ECM7 (DQ146370)	*Lactarius fluvissimus*	(AF204679	90.65	*H. odorata*	PBG
ECM8 (DQ146371)	*Tomentella* sp. 041	(AJ534912)	91.79	*A. costata*	CBG
ECM9 (DQ146372)	*Lepiota* sp. Vellinga 2590	(AY176485)	79.00	*S. obtusa*	PKDK
ECM10 (DQ146370)	*Lactarius fluvissimus*	(AF204679)	91.33	*H. odorata*	PBG
ECM11 (DQ146373)	*Tomentella* sp. J54	(AJ534914)	86.96	*A. costata*	PKDK
ECM12 (DQ146374)	*Scleroderma bovista*	(AB099901)	80.18	*D. baudii*	PBG
ECM13 (DQ146375)	*Inocybe nitidiuseula*	(AJ534934)	79.00	*D. turbinatus*	AMT
ECM14 (DQ146375)	*Inocybe nitidiuseula*	(AJ534934)	79.00	*D. turbinatus*	AMT
ECM15 (DQ146376)	*Tomentella* sp. J54	(AJ534914)	82.08	*S. roxbughii*	TPPNP
ECM16 (DQ146377)	*Scleroderma bovista*	(AB099901)	77.09	*S. roxburghii*	PKDK
ECM17 (DQ146378)	*Scleroderma* sp. CM 1	(AB099900)	76.00	*D. baudii*	PBG
ECM18 (DQ146394)	*Xerocomus pruinatus*	(AF402140)	75.02	*A. curtisii*	PBG
ECM19 (DQ146379)	*Scleroderma bovista*	(AB099901)	79.49	*S. roxburghii*	PKDK
ECM21 (DQ146380)	*Tomentella* sp. 041	(AJ534912)	89.23	*D. alatus*	PKA
ECM21 (DQ146387)	*Tomentella* sp. J54	(AJ534914)	90.25	*H. odorata*	PKA
ECM22 (DQ146381)	*Tomentella* sp. J54	(AJ534914)	89.05	*H. ferrea*	SERS
ECM23 (DQ146382)	*Inocybe pudica*	(AY228341)	73.51	*H. odorata*	WSM
ECM24 (DQ146383)	*Scleroderma bovista*	(AB099901)	77.07	*H. ferrea*	SERS
ECM25 (DQ146384)	*Tomentella ellisii*	(AF272913)	95.00	*H. ferrea*	SERS
ECM26 (DQ146385)	*Pisolithus* sp. DYS3	(AB099920)	72.51	*H. ferrea*	SERS
ECM27 (DQ146386)	*Russular foeteus*	(AF418613)	91.58	*C. lanceolatum*	PBG
ECM29 (DQ146387)	*Tomentella* sp. J54	(AJ534914)	90.63	*S. guiso*	CBG
ECM30 (DQ146388)	*Tomentella* sp. 041	(AJ534912)	88.02	*S. roxburghii*	TPPNP
ECM31 (DQ146389)	*Pisolithus* sp. MURU	(AY179746)	73.50	*D. alatus*	PKDK
ECM33 (DQ146390)	*Tomentella* sp. S69	(AF430289)	88.02	*D. tuberculatus*	TPPNP
ECM34 (DQ146370)	*Lactarius fluvissimus*	(AF204679)	91.42	*P. stellata*	PBG
ECM35 (DQ146391)	*Boletus lupinus*	(AJ296294)	72.06	*D. baudii*	PBG

*Accession number of DNA Data Bank of GenBank, **Same study site as described in Fig. 1

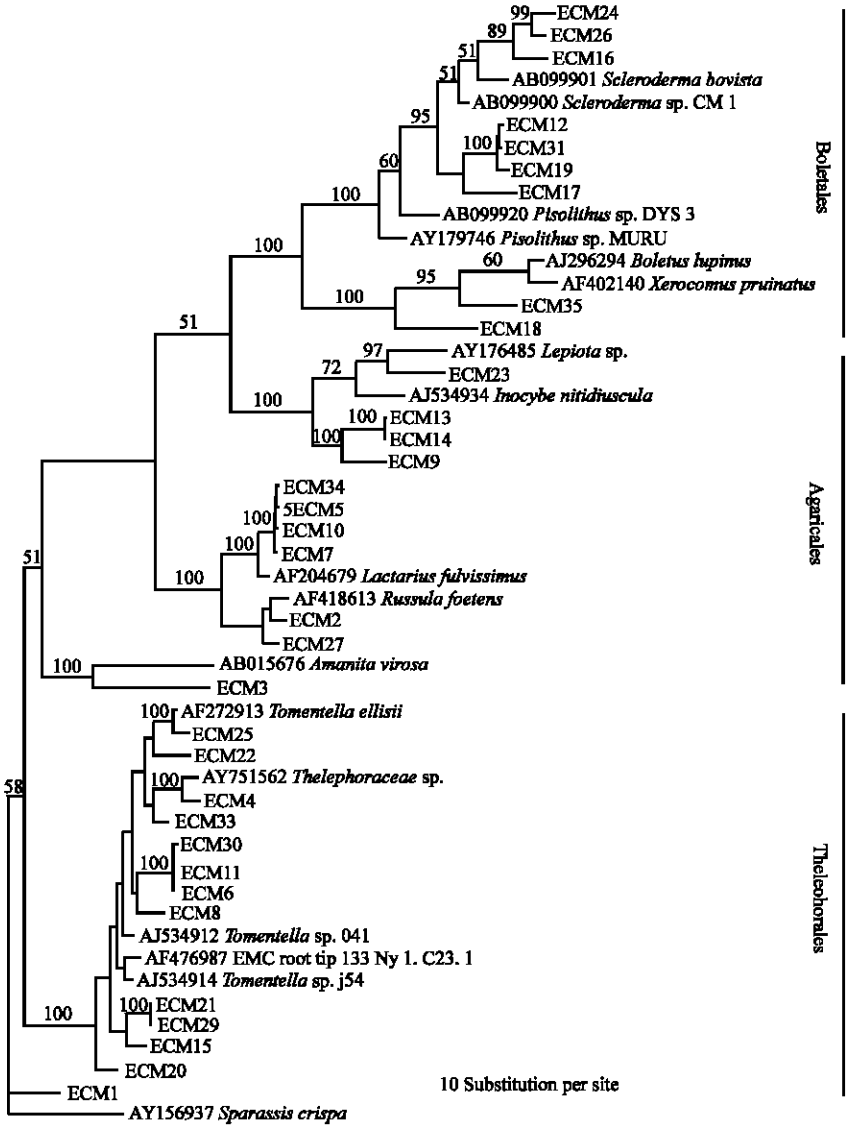

Fig. 2: The strict consensus tree of 12 most parsimonious trees for the ITS regions of ectomycorrhizal fungi on Dipterocarpaceae. The numbers above branches indicate the bootstrap value of 500 bootstrap replicates (lower than 50% are not shown). The total length is 3753. Consistency index and retention index is 0.4121 and 0.2966, respectively. This tree is rooted with *Sparassis crispa* AY156937

and sequenced from 15 dipterocarps species. DNA sequences were submitted to the GenBank database under Accession no. DQ146366-DQ146392 as showed in Table 1. After multiple alignments, phylogenetic trees with the 33 ECMs and sequences from the GenBank database with the highest sequence similarity to the clone sequences were constructed (Fig. 2).

Three main fungal orders were grouped in Boletales, Agaricales and Thelephorales. Most terminal clade within these three orders is well supported with high bootstrap value. The Boletales order includes 9 ECMs. The Agaricales order includes the *Russular*-like ECM2 (Fig. 2). Within the Agaricales order includes ECM5, 7, 10 and 34 in *Lactarius* clade with a high bootstrap. The Thelephorales order includes the ECM25 (*Tomentella*-like species). However, another presumed member of this order (ECM1) that is also similar to *Tomentella* sp. does not group within the *Tomentella* clade. With know affinity, all of 33 ECMs represented basidiomycetes. Only 2 ECMs of ECM2 and ECM25, a Blast search sequence similarity of ≥ 95% were found (Table 1) and could be assigned to the genus of *Russular* and *Tomentella,*

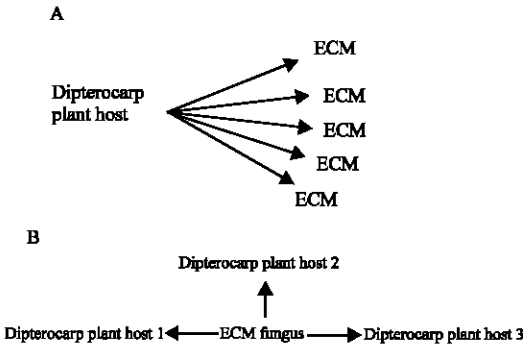

Fig. 3: Association patterns of ectomycorrhizal fungi on dipterocarpaceae

respectively. All of the remaining (31 ECMs) with a low sequence similarity (≤95%) to know ECM fungal sequences were assigned to the family or ordinal level.

Association pattern of ECM fungi on dipterocarp: In this study, it was found that a single plant species associated with more than one fungus. For instant different fungi of ECM3, ECM21 and ECM31 interacted with *D. alatus* (Fig. 3A). Whereas the same fungi such as ECM5, ECM7, ECM10 and ECM34 that are identical (data not show) associated with different host of *D. kerii*, *H. odorata* and *P. stellata* respectively (Table 1, Fig. 3B). From these results, association pattern between ECM fungi and dipterocarp species might be conceptualized as shown in Fig. 3. These two pattern showed a high diversity of ectomycorrhizal on dipterocarp and a low degree of specificity.

DISCUSSION

This is the first diversity study of ectomycorrhizal fungi on dipterocarp in Thailand using molecular identification techniques. The ITS sequence was used to compare with ITS sequence available from GenBank database. Phylogenetic construction of parsimonious trees with 33 ECMs and related GenBank sequences placed most ECMs in three main fungal orders. For 2 ECMs a Blast search sequence similarity of ≥95% was found with GenBank basidiomycetes sequences, enabling identification to the genus level. Fourteen ECMs with 87 to 94% sequence similarities were sufficient for placing to known fungal taxa at family level. Seventeen ECMs with 72 to 86% sequence similarities were assigned to ordinal level (Table 1). Most of case with low similarity of ITS sequence indicated that the fungus belongs to an ECM fungal that is not yet present in the GenBank database.

The diversity of ectomycorrhizal of fungi on dipterocarp species has been presented in studies using both descriptions of the ectomycorrhizae and observations of basidiomes growing under and around parent trees. But these method needs more refinement to identify ectomycorrhizal morphotype and some those ectomycorrhizal fungi do not produce sporocarp. However, 26 ectomycorrhzal types were found on *Shorea pavifolia* (Ingleby *et al*., 1998) and 15 of 28 species of ectomycorrhzal fungi that were found on *Shorea leprosula* were Russulaceae (Lee *et al*., 1997). In this study molecular techniques were used to identify ECM sequences by comparing with the GenBank database and also found a high diversity of ECM on Dipterocarpaceae. As universal symbols, phylogenetic analysis can be conducted from sequence data. The phylogenetic placement ECM samples on three orders of Boletales, Agaricales and Thelephorales by amount of 27.27, 33.33 and 39.39%, respectively.

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