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Genetic variability in intergenic spacers of ribosomal DNA in *Pisolithus* isolates associated with pine, eucalyptus and *Afzelia* in lowland Kenyan forests

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SUMMARY

Basidiocarps of *Pisolithus* associated with indigenous (*Afzelia quanzensis* Welw.) and introduced (*Pinus caribaea* Mor. and *Eucalyptus camaldulensis* Dehnh.) hosts in the lowland forests of the Coast Province of Kenya are morphologically distinct. Genetic variability among 52 *Pisolithus* basidiocarps, collected beneath the various host plants, was examined based on sequence polymorphism within the internal transcribed spacer (ITS) and intergenic spacer (IGS1) of ribosomal DNA genes. Variability in ITS and IGS1 sequences indicated that the three host-associated morphotypes were genetically different. Consensus trees generated by bootstrap analysis of sequence data of *Pisolithus* isolates from Australia and Kenya are polyphyletic and strongly suggest that the three different morphotypes/genotypes present in Kenya represent separate biological species. In addition, our data indicate that little genetic exchange occurs *in silva* between these species.

Key words: *Afzelia*, ectomycorrhizal fungus, eucalypt, internal transcribed spacer, intergenic spacer, pine, *Pisolithus*, ribosomal DNA.

INTRODUCTION

Although considerable variation exists in terms of basidiocarp, spore and isolated mycelium morphology, taxa within the genus *Pisolithus* are widely regarded as conspecific (Watling *et al.*, 1995). However, distinct *Pisolithus* species, including *P. aurantioscabrosus* Watling *et al.*, *P. kisslingi* E. Fisch, *P. microcarpus* (Cke. & Mass.) G. Cunn, *P. pusillum* Pat. and *P. tinctorius* (Pers.) Coker & Couch [Syn.: *P. arhizus* (Scop. per Pers.) Rauschert] have been described based on distinctive basiocarp and basidiospore morphology (Cunningham, 1942; Watling *et al.*, 1995).

Large variations in enzyme activity (Ho, 1987), protein patterns (Burgess, Malajczuk & Dell, 1995), rDNA sequence (Anderson, Chambers & Cairney, 1998), mycorrhizal ability (Tonkin, Malajczuk &

McComb, 1989; Lamhamedi *et al.*, 1990) and the morphology of basidiocarps and basidiospores (Burgess, Dell & Malajczuk, 1994) of *P. tinctorius* isolates have been reported, suggesting that several biological species can be recognized within the group currently described as *P. tinctorius* (Bronchart, Calogne & Demoulin, 1975; Calonge & Demoulin, 1975; Burgess *et al.*, 1995; Watling *et al.*, 1995). The occurrence of a tetrapolar mating system is seen as further evidence that several biological species exist within the current *P. tinctorius* grouping (Kope & Fortin, 1990). Previous studies have stressed that host and geographical origin of isolates may play significant roles in this variability (Marx, 1980; Malajczuk, Lapeyrie & Garbaye, 1990; Burgess *et al.*, 1994).

P. tinctorius has a world-wide distribution and has been found in a range of habitats including roadside areas, orchard and forest sites, as well as eroded and mine-site soils (Marx, 1977). It is an early colonizer

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Figure 1. Mature basidiocarps of *Pisolithus* associated to (a) *Afzelia quanzensis*, (b) *Eucalyptus camaldulensis*, and (c) *Pinus caribaea* collected in the Coast Province of Kenya demonstrating the large variation in form. Magnification: (a), $\times 0.4$; (b), $\times 0.6$; (c), $\times 0.33$.

and has been demonstrated to be a very successful symbiotic partner of a wide range of gymnosperms and angiosperms (Marx, 1977; Cairney & Chambers

1997). This fungus has potential for practical application in afforestation programmes with growth increases following inoculation reported for pines and eucalypts (Marx, Bryan & Cordell, 1977; Garbaye, Delwaulle & Diangana, 1988; Malajczuk *et al.*, 1994). Growth responses of *Eucalyptus* and *Pinus* spp. to inoculation with *P. tinctorius* are, however, variable and strongly influenced by fungal genotype (Tonkin *et al.*, 1989; Burgess *et al.*, 1994; Cairney & Chambers 1997). A thorough examination of phylogeny with *P. tinctorius* and related biological species will probably reveal a genetic basis for differential host specificity and growth response of inoculated plants.

In the Coast Province of Kenya, woodlands of Miombo-like vegetation comprise mainly legume trees of the Caesalpinioideae (e.g. *Afzelia quanzensis* Welw., *Brachystegia spiciformis* Benth., *Julbernardia magnistipulata* (Harms.) Troupin). Large plantations of *Pinus caribaea* Mor. and *Eucalyptus camaldulensis* Dehnh. have also been established in Kenya since 1900, mainly in upland areas. *Pisolithus* appears to associate with pines, eucalypts, and a native leguminous tree (*A. quanzensis*). The basidiocarps exhibit considerable morphological variation and are separable into three distinct morphotypes (Fig. 1), each associated with only one of the above hosts (Ivory *et al.*, 1996).

The present study aimed to provide comparative information on the genetic variability and phylogenetic relationships of the three distinct *Pisolithus* morphotypes found in Kenya. RFLP and sequencing of the intergenic spacers (ITS, IGS1) of the rDNA gene complex of *Pisolithus* collected beneath *A. quanzensis*, *P. caribaea* and *E. camaldulensis* were undertaken because these regions are known to be variable at the inter- and intraspecific level (Gardes *et al.*, 1991; Henrion, Le Tacon & Martin, 1992; Appel & Gordon, 1996; Kretzer *et al.*, 1996). In addition, ITS region sequences can diagnose phylogenetic relationships at many phylogenetic levels (Hershkovitz & Lewis, 1996). Our sampling identified three distinct RFLP haplotypes, each specific to a single host. Pairwise comparison of ITS and IGS1 nucleotide sequences between these haplotypes indicates that divergence among them is extensive and suggest that different *Pisolithus* species are present in the study area.

MATERIALS AND METHODS

Sites and source of collections

Basidiocarps of *Pisolithus tinctorius* were collected in 1993, 1994 and 1995 in Kenya from all its known habitats in the indigenous ectomycorrhizal forests in the lowland coastal region at Arabuko-Sokoke Forest Reserve, in *P. caribaea* and *E. camaldulensis* plantations at Gede Forest Station and the pine

Figure 2. Map of the study area in the Coast Province of Kenya with the sampling sites (●) where *Pisolithus* basidiocarps were collected. F.S., Forest Station.

mycorrhiza trial sites at Arabuko, Kwale Forest Station (Fig. 2). As far as possible, different areas in each locality and 2–12 basidiocarps in each area were sampled. Samples can thus be ranked from sub-

regional (~ 100 km) to area (~ 100 m) and tree scale (~ 10 m). Collection numbers and sampling localities are given in Table 1. The lowland forests occur on infertile, well-drained, deep, sandy soils which are mostly acidic. However, some soils at Gede Forest Station are neutral or slightly alkaline when derived from coral limestone. Mean annual rainfall is *c.* 1000 mm and temperature remains fairly constant around 28–32 °C.

Basidiocarp morphological characteristics including size, shape, and colour were recorded for selected isolates and a basidiocarp of each representative morphotype was retained for producing mycelial cultures and then preserved by drying for taxonomic studies using procedures described previously (Ivory, 1987). Immediately after collection, samples (*c.* 100 mg) for DNA analysis were excised from the central part of basidiocarp stipes (to avoid contamination by other micro-organisms or spore DNA) and fixed in 1 ml of glycerol/ethanol/water (30/30/40).

DNA extraction

DNA was routinely extracted by the cetyltrimethylammonium bromide (CTAB) protocol according to Henrion, Chevalier & Martin (1994). For recalcitrant

Table 1. *Pisolithus* collections used in this study with their host plant and rDNA spacer haplotypes

Sample no.	Year collected	Locality	Host	Haplotype
720	1993	Gede F.S. ^a , office yard	<i>Afzelia quanzensis</i>	A ^b
721	1993	Gede F.S., eucalypt plantation	<i>E. camaldulensis</i>	E
732	1993	Gede F.S., office yard	<i>A. quanzensis</i>	A
737	1993	Arabuko-Sokoke, mixed <i>Afzelia</i> woodland, site 4	<i>A. quanzensis</i>	A
758	1993	Gede F.S., plot 4 (planted 1982)	<i>A. quanzensis</i>	A
824 (1–12) ^c	1994	Gede F.S., plot 1 (planted 1974)	<i>A. quanzensis</i>	A
826	1994	Gede F.S., plot 3 (planted 1982)	<i>A. quanzensis</i>	A
827 (1–2) ^c	1994	Gede F.S., (12 m from plot 3)	<i>A. quanzensis</i>	A
828 (1–2) ^c	1994	Gede F.S., plot 4 (planted 1982)	<i>A. quanzensis</i>	A
862	1994	Kwale F.S., mycorrhiza trial	<i>P. caribaea</i>	P
943	1994	Gede F.S., office yard	<i>A. quanzensis</i>	A
944	1994	Arabuko-Sokoke, mixed <i>Afzelia</i> woodland, site 4	<i>A. quanzensis</i>	A
5072 (1–5) ^c	1995	Gede F.S., office yard	<i>A. quanzensis</i>	A
5103 (1–5) ^c	1995	Gede F.S., office yard	<i>A. quanzensis</i>	A
5105 (1–3) ^c	1995	Gede F.S., <i>Afzelia</i> plot 2	<i>A. quanzensis</i>	A
5106 (1–4) ^c	1995	Gede F.S., <i>Afzelia</i> plot 3	<i>A. quanzensis</i>	A
5107 (1–3) ^c	1995	Gede F.S., <i>Afzelia</i> plot 4	<i>A. quanzensis</i>	A
5110 (1–10) ^d	1995	Gede F.S., roadside plantation	<i>E. camaldulensis</i>	E
5111 (1–10) ^e	1995	Arabuko, mycorrhiza trial	<i>P. caribaea</i>	P
5148 (1–3) ^e	1995	Kwale F.S., mycorrhiza trial	<i>P. caribaea</i>	P

For easy reference in the text, localities or areas sampled are listed. Haplotypes A, E and P stand for *Afzelia*-, *Eucalyptus*-, and *Pinus*- RFLP types (see Figs 3, 6). The numbers in brackets refer to individual basidiocarps collected in the mentioned area.

^a F.S., Forest Station.

^b Haplotypes: A, *Afzelia*; E, *Eucalyptus*; P, *Pinus*.

^c Sample comprises (×) different basidiocarps collected within 10 m of the host tree.

^d Sample comprises 10 different basidiocarps collected along a roadside bordering the eucalypt plantation.

^e Samples comprises (×) different basidiocarps, each collected beneath a different host tree within the plantation.

samples giving no amplification or low amplification yield, DNA was extracted using a guanidinium buffer followed by DNA purification using GeneClean® glassbeads (Grube *et al.*, 1995; Martin *et al.*, 1997).

PCR amplification and RFLP

The ITS and IGS1 regions of the rDNA were amplified in duplicate by PCR using the primers ITS1/ITS4 and CNL12/5SA (Henrion *et al.*, 1992) respectively, *Taq* DNA polymerase, and the buffer provided by the manufacturer (Appligène-Oncor, Illkirch, France) according to Henrion *et al.* (1994). Amplifications were carried out on a GeneAmp® PCR System 9600 (Perkin Elmer). The thermal cycling parameters were an initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 2 min with a final extension at 72 °C for 10 min. Controls with no DNA were included in every series of amplification to test for the presence of contamination of reagents and reaction buffers. For RFLP analysis, one tenth of the amplified DNA was digested for between 1 h and overnight with 5–10 units of various restriction enzymes (*Alu* I, *Hinf* I, *Mbo* I, *Rsa* I) (Promega, BioLabs) according to the manufacturers' instructions. The amplification products were size-fractionated using a 1.5% regular agarose gel, whereas restriction fragments were separated using 2% composite agarose (1.5% wide-range agarose and 0.5% regular agarose) or 10% polyacrylamide gels (Sambrook, Fritsch & Maniatis 1989). Gels were stained with ethidium bromide, and photographed under u.v. light. Size standards were: ϕ X174 DNA, digested with *Hae* III, and BRL 100-bp ladder.

DNA sequencing and phylogenetic analyses

ITS and IGS1 were amplified from two basidiocarps (collection nos 5105, 5110 and 5111) representative of each of the three haplotypes as described above. Double-stranded products were purified with QuickSpin® columns (Qiagen, Dusseldorf, Germany) and sequenced for both strands. The sequencing reactions were performed using the PRISM® Ready Reaction Dye Primer Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA), *Taq* FS polymerase, and ITS1/ITS4 primers for ITS and CNL12/5SA for IGS1. The sequencing reaction products were analysed using a ABI model 373S DNA sequencer (Perkin Elmer Applied Biosystems) at the Sequencing Facilities of Laval University (Québec City, Québec, Canada). Raw sequence data were edited using Sequencher® (Gene Codes Corporation, Ann Arbor, MI) for Macintosh® and were deposited in the National

Center for Biotechnology Information (NCBI) databases under the following GenBank accession nos: no. 5105 ITS: AF003915, no. 5110 ITS: AF003914, no. 5111 ITS: AF003916, no. 5106 IGS1: AF061182, no. 5110 IGS1: AF061183, and no. 5111 IGS1: AF061184.

Search for sequence homology in the NCBI GenBank DNA database was carried out by Gapped BLAST (NCBI) (Altschul *et al.*, 1997) WWW network services. Multiple sequence alignments were initially constructed with the aid of the MultAlin program (Corpet, 1988) on the WWW ProDom server (INRA, Toulouse, France): for the IGS1 sequences, and for the ITS sequences of the Kenyan *Pisolithus* (present study), *P. tinctorius* 441 collected under *Eucalyptus citriodora* in Brazil (GenBank accession no. U62666) (Carnero Diaz, Tagu & Martin, 1997) and six *Pisolithus* isolates from New South Wales (Australia) [CS01 (GenBank accession no. AF004732), LJ07 (AF004733), R01 (AF004735), W15 (AF004736) W16 (AF004737) and WM01 (AF004734)] (Anderson *et al.*, 1998). Owing to short hypervariable areas in the ITS sequences, sequence alignments were hand edited to improve alignments using the SeqPup (D. Gilbert, National Institutes of Health, Bethesda, MD, USA) sequence editor. The PAUP 3.1.1 program (Swofford, 1993) was used to construct parsimony-based trees. Both heuristic searches, using the nearest-neighbour interchange method, and branch and bound method searches were performed with similar results. All alignment gaps were treated as missing data. Validities of the clades were tested using bootstrap analysis (1000 iterations). Trees were drawn using PAUP 3.1.1 or using TreeView (version 1.3, written by Roderic D. M. Page, <http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>).

RESULTS

Basidiocarp collections

Fifty-two basidiocarps of *Pisolithus* were collected at various times between 1993 and 1995 from areas within the localities listed in Table 1. Other localities where likely hosts grow were also searched for the fungus, without success. The three morphotypes therefore appear to have very restricted distributions in the lowland coast region in Kenya. The basidiocarps collected were all readily separated into the three morphotypes associated with particular host trees in Kenya on the basis of macroscopic characters (Fig. 1). The pine-associated taxon has large basidiocarps with a massive stipe, brown context, yellow external hyphae, large peridioles which extend into the stipe, and a thin dark, fragile exoperidium. The eucalypt-associated taxon has smaller basidiocarps with a less-massive stipe, bright orange/yellow stipe context, brown external hyphae, large peridioles not

Figure 3. The amplified ITS and RFLP patterns of *Pisolithus* isolates associated with (a) *Afzelia* (= haplotype A), (b) *Eucalyptus* (= haplotype E), or (c) *Pinus* (= haplotype P) in the lowland forests of the Coast Province of Kenya. Lane 1, uncut ITS; lane 2, 3, and 4: ITS cut with *Mbo* I, *Alu* I and *Hinf* I, respectively. 50-bp bands lanes (a) 2 and 3 are primers-dimers. Uncut ITS remains in lane 3. M, fragment size markers in bp.

extending into the stipe, and a thin, pale, fragile exoperidium. The *Afzelia*-associated taxon has small basidiocarps with a slender stipe, brown context, brown external hyphae, small peridioles not extending into the stipe, and a thick, fragmenting exoperidium. The latter morphotype will be described elsewhere as a separate species (M. Honrubia, University of Murcia, Spain, pers. comm.).

RFLP and sequence of ITS

The rDNA ITS region of 52 basidiocarps of the three different morphotypes of *Pisolithus* were amplified with the conserved fungal primers ITS1 and ITS4. All basidiocarps of the *Pinus*, *Eucalyptus* and *Afzelia* morphotypes produced a single ITS band of c. 600 bp (Fig. 3). All basidiocarps presented restriction sites within the ITS region for each endonuclease used (Table 2), except for *Rsa* I which was excluded from further analysis. After digestion using endonucleases *Mbo* I, *Alu* I or *Hinf* I, each of the 52 basidiocarps produced one of three distinct rDNA haplotypes, referred to as A, E or P, with two to four restricted fragments per pattern (Fig. 3a, b, c). These groups corresponded to collections sampled under *Afzelia*, eucalypt or pine, respectively. No restricted fragments were shared between these groups. Within each group, RFLP patterns of basidiocarps collected under the same tree (e.g. collection no. 5107 including basidiocarps 1, 2 and 3) or geographically-separated basidiocarps (up to 120 km for pine-associated isolates) (e.g. nos 5111/1-10 and 5148) were identical. RFLP patterns of basidiocarps coming from the same localities but collected in different years (Table 1) showed identical patterns.

Restriction digests of the amplified ITS revealed a variability indicative of striking sequence differences among the three distinct *Pisolithus* groups. Both strands of the amplified fragments, containing ITS1, 5.8S, and ITS2, from the three morphotypes/haplotypes were sequenced (GenBank accession no. AF003914, AF003915 and AF003916) and aligned (Fig. 4) with known *Pisolithus* ITS sequences

Table 2. Size of the restriction fragments (in base pairs) of the rDNA ITS and IGS1 obtained using *Alu* I, *Hinf* I and *Mbo* I

Endonuclease	<i>Afzelia</i> -type	<i>Eucalyptus</i> -type	<i>Pinus</i> -type
ITS			
<i>Alu</i> I	85, 120, 480	85, 500	80, 85, 95, 360
<i>Hinf</i> I	95, 110, 140, 190	110, 210, 240	85, 120, 160, 220
<i>Mbo</i> I	120, 140, 170, 290	120, 170, 280	260, 300
IGS1			
<i>Alu</i> I	160, 225	135, 340	160, 215
<i>Hinf</i> I	115, 280	105, 115, 125, 150	110, 145, 160
<i>Mbo</i> I	225, 255	105, 155, 215	220, 250

	1	ITS1 →				50
CS01	TC	-GAGCCGT	GCAGAGGGGG	GACCTTTACC	CCGGTCCCTT	CGAAGCCCTT
R01	TC	-GAAAGGT	GCGGAGGGGG	GACCTTTGCC	---GGTCCTT	CGAAACCCCTT
W15	TC	-GAAAGGT	GCGGAGGGGG	ACCTTTACC-	---GGTCCTT	CGAAGCCCTT
W16	TC	-GAAACGT	GTGGAGGGGG	ACCTTTACT-	---GGTCCTT	CCAAGCCCTT
LJ07	TC	AGAAA--T	TGGAAGGGGG	GCGACGCGCT	TTGCCCCCTT	TTACCTCTCT
WM01	TC	A-AAAA-T	TGGAAGGGGG	GCGACGCGCT	TCACCCCTT	TTACCTCTCT
5110	TC	-GAAA--T	TGGAAGGGGG	GCGACGCGCT	TCGCCCCCTT	TTACCTCTCT
5105	CC	-GAGGCGT	--GGAGGGGG	ACTCGTCCCT	CGAAGCCCTC	GTATACCTCT
5111	TC	AGAGGGGA	TCGGA-GAGA	TCCGTTCCAC	CGAAGCCCTT	CGTTACTCT
Consensus	tC	gAaa gt	tcggAgGgGg	cc ttccc	c gcCCTt	cga cCtcT
	51					100
CS01	TTT	ACTTTGT	CCACACCTCT	GTGCACCCCA	TTCGCGCGAG	GTTCTTCGGA
R01	TTT	ACTTTGT	CCACACCTCT	GTGCACCCCG	TTCGCGCGAG	GTTCTTCGGA
W15	TTT	ACTTTGT	CCACACCTCT	GTGCACCTG	TTCGCGTGAG	GTTCTTCGG
W16	TTT	ACTTTGT	CCACACCTCT	GTGCACCTG	TTCGCGTGAG	GTTCTTCGG
LJ07	----	C-----	--ACACC--	CGTGTGCACC	CATTTCGCAAG	GTCTTCGGG
WM01	----	C-----	--ACACC--	CGTGTGCACC	CATTTCGCAAG	GTCTTCGGG
5110	----	C-----	--ACACC--	CGAGTGCACC	CATTTCGCAAG	GTCTTCGGG
5105	TTG	AC-----	--ACACC--	CGTGTGCACC	CGTTCGCGGG	GTCTTCGGG
5111	TTG	AC-----	--ACACCTCA	CGTGCACCCC	ATTTCGCAAGG	GCCCTTCGGG
Consensus	tt aC		ACACCtc	cgtg Cccc	tt cgcgaG	GtcCttcgGg
	101					150
CS01	ACC	-TGCGTG	ATACC-ATCT	CGAACTCGTA	TGTCTACAGA	ATGTAACCTA
R01	ACC	CTGTGTG	ATGCTTATCT	CGAACTCGTA	TGTCTACAGA	ATGTGACCTA
W15	AAC	CTGCGTG	ATACT-ATCT	CGAACTCGTA	TGTCTACAGA	ATGTAACCTA
W16	AAC	CTGCGTG	ATACT-ATCT	CGAACTCGTA	TGTCTACAGA	ATGTAACCTA
LJ07	A	-CCTGCGCA	TCCTATCGTG	GAACCTCCGCA	TGTCTACAGA	ATGTCATGAA
WM01	A	-CCTGCGCA	TCCTATCGTG	GAACCTCCGCA	TGTCTACAGA	ATGTCATGAA
5110	A	-CCTGCGCA	TCCTATCGAG	GAACCTCCGCA	TGTCTACAGA	ATGTCATGAA
5105	A	-CC-----CT	GCGTCTTTTG	TAACCTCTGCA	TGTCTACAGA	ATGTTGGGTT
5111	A	-CCGCGCG	TTTCATCTCC	GACT--CGCA	TGTCTACAGA	ATGTCGATAG
Consensus	A	Cctgcgcg	t c t tc	aa cGcA	TGTCTACAGA	ATGT a ta
	151		5.8S →			200
CS01	GTGT	--TGGA	AACGAAA--A	TACAACCTTC	AGCAACGGAT	CTCTTGGCTC
R01	GTGT	GTTGGA	AATGGAA--A	TACAACCTTC	AGCAACGGAT	CTCTTGGCTC
W15	GCGT	--TGGA	AAAGAAA--A	TACAACCTTC	AGCAACGGAT	CTCTTGGCTC
W16	GCGT	--TGGA	AAAGAAA--A	TACAACCTTC	AGCAACGGAT	CTCTTGGCTC
LJ07	-ATTTG	----	AAACGAGTAA	TACAACCTTC	AGCAACGGAT	CTCTTGGCTC
WM01	-ATTTG	----	AAACGAGTAA	TACAACCTTC	AGCAACGGAT	CTCTTGGCTC
5110	-ATT	-G-----	AAACGAGTAA	TACAACCTTC	AGCAACGGAT	CTCTTGGCTC
5105	-GGCGT	CCGA	AGACACACCA	TACAACCTTC	AGCAACGGAT	CTCTTGGCTC
5111	CATTTG	GATAT	ATATATAAAA	TACAACCTTAC	AGCAACGGAT	CTCTTGGCTC
Consensus	gt gga		Aaa aaa A	TACAACCTTc	AGCAACGGAT	CTCTTGGCTC
	201					250
CS01	TCGC	ATCGAT	GAAGGACGCA	GCGAATCGCG	GTAAGTAATG	TGAATTGCAG
R01	TCGC	ATCGAT	GAAGGACGCA	GCGAATCGCG	ATAAGTAATG	TGAATTGCAG
W15	TCGC	ATCGAT	GAAGGACGCA	GCGAATCGCG	ATAAGTAATG	TGAATTGCAG
W16	TCGC	ATCGAT	GAAGGACGCA	GCGAATCGCG	ATAAGTAATG	TGAATTGCAG
LJ07	TCGC	ATCGAT	GAAGGACGCA	GCGAATTGCG	ATAAGTAATG	TGAATTGCAG
WM01	TCGC	ATCGAT	GAAGGACGCA	GCGAATTGCG	ATAAGTAATG	TGAATTGCAG
5110	TCGC	ATCGAT	GAAGGACGCA	GCGAATTGCG	ATAAGTAATG	TGAATTGCAG
5105	TCGC	ATCGAT	GAAGAACGCA	GCGAATCGCG	ATAAGTAATG	TGAATTGCAG
5111	TCGC	ATCGAT	GAAGAACGCA	GCGAATCGCG	ATAAGTAATG	TGAATTGCAG
Consensus	TCGC	ATCGAT	GAAGgACGCA	GCGAATcGCG	aTAAGTAATG	TGAATTGCAG
	251					300
CS01	ATTTT	CCGTG	AATCATCGAA	TCTTTGAACG	CACCTTGCGC	TCCTTGGTAT
R01	ATTTT	CCGTG	AATCATCGAA	TCTTTGAACG	CACCTTGCGC	TCCTTGGTAT
W15	ATTTT	CCGTG	AATCATCGAA	TCTTTGAACG	CACCTTGCGC	TCCTTGGTAT
W16	ATTTT	CCGTG	AATCATCGAA	TCTTTGAACG	CACCTTGCGC	TCCTTGGTAT
LJ07	ATTTT	CCGTG	AATCATCGAA	TCTTTGAACG	CACCTTGCGC	TCCTTGGTAT
WM01	ATTTT	CCGTG	AATCATCGAA	TCTTTGAACG	CACCTTGCGC	TCCTTGGTAT
5110	ATTTT	CCGTG	AATCATCGAA	TCTTTGAACG	CACCTTGCGC	TCCTTGGTAT
5105	ATTTT	CCGTG	AATCATCGAA	TCTTTGAACG	CACCTTGCGC	TCCTTGGTAT
5111	ATTTT	CCGTG	AATCATCGAA	TCTTTGAACG	CACCTTGCGC	TCCTTGGTAT
Consensus	ATTTT	CCGTG	AATCATCGAA	TCTTTGAACG	CACCTTGCGC	TCcTtGGTAT
	301			ITS2 →		350
CS01	TCCG	AGGAGC	ATGCCTGTTT	GAGTGTCAAT	GAAAATCTCA	AGCCGAGCTG
R01	TCCG	AGGAGC	ATGCCTGTTT	GAGTGTCAAT	GAAAATCTCA	AGCCGAGCTG
W15	TCCG	AGGAGC	ATGCCTGTTT	GAGTGTCAAT	GAAAATCTCA	AGCCAGCTG
W16	TCCG	AGGAGC	ATGCCTGTTT	GAGTGTCAAT	GAAAATCTCA	AGCCAGCTG
LJ07	TCCG	AGGAGC	ATGCCTGTTT	GAGTGTCTCG	GAAAATCTCGA	ACCGAGCCTC
WM01	TCCG	AGGAGC	ATGCCTGTTT	GAGTGTCTCG	GAAAATCTCGA	ACCGAGCCTC
5110	TCCG	AGGAGC	ATGCCTGTTT	GAGTGTCTCG	GAAAATCTCGA	ACCGAGCCTC
5105	TCCG	AGGAGC	ATGCCTGTTT	GAGTGTCTCG	GAAAATCTCGA	ACCGAGGCTT
5111	TCCG	AGGAGC	ATGCCTGTTT	GAGTGTCTCG	GAAAATCTCAA	ACCAAGCTTT
Consensus	TCCG	AGgAGC	ATGCCTGTTT	GAGTGTcATc	GAAAtctcA	AcC aggcT

Figure 4. For legend see opposite.

Figure 4. Multiple sequence alignment of the ITS region of basidiocarps of *Pisolithus* associated to *Eucalyptus camaldulensis*, *Pinus caribaea*, or *Afzelia quanzensis* in Kenya, together with *Pisolithus* ITS sequences available in the NCBI database, no. 5105 (GenBank accession no. AF003915), no. 5110 (accession no. AF003914), no. 5111 (accession no. AF003916), basidiocarps associated with *A. quanzensis*, *E. camaldulensis*, and *P. caribaea*, respectively; 441, vegetative mycelium of *P. tinctorius* 441 associated with *Eucalyptus citriodora* in Brazil; CS01 (GenBank accession no. AF004732), LJ07 (AF004733), R01 (AF004735), W15 (AF004736), W16 (AF004737) and WM01 (AF004734), isolates of *Pisolithus* collected in New South Wales (Australia) (Anderson *et al.*, 1998; with kind permission). –, gap resulting from insertion/deletion.

(Anderson *et al.*, 1998, Carnero-Diaz *et al.*, 1997). The *Pisolithus* ITS sequences were all clearly related to each other. The actual length of the corresponding

fragment was 589–627 bp, also containing a part of the 3'-end of the 18S rDNA and the 5'-end of the 28S rDNA. Typically, a high degree of similarity

Figure 5. Radial parsimony tree of ITS sequences of *Pisolithus* isolates. The tree was constructed from the sequence alignment data set shown in Figure 4. The illustration is one of three equally parsimonious trees of length 329 which were found using PAUP 3.1.1 to perform a heuristic search with simple addition. Numerical values, shown along the stem of each supported clade, are those deriving from 1000 replicates of heuristic parsimony bootstrap analysis. The scale bar on the bottom right indicates the length of 10 steps in the tree. Outlines and shading indicate particular groupings of ITS referred to in the text.

was present within the 194 bp-5.8S rDNA region in all the *Pisolithus* collections (< 95 %) (Fig. 4) and the *Pisolithus* 5.8S showed a high similarity with basidiomycetous ITS sequences deposited in the NCBI DNA database (data not shown).

The ITS1 and ITS2 sequences of the three distinct Kenyan *Pisolithus* morphotypes differed by several insertions/deletions and base substitutions, but conservation patterns with phylogenetic diagnostic value (Hershkovitz & Zimmer, 1996), could be easily identified. The multiple sequence alignment (Fig. 4) indicated that isolates no. 5110 (eucalypt-associated morphotype), and LJ07 and WM01 from NSW (Australia) had 98 % sequence homology with each other, but only 85 % homology with the other isolates. Their sequence are characterized by the presence of a CT-rich insertion in the 3'-end of the ITS2 region. Similarly, isolates CS01,

Figure 6. The amplified IGS1 and RFLP patterns of *Pisolithus* isolates associated with (a) *Afzelia* (= haplotype A), (b) *Eucalyptus* (= haplotype E), or (c) *Pinus* (= haplotype P) in the lowland forests of the Coast Province of Kenya. Lane 1, uncut IGS1; lane 2, 3, and 4: ITS cut with *Mbo* I, *Alu* I and *Hinf* I, respectively. M, fragment size markers in bp. Partial digestion products are visible above 300 bp in (c) 4.

R01, W15, W16 from NSW and *P. tinctorius* 441 from Brazil showed a high degree of sequence homology, being 95–97 % homologous. The ITS sequences of *Afzelia*- (no. 5105) and Pine- (no. 5111) associated isolates were divergent with each other and strikingly divergent from other investigated *Pisolithus* isolates.

Equally weighted, unordered parsimony analysis of the data set of all ITS sequences resulted in three equally parsimonious trees (differences between the trees were due to branch swapping of haplotypes). The strict consensus radial tree (Fig. 5), deriving from 1000 iterations of heuristic parsimony bootstrap analysis, shows the relationships among the 10 ITS sequences. It supports the clear differentiation of the three major groups revealed by sequence pairwise comparison. The various *Pisolithus* groups were supported by very strong branches (79–100 % of 1000 bootstrap iterations).

Figure 7. Multiple sequence alignment of IGS1 of basidiocarps of *Pisolithus* associated to *Eucalyptus camaldulensis* (5110; accession no. AF0061183), *Pinus caribaea* (5111, accession no. AF0061184) and *Afzelia quanzensis* (5106, GenBank accession no. AF0061182). –, gap resulting from insertion/deletion.

RFLP and sequence of IGS

The basidiocarps were further examined by PCR/RFLP of the rDNA IGS, a rDNA region known as highly variable in fungi (Henrion *et al.*, 1992, 1994; Apel & Gordon, 1996; Selosse *et al.*, 1996). All basidiocarps of the *Pinus*-, *Eucalyptus*- and *Afzelia*-morphotypes produced a single IGS band of 450 bp (Fig. 6). Heteroduplex formation resulting from rDNA heterozygosity (Selosse *et al.*, 1996) was not observed in the amplification of *Pisolithus* rDNA IGS. Three distinct restriction patterns, with two to four restricted fragments per pattern, were detected using *Alu* I, *Hinf* I, and *Mbo* I (Fig. 6; Table 2). The clustering of basidiocarps was identical to the one

achieved by PCR/RFLP of the rDNA ITS. In both analyses, the 52 basidiocarps formed three clusters which correspond to the three morphotypes of *Pisolithus*. By contrast, RFLPs of IGS1 of isolates, collected over a wide geographical scale (100–120 km), were identical within each morphotype/haplotype.

Both strands of the amplified IGS1 were sequenced (GenBank accession no. AF001182, AF0061183 and AF0061184) and aligned (Fig. 7). These sequences were clearly related to each other. The actual length of the corresponding fragment was 446–449 bp, also containing a part of the 3'-end of the 25S rDNA and the 5'-end of the 5S rDNA. The aligned sequences indicated that isolates nos 5110

(eucalypt-associated), and 5111 (eucalypt-associated) and 5105 (*Afzelia*-associated) had 21 % sequence divergence with each other.

DISCUSSION

Because collections within the *Pisolithus* taxon exhibit considerable variability in basidiocarp and basidiospore morphology, it is widely thought that *Pisolithus* is a complex of evolutionarily independent biological species (Bronchart *et al.*, 1975; Kope & Fortin 1990; Burgess *et al.*, 1995; Watling *et al.*, 1995). Various methods based on the PCR have been proposed to characterize ectomycorrhizal fungi (Gardes *et al.*, 1991; Henrion *et al.*, 1992) and to examine phylogenetic relationships in the various taxa (Bruns, White & Taylor, 1991). Direct sequencing of genes coding for nuclear and mitochondrial rRNA genes and intergenic spacers amplified by PCR (Bruns *et al.*, 1989; Bruns & Szaro, 1992; Kretzer *et al.*, 1996; Selosse *et al.*, 1996) and RFLP analysis of these PCR-amplified sequences (Gardes *et al.*, 1991; Henrion *et al.*, 1992, 1994) have been used to characterize ectomycorrhizal fungi at the intraspecific and interspecific phylogenetic levels. In the present investigation, we have used this approach to investigate genetic and phylogenetic relationships between the morphotypes of *Pisolithus* found in plantations and woodlands of the Coast Province of Kenya.

In the area studied, host association appeared to be a key feature in the clustering of *Pisolithus* isolates since all of the basidiocarps, isolated under a specific host plant (*A. quanzensis*, *P. caribaea*, or *E. camaldulensis*), but ranking from subregional to local area scale, produced identical rDNA patterns and sequences. They showed no obvious correlation with geographical origin. This lack of genetic variability within highly polymorphic DNA regions, such as the IGS1, has been confirmed by using micro-satellite-primed PCR (data not shown) and suggests that little breeding occurs between *Pisolithus* associated with pine and eucalypt plantations and the indigenous morphotype associated with a native tree (*Afzelia*) in forest reserves even when basidiocarps of different morphology/haplotype occur in close proximity (~10–100 m). The variation in basidiocarp morphology and the degree of host specificity (Ivory *et al.*, 1996), together with the present genetic analysis suggest that eucalypt-compatible and pine-compatible strains of *Pisolithus* have been introduced to Kenya and now persist in a few pine and eucalypt plantations.

The sequence analysis of the rDNA ITS and IGS1 have confirmed that the *Pisolithus* types in the study area are genetically highly divergent. The three groups formed by analysis of sequence polymorphism of rDNA spacers correlated very well with basidiocarp morphotypes and host range (Ivory

et al., 1996). The estimated pairwise distance among *Pisolithus* ITS sequences (up to 20 %) is extensive when compared to that found in some other fungal species (1–10 %) (Gardes *et al.*, 1991; Hibbett *et al.*, 1995; Bruns *et al.*, 1991; Hseu *et al.*, 1996). Comparable levels of sequence divergence between distinct species and species groups within the basidiomycetes (e.g. *Lentinus*; Hibbett *et al.* (1995)) and ascomycetes (e.g. *Cenococcum geophilum*; Lobuglio, Rogers & Wang (1991)) have been interpreted as indicating significant evolutionary divergence within these species.

Similarly, the high degree of rDNA ITS divergence among *Pisolithus* from Kenya (present study) and Australia (Anderson *et al.*, 1998) suggests that we are dealing with a complex of evolutionarily independent biological species. Although highly variable at the interspecific level, the ITS region contains conserved patterns and could be used to diagnose phylogenetic relationships (Hershkovitz & Lewis, 1996; Hershkovitz & Zimmer, 1996). A parsimony analysis of the data set of all ITS sequences of *Pisolithus* available in DNA databases was therefore performed. The phylogenetic analysis clearly separated a group of isolates including the eucalypt-associated no. 5110 (Kenya), LJ07 and WM01 (Australia) from the cluster containing other Australian isolates (W16, W15, CS01, R01) and no. 441 from Brazil. These findings confirm and extend a study, based on RAPD and ITS sequence analyses, of NSW *Pisolithus* (Anderson *et al.*, 1998). Separation of the eucalypt-associated isolates on two strongly supported branches (100 % bootstrap values from 1000 iterations) indicates that these two groups might represent two different species. Basidiocarp and basidiospore morphology of the Australian isolates CS01, R01, W15 and W16 conformed to Cunningham's description (Cunningham, 1942) of *P. tinctorius* (Anderson *et al.*, 1998). This suggests that the group, including isolates CS01, R01, W15, W16 and no. 441, probably represents *P. tinctorius*, whereas the other cluster (no. 5110, LJ07 and WM01) represents another, probably undescribed *Pisolithus* species. Isolates of *Pisolithus* associated to *Afzelia* (no. 5105) or pine (no. 5111) were related, but widely separated from other clades. They might also represent additional species, but more isolates need to be included in future studies to fully resolve the genetic heterogeneity within these two *Pisolithus* types. Interestingly, ITS sequence of *Pinus*-associated basidiocarps (no. 5111) found in Kenya was identical to the sequence of isolates of *Pisolithus* collected beneath pine species on different continents suggesting a common origin for this morphotype (Dell *et al.*, unpublished).

It appears that distinct evolutionary lineages occur amongst the *P. tinctorius* populations world-wide (Bronchart *et al.*, 1975; Calogne & Demoulin, 1975; Burgess *et al.*, 1995; Watling *et al.*, 1995; Anderson

et al., 1998; present study). An analysis of rDNA ITS sequence variation amongst these world-wide populations would be useful for exploring the phylogenetic relationships existing between these lineages.

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