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The early-stage ectomycorrhizal Thelephoroid fungal sp. is competitive and effective on *Afzelia africana* Sm. in nursery conditions in Senegal

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Abstract This study was conducted to evaluate the competitiveness and effectiveness of Thelephoroid fungal sp. ORS.XM002 against native ectomycorrhizal fungal species colonizing potted *Afzelia africana* seedlings during 3 months of growth in different forest soils collected from under mature trees. Using morphotyping and restriction fragment length polymorphism (RFLP) analysis of the nuclear rDNA internal transcribed spacer (ITS), we were able to distinguish the introduced Thelephoroid fungal sp. ORS.XM002 among native ectomycorrhizal fungal species that form ectomycorrhizae in *A. africana* seedlings. The morphotype (MT) of the introduced fungus showed some color variation, with a shift from light- to dark-brown observed from younger to older mycorrhizal tips. We were able to differentiate the ITS type xm002 of the introduced fungus from the 14 ITS-RFLP types characterizing the 9 native MT that occurred in forest soils. The frequency of ITS type xm002 ranged from 40% to 49% depending on the forest soil used, and was always higher than those of ITS types from native dark-brown MT that occurred in inoculated seedlings 3 months after inoculation. We considered Thelephoroid fungal sp. ORS.XM002 to be responsible for stimulation of mycorrhizal colonization of inoculated *A. africana* seedlings when compared with control seedlings in forest soils. This fungus appeared to be more effective in increasing the root dry weight of *A. africana* seedlings. To identify the unknown introduced fungal species and native MT, we sequenced the ML5/ML6 region of the mitochondrial large subunit rRNA. Sequence analysis showed that these fungi belong to three ML5/ML6 groups closely related to the Cortinarioid, Thelephoroid, and Sclerodermataceous taxa. The molecular evidence for the persistence of Thelephoroid fungal sp. ORS.XM002 despite competition from native fungi argues in favor of using this fungus with *A. africana* in nursery soil conditions in Senegal.

Keywords Ectomycorrhizas · Thelephoroid fungal sp. · Morphotyping · ITS-RFLP typing · Sequencing

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Introduction

Ectomycorrhizal symbiosis is a mutualistic plant-fungus association, well known among European, North American, South American, Asian and Australian forest species (Smith and Read 1997). Many trees species, such as those in the *Pinaceae*, *Fagaceae*, *Caesalpinioideae*, *Betulaceae*, *Dipterocarpaceae*, *Myrtaceae, Casuarinaceae* and *Acacieae*, are associated with a high diversity of ectomycorrhizal fungi, which have been estimated at a global level of 5,000–6,000 species (Molina et al. 1992). These ectomycorrhizal fungi play a major role in the biology and ecology of forest trees, affecting growth, water and nutrient absorption, and protection against pathogens (Smith and Read 1997). In contrast, data are limited in African tropical forests, particularly in West Africa where native ectomycorrhizal trees are distributed

in dry woodlands and gallery forests (Thoen and Bâ 1989; Thoen and Ducousso 1989; Sanon et al. 1997). These native ectomycorrhizal tree species include some members of cesalpinoid legumes, *Euphorbiaceae* and *Dipterocarpaceae*. Among ectomycorrhizal African trees, *Afzelia africana*, a cesalpinoid legume, is considered one of the most important timber species for reforestation in West Africa (Thies 1995). Field observations indicate that mature trees of *A. africana* are associated with a great diversity of ectomycorrhizal fungi species in dry woodlands and gallery forests of Senegal and Burkina Faso (Thoen and Bâ 1989; Sanon et al. 1997). Only some early-stage ectomycorrhizal fungi, isolated from sporocarps, ectomycorrhizas and sclerotia, are able to form ectomycorrhizas on *A. africana* seedlings (Bâ and Thoen 1990; Sanon et al. 1997).

These fungi also colonize the root system, following a characteristic time sequence on lateral roots of *A. africana* Sm. seedlings grown in cores of soil taken beneath mature trees in Bayottes forest in southern Senegal (Bâ et al. 1991). Of the morphotypes (MT) observed in the natural soil tested over a 6-month period, some are present on the roots soon after germination of the seed and before the first leaf is formed, while other MT appear later. All MT that appear in this experiment are considered as early-stage fungi according to Deacon et al. (1983). However, they colonize in sequence, which permits those fungi that occur first in the colonization process to be distinguished from those observed later. One dark-brown MT, identified as Thelephoroid fungal sp. ORS.XM002 by determining the sequence of the ML5/ML6 region of the mitochondrial large subunit rRNA (mt-LrRNA) gene (see below), is recorded as early as 3 weeks and dominates on *A. africana* seedlings, in terms of mycorrhizal colonization rate, 6 months after sowing (Bâ et al. 1991). One explanation for this may be that Thelephoroid fungal sp. ORS.XM002 possesses vegetative propagules allowing immediate opportunistic root colonization and long-term survival in the soil (Bâ et al. 1991). This fungus improves growth and nutrient concentrations of different provenances of *Afzelia* spp. seedlings in sterilized soils or in ectomycorrhizal propagule-free soils (Bâ 1990; Bâ et al. 1999, 2002). Therefore, Thelephoroid fungal sp. ORS.XM002 is a potential candidate for artificial inoculation of *A. africana* seedlings in forest nurseries and plantations. However, the essential criterion of selection is the competitiveness of fungal strains as defined by Garbaye (1991). This includes the ability of the selected fungal strain to compete and to enhance host performance to a greater degree than the naturally occurring ectomycorrhizal fungi in forest nurseries and plantations. It is necessary in this respect to ascertain whether the introduced fungus is able to disseminate and persist in natural ectomycorrhizal communities of *A. africana*.

Although identification of some MT has been achieved (e.g., MT of *Cenococcum geophilum*), a considerable number of fungal symbionts in ectomycorrhizas described on the basis of morphological and structural characters remain unidentified (Agerer 1987–1996; Thoen and Bâ 1989; Bâ et al. 1991; Dahlberg and Stenlid 1995). Detection and identification of the introduced strains need to be evaluated in natural ectomycorrhizal communities (Gardes et al. 1991; Henrion et al. 1994; Selosse et al. 1998, 1999). In this respect, molecular tools offer a more sensitive means than morphotyping for identifying ectomycorrhizal fungi from a root tip (Gardes et al. 1991; Henrion et al. 1994; Erland et al. 1999; Jonsson et al. 1999a). Typing using PCR-amplification and restriction fragment length polymorphism (RFLP) analysis of the nuclear rDNA internal transcribed spacer (ITS) region of the mycobiont is among the most useful approaches to analysis of molecular differences in intraspecific variation in a genus, or among fungal species in single mycorrhizas (Gardes et al. 1991; Jonsson et al. 1999b; Erland et al. 1999). Such analysis has allowed comprehensive surveys of ectomycorrhizal communities and also discrimination of introduced fungi within communities (Gardes and Bruns 1993, 1996; Henrion et al. 1994; Guidot et al. 2001). However, although PCR-RFLP methods permit identification of mycobionts in MT either by matching with mycorrhizal RFLP databases, or by corresponding fruitbody RFLP patterns, unmatched MTs remain unknown (Gardes and Bruns 1996; Glen et al. 2001a, 2001b; Guidot et al. 2001). To identify unknown MT, the ML5-ML6 region of the mt-LrRNA gene is sequenced and compared to known sequences in a database available for ectomycorrhizal fungi (Bruns et al. 1998; Horton and Bruns 2001).

A research program is underway in Senegal to select ectomycorrhizal fungi to improve the growth of *A. africana* seedlings in forest nurseries (Bâ et al. 1999). Forest soils from native stands of *A. africana* are often used as nursery substrates for seedlings by the Forest Services in Senegal. This raises the question of how the selected Thelephoroid fungal sp. ORS.XM002 colonizes the root system of *A. africana* seedlings and competes with the indigenous mycorrhizal populations in nursery soils. To address this question, the introduced Thelephoroid fungal sp. ORS.XM002 needs to be evaluated in nursery conditions where natural propagules exist and before its use for large-scale inoculation programs. The present study aimed at evaluating the competitiveness and effectiveness of the introduced Thelephoroid fungal sp. ORS.XM002 in three sampled forest soils where it was in competition with native ectomycorrhizal fungi in nursery conditions. A combination of morphological and molecular (PCR-RFLP and sequencing) analysis was used to distinguish and identify the introduced Thelephoroid fungal sp. ORS.XM002 among fungal species that spontaneously form ectomycorrhizae in *A. africana* seedlings.

Materials and methods

Soil preparation

Three soils were collected from native stands of *A. africana* in the Kolda (12°44′N, 14°32′W), Bignona (12°50′N, 16°8′W) and

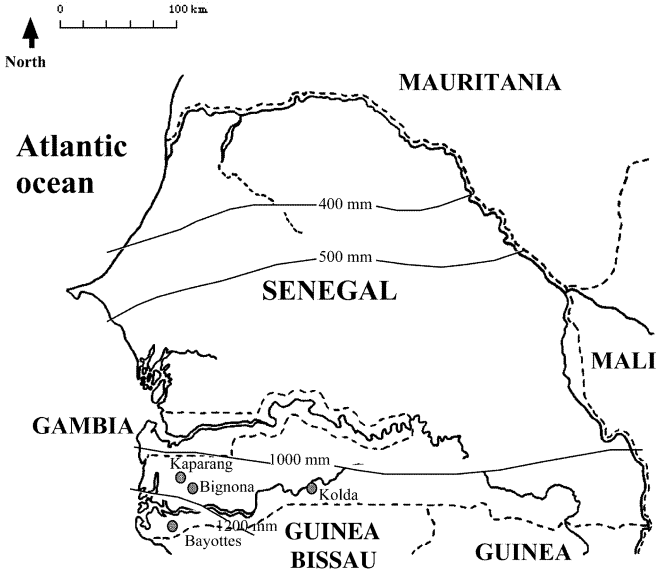

Fig. 1 Location of the sampling sites in a forest ecosystem (●). Annual mean rainfall (—mm—) is indicated

Table 1 Some physical and chemical properties of the forest soils used in this study

	Bignona	Kaparang	Kolda
Clay (%)	8.7	6.1	7.7
Silt (%)	10.5	14.9	13.7
Sand (%)	74.8	76.6	76.5
Total C (%)	0.5	0.5	0.5
Total N (%)	0.05	0.05	0.04
C/N	10	10	12.5
K (meq100 g^{-1} soil)	0.3	0.1	0.1
Ca (meq100 g^{-1} soil)	2.6	2.7	2.6
Mg (meq100 g^{-1} soil)	1.2	1.4	0.7
P-Olsen (ppm)	4.2	8.8	13.6
pH (H_2O)	6.2	6.2	7.0
pH (KCl)	5.3	5.4	6.0

Kaparang (12°49′N, 16°19′W) forests in southern Senegal (Fig. 1). Some chemical and physical properties of these soils are indicated in Table 1. They were NPK-deficient soils according to regional standards for growing plants. These forest soils were collected in February 2001 from under three mature trees in each study site. Ten soil cores (30-cm in depth; 10-cm in diameter) were taken around each mature tree, pooled per site and stored at room temperature (20–25°C). All soils were crushed and passed through a 2-mm sieve before use.

Fungus and fungal inoculum

Culture of Thelephoroid fungal sp. ORS.XM002 was isolated from dark-brown ectomycorrhizas collected on *A. africana* seedlings grown in the Bayottes forest (12°29′N, 16°17′W) soil collected in southern Senegal (Fig. 1) (Bâ and Thoen 1990). Cultures are maintained in the culture collections of IRD-Dakar in Senegal, and LSTM-Montpellier and INRA-Champenoux in France. Fungal cultures were kept in the dark at 30°C on MMN medium (Marx 1969). Thelephoroid fungal sp. was cultured in 100 ml liquid MMN medium in previously autoclaved (120°C, 20 min) 250 ml Erlenmeyer flasks. Ten plugs (0.5 cm diameter) of Thelephoroid fungal sp. ORS.XM002 were inoculated per Erlenmeyer flask. The liquid medium was colonized by the fungus after 1 month of growth at 30°C without shaking. The mycelium was gently washed twice and blended in 100 ml sterile distilled water for approximately 30 s using an Ultraturax blender. Inoculum liquid was kept at –4°C for 2 weeks before use.

Plant culture and experimental design

Seeds of one provenance of *A. africana* from Kolda were obtained from the Centre National des Recherches Forestières in Senegal. They were scarified in 95% sulfuric acid for 2 h, rinsed with tap water and transferred into plastic bags (2 l) containing sieved forest soil. Two seeds were sown per plastic bag. Five days after sowing, pre-germinated seeds were inoculated with 3 ml mycelial suspension. The uninoculated controls received 3 ml autoclaved mycelial suspension of fungal cultures after sowing. After emergence, seedlings were thinned to one plant per plastic bag. Plants were watered daily with tap water without nutrient solution and grown in a greenhouse between March and May 2001, at 25–35°C with a day-length of about 12 h. The experiment in each forest soil was set up as a 1×2 factorial design consisting of one provenance of *A. africana* and one ectomycorrhizal Thelephoroid fungal sp. ORS.XM002 plus a non-inoculated control. In each forest soil, experiments were arranged in a randomized design with ten replicates per treatment combination.

Sampling and seedling measurement

Root tips with mycorrhiza of *A. africana* seedlings in forest soils were classified into MT based on distinctive macroscopic and microscopic features following several criteria: mantle color and texture; emanating hyphae; presence or absence of clamp connections at the septa of hyphae, mycelial strands and sclerotia linked to ectomycorrhizas. Mycorrhizal colonization was confirmed by microscopic (×400) examination of root tips to determine the presence of a mantle and a Hartig net. Mycorrhizal colonization of each MT was quantified monthly over a period of 3 months in each forest soil following sowing. For this purpose, ten plants were fully destroyed each month and mycorrhizal colonization was estimated on ten randomly sampled lateral roots along the tap-root of each plant. These lateral roots were washed gently and dispersed in a dish of water under a stereobinocular microscope at ×160 magnification. Numbers of root tips of different MT and noncolonized roots were counted for each lateral root to determine the percentage of mycorrhizal colonization (number of mycorrhizal roots/total number of roots ×100) of each MT. Mycorrhizal colonization of each MT was evaluated according to the grid-line intersection method modified by Brundrett et al. (1996). In all, we morphotyped 9,000 mycorrhizal and non-mycorrhizal root tips from 540 seedlings in three forest soils. MT samples were placed on a cotton layer in 10 ml tubes half-filled with silica gel (Prolabo, Strasbourg, France) for rapid drying, and stored at room temperature before DNA extraction.

To evaluate growth variables and total mycorrhizal colonization, plants were harvested in each forest soil after 3 months of growth. Height, collar diameter and dry weight (7 days at 80°C) of shoots and roots, and total mycorrhizal colonization were measured.

DNA extraction and amplification of the ITS region

DNA was extracted from a single mycorrhiza of each MT, or from 10 mg dry weight of a mycelial culture of Thelephoroid fungal sp. ORS.XM002, using a DNeasy Plant mini-kit following the manufacturer's recommendations (Qiagen, Courtaboeuf, France). At least five samples were extracted from each MT. The ITS region was amplified by PCR from DNA extracted from each MT. Each 25 μl PCR reaction mixture contained a 2 μl aliquot of DNA, 1 mM each of primers ITS1 (5′-TCCGTAGGTGAACCTGCGC-3′) and ITS4

(5′-TCCTCCGCTTATTGATATGC-3′), and a Ready-To-Go PCR bead (Amersham Pharmacia Biotech) for individual PCR reactions. When brought to a final volume of 25 µl, each bead contains 1.5 U *Taq* DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM of each dNTP and stabilizers including BSA. Amplifications were performed with a DNA thermal cycler (GenAmp PCR System 2400, Perkin Elmer, Foster City, Calif.) programmed as follows: 1 cycle for 5 min at 95°C followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min 30 s, and extension at 72°C for 7 min. Negative controls (no DNA template) were included to test for the presence of DNA contamination in the reagents. Up to five template dilutions were assayed before discarding a sample as not amplified. PCR products were separated by electrophoresis in 1% (w/v) agarose gels (Sigma) in 1×TBE (0.9 M Tris-borate, 0.9 M boric acid, 20 mM EDTA) with ethidium bromide at 10 µg/ml in the running buffer. DNA bands were visualized by fluorescence under UV light and photographed.

Restriction analysis of the ITS region

Restriction digests with the endonucleases *Hae*III, *Hinf*I and *Taq*I (Pharmacia) were used to differentiate MT into different RFLP types. RFLP patterns of at least four samples of each MT were determined. Per each 20 µl restriction digest, 10 µl amplified PCR product was mixed with the appropriate restriction buffer and 1 µl of the appropriate enzyme and then incubated for 2 h at 37°C (*Hae*III or *Hinf*I digests), or at 65°C (*Taq*I digests). Restriction fragments were separated by electrophoresis in 2.5% (w/v) MetaPhor agarose gels (TEBU) in 1×TBE with ethidium bromide at 10 µg/ml in the running buffer. DNA bands were visualized by fluorescence under UV light and photographed. Restriction fragment lengths were estimated using the computer program Biogene (Vilbert Lourma Biotechnology, version 99.04, no. 2630).

MT were ITS-typed with the most discriminating endonuclease, *Hinf*I, to determine the frequencies of the introduced Thelephoroid fungal sp. and native MT on *A. africana* seedlings 1 and 3 months after sowing in each forest soil. After 1 and 3 months, 20 samples were taken per MT and per treatment from five sampled seedlings for ITS-RFLP analysis. In all, we analyzed 200 samples at 1 month, and 360 samples at 3 months. If RFLP analysis failed with a single mycorrhiza, another single mycorrhiza from the same subsample was randomly drawn until an unambiguous RFLP pattern was obtained. Thereafter, a quantitative evaluation was made by calculating the frequency (number of ITS-RFLP types/number of MT ×100) of the different ITS-RFLP types corresponding to each MT collected in each forest soil.

Sequencing and identification of an amplified mitochondrial region

We amplified one region of an approximately 400-bp fragment of the mt-LrRNA gene by PCR from DNA isolated from each MT as described above. Sequencing with primers ML5 (5′-CTCG-GCAAATTATCCTCATAAG-3′) and ML6 (5′-CAGTAGAAGCT-GCATAGGGTC-3′) allowed comparison with mt-LrRNA gene sequence data in GenBank. Sequences of sporocarps of *Scleroderma verrucosum* IR 500 and *S. dictyosporum* IR 109 collected from under *Uapaca somon* and *A. africana* stands, respectively, in Burkina Faso (Sanon et al. 1997), were also added to the database. Each PCR product was extracted and purified using a QIA quick Gel Extraction kit following the manufacturer's recommendations (Qiagen). Sequencing was performed with an ABI Prism BigDye Terminator Cycle sequence kit (Applied Biosystems, Foster City, Calif.) and analyzed on an Applied Biosystems model 310 DNA sequencer (Perkin-Elmer). Sequence chromatograms were corrected by hand for errors in the automated nucleotide detection. The corrected forward and reverse DNA sequences of each MT were aligned and the resulting consensus DNA sequences were further analyzed. ML5/ML6 consensus DNA sequences were aligned using the Fasta 3.0 (Pearson and Lipman 1988) and Clustal X (Thompson et al. 1997) programs. A neighbor-joining tree based on patristic distances generated using PAUP* 4.0b (Swofford 1998) was constructed and a bootstrap confidence analysis was performed on 1,000 replications to determine the reliability of the tree topology obtained (Felsenstein 1985). At least 95% sequence identity in this region generally corresponds to sets of closely related genera (Gardes and Bruns 1996)

Statistical analysis

All data were subjected to one-way analysis of variance, and mean values were compared using Newman-Keuls multiple range test (Gagnon et al. 1989). Data of mycorrhizal colonization and ITS-RFLP frequencies were transformed by arc sin (square root) before analysis.

Results

Typing using macroscopical features

Ectomycorrhizas of the introduced Thelephoroid fungal sp. ORS.XM002 are characterized by a change from a light-brown mantle color in younger roots to dark-brown in older roots, a hairy mantle with clamp connections at the septa of hyphae, and abundant mycelial strands (Bâ 1990; Bâ et al. 1991). The introduced (xmbrf) and native dark-brown MT from Kolda (kmtbrf), Bignona (bmtbrf) and Kaparang (kamtbrf) possess similar features. The texture of the sheath of white MT varies from smooth (kmtb1) to felty (kmtb2), hairy (kmtb3) or bristly (kmtb4), with glossy or waxy aspect. All white MT are also characterized by emanating hyphae, absence of clamp connections at the septa of hyphae, mycelial strands, and sclerotia linked to ectomycorrhizas. However, these white MT were grouped into one MT because there are not enough features to distinguish them clearly. Regardless of the forest soil used, the texture of the sheath of introduced (xmlb) and native light-brown MT from Kolda (kmtlb), Bignona (bmtlb) and Kaparang (kamtlb) is hairy with dull aspect, emanating hyphae, clamp connections at the septa of hyphae, and mycelial strands linked to ectomycorrhizas. In Bignona and Kaparang forest soils, brown MT (bmtbr and kamtbr, respectively) are surface downy with emanating hyphae, presence or absence of clamp connections at the septa of hyphae, and mycelial strands linked to ectomycorrhizas.

Mycorrhizal root tips were grouped into MT in each forest soil (Table 2). In Kolda forest soil, there were a total of three MT appearing on uninoculated seedlings following a time sequence of ectomycorrhizal colonization. Light-brown, white and dark-brown MT were recorded in the first month, reaching 5%, 6% and 10.5% of the root tips, respectively. At 3 months, the dark-brown MT was dominant and comprised 48.7% of the root tips in control seedlings. In Bignona forest soil, three MT also appeared on root tips of uninoculated seedlings following a sequence (Table 2). One month after transplanting, only light-brown MT was present, colonizing 22.6% of root tips. This MT was then in part replaced by brown and dark-brown MT after 2 and

Table 2 Percentage of mycorrhizal colonization in *Afzelia africana* seedlings 1, 2 and 3 months after inoculation with Thelephoroid fungal sp. ORS.XM002 in Kolda, Bignona and Kaparang forest soil. Mean values were compared in each forest soil. In each column, means not followed by a common letter differ significantly ($P<5\%$) from each other

Morphotypes	Soil	Month 1	Month 2	Month 3
Kolda				
Light brown	Inoculated	6.12 a	3.86 a	4.68 a
	Control	5.00 a	4.04 a	5.62 a
White	Inoculated	4.82 a	8.04 a	6.88 a
	Control	6.00 a	8.00 a	8.68 a
Dark brown	Inoculated	10.42 b	42.08 c	72.30 c
	Control	10.52 b	26.92 b	48.70 b
Bignona				
Light brown	Inoculated	39.75 b	7.11 a	3.82 a
	Control	22.60 c	8.00 a	6.22 a
Brown	Inoculated	0.00 a	35.30 b	33.14 c
	Control	0.00 a	33.90 b	27.56 b
Dark brown	Inoculated	0.00 a	35.55 b	52.83 d
	Control	0.00 a	10.40 a	39.26 c
Kaparang				
Light brown	Inoculated	34.26 c	2.34 a	4.22 a
	Control	5.49 b	3.30 a	3.90 a
Brown	Inoculated	0.00 a	30.95 b	19.11 b
	Control	0.00 a	27.51 b	18.02 b
Dark brown	Inoculated	0.00 a	46.42 c	68.80 d
	Control	0.00 a	28.48 b	58.00 c

3 months. However, the dark-brown MT was superior to light-brown and brown MT in terms of mycorrhizal colonization in control seedlings 3 months after sowing. In Kaparang forest soil, three MT were identified according to the color of mantle: light-brown, brown and dark-brown MT (Table 2). Only light-brown MT appeared on roots of uninoculated seedlings 1 month after transplanting. This MT was then dominated by brown and dark-brown MT 2 and 3 months after sowing. Dark-brown MT was significantly superior to light-brown and brown MT in terms of mycorrhizal colonization in uninoculated seedlings 3 months after sowing.

Native dark-brown MT were dominant in control seedlings whatever the forest soil considered. However, differences in terms of mycorrhizal colonization between the inoculated and uninoculated seedlings may be due to the introduced Thelephoroid fungal sp. ORS.XM002 in the three forest soils 3 months after sowing (Table 2). Nevertheless, using the morphotyping approach, it has not been possible to ascertain whether the introduced fungus was more frequent than native dark-brown MT; one reason for this may be that these dark-brown MT lack distinctive features and, consequently, a number of fungi share the few characters that we used to define it. Thus, subsequent growth of inoculated seedlings may not be attributed to the introduced fungus. MT were therefore further analyzed by RFLP typing of the amplified ITS.

Table 3 Sizes of PCR-restriction fragment length polymorphism (RFLP) fragments (only band sizes larger than 50 bp were considered) obtained from nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS) products amplified using primers ITS1 and ITS4 (see Materials and methods)

Origins of forest soil	Morphotypes	ITS-RFLP types	Band sizes (bp)			
			Uncut	*Taq*I	*Hinf*I	*Hae*III
Kolda	Light brown	a5	689	211, 272	299, 360	221, 440
		xm	701	221, 340	101, 242, 292	605
	White	a3	711	58, 109, 125	100, 269, 342	64, 90, 158, 320
		a1	632	254	103, 222, 305	597
		a2	675	330	328, 340	681
		a4	689	274, 345	243, 293	221, 440
	Dark brown	a5	689	211, 272	299, 360	221, 440
		xm	701	221, 340	101, 242, 292	605
Bignona	Light brown	b3	689	274, 345	243, 293	219, 436
		b4	689	211, 272	299, 360	221, 440
		b5	654	221, 340	243, 293	605
		xm	701	221, 340	101, 242, 292	605
	Brown	b2	725	298, 348	202, 367	174, 524
		b1	575, 780	184, 210, 248	196, 279, 375	96, 151, 297, 351, 441
	Dark brown	b4	689	211, 272	299, 360	221, 440
		b5	654	221, 340	243, 293	605
		xm	701	221, 340	101, 242, 292	605
Kaparang	Light brown	c2	571	195, 217	194, 268	450
		c5	695	221, 340	215, 235, 243	605
		c4	708	274, 345	321, 348	219, 384
		xm	701	221, 340	101, 242, 292	605
	Brown	c1	791	170, 282, 339	nd[a]	nd
		c2	571	195, 217	194, 268	450
		c3	795	225, 240, 315	225, 265, 300	605
	Dark brown	c5	695	221, 340	215, 235, 243	605
		xm	701	221, 340	101, 242, 292	605

[a] Not determined

Table 4 ITS-RFLP type frequencies of the introduced Thelephoroid fungal sp. XM002 morphotype (MT) and native MT occurring on inoculated or uninoculated *A. africana* seedlings growing in Kolda, Bignona and Kaparang forest soils 1 and 3 months after sowing. Mean values were compared in each forest soil. Different letters within a column indicate significantly different values at $P<1\%$. *W* White, *Lb* light brown, *Db* dark brown, *B* brown

A. africana inoculated or not	MT	ITS-RFLP types	Frequency of the ITS-RFLP 6 types in mycorrhizas (%)	
			Month 1	Month 3
Kolda				
Inoculated	W	a1	6.6 cd	8.3 c
	W	a2	6.6 cd	13.3 bc
	W	a3	9.9 cd	3.3 c
	W	a4	11.6 cd	1.6 c
	Lb and Db	a5	34.9 a	26.6 b
	Lb and Db	xm	28.3 ab	43.3 a
Control	W	a1	14.9 bcd	4.9 c
	W	a2	13.3 bcd	11.6 bc
	W	a3	13.3 bcd	13.2 bc
	W	a4	23.2 abc	26.6 b
	Lb and Db	a5	34.9 a	43.3 a
	Lb and Db	xm	0.0 d	0.0 c
Bignona				
Inoculated	B	b1	0.0 c	6.6 bc
	B	b2	0.0 c	9.9 bc
	Lb	b3	20.0 abc	1.6 c
	Lb and Db	b4	15.0 abc	16.6 bc
	Lb and Db	b5	35.0 ab	21.6 b
	Lb and Db	xm	30.0 ab	40.0 a
Control	B	b1	0.0 c	8.3 bc
	B	b2	0.0 c	4.9 c
	Lb	b3	30.0 ab	16.6 bc
	Lb and Db	b4	40.0 a	34.9 a
	Lb and Db	b5	30.0 ab	35.0 a
	Lb and Db	xm	0.0 c	0.0 c
Kaparang				
Inoculated	B	c1	0.0 b	3.3 c
	B	c3	0.0 b	8.3 bc
	Lb	c4	20.0 ab	9.9 bc
	Lb and B	c2	20.0 ab	3.3 c
	Lb and Db	c5	30.0 ab	24.9 b
	Lb and Db	xm	30.0 ab	49.9 a
Control	B	c1	0.0 b	11.6 bc
	B	c3	0.0 ab	6.6 c
	Lb	c4	25.0 ab	24.9 b
	Lb and B	c2	25.0 ab	13.3 bc
	Lb and Db	c5	50.0 a	43.3 a
	Lb and Db	xm	0.0 b	0.0 c

Analysis of individual ITS restriction fragments

After restriction with the enzymes *Taq*I, *Hinf*I and *Hae*III, the MT collected in inoculated and uninoculated seedlings could be further divided into several ITS types (Table 3). Of the three MT collected in Kolda forest soil, four ITS types were found in white MT and two were found in light-brown and dark-brown MT (Table 3). This suggests a change in mantle color of MT from light-brown to dark-brown. However, the ITS type frequency of introduced MT was higher, reaching 43.3% on inoculated seedlings (Table 4). In Bignona forest soil, six different ITS types were recognized in all (Table 3). Of these six ITS types, three were found in light-brown and dark-brown MT, suggesting a change in mantle color. Two other MT were recorded in brown MT, and one in light-brown MT (Table 3). At 3 months, the ITS type frequencies of native MT decreased when plants were inoculated with the introduced fungus (Table 4). In Kaparang forest soil, three MT were differentiated into six different ITS types (Tables 2, 3). Two ITS types that occurred first in light-brown MT (c2 and c5) had similar ITS patterns in brown and dark-brown MT, respectively, appearing later. These results suggested that the light-brown mantle color change in ageing mycorrhizas. The ITS type c5 belonging to dark-brown MT accounted for 24% of the mycorrhizas found in the inoculated seedlings, where the ITS type xm was higher, reaching 49% of mycorrhizas (Table 4).

Molecular identification of the unknown fungi

Sequence analysis and phylogenetic grouping of the ML5/ML6 region of the mt-LrRNA was further used to identify the unknown fungal symbionts by comparing to a

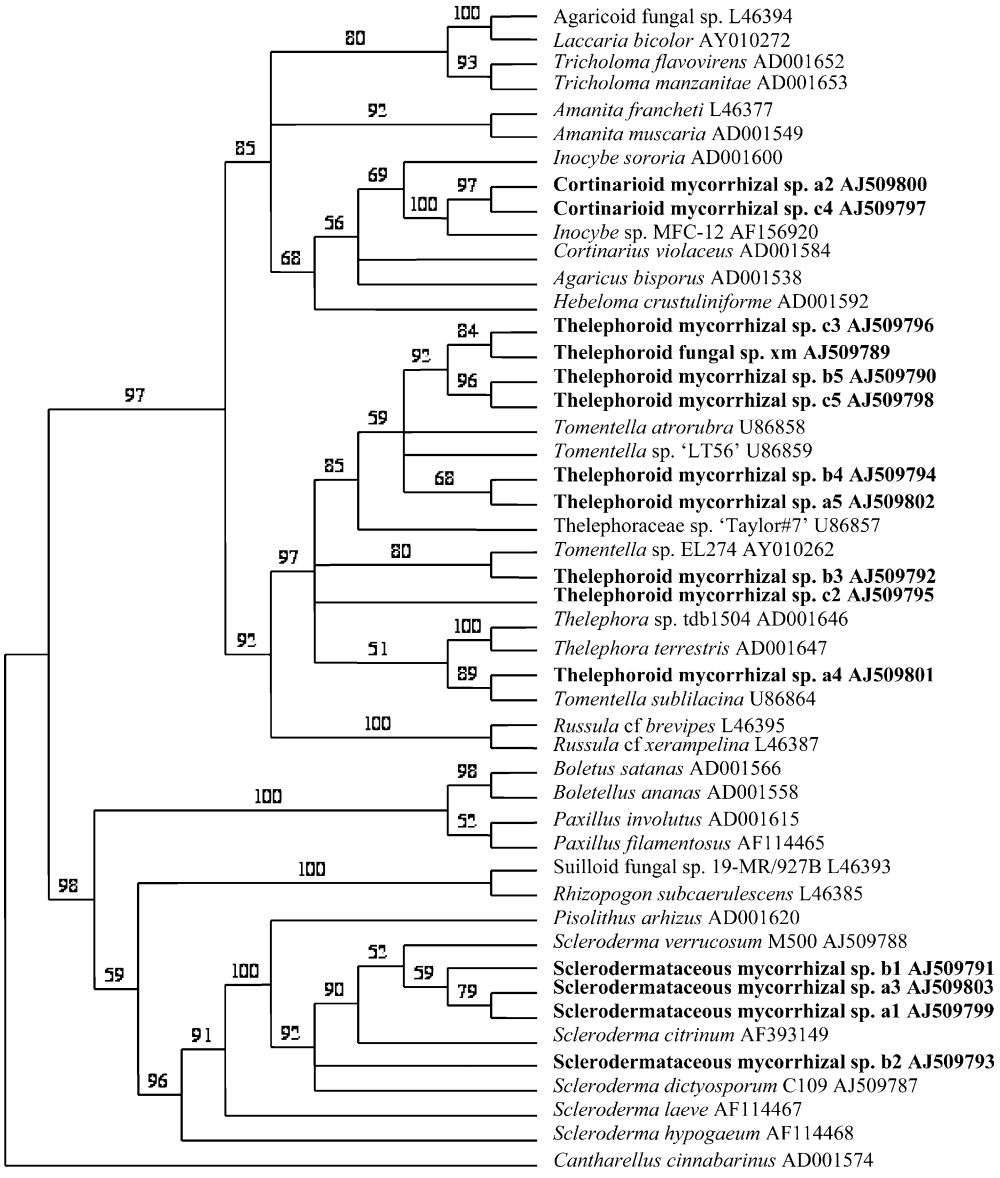

Fig. 2 Phylogenetic placement of unknown mycorrhizal types (indicated in *bold*) compared to sequences in GenBank. Sequence alignment was performed using Clustal X. The aligned sequences were analyzed using PAUP* version 4.0b. Pairwise distances were calculated for all taxa by neighbor-joining analysis using uncorrected ('p') distances. Confidence in the branches of the neighbor-joining tree was assessed by bootstrap analysis using 1,000 replicates. Branches supported by more than 50% of bootstrap replicates are indicated

previously published database of sequences from that region in GenBank (Bruns et al. 1998).

Results from partial sequence analysis of the ML5/ML6 region provided genera-level identification of unknown MT. No amplification was obtained from c1. The 15 sequences were compared to the GenBank database using the algorithm BLASTN to identify the most similar ML5/ML6 sequences. In all cases, sequence similarity was 96–100% between unknown MT and their closest known genera in a family or subfamily of basidiomycete sequences in the GenBank database. Nine types were closely related to the Thelephoroid taxa, four to the Sclerodermatales, and two to the Cortinarioid taxa (Fig. 2). Phylogenetic analysis as displayed by the high bootstrap values shown in the neighbor-joining based tree in Fig. 2 also demonstrates that the different species fall into three groups.

Seedling growth

Thelephoroid fungal sp. ORS.XM002 appeared effective in increasing the root dry weight of *A. africana* seedlings when compared with control seedlings in each forest soil (Table 5). However, *A. africana* seedlings differed in their response to inoculation with the introduced fungus for the other measured variables (Table 5). In Kolda forest soil, other variables of inoculated seedlings were not affected by inoculation. In Bignona forest soil, inoculated seedlings had greater total dry weight than control seedlings. In Kaparang soil, height and collar diameter were stimulated in inoculated seedlings, whereas shoot dry weight were not (Table 5).

Table 5 Effect of inoculation with Thelephoroid fungal sp. ORS.XM002 on growth variables and mycorrhizal colonization of *A. africana* seedlings 3 months after sowing in Kolda, Bignona and Kaparang forest soils. Mean values were compared in each forest soil. Different letters within a column indicate significantly different values at $P<5\%$

A. africana inoculated or not	Height (cm)	Collar diameter (mm)	Shoot dry weight (g)	Root dry weight (g)	Total dry weight (g)	Total mycorrhizal colonization (g)
Kolda						
Inoculated	23.88 a	5.76 a	1.85 a	0.57 b	2.42 a	83.86 b
Control	22.48 a	5.40 a	1.70 a	0.43 a	2.13 a	63.00 a
Bignona						
Inoculated	23.10 a	5.40 a	1.91 a	0.54 b	2.45 b	89.79 b
Control	22.76 a	5.46 a	1.68 a	0.39 a	2.07 a	73.04 a
Kaparang						
Inoculated	30.24 b	6.36 b	1.86 a	0.58 b	2.44 a	92.13 b
Control	24.16 a	5.10 a	1.76 a	0.47 a	2.23 a	79.92 a

Discussion

One of the major questions addressed in the present study was whether the introduced Thelephoroid fungal sp. ORS.XM002 was able to become established early in root systems of *A. africana* seedlings and then to compete in the course of time with natural ectomycorrhizal fungi found in three different forest soils collected from under native stands of *A. africana*. To answer this question, we used a combination of three approaches to detect and identify the introduced fungus among native ectomycorrhizal fungi revealed in bioassay using baiting seedlings in cores of soils taken in three different sites: sorting of MT based on morphological characters, grouping of fungi by RFLP analysis and identification of unknown fungi by phylogenetic placement against a reference database in GenBank (Gardes et al. 1991; Bruns et al. 1998; Pritsch et al. 2000; Horton and Bruns 2001).

The time sequence of the colonization process by the native ectomycorrhizal fungi indicated by MT surveys in control seedlings was similar in all forest soils, with a predominance of light-brown MT at first, followed by dark-brown MT that dominated later. This supports an earlier report of Bâ et al. (1991) indicating a general pattern in which dark-brown MT dominates over a 6-month period, with some other MT appearing in baiting seedlings. As shown by the results of ITS-RFLP and sequence analysis in the present study, a morphological approach cannot be used to identify fungal symbionts from mycorrhizas and to evaluate subsequent growth differences of seedlings that could be due to application of the fungus.

In our study, the primer pairs ITS1/ITS4 specifically amplified the ITS from fungal symbionts in MT, but not the host symbiont. RFLP variation in the ITS region could be used to discriminate fungal symbionts in MT from the different forest soils. Indeed, the number of ITS types was greater than the number of MT whatever the forest soil considered. Each MT observed in control and inoculated seedlings consisted of between one and four ITS types. Identical ITS types could also be found in roots assigned to different MT. For instance, some ITS types found in light-brown MT were also present in brown or dark-brown MT. MT occurring in different forest soils were comparable, whereas their ITS RFLP patterns differed greatly. Only the ITS type a5 in the dark-brown MT from Kolda was found to be identical to the ITS type b4 in the dark-brown MT from Bignona. The correlation between the occurrence of MT and ITS types has, however, been shown to be poor. Several studies support the view that not only can one MT include more than one ITS type, but also that most ITS types can appear in more than one MT (Gardes and Bruns 1996; Jonsson et al. 1999a, 1999b). Sometimes, two ITS regions were amplified from the same mycorrhiza. This explains why the sum of the bands in the digests is greater than the uncut ITS fragment of the ITS type b1 (Table 3). This phenomenon is possibly due to having detected either a double colonization of two different species on one mycorrhizal root tip, or ITS heterogeneity within a single genome of one fungal species (Gardes and Bruns 1996; Erland et al. 1999).

Variation in the ITS region was also used to detect early colonization and persistence of the introduced fungus directly. Thelephoroid fungal sp. ORS.XM002 on *A. africana* seedlings. This fungus was not found in any of the three soils, and did not provoke any consistent change in species composition following inoculum application. This result is not in agreement with some studies indicating that fungi introduced in nurseries could either decrease the composition of native fungal species, or completely replace some indigenous ectomycorrhizal fungi of the same species (Villeneuve et al. 1991; Henrion et al. 1994). Furthermore, the introduced Thelephoroid fungal sp. ORS.XM002 significantly increased mycorrhizal colonization rates in inoculated seedlings when compared to control seedlings. The frequency of ITS type xm ranged from 40% to 49% depending on the forest soil used and was higher than those of ITS types from native dark-brown MT occurring on inoculated seedlings in the three forest soils. For these reasons, we considered the introduced fungus responsible for the stimulation of ectomycorrhizal colonization rates in inoculated seedlings

compared to those of control seedlings. The introduced fungus Thelephoroid fungal sp. ORS.XM002 may therefore be considered as a good competitor.

To identify unknown fungi, we sequenced the ML5/ML6 region of the mt-LrRNA gene and compared the sequences obtained with a database of sequences from that region in GenBank. Sequence analysis of this region was not always consistent with results of ITS-RFLP matching. ITS types a5 and b4 were similar, whereas their ML5/ML6 regions of the mt-LrRNA gene were different in 9 bp (results not shown). However, sequence analysis provided genera-level identification of unknown fungal symbionts in native MT. Placements of our ML5/ML6 groups 1, 2 and 3 were closely related to Cortinarioid, Thelephoroid, and Sclerodermataceous taxa, respectively. Group 1 is closely related to *Inocybe* spp., group 2 to *Thelephora* spp. and *Tomentella* spp. but without a clear attribution to either genus, and group 3 to *Scleroderma* spp. Thelephoroid fungal spp., *Inocybe* spp. and *Scleroderma* spp., were recorded as early-stage ectomycorrhizal over a 3-month period. However, Thelephoroid taxa were dominant on seedlings in this study. Our results are consistent with those of other studies supporting the view that Thelephoroid taxa are abundant and particularly good competitors on root systems of seedlings in nursery soils (Bâ et al. 1991; Villeneuve et al. 1991; Taylor and Bruns 1999).

A second major question addressed in this study was whether the introduced Thelephoroid fungal sp. ORS.XM002 improves growth of *A. africana* seedlings in nursery soils conditions. To answer this question, we evaluated the effect of the introduced strain on the growth of seedlings in plastic bags containing three sampled forest soils. The development of the root system was improved by inoculation irrespective of the origin of the forest soils. Thus, the increased root dry weight could be reflected in the total dry weight of inoculated seedlings in Bignona forest soil. Our results are in agreement with previous studies, which showed that *Afzelia* spp. root systems increased in response to inoculation with Thelephoroid fungal sp. ORS.XM002 in soils with low available NPK (Bâ 1990; Bâ et al. 1999, 2001). Several studies have shown that development of the root system appears to be a key factor in seedling growth improvement after outplanting (Garbaye and Wilhelm 1984; Al-Abras et al. 1988; Villeneuve et al. 1991). Therefore, Thelephoroid fungal sp. ORS.XM002 appears to be an early colonizer, a good competitor, and an efficient fungus in promoting seedling growth. It is unclear what mechanism allows Thelephoroid fungal sp. ORS.XM002 to colonize first and to persist in the forest soils. However, results should be interpreted cautiously as the duration of our study was limited to a 3-month period. Further work is required to evaluate the persistence and the dissemination of the introduced Thelephoroid fungal sp. ORS.XM002 after outplanting of the mycorrhizal seedlings.

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