—*Proceeding*—

Molecular identification of ectomycorrhizal fungi associated with Dipterocarpaceae

Supaart SIRIKANTARAMAS[1)], Norimitsu SUGIOKA[1)], Su S. LEE[2)], Latiff A. MOHAMED[3)], Hua S. LEE[4)], Alfred E. SZMIDT[1)] and Tsuneyuki YAMAZAKI[1,5,6)]

[1)] Graduate School of Sciences, Department of Biology, Kyushu University, Japan.
[2)] Forest Research Institute Malaysia.
[3)] Institute for Environment Development, University Kebangsaan Malaysia.
[4)] Department of Forestry, Kuching, Malaysia.
[5)] Present Address: Research Institute of Evolutionary Biology, 4–28, Kamiyoga 2, Setagaya, Tokyo.
[6)] Corresponding author.
Email: tsuneyukiyamazaki@ybb.ne.jp

ABSTRACT We identified both the fungi and plants of ectomycorrhizal root tips of Dipterocarpaceae using the sequence of mitochondrial large (ML) subunit ribosomal DNA, internal transcribed spacer (ITS) sequence of nuclear ribosomal DNA, partial *matK* sequence of chloroplast DNA and *trnL-trnF* spacer sequence of chloroplast DNA. We compared the DNA sequences from ectomycorrhizal roots with the sequences from the available databases using phylogenetic analysis. The ectomycorrhizal fungi identified by ML and/or ITS sequences belonged to the following seven families: Russulaceae, Boletaceae, Cortinariaceae, Thelephoraceae, Hygrophoraceae, Sclerodermataceae, Cantharellaceae and Amanitaceae. The Dipterocarpaceae species identified by partial *matK* and/or *trnL-trnF* sequences belonged to the following genera: *Dryobalanops*, *Hopea*, *Vatica*, *Cotylelobium*, *Anisoptera*, *Upuna*, *Dipterocarpus* and *Shorea*. Members of Thelephoraceae, which have never been reported to form symbiotic roots with Dipterocarpaceae, appeared to be the most abundant and were usually associated with *Shorea* species. Analyses of other Dipterocarpaceae species are needed to confirm these results.

Key words: Dipterocarpaceae, ectomycorrhiza, DNA, phylogeny

INTRODUCTION

Plants of the Dipterocarpaceae family are distributed in the tropics and predominantly in the rain forests of South East Asia. Dipterocarpaceae consists of three subfamilies: Dipterocarpoideae in Asia, Monotoideae in Africa and South Africa, and Pakaraimoideae in South America. The number of genera and species of Asian dipterocarps is much greater than in the African and South American subfamilies. There are approximately 470 species in 13 genera in Dipterocarpoideae (Ashton, 1982). Many of these species are major sources of timber, comprising about 80% of the timber exports in South East Asia (Ashton *et al.*, 1988).

Dipterocarpaceae are believed to often form symbiotic roots with ectomycorrhizal (EM) fungi (Smits, 1992). Now, the important role of EM fungi in soil ecology is widely accepted (Dahlberg, 2001). This kind of symbiosis involving nutrient exchange between fungi and trees is important not only for normal tree growth, but also for the survival of seedlings (Smits, 1994). EM roots are very easy to observe because of their distinctive characteristics. They usually maintain juvenile roots, have very few, or no, root hair, and they tend to be short and thick with various colors such as brown, black, white or yellow. Within the root, the fungus forms a complex branching structure, known as the Hartig net, which mediates nutrient transfer between fungus and plant (Smits, 1994).

Although the relationship between dipterocarps and EM fungi has been reported as early as the 1920s (Smits, 1992), the EM fungal community in this group of plants has not been thoroughly examined. Some studies identified the dominant EM fungi using fruiting body or root morphotype-based identification (Beaker, 1983; Lee, 1992; Watling, 1995). Most observations suggest that the symbiotic fungi of dipterocarps belong to Basidiomycota (Lee, 1998). However, observations based solely on fruiting bodies may not be sufficient when studying ectomycorrhizal communities, which are composed of extremely diverse array of species. Moreover, different species are known to form similar morphological characteristics on an EM root (Jonsson *et al.*, 1999). The results of recent studies on the EM fungal community of pines showed that there is a

low correspondence between fungi that appear to be the most abundant as sporocarps and those that appear to be the most abundant on roots (Gardes & Bruns, 1996a; Yamada & Katsuya, 2001). Typically, the species that are abundant below-ground will seldom produce fruiting bodies, conversely the species that commonly form fruiting bodies may not be well represented below-ground (Dahlberg, 2001). Therefore, fruiting body observation alone is insufficient for assessing the EM fungal communities.

Molecular identification has been recently applied to characterize EM community structure (Buscot *et al.*, 2000; Horton & Bruns, 2001). In this study, we collected and identified the dipterocarp EM roots for both dipterocarps and EM fungi using phylogenetic analysis. Our EM fungi identification was based on the ML region of the mitochondrial genome and the internal transcribed spacer (ITS) region of nuclear genome while Dipterocarpaceae identification was based on the *matK* and the *trnL-trnF* spacer region of the chloroplast genome. The objective of this study was to assess the presence and abundance of EM fungi associated with Dipterocarpaceae species in Malaysia.

MATERIALS AND METHODS

Study sites

EM root samples were collected in Malaysia from the following five locations: Lambir National Park, Sarawak; Engkabang, Sarawak; Semengoh, Sarawak; Kubah National Park, Sarawak; and Dipterocarp Arboretum, Forest Research Institute Malaysia (FRIM), Kuala Lumpur (Table 1).

Table 1. List of sampling sites, number of root samples and DNA region used to identify Dipterocarpaceae and EM fungi.

Location	No. of samples	DNA region	No. of identified samples	Sample designation
Lambir National Park, Sarawak 1998	25	trnL-trnF, matK, ML, ITS	20	L1-L20
Engkabang, Sarawak 2001	18	trnL-trnF, ML	9	E1-E9
Semengoh, Sarawak 2001	15	trnL-trnF, ML	9	S1-S9
Kubah National Park, Sarawak 2001	28	trnL-trnF, ML	26	K1-K26
Dipterocarp Arboretum, FRIM, Kuala Lumpur 2001	30	trnL-trnF, ML	24	F1-F24

Sampling

The roots from different species of Dipterocarpaceae were randomly sampled to investigate the diversity of associated EM fungi. EM roots were taken from soil not more than 60 cm away from the trees. We collected one or several root sample(s) from different places around each tree. The species of the trees growing close to the root sampling site were also identified and recorded. We collected 116 root samples in total. The roots were cleaned and examined under a dissecting microscope to confirm the presence of typical structures of the EM roots. The EM roots were kept in 100% ethanol at 4℃ until further analysis. In addition, we collected fruiting bodies of the following five fungi: *Scleroderma sinnamariense*, *Scleroderma columnare*, *Amanita fritillaria*, *Russula alboareolata* and *Russula* sect. Ilicinae.

DNA extraction

DNA was extracted from the root samples and from fruiting bodies with the CTAB method (Doyle & Doyle, 1987) with minor modifications. Samples were ground to a powder in liquid nitrogen and incubated in 2xCTAB buffer for 2 hours. After chloroform-isoamyl alcohol extraction and RNAse digestion, DNA was precipitated overnight with isopropanol.

PCR and direct DNA sequencing

PCR amplification was performed using plant and fungi specific primers. For the identification of Dipterocarpaceae, the *matK* and *trnL-trnF* regions were amplified using universal primers, matK-AF/matK-8R (Ooi *et al.*, 1995), and trn-e/trn-f (Taberlet *et al.*, 1991), respectively. For the identification of mycorrhizal fungi, the ITS and ML regions were amplified using ITS-1f/ITS-4b primers (Gardes & Bruns, 1993) and ML-5/ML-6 primers (White *et al.*, 1990), respectively. Amplified products were purified with GeneCleanIII (Bio 101, La Jolla, CA) following the manufacturer's protocol. DNA sequences were determined by cycle sequencing of double stranded products using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit following the supplier's instruction with an Applied Biosystems 3100 sequencer. The sequencing primers for *trnL-trnF* spacer region, ITS region and ML region were the same as those used as PCR primers. For the *matK* region, which is about 1.5 kb in length, the partial *matK* region, about 800 bp in length, was obtained using a pair of 8R and 1125R primers (Kajita *et al.*, 1998).

Cloning of PCR product

For some samples, the 3' PCR primer of *matK* region did not produce good results in sequencing. Therefore, purified PCR products were ligated to pGEM-T vector (Promega) following the manufacturer's instructions and the resultant plasmids were transformed into *Escherichia coli* (XL1-Blue, Stratagene) following standard protocols (Sambrook *et al.*, 1989). After the resultant plasmids were amplified in XL1-Blue and purified (Sambrook *et al.*, 1989), the inserted sequences were determined using both the T7 and SP6 primers.

Phylogenetic analyses

Sequences obtained from both ectomycorrhizal roots and identified fruiting bodies were edited and assembled using Sequence Navigator software for Macintosh. Phylogenetic trees were constructed based on each DNA region listed in Table 1. The sequences from each region were added into the following databases: 23 sequences of *matK* sequence database (Kajita *et al.*, 1998), 52 sequences of *trnL-trnF* spacer sequence database (Kamiya *et al.*, 1998), 130 sequences of ITS sequence database (available at NCBI) and 175 sequences of ML sequence database (by Tom Horton, available at http://plantbio.berkeley.edu/~bruns/). Alignments were performed using Clustal X program (Thompson *et al.*, 1997). Phylogenetic trees were constructed using the neighbor-joining method (Saitou & Nei, 1987) of the Clustal X program (Thompson *et al.*, 1997). The bootstrap analysis was performed based on 1000 replications.

RESULTS

Phylogenetic analyses

The sequences of 22 samples were not determined because the ML-5/ML-6 did not yield good results. Thus, these samples were excluded from further analysis. The sequences of the *trnL-trnF* IGS region of chloroplast DNA and ML5-ML6 region of mitochondrial large subunit rDNA gene in 94 out of 116 EM root samples collected in the vicinity of more than 50 trees were successfully determined. In addition, the samples collected in Lambir were also analyzed based on partial sequences of *matK* region of chloroplast DNA and ITS region of nuclear small subunit rDNA gene (Table 1). When using trn-e/trn-f and ML-5/ML-6 primers, PCR products could be easily amplified and their sequence determined by direct sequencing. The sequences of identified fruiting bodies of five Basidiomycetes fungi were also determined. Phylogenetic trees were constructed to identify unknown samples based on the *trnL-trnF* spacer region for all samples, the *matK* region and combined *matK/trnL-trnF* for the samples from Lambir, the ML-5/ML-6 region for all samples and ITS region for the samples from Lambir (Table 1). The neighbor-joining tree based on the *trnL-trnF* spacer region had low bootstrap support (data not shown). On the other hand, higher bootstrap values were obtained for the tree based on *matK* sequences and combined *matK/trnL-trnF* sequences from Lambir (data not shown). The neighbor-joining trees based on ML sequences had somewhat better bootstrap support for the cluster containing unknown root samples. We could not determine ITS sequence for one sample (L4) from Lambir. The ITS sequences were difficult to align among fungi families. Therefore, individual alignments were made separately for each family and used for tree construction. We could construct trees based on ITS region of Thelephoraceae, Amanitaceae and Cortinariaceae. On the other hand, Russulaceae and the other families could not be analyzed because of a lack of available sequences in the database. The trees based on the ITS sequence had strong

bootstrap support (data not shown).

The placement of identified fruiting bodies and unknown root samples

Analyses based on *trnF-trnL* and *matK* sequences

On a phylogenetic tree based on *trnL-trnF* spacer regions, six of the unknown root samples were clearly separated from the group containing dipterocarps (data not shown). These sequences were submitted to the BLAST search. The search result confirmed that they do not belong to Dipterocarpaceae (data not shown). Therefore, these six samples were excluded from further analysis leaving 88 unknown samples.

The placement of the unknown samples from Lambir on the phylogenetic trees based on *trnL-trnF* spacer and partial *matK* region was the same (data not shown). The identities of root samples determined using the *trnL-trnF* spacer sequence were subsequently compared with the field identification of trees growing close to the root sampling site. In most cases, the identity established based on the *trnL-trnF* spacer sequence agreed with the field data. The identified dipterocarps belonged to the following genera: *Dryobalanops*, *Hopea*, *Vatica*, *Cotylelobium*, *Anisoptera*, *Upuna*, *Dipterocarpus* and *Shorea*. Most samples (60 out of 88) belonged to the *Shorea* genus.

Analyses based on ML and ITS sequences

The placement of unknown root samples, collected from Lambir on trees based on ML and ITS region, was similar (data not shown). An outline of the neighbor-joining tree based on the ML region showing identified fungi families is shown in Figure 1. The EM fungi identified with the ML sequences belonged to the following families: Russulaceae, Boletaceae, Cortinariaceae, Thelephoraceae, Hygrophoraceae, Sclerodermataceae, Cantharellaceae and Amanitaceae. Parts of the tree corresponding to individual families are shown on Figures 2, 3, and 4. Members of Thelephoraceae were the most prevalent EM fungi and were often found associated with *Shorea* species. All five fruiting body samples: *Scleroderma sinnamariense*, *Scleroderma columnare*, *Amanita fritillaria*, *Russula alboareolata* and *Russula* sect. Ilicinae, were correctly placed within their groups (Figs 2 and 3).

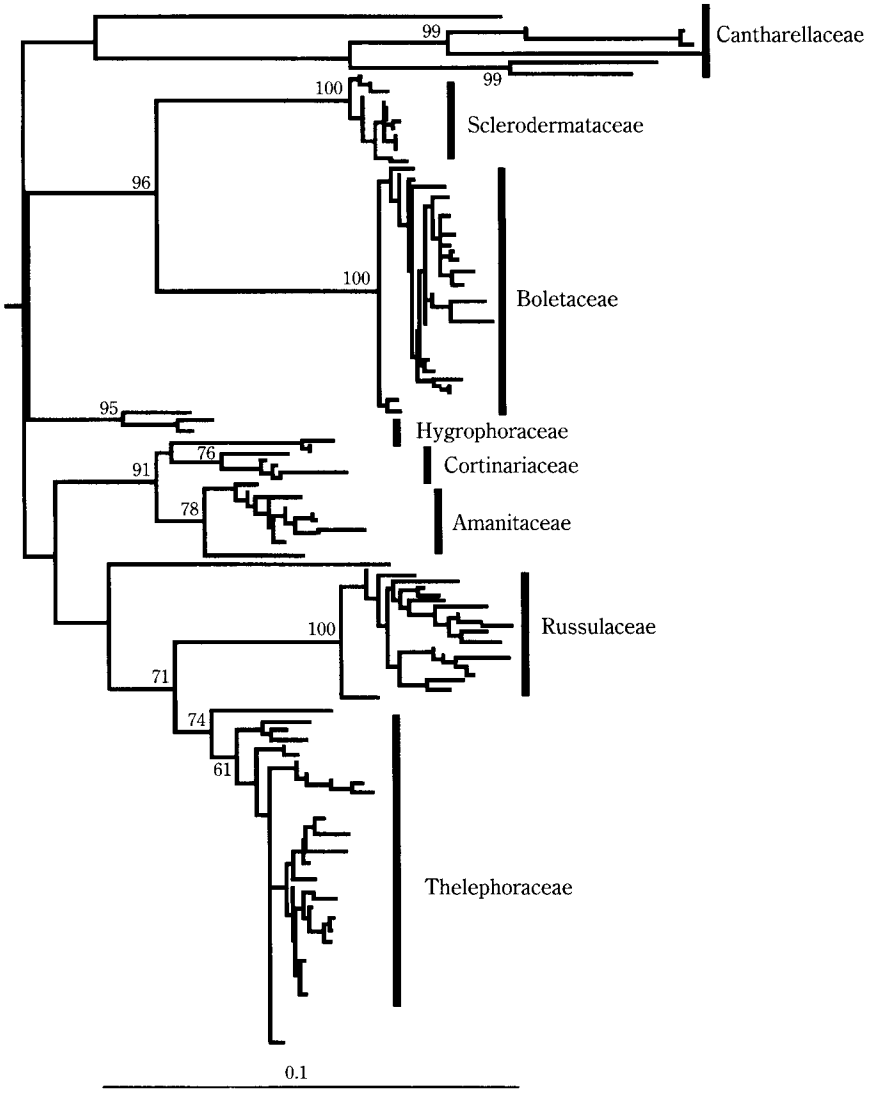


Fig. 1. An outline of the neighbor-joining tree based on the ML region showing identified fungi families. The numbers at branches show the percentage of bootstrap value based on 1000 replicates (only values >50% are shown).

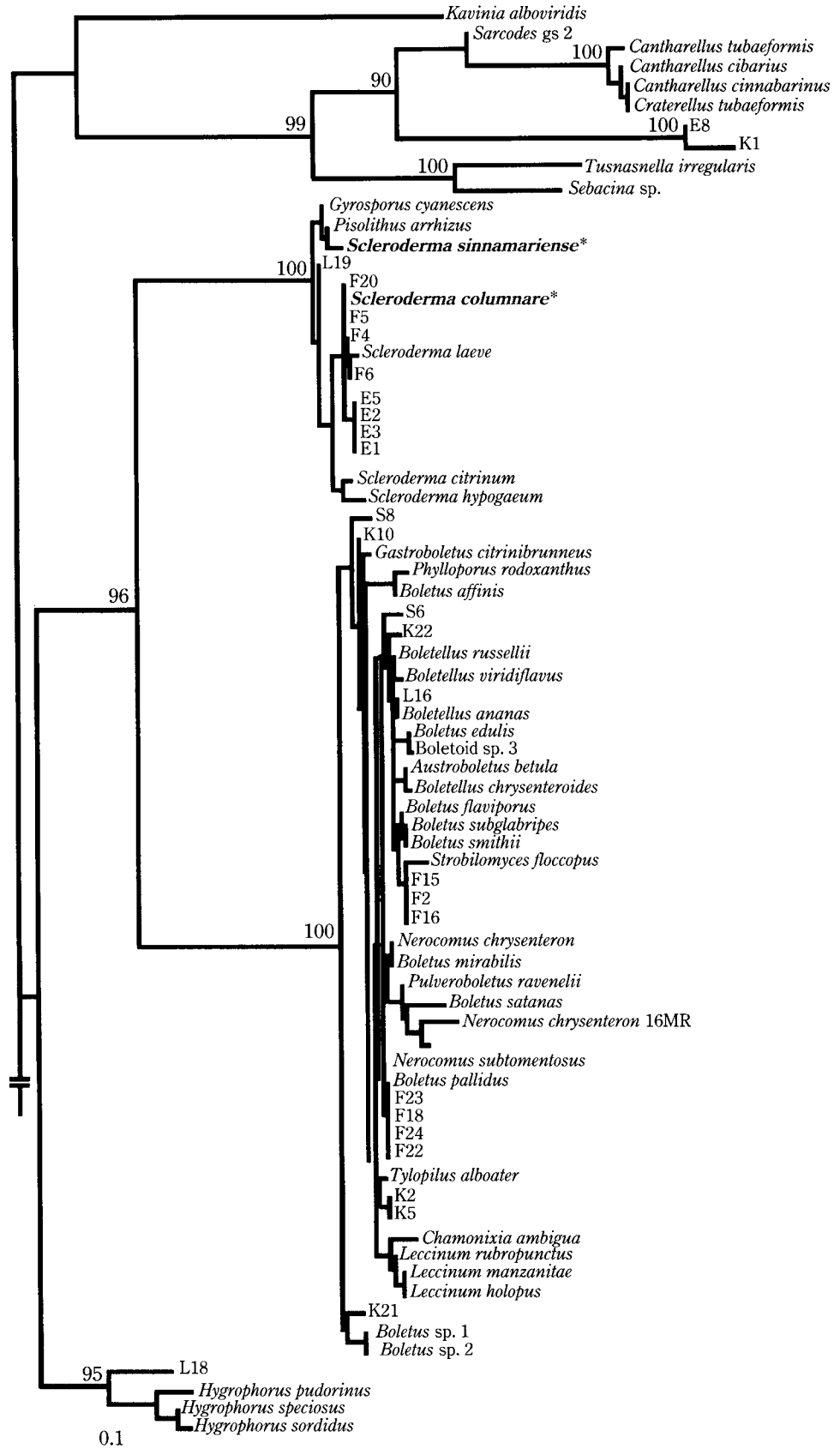
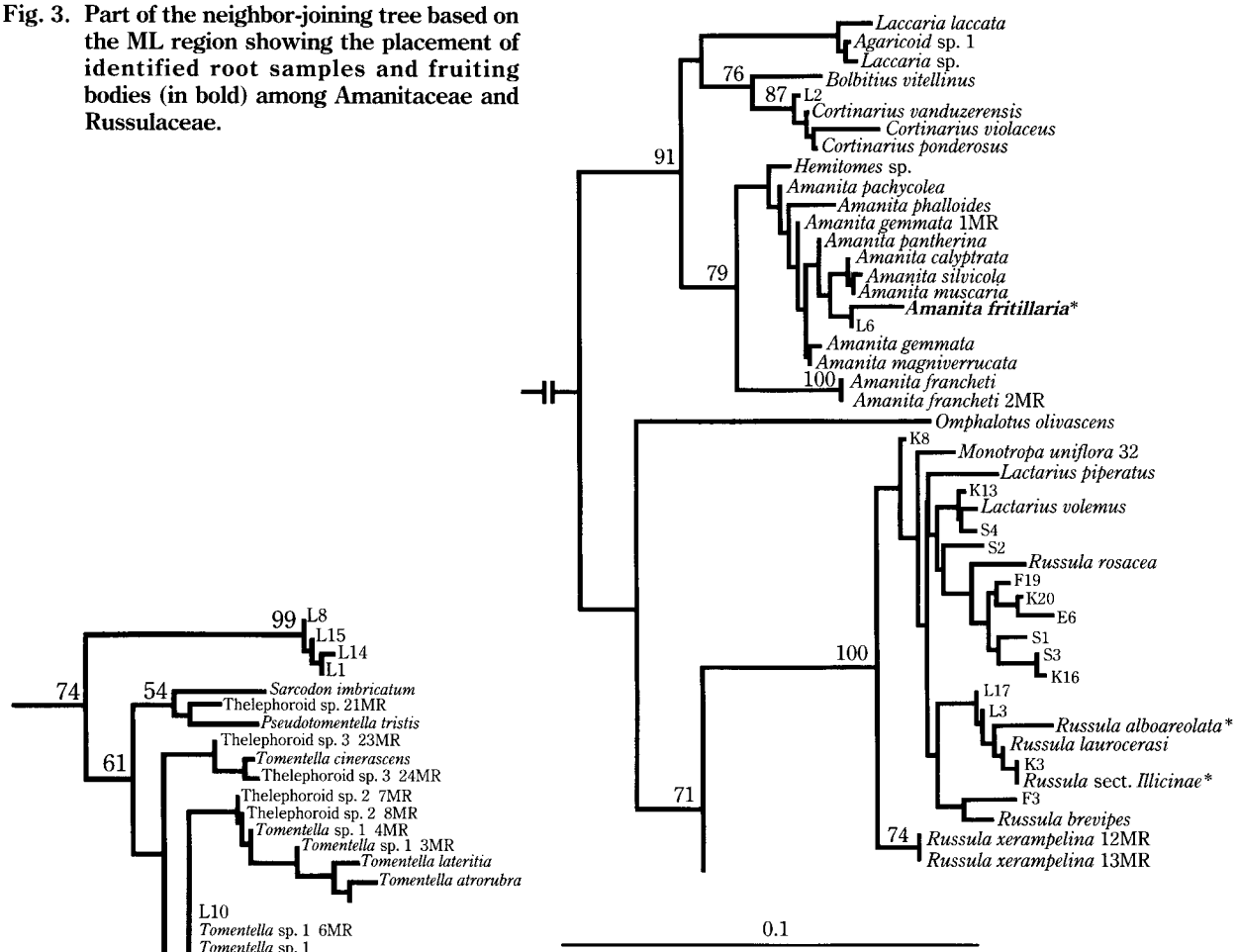


Fig. 2. Part of the neighbor-joining tree based on the ML region showing the placement of identified root samples and fruiting bodies (in bold) among Boletaceae, Sclerodermataceae, Hygrophoraceae and Cantharellaceae.

Fig. 3. Part of the neighbor-joining tree based on the ML region showing the placement of identified root samples and fruiting bodies (in bold) among Amanitaceae and Russulaceae.



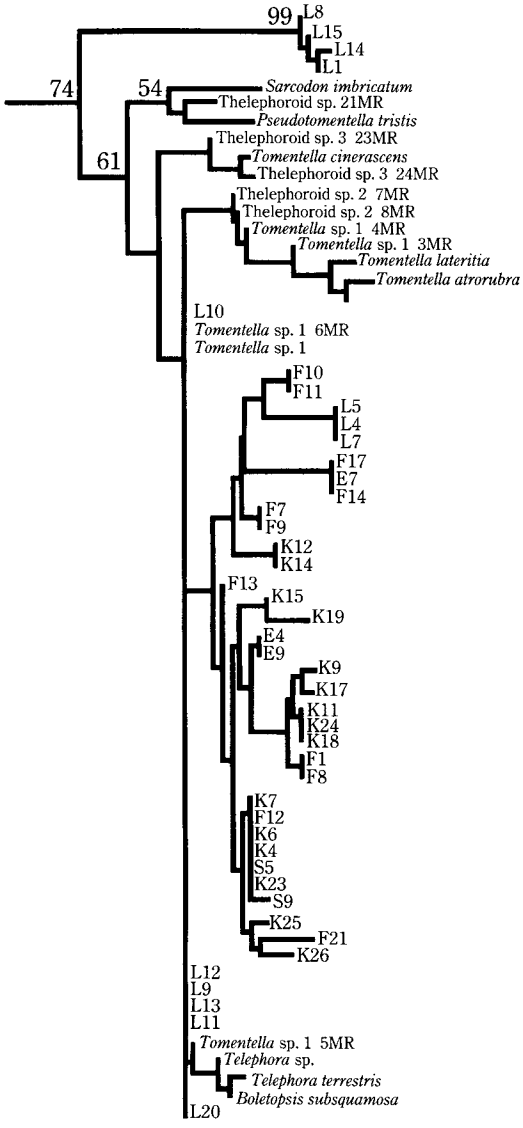


Fig. 4. Part of the neighbor-joining tree based on the ML region showing the placement of identified root samples among Thelephoraceae.

DISCUSSION

The aim of this study was to determine the most abundant EM fungi species associated with Dipterocarpaceae. Many aspects of the EM fungi communities are still unknown including the number and abundance of species in the EM communities and the factors that affect the communities (Horton & Bruns, 2001). In this study, to obtain a general picture of the EM community structure in dipterocarp forests, root samples from a large number of different tree species growing at several distant locations were collected. Because one or only a few root samples from each tree were collected, the results can underestimate the number of EM fungal species associated with dipterocarps. However, identification of the most common EM fungi, which can be often found in dipterocarp forests, was not negatively affected by the sampling method.

Recently, molecular identification has been widely applied to study below-ground community structure. By comparing the restriction fragment length polymorphisms (RFLP) patterns or DNA sequences with the database constructed from identified samples, the identification can be done easily with high reliability (Bidartondo *et al.*, 2000; Bruns *et al.*, 1998; Gardes & Bruns, 1993; Gardes & Bruns, 1996b; Horton & Bruns, 1998; Landeweert *et al.*, 2003; Linder *et al.*, 2000). The PCR-RFLP method is more often used because of its low cost, but when the RFLP patterns that are not included in the database are obtained, the comparison and identification cannot be done. Those samples are usually subsequently examined using phylogenetic analysis. Identification using phylogenetic analysis has been successful in many studies and samples can be identified to the family, genus or species level depending on the DNA region used (Bruns *et al.*, 1998; Horton & Bruns, 1998). Therefore, the identification using phylogenetic analysis was employed for this study.

Good DNA sequences of ML region from 22 samples were not obtained. Contamination by other soil fungi or bacteria that were homologous to the primers could be one possible cause. It is also possible that those unknown root samples contained more than one species of EM fungi whose morphology was almost the same making it difficult to distinguish them under the microscope. However, Dipterocarpaceae and EM fungi in a total of 88 EM root samples were successfully identified. The *trnL-trnF* and *matK* regions were used to construct phylogenetic trees for dipterocarps identification. These two regions evolve more rapidly than other regions in the chloroplast genome and are often used to study phylogenetic relationships (Kajita *et al.*, 1998; Wang *et al.*, 1999). The *matK* region provided better resolution at the species level because of its large size and synonymous sites, which have evolved about 1.8 times faster than the spacer region. However, we used the *trnL-trnF* spacer region to analyze all samples because it is more easily sequenced. The tree obtained based on the chloroplast DNA sequences had weak bootstrap support. However, our purpose was not to reconstruct dipterocarps phylogeny but to confirm whether the root belonged to a member of the Dipterocarpaceae. We, therefore, believe that identification based on the *trnL-trnF* IGS region is sufficient for this purpose. The placement of unknown samples on the tree based on combined partial *matK/trnL-trnF* sequences was similar to that on the tree based on only the *trnL-trnF* spacer region. This further supports our identification of dipterocarps. In addition, in most cases the identity of root samples inferred from the DNA sequence information agreed with the identity of trees growing close to the sampling site.

For the EM fungi, all samples were identified based on the ML region because of the large number of sequences in each family available in the database. The number of available DNA sequences of the ITS region for some families is small making phylogenetic analysis difficult. Unlike ITS region, which can identify individual species, the ML region can identify only individual families or genera. However, the ML5-ML6 region is advantageous because: it is small, easy to align and fungal-specific primers to amplify it are available (Bruns *et al.*, 1998). Fungi identification was confirmed by the congruence of the phylogenetic trees based on the ML region and the ITS region of samples taken from Lambir and the high bootstrap support for the branches on which the unknown samples were placed. In addition, the placement of morphologically identified fruiting bodies was correct when the identification was based on only the ML region. None of the samples were grouped with the non-mycorrhizal fungi. Biased selection of taxa in both Dipterocarpaceae and EM fungi could be a problem if the main purpose is to study phylogenetic relationships (Bruns *et al.*, 1998). However, it should not affect the identification process.

The results showed that Thelephoraceae is one of the most common and abundant fungi families associated with Dipterocarpaceae. However, previous studies based on the abundance of fruiting bodies and root morphology suggested that species of Russulaceae, Amanitaceae and Boletaceae are the most common EM fungi of Dipterocarpaceae (Lee, 1998). Thelephoraceae family has never been reported to form ectomycorrhizas with Dipterocarpaceae. On the other hand, members of Thelephoraceae have been reported as one of the most abundant EM fungi in boreal forest ecosystems (Horton & Bruns, 2001). The low correspondence between the EM fungi present above- and under-ground has often been observed in the other studies on ectomycorrhizal communities (Gardes & Bruns, 1996b; Horton & Bruns, 2001). Furthermore, root

morphology alone is not a good identification method because different species of fungi can have the same morphology. Recently, EM roots of dipterocarp species were cultured *in vitro*. The obtained fungus was identified as the member of Thelephoraceae (Lee, S.S. personal communication). In addition, results of studies on Dipterocarpaceae in Thailand based on molecular identification also suggest that members of Thelephoraceae are one of the most abundant EM fungi (Vichitsoonthonkul, T. personal communication). Therefore, we believe that members of Thelephoraceae are indeed among the most common EM fungi in Dipterocarpaceae.

Recently, research on EM communities has been advanced, especially in pine forests (Gardes & Bruns, 1996b; Horton & Bruns, 1998; Yamada & Katsuya, 2001). However, EM community structure in dipterocarp forests has been little studied. This study is the first attempt using molecular techniques to investigate the EM fungi associated with Dipterocarpaceae. Most of the samples identified in this study belonged to *Shorea* genus, which is the most abundant genus in Malaysia. Studies on other genera, such as *Dipterocarpus*, *Dryobalanops*, *Hopea*, etc., are still needed to complete the EM fungal investigation on Dipterocarpaceae. Most of the studies suggested that EM fungi can associate with the multiple hosts (Horton & Bruns, 1998). Therefore, further investigations will focus on the host specificity of the EM fungi.

For rehabilitation and reforestation purposes, it is necessary to identify EM fungi at the species level. Unfortunately the available ITS database is too small for this purpose and it does not contain sequences of Thelephoraceae species found in dipterocarp forests. In fact, most of the fungal ITS sequences in the database were obtained from sporocarps found in pine forests. Thelephoroid sporocarps taken from Dipterocarpaceae forests are needed to construct a better database for the identification of EM fungi associated with Dipterocarpaceae.

In conclusion, we believe that the members of Thelephoraceae could be some of the most abundant EM fungi associated with Dipterocarpaceae. However, the database containing only limited number of sequences for fungi occurring in dipterocarp forest is still one problem that makes identification at the species level difficult. In addition, in order to cover all genera in Dipterocarpaceae, additional samples from *Hopea*, *Vatica*, *Dipterocarpus*, *Neobalanops*, *Dryobalanocarpus* etc. must be analyzed.

ACKNOWLEDGMENTS This study was supported by grants Nos. 11490026 and 12575002 to T. Y., and 13490022 and 13575002 to AES from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

Ashton, P.S. 1982. Dipterocarpaceae. *Flora Malesiana ser. 1 Spermatophyta* **9:** 251-552.

Ashton, P.S., Givnish, T.J. & Appanah, S. 1988. Staggered flowering in the Dipterocarpaceae: New insights into floral induction and the evolution of mast fruiting in the aseasonal tropics. *The American Naturalist* **132:** 44-66.

Beaker, P. 1983. Mycorrhizas of *Shorea* (Dipterocarpaceae) seedlings in lowland Malaysian rainforest. *Malaysian Forester* 46: 146-170.

Bidartondo, M.I., Kretzer, A.M., Pine, E.M. & Bruns, T.D. 2000. High root concentration and uneven ectomycorrhizal diversity near *Sarcodes sanguinea* (Ericaceae): A cheater that stimulates its victims? *American Journal of Botany* **87:** 1783-1788.

Bruns, T.D., Szaro, T.M., Gardes, M., Cullings, K.W., Pan, J.J., Taylor, D.L., Horton, T.R., Kretzer, A., Garbelotto, M. & Li, Y. 1998. A sequence database for the identification of ectomycorrhizal basidiomycete by phylogenetic analysis. *Molecular Ecology* **7:** 257-272.

Buscot, F., Munch, J.C., Charcosset, J.Y., Gardes, M., Nehls, U. & Hampp, R. 2000. Recent advances in exploring physiology and biodiversity of ectomycorrhizas highlight the functioning of these symbioses in ecosystems. *FEMs Microbiology Review* **24:** 601-614.

Dahlberg, A. 2001. Community ecology of ectomycorrhizal fungi: an advancing interdisciplinary field. *New Phytologist* **150:** 555-562.

Doyle, J.J. & Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19:** 11-15.

Gardes, M. & Bruns, T.D. 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2:** 113-118.

Gardes, M. & Bruns, T.D. 1996a. Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: Above- and below-ground views. *Canadian Journal of Botany* **74:** 1572-1583.

Gardes, M. & Bruns, T.D. 1996b. ITS-RFLP matching for identification of fungi. *Species Diagnostics Protocols* **50:** 177-186.

Horton, R.T. & Bruns, T.D. 1998. Multiple-host fungi are the most frequent and abundant ectomycorrhizal types in a mixed stand of Douglas fir (*Pseudotsuga menziesii*) and bishop pine (*Pinus muricata*). *New Phytologist* **139:** 331-339.

Horton, T.R. & Bruns, T.D. 2001. The molecular revolution in ectomycorrhizal ecology: peeping into the black-box. *Molecular Ecology* **10:** 1855-1871.

Jonsson, L., Dahlberg, A., Nilsson, M.C., Karen, O. & Zackrisson, O. 1999. Continuity of ectomycorrhizal fungi in self-regenerating boreal *Pinus sylvestris* forests studied by comparing mycobiont diversity on seedlings and mature trees. *New Phytologist* **142:** 151-162.

Kajita, T., Kamiya, K., Nakamura, K., Wickneswari, W., Yoshimaru, H., Tsumura, Y., Tachida, H. & Yamazaki, T. 1998. Molecular phylogeny of Dipterocarpaceae in Southeast Asia based on nucleotide sequence of *matK*, *trnL* intron and *trnL-trnF* intergenic spacer region in chloroplast DNA. *Molecular Phylogenetics and Evolution* **10:** 202-209.

Kamiya, K., Harada, K., Ogino, K., Kajita, T., Yamazaki, T., Lee, H-S. & Ashton, P. 1998. Molecular phylogeny of dipterocarp species using nucleotide sequences of two non-coding regions in chloroplast DNA. *Tropics* **7:** 195-207.

Landeweert, R., Leeflang, P., Kuyper, T.W., Hoffland, E., Rosling, A., Wernars, K. & Smit, E. 2003. Molecular identification of ectomycorrhizal mycelium in soil horizons. *Applied Environmental Microbiology* **69:** 327-333.

Lee, S.S. 1992. *Some aspects of the biology of mycorrhizas of the Dipterocarpaceae*. PhD Thesis, University of Aberdeen.

Lee, S.S. 1998. Root symbiosis and nutrition. In: *A review of dipterocarps: Taxonomy, ecology and silviculture* (S. Appanah eds), Center for International Forestry Research, Indonesia.

Linder, C.R., Goertzen, L.R., Heuvel, B.V., Francisco-Ortega, J. & Jansen, R.K. 2000. The complete external transcribed spacer of 18S-26S rDNA: Amplification and phylogenetic utility at low taxonomic levels in Asteraceae and closely allied families. *Molecular Phylogenetics and Evolution* **14:** 285-303.

Ooi, K., Endo, Y., Yokoyama, J. & Murakami, N. 1995. Useful primer designs to amplify DNA fragments of the plastid gene *matK* from angiosperm plants. *Journal of Japanese Botany* **70:** 328-331.

Saitou, N. & Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4:** 406-425.

Sambrook, J., Fritsh, E.F. & Maniatis, T. 1989. Molecular cloning: A laboratory manual. 1-545 pp.

Smits, W.T.M. 1992. Mycorrhizal studies in dipterocarp forests in Indonesia. In: *Mycorrhizas in ecosystems* (D.J. Read, D.H. Lewis, A.H. Fitter & I.J. Alexander eds), CAB International, University Press, Cambridge.

Smits, W.T.M. 1994. *Dipterocarpaceae: mycorrhizae and regeneration*. PhD Thesis, Agricultural University Wageningen.

Taberlet, P., Gielly, L., Pautou, G. & Bouvet, J. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* **17:** 1105-1109.

Thompson, J.D., Gibson, T.J., Plewniak, F. & Higgins, D.G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24:** 4876-4882.

Wang, X.R., Tsumura, Y., Yoshimaru, H., Nagasaka, K. & Szmidt, A.E. 1999. Phylogenetic relationships of Eurasian pines (*Pinus*, Pinaceae) based on chloroplast rbcL, matK, rpL20-rpS18 spacer, and trnV intron sequences. *American Journal of Botany* **86:** 1742-1753.

Watling, R. 1995. Assessment of fungal diversity: Macromycetes, the problems. *Canadian Journal of Botany* **73:** S15-S24.

White, T.J., Bruns, T., Lee, S.S. & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocals, A guide to Methods and Applications* (M.A. Innis, D.H. Gelfand, J.J. Sninsky & T. J. White eds), 315-323, Academic Press, San Diego.

Yamada, A. & Katsuya, K. 2001. The disparity between the number of ectomycorrhizal fungi and those producing fruit bodies in a *Pinus densiflora* stand. *Mycological Research* **105:** 957-965.

Received 4th Oct. 2003
Accepted 1th Dec. 2003