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FULL PAPER

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Molecular phylogeny of ectomycorrhizal *Pisolithus* fungi associated with pine, dipterocarp, and eucalyptus trees in Thailand

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Abstract The phylogenetic relationships among 135 *Pisolithus* basidiomes and two isolates collected from three pine forests, a pine-dipterocarp forest, two dipterocarp forests, and 29 eucalyptus plantations in Thailand were investigated. Internal transcribed spacer (ITS) polymorphism analyses, including terminal RFLP, divided them into 26 groups. The ITS in a representative basidiome of each group was sequenced, and a phylogenetic analysis was performed. The dendrogram suggested that at least three *Pisolithus* species are present in Thailand. *Pisolithus* basidiomes collected in the pine forests and under some *Shorea roxburghii* trees in a pine-dipterocarp forest corresponded to species 5 as previously described by Martin et al. in 2002. Those collected under *S. roxburghii* and *Dipterocarp alatus* trees in the dipterocarp forests did not match any previously reported species. Basidiomes collected from the eucalyptus plantations were all identified as *Pisolithus albus*.

Key words Internal transcribed spacer (ITS) · Phylogeny · *Pisolithus* · Terminal RFLP

Introduction

Pisolithus is a group of gasteromycetes with worldwide distribution that form ectomycorrhizas with a wide range of angiosperm and gymnosperm tree species (Marx 1977). Inoculation with certain *Pisolithus* species has been shown to promote seedling growth of many tree species and has been used to enhance tree establishment and growth in plantations of economically significant trees such as *Eucalyptus* and *Pinus* (Garbaye et al. 1988).

Many investigators have been interested in the phylogeny of *Pisolithus* (Anderson et al. 1998, 2001; Martin et al. 1998, 2002; Gomes et al. 2000; Díez et al. 2001). Formerly, most *Pisolithus* isolates were classified as *P. tinctorius* (Pers.) Coker and Couch. However, variations within the former *P. tinctorius* were found in various enzyme activities, growth rates (Ho 1987), sexual incompatibility (Kope and Fortin 1990), mycorrhiza-forming ability (Lamhamedi et al. 1990), electrophoretic protein profiles (Burgess et al. 1995), and host specificity (Anderson et al. 1998, 2001; Martin et al. 1998, 2002; Díez et al. 2001). In addition, considerable large variations in morphology in basidiomes, basidiospores, and cultured mycelia also occur within *P. tinctorius* (Chambers and Cairney 1999). Thus, this species has been regarded to be conspecific. Several new species such as *P. aurantioscabrosus* Watling, Taylor, Lee, Sims & Alexander., *P. kisslingi* E. Fisch, *P. microcarpus* (Cke. & Mass.) G. Cunn., and *P. pusillum* Pat. have been described (Watling et al. 1995).

Progress in molecular biology has led to a better understanding of the genetic variation among *Pisolithus* isolates. Random amplification of polymorphic DNA (RAPD) analyses indicate that large genetic variations exist within *Pisolithus* isolates from Australia (Anderson et al. 1998), Brazil, United States, and France (Junghans et al. 1998). Variation in restriction fragment length polymorphism (RFLP) of ribosomal DNA and internal transcribed spacer (ITS) regions and nucleotide sequence difference in those regions have also been used to investigate phylogeny within *Pisolithus*. Such investigations have been reported on *Pisolithus* in Australia, Kenya, Europe, North America, South East Asia, and Brazil (Anderson et al. 1998, 2001; Martin et al. 1998; Sims et al. 1999; Gomes et al. 2000; Díez et al. 2001). Recently, an overall *Pisolithus* phylogeny by ITS sequence analysis has been reported, showing that a

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number of *Pisolithus* species exist worldwide (Martin et al. 2002). Nevertheless, there are still almost blank regions, especially in Asia, to be filled by additional analysis.

In Thailand, *Pisolithus* has been reported to associate with pines (Boonthavikoon 1998), dipterocarps (Boonthavikoon 1998; Chalermpongse 1999), and eucalyptus (Chalermpongse 1995; Phosri et al. 1999). In these reports, all *Pisolithus* were regarded as the same species, *P. tinctorius*. However, the morphology varied among *Pisolithus* basidiomes under different host trees. Also, a previous study showed that Thai *Pisolithus* isolates from basidiomes occurring under *Pinus kesiya* are poor colonizers of *Eucalyptus camaldulensis* and vice versa (Phosri et al. 1999). These results suggest that the species of *Pisolithus* in different host forests could be phylogenetically distinct. In this article, we have analyzed phylogenetic relationships among *Pisolithus* basidiomes collected from pine forests, a pine-dipterocarp forest, dipterocarp forests, and eucalyptus plantations in Thailand based on ITS sequences.

Materials and methods

Sample sources and DNA extraction

One hundred and thirty-five basidiomes of *Pisolithus* were collected in 2000, 2001, and 2002 from pine forests mainly composed of *P. kesiya* Royle ex Gordon., a pine-dipterocarp forest, dipterocarp forests mainly composed of *Shorea roxburghii* G. Don and *Dipterocarpus alatus* Robx., and *E. camaldulensis* Dehnh. plantations in Thailand. In each forest or plantation, 2–13 basidiomes were sampled. Pieces of stipe tissue were excised from each basidiome, dried by silica gel, and stored at 4°C until use. Cultured mycelia of two *Pisolithus* isolates (PPBN1 and PPBN2) collected in 1996 under *P. kesiya* and two from *Pinus densiflora* Sieb. et Zucc. were obtained from collections of the Department of Microbiology, Chulalongkorn University, Bangkok, Thailand and Symbiotic Function Research Unit, Asian Natural Environmental Science Center, the University of Tokyo, Tokyo, Japan, respectively. *Scleroderma* basidiomes collected on the foot of Mt. Fuji in Japan (*Scleroderma bovista* Fr.) and from the *P. kesiya* forest in Thailand (*Scleroderma* sp.) were used as outgroup species.

Genomic DNA was prepared from the dried samples of *Pisolithus* by homogenization in 1.5-ml tubes with a FastPrep FP120 homogenizer (Savant, faxmingdale, NY, USA) and extraction with cetyltrimethylammonium bromide (CTAB) as described in Zhou et al. (1999).

ITS amplification and terminal RFLP analysis

The ITS region of each sample was amplified with two of the primers ITS1f (Gardes and Bruns 1993), ITS3, and ITS4 (White et al. 1990). One primer was labeled with Texas red fluorescent dye (Genset KK, Kyoto, Japan) at the 5′-end for analysis with a sequencer. The primer pairs comprised labeled ITS1f and ITS4, ITS1f and labeled ITS4, or ITS3 and labeled ITS4. Fragments amplified by primers ITS1f and ITS4 and by ITS3 and ITS4 were designated as $ITS_{1f\text{-}4}$ and $ITS_{3\text{–}4}$, respectively. Twenty microliters of reaction mixture contained 5 ng template DNA, 0.2 mM each dNTP, 1 × PCR buffer, 1.5 mM Mg^{2+}, 0.5 U Ampli Taq Gold (Ampli Taq Gold kit; Perkin Elmer, Branchburg, NJ, USA), and 0.5 μM of the primer pair. The amplification reactions were performed in a thermal cycler (TP 3000; Takara Shuzo, Tokyo, Japan). Amplification was started at 94°C for 9 min, followed by 38 cycles of a denaturing step at 94°C for 1 min, an annealing step at 51°C for 1 min, and an extension step at 72°C for 1 min, and ended with an additional 5-min extension step at 72°C. Two kinds of labeled $ITS_{1f\text{-}4}$ were used in the following terminal-RFLP analysis (Zhou and Hogetsu 2002). Three microliters $ITS_{1f\text{-}4}$ was digested with 5 U restriction endonuclease (*Alu*I or *Hin*fI) at 37°C for 8 h. After tenfold dilution, polymerase chain reaction (PCR) products of ITS and their restricted fragments were denatured at 94°C for 5 min and electrophoresed on 6% Long Ranger acrylamide gels (FMC Bioproducts, Rockland, ME, USA), with 6.1 M urea, and 1.2 × TBE [0.1 M Tris (hydroxymethyl) aminomethane, 3.0 mM ethylene diaminetetraacetic acid (EDTA), and 0.1 M boric acid], in a sequencer (SQ-5500E; Hitachi, Tokyo, Japan).

Band patterns were analyzed using FRAGLYS 2.0 software (Hitachi). According to the labeled primer and restriction endonuclease used, four kinds of restricted fragments were obtained in the terminal RFLP: those labeled at the ITS_{1f} end and digested with *Alu*I (*Alu*I_{1f}), those labeled at the ITS_4 end and digested with *Alu*I (*Alu*I_4), those labeled at the ITS_{1f} end and digested with *Hin*fI (*Hin*fI_{1f}), and those labeled at the ITS_4 end and digested with *Hin*fI (*Hin*fI_4) (Zhou and Hogetsu 2002). Samples showing the identical sizes of $ITS_{1f\text{-}4}$, $ITS_{3\text{–}4}$, and restricted fragments of $ITS_{1f\text{-}4}$ were considered to belong to the same genotype. From each group, one sample was arbitrarily chosen and used for sequence analysis.

Sequencing and phylogenetic analysis

$ITS_{1f\text{-}4}$ regions were amplified from the representative sample of each genotype and from two Japanese *Pisolithus* isolates and *Scleroderma* basidiomes. Amplified $ITS_{1f\text{-}4}$ fragments were cloned using pT7 Blue vectors (Novagen, Madison, WI, USA) and transformed into *Escherichia coli* strain XL1-Blue MRF. Ligation and transformation were performed according to the manufacturer's protocol. Plasmid DNA was extracted from positive clones and sequenced with a Thermo Sequence Pre-mixed Cycle Sequencing kit (Hitachi) using the T7 and M13 forward primers labeled with Texas Red (Hitachi) in an SQ-5500E sequencer.

$ITS_{1f\text{-}4}$ sequences were automatically aligned with some *Pisolithus* ITS sequences obtained from GenBank DNA database (http://www.ddbj.nig.ac.jp) (Table 1). They were chosen to cover 11 phylogenetic species identified by Martin et al. (2002). The alignment was carried out using Clustal X (http://inn-protweiznann.ac.il/solfware/ClustalX.html), and manually improved in MacClade 4 (Maddison and

Table 1. List of *Pisolithus* sequences obtained from GenBank database

Strain	GenBank accession no.	Host	Origin	Reference
5105	AF003915	*Afzelia quanzensis*	Kenya	Martin et al. (1998)
K915	AF228653	*A. quanzensis*	Kenya	Díez et al. (2001)
MU98/105	AF374665	*Eucalyptus patens*	Australia	Martin et al. (2002)
KS781	AF374719	*E. calophylla*	Australia	Martin et al. (2002)
cab01	AF228644	*Cistus ladanifer*	Spain	Díez et al. (2001)
cr04	AF228643	*C. ladanifer*	Spain	Díez et al. (2001)
PtJap	AF374629	*Pinus pumila/Betula ermanii*	Japan	Martin et al. (2002)
MP9812	AF374627	*Pinus* sp.	South Africa	Martin et al. (2002)
pt03	AF228648	*Quercus ilex, Q. coccifera*	Spain	Díez et al. (2001)
MH728	AF374679	*Eucalyptus* sp.	China	Martin et al. (2002)
H614	AF374710	*P. elliotii*	China	Martin et al. (2002)
5111	AF003916	*Pinus caribaea*	Kenya	Martin et al. (1998)
MARX270	AF374632	*P. elliotii*	USA	Martin et al. (2002)
ch01	AF228645	*Q. ilex, Q. coccifera*	Spain	Díez et al. (2001)
COI24	AF374622	*Acacia holosericea*	Senegal	Martin et al. (2002)
H4937	AF374670	*E. tereticornis, E. tesselaris*	Australia	Martin et al. (2002)
MU98/101	AF374661	*E. camaldulensis*	Australia	Martin et al. (2002)
MH56	AF374708	*Eucalyptus/Acacia*	Australia	Martin et al. (2002)
UFSC132	AF374704	*E. dunni*	Brazil	Martin et al. (2002)
441	U62666	*E. citriodora*	Brazil	Carnero-Diaz et al. (1997)
CSH4461	AF374624	*Acacia* sp.	Australia	Martin et al. (2002)
MU98/6	AF374646	*E. globulus*	Australia	Martin et al. (2002)
Pash01	AF415226 & AF415227	*Shorea macroprepa*	Malaysia	Martin et al. (2002)

Maddison 2001). ITS sequences of *S. bovista* and an unidentified *Scleroderma* species were included as outgroup species. Phylogenetic dendrograms were constructed in neighbor-joining (NJ) modes of PAUP* 4.08b (PPC) (Swofford 1999). Neighbor-joining analysis was performed after Kimura's two-parameter model. Bootstrap values were calculated by 1000 replications.

Results

ITS polymorphism analysis

The size of ITS_{3-4} fragments varied in length from 395 to 447 bp. One hundred and eight samples produced a single ITS_{3-4} fragment and 29 samples produced 2 fragments (Table 2).

The 137 *Pisolithus* samples were grouped into 26 genotypes by combination of banding pattern polymorphisms in ITS_{3-4} and the terminal-RFLP fragments *Alu*I_{1f}, *Hin*fI_{1f}, *Alu*I_4, and *Hin*fI_4 (Table 2). Basidiomes in the pine forests, pine-dipterocarp forest, dipterocarp forests, and eucalyptus plantations were composed of 11, 1, 3, and 11 genotypes, respectively. In two pine forests, only 1 genotype appeared, and more than 2 genotypes appeared in other forests and plantations. Although some genotypes commonly occurred in several forests, no genotype found in eucalyptus plantations appeared in pine forests and dipterocarp forests, and vice versa.

Phylogenetic analysis

ITS_{1f-4} sequences of 26 Thai *Pisolithus* samples representing ITS genotypes were determined (Table 2). The sampling localities of each representative genotype are given in Table 3. The ITS sequence data reported in this study have deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases (Table 3). Sequences ranged from 624 to 692 bp, including the entire part from the 3′-end of the 18S rDNA to the 5′-end of the 28S rDNA. The ITS1 and ITS2 regions of these samples included insertions, deletions, and base substitutions, which indicated that the high length variation in ITS_{3-4} and terminal-RFLP fragments resulted from deletions and insertions in the ITS region.

A phylogenetic dendrogram was drawn in a neighbor-joining mode with our ITS sequences and those registered in the database (Fig. 1). All sequences in the dendrogram were divided into three major clades (clade I, clade II, and clade III) with high bootstrap values as in Martin et al. (2002). Sequences obtained from the database structured the same phylogenetic dendrogram as in Martin et al. (2002). Sequences of all basidiomes collected in Thailand were distributed in clades I and II (Fig. 1). Three sequences of all basidiomes collected under *S. roxburghii* and *D. alatus* trees in dipterocarp forests in Thailand were grouped into a species that was different from any species reported previously. The other 12 sequences of our samples in clade I were grouped together with sequences of species 5 in Martin et al. (2002). These samples comprised all samples collected under *P. kesiya* trees in pine forests and three samples collected under *S. roxburghii* trees in a pine-dipterocarp forest. In addition, two isolates collected under *P. densiflora* trees from Japan (AB106874 and AB106875) had 97%–99% sequence similarity with all samples in clade I. All 11 sequences representing samples collected from Thai eucalyptus plantations were contained in clade II and grouped together with sequences of *P. albus*.

Table 2. Internal transcribed spacer ($ITS_{3–4}$) and ITS terminal-restriction fragment length polymorphism (T-RFLP) of *Pisolithus* samples from *E. camaldulensis* plantations, pine forests, a pine-dipterocarp forest, and dipterocarp forests

Genotype	$ITS_{3–4}$ (bp)	*Alu*I_{1f} (bp)	*Hin*fI_{1f} (bp)	*Alu*I_4 (bp)	*Hin*fI_4 (bp)	Number of samples
1	401	413	291	93	336	24
2	395, 397	413	291	92	329, 331	11
3	395	413	291	92	329	6
4	402	413	291	93	335, 336	19
5	401	413	291	93	335	3
6	397, 401	413	291	92, 93	331, 336	6
7	402	413	291	93	336	1
8	402	413	291	93	335	22
9	395	413	291	92	331	2
10	395, 402	413	291	92, 93	329, 335	1
11	401	413	291	93	335, 336	5
12[a]	445	587	201	138, 141	252, 256	1
13[a]	445	587	201	140	254	5
14[a]	443	587	201	141	255	1
15[a]	444	587	201	140	252	1
16[a]	444	587	201	140	253	1
17[a]	443	587	201	139	252	1
18[a]	443, 446	587	201	137, 141	252, 256	2
19[a]	447	587	201	143	257	5
20[a]	436, 442	587	201	129, 138	245, 251	3
21[a]	436, 443	587	201	130, 139	245, 254	1
22[a]	435	587	201	130	245	1
23[b]	446	587	201	139	254	3
24[c]	412, 414	586	180	77, 79	234, 236	2
25[c]	412, 414	587	180	82	234, 236	3
26[c]	412	587	180	82	234	7

[a–c] Genotypes obtained from *Pisolithus* samples from pine forests, a pine-dipterocarp forest, and dipterocarp forests, respectively

Relationship between terminal-RFLP and sequence analyses

The restriction site of *Alu*I in the ribosomal RNA gene region was located within ITS2 and sites of *Hin*fI within ITS2 and 5.8S rDNA (Fig. 2). Sizes of *Hin*fI_{1f} were identical among genotype groups within the same species, but were different among the three species (Table 2). *Alu*I_{1f} sizes of all the samples in clade I were the same within each species, but variable within *P. albus*. Sizes of *Hin*fI_4 and *Alu*I_4 were variable within the same species. $ITS_{3–4}$ was also variable within species. Sequence analyses demonstrated that more interspecific variation was located in ITS1 than ITS2, whereas there was more intraspecific variation in ITS2 than ITS1.

Discussion

ITS polymorphism analysis

Comparison between ITS sequences of all terminal-RFLP groups revealed that the intra- and interspecific size differences in the terminal-RFLP fragments were caused by deletions or insertions mainly in ITS2 and ITS1, respectively.

Three species were identified among Thai *Pisolithus* basidiomes. Size of the terminal-RFLP fragment *Hin*fI_{1f} was identical among basidiomes of the same species. Analysis of restriction sites by *Hin*fI in ITS sequences of our samples and database-registered samples also indicated that *Hin*fI_{1f} differed between *Pisolithus* species in the present study and the study of Martin et al. (2002). This result suggests that *Hin*fI_{1f} could be used as an indicator for *Pisolithus* species classification.

Phylogenetic analysis

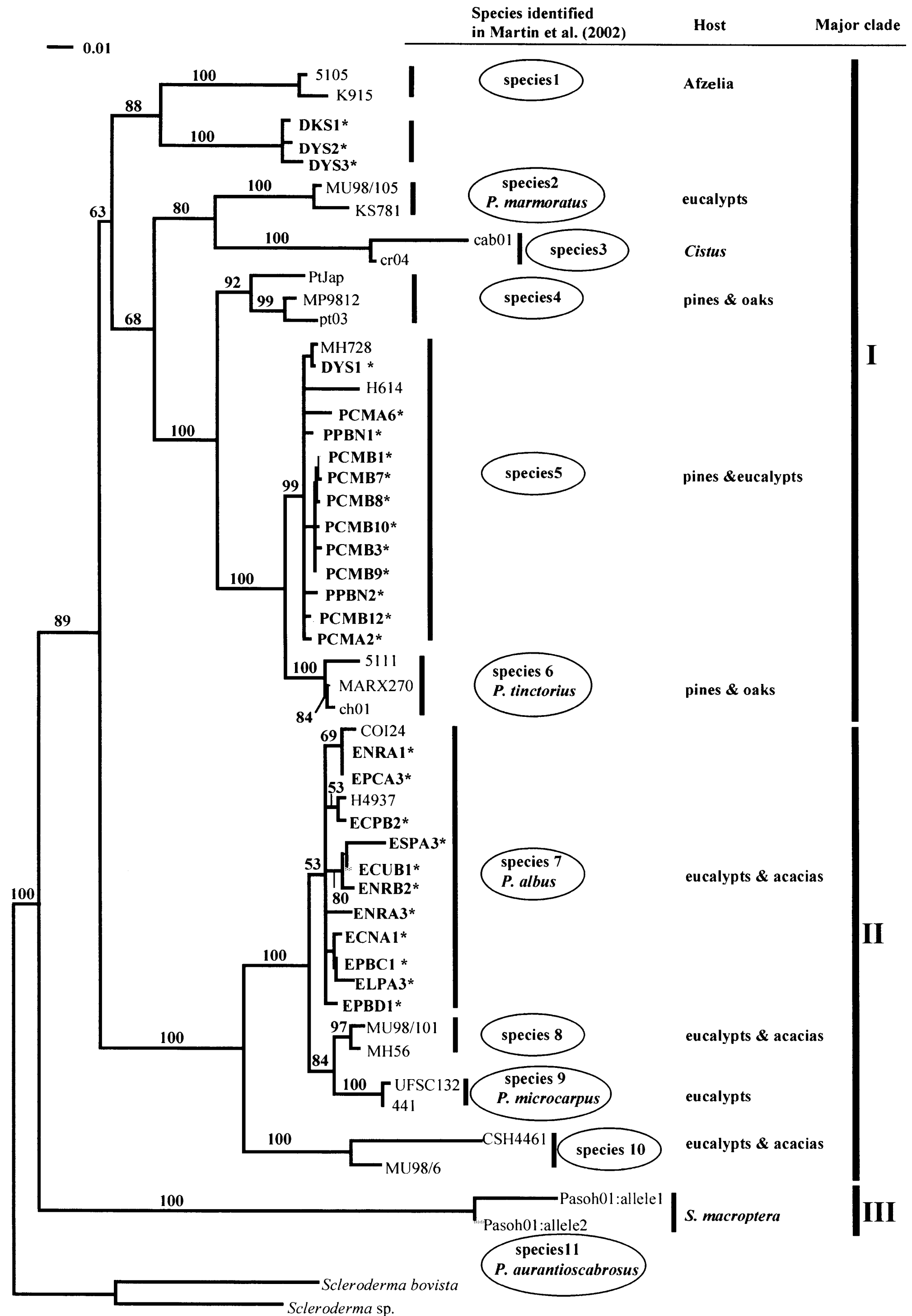
Phylogenetic analysis based on ITS sequences clearly separated *Pisolithus* samples in Thailand into three groups within two clades (see Fig. 1). Phylogenetic analysis showed that all samples from dipterocarp forests were not classified into any *Pisolithus* species in Martin et al. (2002) including their species 11 (putative *P. aurantioscabrosus*) that was collected under a dipterocarp, *Shorea macroptera*. Although these samples seem to be closely related to species 1, the high bootstrap value indicates their phylogenetic difference (Fig. 1); thus, they may represent an additional new species. Voucher specimens were deposited in Royal Botanic Garden, Edinburgh, UK, and in Symbiotic Function Research Unit, Asian Natural Environmental Science Center, the University of Tokyo, Japan. Further identification by morphology is in progress.

Our result showed all 22 samples collected in pine forests and 3 samples under *S. roxburghii* trees in a

Table 3. List of *Pisolithus* samples whose ITS sequences were obtained in this study with their host plant and ITS T-RFLP genotype

Representative sample	ITS T-RFLP genotype	GenBank accession no.	Locality	Host	Geographic location	Year collected
ECPB2	1	AB099910	Nong bua ra haeo District, Chaiyaphum Province	*Eucalyptus camaldulensis*	15°47′ N 101°33′ E	2001
ECUB1	2	AB099911	Pa thiu District, Chumphon Province	*E. camaldulensis*	10°45′ N 99°16′ E	2001
ESPA3	3	AB099912	Doembang nang buat District, Supanburi Province	*E. camaldulensis*	Unknown	2000
ENRA1	4	AB099914	Si khia District, Nakhonratchasima Province	*E. camaldulensis*	14°48′ N 101°53′ E	2001
ELPA3	5	AB099913	Li District, Lumphun Province	*E. camaldulensis*	17°57′ N 98°53′ E	2001
EPBC1	6	AB099917	Phetchabun Province	*E. camaldulensis*	15°50′ N 100°55′ E	2001
ENRA3	7	AB099915	Si khia District, Nakhonratchasima Province	*E. camaldulensis*	14°48′ N 101°53′ E	2001
EPCA3	8	AB099918	Ban khaosai, Thap kho District, Pichit Province	*E. camaldulensis*	16°08′ N 100°38′ E	2001
ENRB2	9	AB099908	Non thai District, Nakhonratchasima Province	*E. camaldulensis*	15°09′ N 102°06′ E	2001
EPBD1	10	AB099916	Phetchabun Province	*E camaldulensis*	15°49′ N 101°10′ E	2001
ECNA1	11	AB099909	City District, Chainat Province	*E. camaldulensis*	Unknown	2000
PCMB1	12	AB099904	Mae Sa Nam, Hot District, Chiangmai Province	*Pinus kesiya*	18°09′ N 98°17′ E	2001
PCMB3	13	AB099906	Mae Sa Nam, Hot District, Chiangmai Province	*P. kesiya*	18°09′ N 98°17′ E	2001
PCMB7	14	AB099907	Mae Sa Nam, Hot District, Chiangmai Province	*P. kesiya*	18°09′ N 98°17′ E	2001
PCMB8	15	AB099905	Mae Sa Nam, Hot District, Chiangmai Province	*P. kesiya*	18°09′ N 98°17′ E	2001
PCMB9	16	AB099902	Mae Sa Nam, Hot District, Chiangmai Province	*P. kesiya*	18°09′ N 98°17′ E	2001
PCMB10	17	AB099903	Mae Sa Nam, Hot District, Chiangmai Province	*P. kesiya*	18°09′ N 98°17′ E	2001
PCMA12	18	AB099846	Mae Sa Nam, Hot District, Chiangmai Province	*P. kesiya*	18°09′ N 98°17′ E	2001
PCMA6	19	AB099847	Boa keaw, Hot District, Chiangmai Province	*P. kesiya*	18°09′ N 98°23′ E	2001
PCMA2	20	AB099845	Boa keaw, Hot District, Chiangmai Province	*P. kesiya*	18°09′ N 98°23′ E	2001
PPBN1	21	AB099843	Namnao Distict, Phetchabun Province	*P. kesiya*	Unknown	1996
PPBN2	22	AB099844	Namnao Distict, Phetchabun Province	*P. kesiya*	Unknown	1996
DYS1	23	AB099919	Yasothron Province	*Shorea roxburghii*	Unknown	2002
DYS2	24	AB099921	Yasothron Province	*S. roxburghii*	Unknown	2002
DYS3	25	AB099920	Yasothron Province	*S. roxburghii*	Unknown	2002
DKS1	26	AB099922	Kao Hin Saon, Nakhonnayok	*Dipterocarpus alatus*	13°44′ N 101°30′ E	2002

Fig. 1. Neighbor-joining (NJ) phylogenetic dendrogram of *Pisolithus* species based on internal transcribed spacer (ITS) sequences. *Scleroderma bovista* and another *Scleroderma* species were used as an outgroup. Numerical values on branches are the bootstrap values as percentage bootstrap replication from a 1000-replicate analysis. *Scale bar*, indicates 0.1 genetic distance between samples; *asterisks*, Thai samples collected in the present study; *encircled species names*, those designated by Martin et al. (2002)



pine-dipterocarp forest in Thailand were classified into a species corresponding to species 5 designated by Martin et al. (2002) (see Fig. 1). In the dendrogram by Martin et al. (2002), species 5 contains two Asian isolates from pine forests, one from a pine forest in Thailand and one from a pine forest in China. The dendrogram also shows that a *Pisolithus* basidiome collected under Japanese *Pinus pumila* belongs to species 4. In this study, we found two Japanese isolates from *P. densiflora* were grouped into species 5. In spite of the exceptional example, species 5 may usually colonize Asian pine species.

All samples from Thai *Eucalyptus* plantations were classified into *P. albus* (see Fig. 1). Phylogenetic groups corresponding to *P. albus* in previous dendrograms reported by several investigators contains many isolates from *Eucalyptus* trees in Australia as well as Senegal (Bougher and Syme 1998; Anderson et al. 2001; Martin et al. 2002). In Thailand, *Eucalyptus* trees have been introduced from Australia since

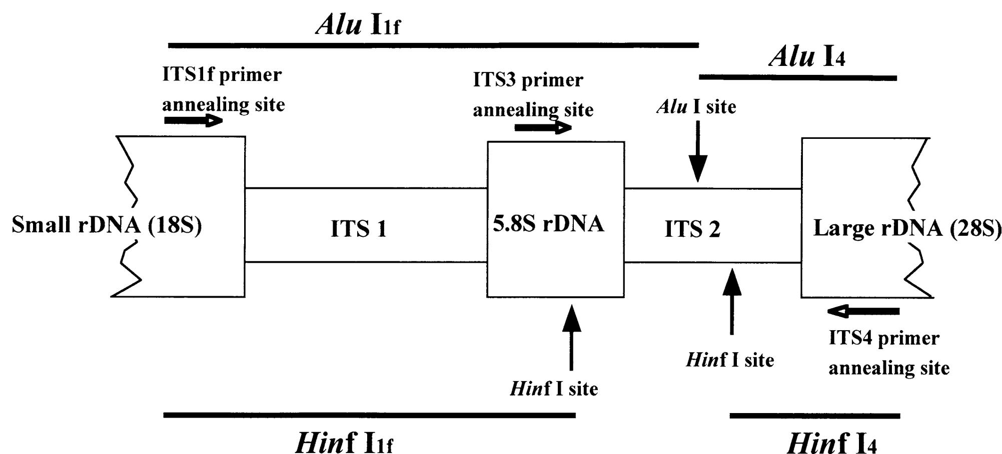


Fig. 2. Diagram of annealing sites of primers and restriction sites of *Alu*I and *Hin*fI enzymes in the ribosomal RNA gene region. *Horizontal lines* represent the terminal-RFLP fragments *Alu*I_{1f}, *Alu*I_4, *Hin*fI_{1f}, and *Hin*fI_4

1946 for reforestation and paper pulp production (Saardarvudh 1984). The best growing species was *E. camaldulensis*, and at present *E. camaldulensis* stands have been widely spread all over the country. Taking into consideration the fact that Thailand initially imported many *Eucalyptus* seedlings from Australia, it seems likely that *P. albus* in Thai *E. camaldulensis* stands has been introduced from Australia together with *Eucalyptus* seedlings, similar to the situation in South Africa, Brazil, and Kenya (Burgess et al. 1995; Martin et al. 1998). In *E. camaldulensis* stands, a number of *Pisolithus* basidiomes emerge in the rainy season, and these basidiomes may disperse an enormous amount of spores in a wide range. The vigorous spore dispersal may spread the species distribution to many *E. camaldulensis* stand in a wide area.

Although species 5 usually associated with *Pinus* species was also found under dipterocarp trees, its basidiome was not found under *E. camaldulensis* trees in this study. The present new species was found only under dipterocarp trees. *Pisolithus albus* usually associated with eucalyptus trees was not found in pine and dipterocarp forests. Thus three *Pisolithus* species in Thailand seem to have clearly limited host ranges in nature. The observation that it is difficult for Thai *Pisolithus* isolates from basidiomes under *P. kesiya* trees to colonize *E. camaldulensis* and vice versa (Phosri et al. 1999) may also support the narrow host ranges of Thai *Pisolithus* species. The host range of *Pisolithus* fungi has been generally considered to be relatively wide, because *Pisolithus* species develop their basidomes in association with many tree species, and inoculation experiments with seedlings show wide host ranges (Marx 1977; Martin et al. 1998; Chambers and Cairney 1999). The host range in *Pisolithus* species should be reevaluated on the basis of molecular identification of species of the basidiomes and isolates in nature.

In conclusion, the present study demonstrated that there are three *Pisolithus* species in Thailand; one is a species inhabiting in pine forests and the pine-dipterocarp forest, one is a new species colonizing *S. roxburgii* and *D. alatus*, and the other is *P. albus* colonizing *E. camaldulensis*. Their host ranges seem to be relatively narrow.

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