Mycoscience (2008) 49:334–338
DOI 10.1007/s10267-008-0420-x

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Morphological and molecular characterization of *Pisolithus* occurring in Hokkaido Island, Northern Japan

Received: January 23, 2007 / Accepted: April 10, 2008

Abstract *Pisolithus* basidiomes were found under different forest trees in Hokkaido Island, Japan. These basidiomes were characterized morphologically and molecularly. Although presenting different basidiome morphology and growing under different hosts, specimens presented similar spores ornamentation, and diameters. These spores had coarse, crowded, and blunted spines with three to eight basidiospores per basidium. Ribosomal DNA-based phylogenetic analysis indicated that variability of *Pisolithus* in this area is low. Phylogenetic analysis showed that *Pisolithus* analyzed in this study did not group with *Pisolithus* specimens from other geographical origins. These results suggest that *Pisolithus* from this area should be taxonomically distinguished from other *Pisolithus*.

Key words Ectomycorrhizal fungus · Phylogenetic analysis · *Pisolithus* sp. · Ribosomal DNA · Taxonomy

Pisolithus is often regarded as a cosmopolitan ectomycorrhizal fungus with a wide host range, establishing mycorrhizae with angiosperms and gymnosperms (Marx 1977). However, a great variation in the effects of inoculation with different strains of *Pisolithus* on the growth of forest trees, as well as compatibility among host and *Pisolithus* species, has been reported (Burgess et al. 1994; Pereira et al. 2005). There is considerable polymorphism in terms of morphology and size of *Pisolithus* basidiomes and spores. Large variations in colony growth rates, enzyme activities, polypeptide patterns, and mycorrhizal ability have also been reported (Kope and Fortin 1990; Burgess et al. 1994, 1995).

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Since *Pisolithus* was described, several taxa have been proposed based on the distinctive morphology of basidiomes and basidiospores (Marx 1977). Morphological differences were considered to be nondiagnostic, and taxa within the genus *Pisolithus* were regarded as conspecific [Coker and Couch 1928; Pilát 1958 (reviewed in Díez et al. 2001)]. Genetic polymorphism investigation of *Pisolithus* has also shown that they are quite different, additionally showing the correlation between geographic location and *Pisolithus* basidiospore morphology (Burgess et al. 1995) and molecular profile (Anderson et al. 1998; Junghans et al. 1998; Cairney et al. 1999; Gomes et al. 1999; Martin et al. 1998, 2002). Therefore, although worldwide groups of specimens have commonly been referred to as *P. tinctorius*, they actually comprise a number of distinct species (Bronchart et al. 1975; Calogne and Demoulin 1975; Anderson et al. 1998; Junghans et al. 1998; Martin et al. 1998, 2002; Cairney et al. 1999; Gomes et al. 1999, 2000; Sims et al. 1999).

Pisolithus is also found in Hokkaido Island (Hokkaido), Northern Japan, growing under different forest tree species. The objective of this work was to provide information on the morphological and genetic variability among these specimens and compare their sequences of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (rDNA) to others described in the literature.

Fourteen basidiomes of *Pisolithus* from six locations in Hokkaido were selected to be analyzed in this study. The basidiomes were collected during the period from July to September 2005 from different host trees (Table 1).

Pieces from intact, but mature, basidiomes were cut using a cryomicrotome for observation of basidia and basidiospores under a fluorescence microscope, after staining with calcofluor white with 10% KOH.

For scanning electron microscopy, mature spores were air-dried and sputter-coated with gold plus palladium before observation under a JSM5310 scanning electron microscope (SEM), at low vacuum, using an accelerating voltage of 15 kV. Mean basidiospore diameter ($n = 20$) was determined for each specimen by measuring from scanning electron micrographs, and basidiospore spine morphology was

Table 1. Basidiome identification, substitute locality, forest tree species, basidiome morphological characteristics, and GenBank accession of Japanese *Pisolithus* specimens used in this study

Basidiome identification	Locality[a]	Tree species in collection site	Basidiome		GenBank
			Stipe height[b]	Height × width (mm)	accession number
FORBs05006	Rankoshi	*Betula maximowicziana, B. platyphylla, Abies sachalinensis, Larix leptolepis*	Long	33 × 42	EF192104
FORBs05007	Shiretoko (Iozan)	*B. ermanii*	Short	21 × 22	ns
FORBs05008	Shiretoko (Iozan)	*B. ermanii*	Short	34 × 38	EF192105
FORBs05009	Shiretoko	*Pinus pumila*	Short	34 × 38	EF192106
FORBs05010	Akan	*P. pumila, B. ermanii*	Short	53 × 46	EF192107
FORBs05011	Chitose	Mixed forest	Short	63 × 44	EF192108
FORBs05012	Sapporo	Mixed forest	Short	52 × 96	ns
FORBs05013	Tokachi-Dake	*B. ermanii, B. platyphylla, Picea glehnii*	Short	59 × 61	ns
FORBs05014	Tokachi-Dake	*B. ermanii, B. platyphylla, P. glehnii*	Long	33 × 46	ns
FORBs05015	Tokachi-Dake	*B. ermanii, B. platyphylla, P. glehnii*	Long	60 × 53	EF192109
FORBs05016	Tokachi-Dake	*B. ermanii, B. platyphylla, P. glehnii*	Short	65 × 55	ns
FORBs05017	Sapporo	Mixed forest	Short	38 × 39	ns
FORBs05018	Sapporo	Mixed forest	Short	63 × 60	EF192110
FORBs05019	Rankoshi	*B. maximowicziana, B. platyphylla, A. sachalinensis, L. leptolepis*	Long	43 × 60	ns

ns, not sequenced
[a] All specimens are located in Hokkaido Island, Japan
[b] Short, <10 mm

Table 2. *Pisolithus* specimens with information concerning their geographic locations, hosts, and GenBank accession numbers

Specimens	Locality	Host plants	GenBank accession number
PTJap (MH175)	Mt. Iou, Shiretoko Peninsula, Hokkaido, Japan	*Pinus pumila / Betula ermaniii*	AF374629[1]
MP9812	Voeltjiesdord, South Africa	*Pinus* sp.	AF374627[1]
gr13	Granada, Spain	*P. halepensis / Quercus coccifera*	AF228650[2]
pt03	Valencia, Spain	*Q. ilex / Q. coccifera*	AF228648[2]
pt04	Fuentidueña, Spain	*Q. ilex*	AF228649[2]
pt05	Fuentidueña, Spain	*Q. ilex*	AF228651[2]
m14	Moratalla, Murcia, Spain	*P. halepensis*	AF228652[2]
MARX270	Georgia, USA	*P. elliottii*	AF374632[1]
PT301	Georgia, USA	*Pinus* sp.	AF143233[3]
PT90A	Viçosa, Brazil	*Eucalyptus* sp.	AF140547[3]
RS26	Viçosa, Brazil	*Eucalyptus* sp.	AF142991[3]
MU98/5A	Kudardup, Western Australia	*E. globulus*	AF374644[1]
MU98/9	Scott R., Western Australia	*E. globulus*	AF374649[1]
MU98/2	Augusta, Western Australia	*E. marginata*	AF374641[1]
MU98/12	Manjimup, Western Australia	*Eucalyptus* sp.	AF374652[1]

Sources: [1]Martin et al. (2002); [2]Díez et al. (2001); [3]Gomes et al. (2000)

recorded according to the terminology of Kope and Fortin (1990).

Samples of *Pisolithus* spores from the internal part of dried mushrooms were used for extracting DNA. DNA was isolated using the DNeasy Kit (Qiagen, Dusseldorf, Germany). The ITS region of rDNA was amplified with ITS1f (Gardes and Bruns 1993) and ITS4 primers (White et al. 1990). Amplifications were performed on a GeneAmp PCR System 2400 (Perkin-Elmer, Waltham, MA, USA). Samples were run at an initial denaturation for 30 s at 94°C, followed by 40 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 50°C, and extension for 2 min at 72°C.

Samples of PCR products were purified using the QIAquick polymerase chain reaction (PCR) purification kit and sequenced using the BigDyeTM Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. Sequences were generated for both strands using the ITS1f and ITS4 primers for comparison and to assure fidelity.

The sequences were deposited in the GenBank DNA sequence database (Table 1) and compared with other sequences deposited in the GenBank database (Table 2). The sequences were imported into the MEGA program, version 3.1 (Kumar et al. 2004) and aligned using the Clustal W program (Higgins et al. 1994). The resulting multiple alignments were optimized visually. The final alignment of this work is available on request. ITS sequences of *Paxillus involutus* (AF167700) and *Suillus luteus* (L54110) were used as outgroup taxa. Phylogenetic analysis was performed by

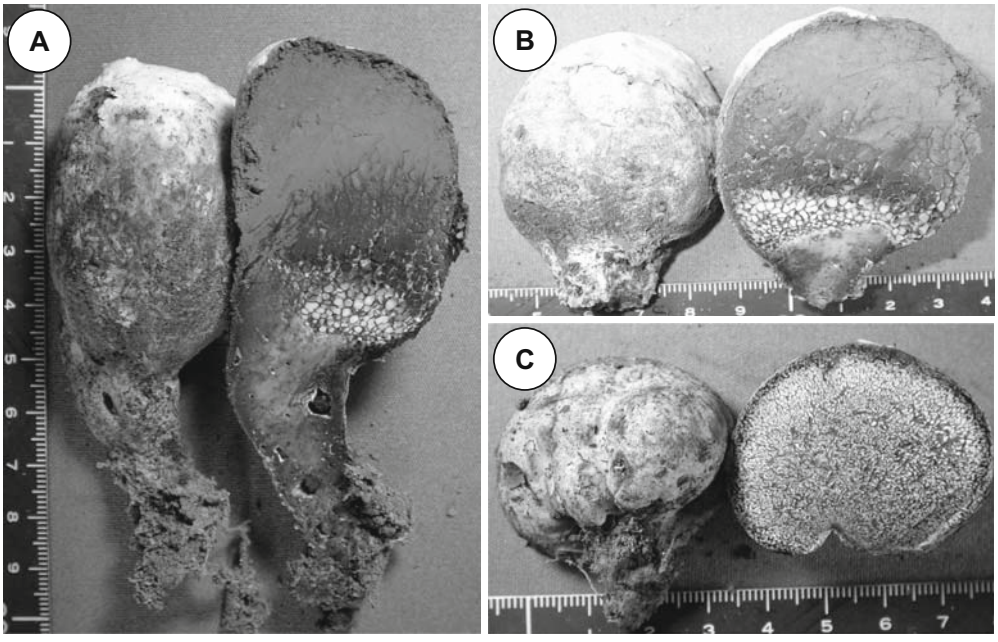

Fig. 1. *Pisolithus* species from Hokkaido Island. **A** Mature basidiome with a long stipe (FORBs05015). **B** Mature basidiome with a short stipe (FORBs05010). **C** Immature basidiome (FORBs05017)

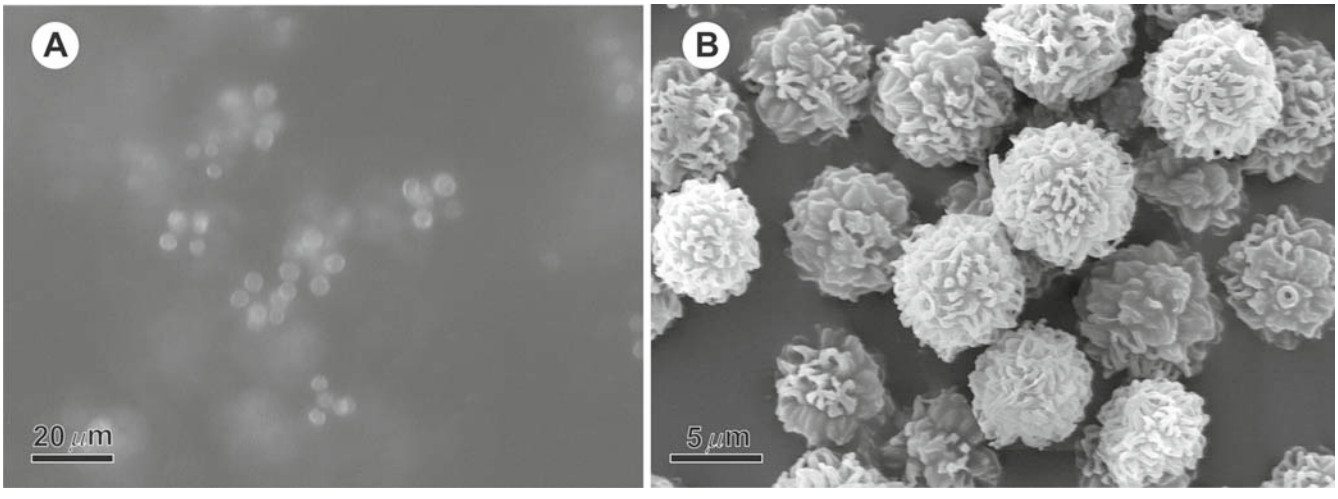

Fig. 2. Micrographs of *Pisolithus* species from Hokkaido Island. **A** Basidia with three to five basidiospores stained with calcofluor, under fluorescence microscopy; by changing focus, eight-spored basidia were observed. **B** Basidiospores with coarse spines, under scanning electron microscopy (SEM)

the neighbor-joining (NJ) method using the p-distance model with MEGA (Kumar et al. 2004). The robustness of each branch was determined using the nonparametric bootstrap test (Felsenstein 1985) with 1000 replicates.

Basidiomes varied considerably in size [22–96 × 21–65 mm (height × width)], shape (globose or pisiform), peridium color (brown to yellow), and peridium features (smooth to rugulose) (Fig. 1A–C). All analyzed specimens presented as many as eight basidiospores per basidium (Fig. 2A), and spore size, including spines, varied from 6.95 to 10.33 μm, with coarse, crowded, and blunted spines, more than 1.5 μm tall (Fig. 2B). Spore morphology showed a diameter similar to *Pisolithus microcarpus* (5.7–7.8 μm, excluding spines) but smaller than *P. tinctorius* (9.6–13.0 μm, excluding spines) (Grgurinovic 1997), *P. albus* (8.5–13.5 μm, including spines), and *P. marmoratus* (9–13 μm, including spines 2.5 μm tall). However, spine ornamentations are very similar to those of *P. tinctorius* (Grgurinovic 1997), *P. aurantioscabrosus* (Watling et al. 1999), and *P. hypogeous* (Thomas et al. 2003), while *P. microcarpus*, *P. albus*, and *P. marmoratus* present spines that are sharp tipped and erect (Grgurinovic 1997; Bougher and Syne 1998).

Basidiome morphology has been used in *Pisolithus* taxonomy (Burgess et al. 1995; Watling et al. 1995; Kanchanaprayudh et al. 2003; Thomas et al. 2003). However, these characteristics were not useful for identifying *Pisolithus* in Hokkaido. We can conclude that spore morphology is more important than basidiome morphology because the latter may be influenced by soil and environmental conditions.

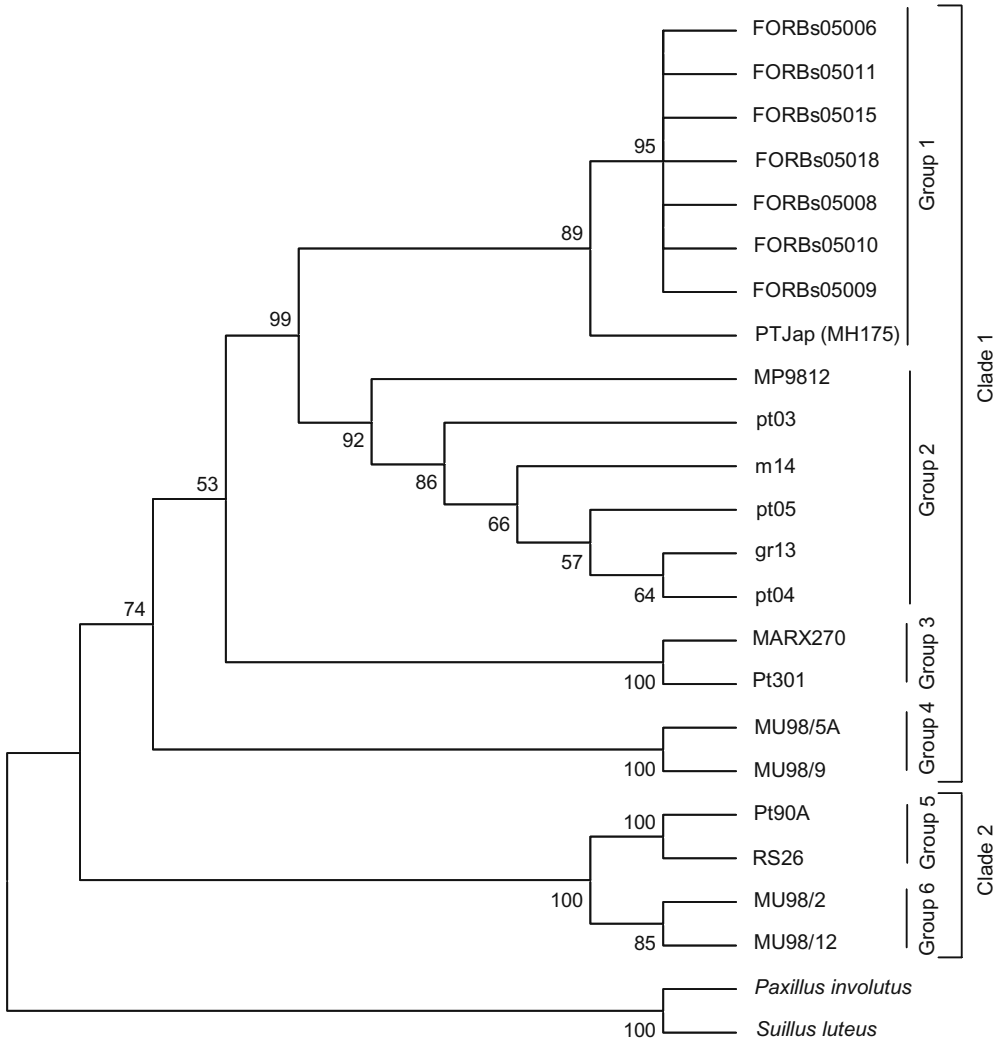

Fig. 3. Neighbor-joining tree based on rDNA internal transcribed spacer (ITS) sequences, showing phylogenetic relationship among *Pisolithus* species. Tree was rooted using *Paxillus involutus* and *Suillus luteus* as outgroups. *Numbers in the branches* indicate the bootstrap support obtained with 1000 replicates (where exceeds 50%)

PCR amplification with the specific primers for the ITS region generated only one band of 700 bp. The topology of the tree separated the specimens into two major clades (Fig. 3). Clade 1 was separated into four groups. Although specimens from Hokkaido had been collected from forests with different tree species, all of them and PTJap [MH175; an isolate from one of the regions mentioned this study (Martin et al. 2002)] were included into group 1 (Fig. 3). These results show low diversity of *Pisolithus* species in Hokkaido, and the specificity of *Pisolithus* for plant hosts is not a rule. This finding is very surprising, as some specificity has been observed according to tree species (Kanchanaprayudh et al. 2003; Pereira et al. 2005). Also, in New South Wales, Australia, Anderson et al. (1998) identified three groups of *Pisolithus* species, whereas in India four groups were found (Singla et al. 2004).

According to Martin et al. (2002), the isolate PTJap (MH175) and MP9812, an isolate from South Africa, and isolates pt03, gr13, pt04, m14, and pt05 from Spain, belong to the species 4. However, our analyses suggest that those isolates from South Africa and Spain, which were included into group 2, are distinct species from Hokkaido's group 1 (Fig. 3).

Group 3, formed by isolates MARX270 and PT301, was identified as *P. tinctorius* (see Fig. 3), from both the United States and isolates from *Pinus* forests (Martin et al. 2002). The group 4 is formed by isolates MU98/5A and MU98/9, both *P. marmoratus* (Martin et al. 2002).

Clade 2, which includes Brazilian and some Australian isolates, all from *Eucalyptus* sp., consisted of two groups. Group 5 included Brazilian isolates (PT90A and RS26) and were identified as *P. microcarpus* (Martin et al. 2002), while group 6 included Australian isolates (MU98/2 and MU98/12), identified as *P. albus* (Martin et al. 2002).

The clustering together of all specimens from *Pinus* is consistent with the view that a degree of host specificity may exist within the *Pisolithus* taxa (Martin et al. 1998). Several previous studies have suggested that *Pisolithus* isolated from basidiomes occurring under *Pinus* spp. are poor colonizers of *Eucalyptus* spp. (Chilvers 1973; Malajczuk et al. 1990; Burgess et al. 1994; Junghans et al. 1998; Pereira et al. 2005). Moreover, in a molecular study of *Pisolithus* in Kenya, three taxa were identified as occurring only in either pine plantations, eucalypt plantations, or native *Afzelia* vegetation (Martin et al. 1998).

Our results related to morphological and molecular characteristics confirm that *Pisolithus* in Hokkaido should be distinguished taxonomically from those found in other regions, and detailed studies need to be done to classify *Pisolithus* in Hokkaido Island, Japan.

Acknowledgments We acknowledge CNPq and Capes (Brazilian institutions) and the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant-in-Aid-16208032) for financial support. We also thank Dr. Tsuneo Igarashi for having collected basidiomes.

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