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REVIEW

Morphological and molecular analyses in *Scleroderma* species associated with some Caesalpinioid legumes, Dipterocarpaceae and Phyllanthaceae trees in southern Burkina Faso

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Abstract A combination of morphotypes, polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) analyses and internal transcribed spacer (ITS) sequencing was used to investigate *Scleroderma* species that were collected from woodlands in Burkina Faso. We harvested 52 specimens from 20 sites during rainy seasons between 1997 and 2000. According to their morphological features, these specimens were initially characterised, and we then identified six species of *Scleroderma*. Two of the species were clearly determined as *Scleroderma dictyosporum* Pat. and *S. verrucosum* Pers. The four remaining species were characteristically described as (1) displaying big spores with spines up to 2 μm (*Scleroderma* sp1), (2) producing spores without ornamentation (*Scleroderma* sp2), (3) spores with very small spines (*Scleroderma* sp3) and (4) with yellow sporocarps and sub-spherical spores (*Scleroderma* sp4). The specimens were then analysed using PCR/RFLP of the intergenic regions of rDNA, ITS and IGS1 and ITS sequencing. The restriction fragments obtained with two endonucleases, *Hinf*I and *Mbo*I on ITS and IGS1 regions, showed that some isolates of *S. dictyosporum* had the same patterns as isolates and basidiocarps of *Scleroderma* sp4 (IR265, IR408, SP4-2903). Isolates of *Scleroderma* sp3 (IR252) had common restriction fragments as isolates of *S. verrucosum* (IR500, IR600). Intraspecific differences were observed in the two previously determined species, as well as in *Scleroderma* sp2. The ITS sequencing and phylogenetic analyses showed that the ribotypes identified by PCR/RFLP within these species might be phylogenetic species. Combining these molecular results allowed regrouping the six morphological species in three sets of cryptic species: a first set with two species including *S. dictyosporum* Pat., a second set with four species, including both *S. verrucosum* Pers. and *Scleroderma* sp1 and a third set with two species, including *Scleroderma* sp2. These investigations and the combined morphological and molecular analyses used to sort out species paved the way for identifying larger populations of *Scleroderma* species in Burkina Faso and other tropical zones.

Keywords *Scleroderma* · Morphology · Identification · PCR/RFLP · ITS sequencing · Burkina Faso

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Introduction

The genus *Scleroderma*, a gasteromycetous belonging to the family of Sclerodermataceae, is distributed worldwide in temperate and tropical regions (Sims et al. 1997). It is a common and important fungal symbiont of many tropical and temperate trees including several species of economic importance in families such as Pinaceae, Myrtaceae, Fagaceae, Dipterocarpaceae and Caesalpinioid legumes (Munyanziza and Kuyper 1995; Sims et al. 1997). Mycelia of *Scleroderma* species are easy to grow, and this makes them suitable symbionts for inoculation in afforestation programmes using eucalypts and pines (Dell et al. 2002; Chen 2006).

More than 25 species have been determined within the genus *Scleroderma*, according to the morphological characteristics of their basidiocarps, basidiospores and mycelia (Sims et al. 1999; Kuo 2004; Guzman et al. 2004). However, the taxonomy of the genus *Scleroderma* is understudied particularly in tropical Africa (Sanon et al. 1997) and in Asia (David and David 1998; Sims et al. 1999). Nevertheless, a revised key of the genus *Scleroderma* was conceived for the identification of 25 Asian species, which includes two indeterminate species (Sims et al. 1995). Because it can be a useful tool in studying population structure in the forest and identifying and monitoring specific strains to introduce into the soil in controlled mycorrhization programmes, it is important to continue investigating the taxonomy of this genus in various ecosystems, mainly of tropical Africa. Although sporocarps allow description and identification of some *Scleroderma* species, molecular tools offer a more sensitive means than morphological description in providing more accurate species differentiation within ectomycorrhizal (ECM) fungi (Hansen et al. 2002; Rivière et al. 2007; Tedersoo et al. 2007).

Many studies have shown that both the polymerase chain reaction (PCR) amplification and restriction fragment length polymorphism (RFLP) and the sequencing of the internal transcribed spacer (ITS) and intergenic spacer (IGS) of the nuclear rDNA are among the most dominant tools used to analyse the inter- and intra-specific variation in fungal symbionts (Gardes et al. 1991; Liang et al. 2004; Matsushita et al. 2005; Ruiz-Diez et al. 2006; Sica et al 2007). The ribosomal DNA spacers (e.g. ITS and IGS1) are known to be inter- and intra-specific variables and have been used as markers to differentiate species of several ectomycorrhizal fungi and/or isolates (Karen et al. 1997; Peter et al. 2001; Horton 2002; Gomes et al. 2002). Several studies have also used sequencing data of nuclear rDNA or ML5-ML6 region of the mitochondrial large subunit rDNA gene for phylogenetic analyses at different levels (e.g. isolates, species, genus, family) to improve the ECM species delimitation (Bruns et al. 1998; Lindemuth et al. 2001; Hong et al. 2002; Rivière et al. 2007).

In West Africa, previous fungal inventories were focused on the genus *Scleroderma*, (Thoen and Bâ 1989; Bâ and Thoen 1990; Sanon et al. 1997). *Scleroderma verrucosum* Pers. and *S. dictyosporum* Pat. were found widely distributed and associated with African tree species of families Caesalpinioid legumes, Phyllanthaceae and Dipterocarpaceae (Sanon et al. 1997). These species were easily isolated in pure culture and efficient in controlled mycorrhization of African woody species in nursery conditions (Bâ et al. 1999, 2002; Diédhiou et al. 2005).

In our previous investigations, we collected abundant sporocarps of *Scleroderma* species under host trees in woodlands of Burkina Faso. Two of them were morphologically identified as *S. dictyosporum* Pat. and *S. verrucosum* Pers. (Sanon et al. 1997; Sanon 1999). In this paper, we reexamined the diversity of *Scleroderma* species using both morphological and molecular analyses of sporocarps and mycelial cultures, and we attempted to accurately identify them.

Materials and methods

Study sites

Twenty sites, distant from 2 to about 1000 km from each other, were visited in the Southern, South-western and Eastern regions of Burkina Faso (Fig. 1). Mean annual rainfall of these regions range from 800 to 1,100 mm with a marked 6-month dry season from November to April. Soil profiles of 0–20 cm are sandy–silt and slightly acidic or neutral (Sanon et al. 1997). All the sites were visited during the rainy season between May and September, from 1994 to 1997 and in 2000. The vegetation is typical open and semi-deciduous forests, savannah, as well as riverside gallery forests. These vegetations are dominated by ECM trees belonging to the families Caesalpinioid legumes (e.g. *Afzelia africana* Sm., *Berlinia grandiflora* (Vahl) Hutch. & Dalziel, *Isoberlinia doka* Craib. & Stapf., and *I. dalziellii* Craib. & Stapf), Phyllanthaceae (*Uapaca guineensis* Mull. & Arg. and *U. somon* Aub. & Léan) and Dipterocarpaceae (*Monotes kerstingii* Gilg.; see Table 1).

Sampling of sporocarps

Sporocarps of *Scleroderma* were collected to cover most of the morphological variation observed under several ECM trees in the field. They were photographed, described (size and colour), dried in an oven at 50°C for at least 24 h and stored at room temperature in the herbarium of the Laboratory of Microbiology (INERA/DPF, Burkina Faso). Morphological descriptions of representative specimens were based on the spore characteristics (size, shape) using

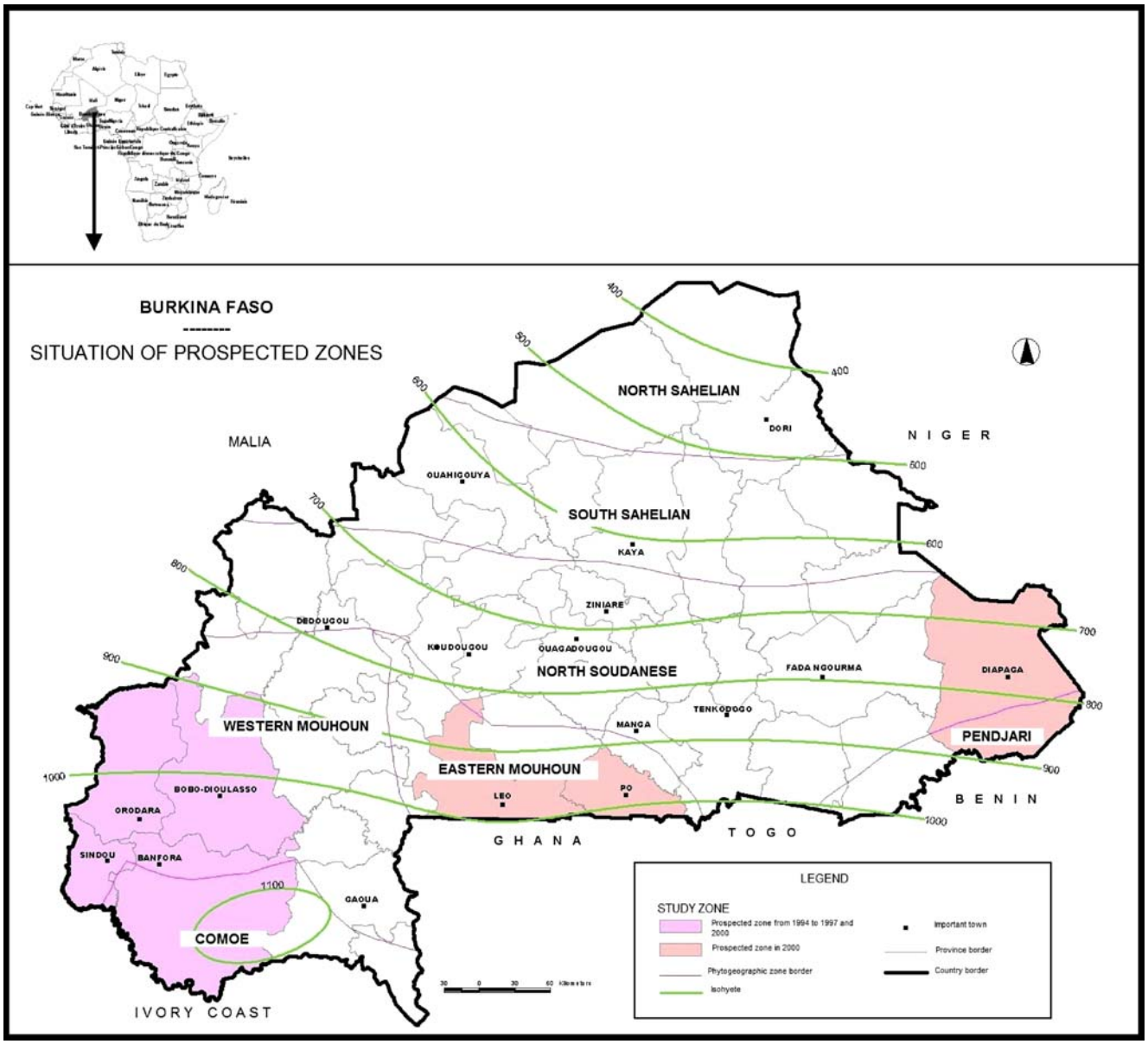

Fig. 1 Locations of the prospected areas

light microscope and spore mass colour (Table 2). Small pieces of fresh sporocarps were aseptically transferred on modified Melin-Norkrans agar medium (Marx 1969) and incubated until fungal growth was observed. Fungal cultures were kept at 30°C on modified Melin-Norkrans agar medium. Few basidiocarps of non-isolated samples were mainly air-dried and maintained in a dry place (ambient temperature) until they were used for DNA extraction.

DNA extraction and amplification

DNA were extracted from mycelia or dry basidiocarps using either the methods of Grube et al. (1995) and Martin et al. (1997) or the DNeasy Kit, according to manufacturer's instructions (Qiagen, France).

DNA were diluted 25 folds for the mycelia and 10 folds for basidiocarps before their amplification. The nuclear rDNA ITS were amplified by PCR with the primers ITS1/ITS4 (White et al. 1990), whilst IGS1spacers amplification was performed by CNL12/5SA (Henrion et al. 1992). All amplifications were performed in 25 µl of 1x PCR buffer (10x), 0,2U *Taq* DNA polymerase (5U/µl; Appligene-Onchor, Illkirch, France), 10 mM DNTP, 10 µM of each primer, 1 to 2 µl DNA and H_2O ultra-pure sterile (qsp 25 µl). The amplifications were performed with a DNA thermal cycler (GeneAmp PCR System 9600, Perkin Elmer) programmed as follows: initial denaturation for

Table 1 Sporocarps and mycelial cultures of *Scleroderma* species studied with their collecting period, host tree and geographical origin

Species /N of voucher material	Collecting period	Host-trees	Locality (Provinces)	Ribotypes
S. dictyosporum				
IR109	1994	*A. africana*	SW (Houet)	A
IR215	1994	*I. doka, I. dalziellii*	SW (Houet)	B
IR250	1996	*I. doka*	SW (Comoé)	A
IR412	1996	*U. guineensis*	SW (Houet)	A
IR602	1996	*M. kerstingii, I. dalziellii*	SW (Comoé)	A
SD-2871	1997	*I. doka, I. dalziellii*	SW (Comoé)	A
SD-3601	1997	*U. guineensis*	SW (Comoé)	A
SD-2872	1997	*I. doka*	SW (Houet)	A
SD-6871	1997	*M. kerstingii, I. dalziellii*	SW (Comoé)	A
SD-4901	2000	*U. somon*	SW (Comoé)	B
ORS7731[a]	1989	*A. africana*	Senegal	A
S. verrucosum				
IR256	1996	*I. doka, I. dalziellii*	SW (Comoé)	C
IR500	1994	*U. somon*	SW (Comoé)	D
IR600	1996	*M. kerstingii, I. dalziellii*	SW (Comoé)	D
IR114	2000	*A. africana*	S (Nahouri)	C
IR110	2000	*A. africana*	S (Nahouri)	C
IR118	2000	*A. africana*	S (Nahouri)	C
IR261	2000	*I. doka*	S(Nahouri)	C
SV-3871	1997	*U. guineensis*	SW (Comoé)	C
SV-5871	1997	*I.. doka*	SW (Comoé)	C
SV-2871	1997	*I. doka, I. dalziellii*	SW (Comoé)	C
SV-5872	1997	*I. doka*	SW (Houet)	C
SV-2873	1997	*M. kerstingii, I. dalziellii*	SW (Comoé)	C
SV-1801	2000	*A. africana*	S (Nahouri)	E
SV-2802	2000	*I. doka*	S (Nahouri)	C
SV-1803	2000	*A. africana*	S (Nahouri)	C
SV-1804	2000	*A. africana*	E (Tapoa)	E
SV-1902	2000	*A. africana*	E (Tapoa)	C
SV-2811	2000	*I. doka*	S (Nahouri)	C
SV-5602	2000	*U. guineensis*	SW (Houet)	C
Scleroderma sp1				
IR406	1994	U. guineensis	SW (Houet)	F
IR409	1995	*U. guineensis*	SW (Houet)	F
IR410	1996	*U. guineensis*	SW (Houet)	F
SP1-4871	1997	*U. guineensis*	SW (Houet)	F
Scleroderma sp2				
IR100	1994	*A. africana*	SW (Houet)	G
IR510	1996	*U. somon*	SW (Comoé)	H
IR134	2000	*A. africana*	SW (Houet)	G
IR263	2000	*I. doka, I. dalziellii*	SW (Comoé)	H
SP2-1802	2000	*A. africana, I. doka*	S (Nahouri)	H
SP2-2801	2000	*I. doka*	S (Nahouri)	H
SP2-1806	2000	*A. africana, I. doka*	E (Tapoa)	H
SP2-2803	2000	*I. doka*	S (Sissili)	H
SP2-2901	2000	*I. doka*	E (Tapoa)	H
SP2-4902	2000	*U. somon*	SW (Comoé)	H
SP2-2902	2000	*I. doka*	SW (Comoé)	H

Table 1 (continued)

Species /N of voucher material	Collecting period	Host-trees	Locality (Provinces)	Ribotypes
SP2-7901	2000	*I. doka, I. dalziellii, A. africana, U. somon, M. kerstingii*	SW (Comoé)	G
SP2-3901	2000	*M. kerstingii*	SW (Comoé)	H
SP2-1901	2000	*A. africana*	E (Tapoa)	H
SP2-5601	2000	*U. guineensis*	SW (Houet)	H
Scleroderma sp3				
IR252	1996	*U. somon*	SW (Comoé)	D
Scleroderma sp4				
IR408	1995	*U. guineensis*	SW (Houet)	A
IR265	2000	*I. doka*	SW (Houet)	A
SP3-2903	2000	*I.. doka*	SW (Houet)	A

Mycelial cultures are referred to as "IR'" and sporocarps are labelled as SD, SV, SP1, SP2, SP3 or SP4

SW south-west, *S* south, *E* east

[a] Isolate from South Senegal (Bâ 1990)

3 min at 94°C, followed by 30 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 2 min and final extension at 72°C for 10 min. Negative control (no DNA template) were included to test for the presence of DNA contamination in the reagents. PCR products were separated by electrophoresis in 1% (*w*/*v*) agarose gels (Sigma) in 1x TBE (0.9 M Tris-borate, 0.9 M boric acid, 20 mM EDTA). DNA bands were stained with ethidium bromide (10 µg/ml) visualised by fluorescence under UV light and photographed.

RFLP analysis

For RFLP analysis, approximately 10 ng of amplified ITS and IGS1 were digested separately overnight at 37°C with 2.5 units of the endonucleases *HinfI* and *MboI* (Biolabs and Promega). Restriction fragments were separated by electrophoresis in 2% Nusieve agarose gels in 1x Tris-acid Boric EDTA (TBE) buffer. DNA bands were visualised by fluorescence under UV light and photographed after staining with ethidium bromide at 10 µg/ml. Restriction fragment lengths were estimated according to the molecular weight markers (100 bp).

Table 2 Description of basidiocarps and basidiospores of the *Scleroderma* species

Species	Basidiocarp colour	Spore size (µm)	Spore ornamentation
S. dictyosporum	Dark brown	7–9	Reticulated
S. verrucosum	Brown	5–9	Blunt spines, 1–2 µm
Scleroderma sp1	Pinkish-brown	9–13	Spines, 2–3 µm
Scleroderma sp2	Brown	5–7	None
Scleroderma sp3	Brown	5–10	Very small spines
Scleroderma sp4	Yellowish	7–9	None

ITS sequencing

At least one representative mycelial culture and basidiocarp of each ribotype (instead of one ribotype of *Scleroderma* sp2) were amplified with primers ITS1/ITS4 and purified in ammonium acetate (Martin and Voiblet 1998). Both strands of amplified products were sequenced using the Big Dye Terminator kit (PE ABI), *Taq* FS polymerase and the primers ITS1/ITS4. The sequencing reaction products were analysed using the automatic sequencer GenoTyper ABI 310 (PE ABI) at the sequencing laboratory of INRA-Nancy (France). The sequence data were revealed with the software Sequence Analysis (PE ABI) and edited using Sequencher (Gene Codes Corporation, Ann Arbor, MI) for Macintosh. In some case, it was necessary to use internal primers (ITS2) and its reverse complement (ITS3) where sequences from ITS1 and ITS4 were short (White et al. 1990).

Phylogenetic analysis

Sequences were aligned using multiple sequence alignment programme MultAlin (Corpet 1988) on the web site (http://www.toulouse.inra.fr/multalin.html). The resulting multiple alignments were optimised visually, and only unambiguous alignments were used in the phylogenetic analyses. Search of sequence homology in the NBC GenBank DNA database was carried out by Gapped BLAST (NCBI) (Altschul et al. 1997). Phylogenetic and distance analyses were conducted

by neighbor-joining (NJ) method using Kimura 2-parameter distances of MEGA version 3.1 (Kumar et al. 2004), and phylogenetic trees were drawn. Confidence in the branches of the neighbor-joining tree was evaluated by bootstrap analysis using 1,000 replicates. Branches supported by more than 50% of bootstrap replicates are indicated on the tree. Five sequences of *Scleroderma* species from GenBank (EU819438, EU784416, DQ453694, AB211267, AJ629886 ; Palmer et al. 2008; Brock et al. 2009; Frank 2006; Nara 2006; Phosri et al 2007) having the highest degree of similarity (88% to 89%) with some sequences of *S. verrucosum* samples were added to the database with two *Pisolithus tinctorius* sequences (AF003915, AF374622 ; Martin et al. 1998, 2002) and two other sequences of *Astraeus odorata* (AJ629874, AJ629882; Phosri et al. 2007). One sequence of *Rhizopogon occidentalis* (FJ197207; Peay et al. 2009) was added as an outgroup.

Results

Description of sporocarps and spore ornamentation

Based on morphological characters of basidiocarps and spores, we identified six species from the collected specimens (Table 1). The specimens made up six species that were *S. dictyosporum* (in ten specimens), *S. verrucosum* (19 specimens), *Scleroderma* sp1 (four specimens), *Scleroderma* sp2 (15 specimens), *Scleroderma* sp3 (one specimen) and *Scleroderma* sp4 (three specimens). Spores of *S. dictyosporum*, *S. verrucosum*, *Scleroderma* sp1 and *Scleroderma* sp3 fell into two morphological types, i.e. reticulate (section *Scleroderma*) and spiny (section Aculeatispora) of the general types of *Scleroderma* spores as described by Sims et al. (1995). *Scleroderma* sp2 and *Scleroderma* sp4 presented particular spore morphology without ornamentation (Table 2, Figs. 2 and 3) and might be new species never described. During the collections between 1994 and 2000, we observed that the basidiocarps were not generally distributed in restricted areas or associated with particular host trees. The colours of the six different basidiocarps ranged from brown, dark-brown to fairly yellow. Their spores varied in sizes from 5 to 13 μm (Table 2).

Analysis of individual ITS and IGS1 restriction fragments

PCR analyses were performed on 52 samples consisting of 30 basidiocarps and 22 mycelial cultures. A low polymorphism of amplified ITS and IGS1 fragments were observed between species and within samples of the same species (data not shown). Amplified ITS and IGS1 ranged from 590 bp to 760 bp and from 440 bp to 550 bp, respectively. ITS and IGS1 regions of basidiocarps and mycelial cultures were digested with the endonucleases *Hinf*I and *Mbo*I. Patterns of the restrictions fragments with these endonucleases were further used to differentiate species into

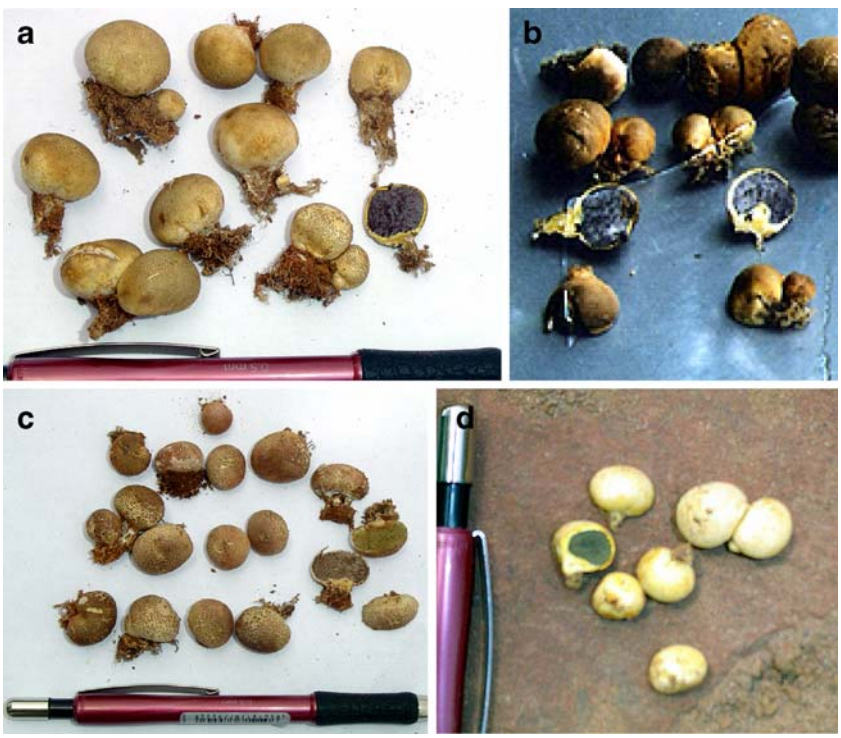

Fig. 2 Basidiocarps of *Scleroderma* spp. **a** *S. verrucosum*, **b** *S. dictyosporum*, **c** *Scleroderma* sp1, **d** *Scleroderma* sp2

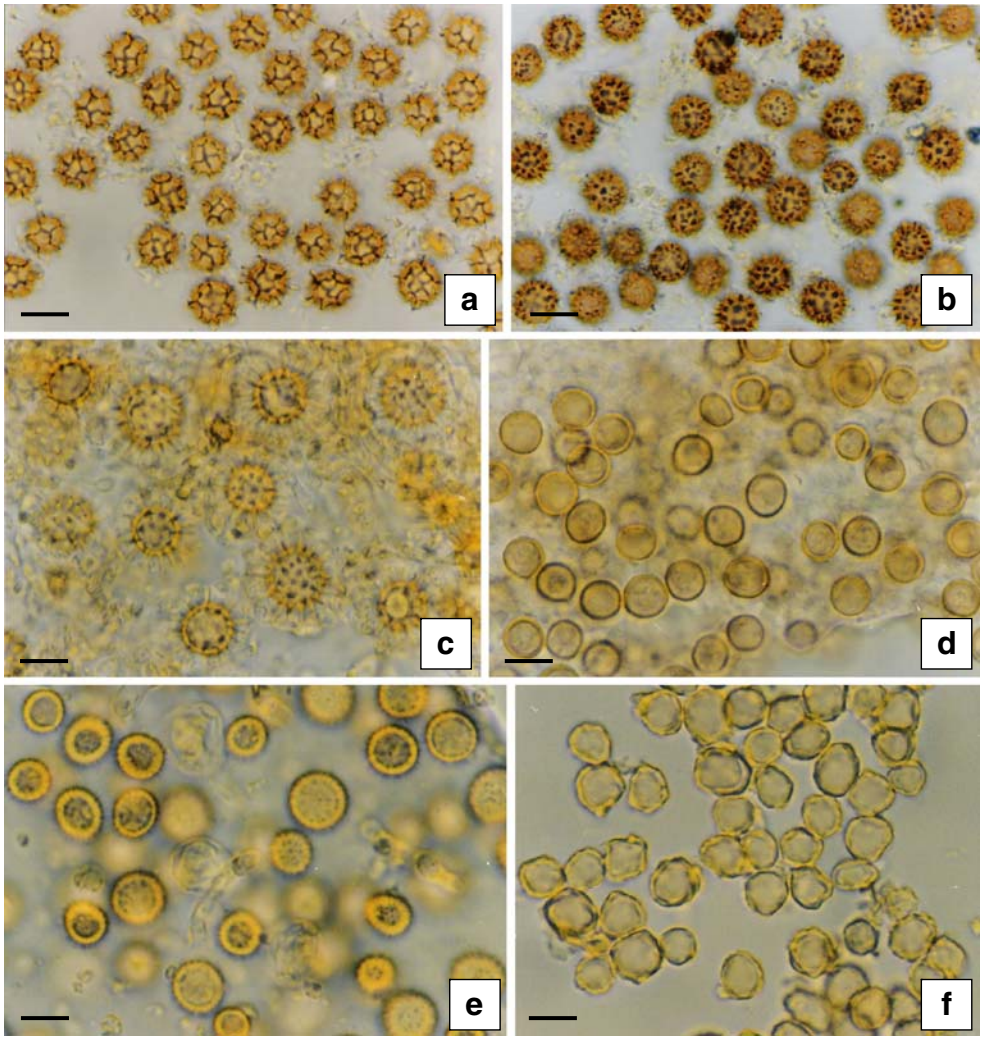

Fig. 3 Basidiospores of *Scleroderma* spp. under light microscope (100 x*g*). **a** *S. dictyosporum*; **b** *S. verrucosum*; **c** *Scleroderma* sp1; **d** *Scleroderma* sp2; **e** *Scleroderma* sp3; **f** *Scleroderma* sp4. Scale bar, 10 µm

ribotypes. Both ITS and IGS1 regions displayed eight common ribotypes referred to as A, B, C, D, E, F, G and H (Table 3). One to three ribotypes were obtained with *Hinf*I and *Mbo*I in each morphotype (Figs. 4a, b and 5a, b). Two ribotypes (A, B) were found in *S. dictyosporum*, three (C, D, E) in *S. verrucosum*, one (F) in *Scleroderma* sp1 and two (G, H) in *Scleroderma* sp2 (Table 3). Ribotype A was recognised in four mycelial cultures (IR109, IR250, IR412, IR602) and in four sporocarps (SD-2871, SD-3601, SD-2872, SD-6971) independent of host trees, geographical origin or year of collection. The ribotype B was found in one mycelial culture (IR215) and in one sporocarp (SD4901). Within *S. verrucosum*, the majority of samples (five mycelial culture and ten sporocarps) were found to belong to the ribotype C. The ribotypes D and E occurred in two mycelial cultures (IR500 and IR600) and basidiocarps (SV1801, SV1804), respectively (Figs. 4a, b and 5a, b, Table 3). The ribotype F of *Scleroderma* sp1 was found in three mycelial cultures (IR406, IR409, IR410) and one basidiocarp (SP1-4871). Digested *Scleroderma* sp2 samples by endonucleases generated two ribotypes, the first one (G) was found in two fungal cultures (IR100 and IR134) and one basidiocarp (SSP2-7901) while the second, (H), occurred in two other mycelial cultures (IR510 and IR263) and ten basidiocarps. The samples (mycelial culture and basidiocarps) of *Scleroderma* sp3 and *Scleroderma* sp4 showed similar RFLP patterns to those of *S. verrucosum* and *S. dictyosporum,* respectively (Table 3).

ITS sequence analysis

Of the 25 sequences obtained, nine were from sporocarps and 16 from mycelial cultures. Both strands of the amplified fragments (569 to 644 bp), containing ITS1, 5.8 S and ITS2 from seven of the eight ribotypes, were aligned with *Scleroderma*, *Pisolithus* and *Astraeus* species sequences from GenBank and with the outgroup *R. occidentalis* strain MF8 RT11-3 (Peay et al 2009). These sequences were corrected manually by excluding the 3'-end of the 18 S rDNA and the 5'-end of the 28 S rDNA, leading

Table 3 Size of the restriction fragments (in base pairs) of the rDNA ITS and IGS1 obtained with *Hinf*I and *Mbo*I. Mycelial cultures were referred to as IR and sporocarps to as SD, SV, SP1, SP2, SP3 or SP4

Species	ITS		IGS1		Ribotypes
	*Hinf*I	*Mbo*I	*Hinf*I	*Mbo*I	
S. dictyosporum					
(4IR and 4SD)	174, 127, 113, 87, 60	208, 159, 135, 119	143, 135, 128	228, 153, 86	A
1IR and 1SD	287, 240	218, 198, 167	160, 151, 82	280, 240	B
S. verrucosum					
(5IR and 10SV)	311, 246, 93	171, 162, 153, 119	182, 167, 119	238, 151, 54	C
(2IR)	285, 146, 110, 66	254, 146, 124, 98, 77	182, 147, 63	228, 178	D
(2SV)	264, 114, 104, 94	208, 159, 135, 92	182, 139, 119	238, 210	E
Scleroderma sp1					
(All samples)	322, 149, 110, 66	241, 153, 140, 119, 102	286, 143	228, 196, 54	F
Scleroderma sp2					
(1SP2 and 2IR)	331, 294, 87	284, 227, 91	232, 143, 88	228, 153	G
(10SP2 and 2IR)	206, 186, 99	152, 137, 113, 106	173, 139, 124	231, 209	H
Scleroderma sp3					
(1IR)	285, 146, 110, 66	254, 146, 124, 98, 77	182, 147, 63	228, 178	D
Scleroderma sp4					
(2IRand 1SP4)	174, 127, 113, 87, 60	208, 159, 135, 119	143, 135, 128	228, 153, 86	A

The numbers of voucher materials are indicated in Table 1. The total of restriction fragments were sometimes inferior to the amplified ITS or IGS1 because fragments smaller than 50 pb were not taken into account

to 643 bp matrix alignment. The alignment of sequences revealed a high degree of variability in ITS1 and ITS2 regions and a low degree of variability in 5.8 S region for our *Scleroderma* samples (data not shown). The 25 sequences were compared with the GenBank database using the algorithm Blastn to identify the most similar ITS sequences. Seven different genotypes were identified, and their partial sequences were deposited in GenBank (Table 4). However, these sequences showed little similarity with available sequences of *Scleroderma* in GenBank database (Table 4).

The constructed neighbor-joining tree split species analysed into three distinct clades (Fig. 6). The clade 1 included the morphological species *S. verrucosum* and *Scleroderma* sp1 while clade 2 contained *S. dictyosporum* morphotype and clade 3 consisted of *Scleroderma* sp2 morphotype. Within these three clades, the PCR/RFLP ribotypes were supported by the weak bootstrap values shown in the neighbor-joining base tree in Fig. 6, suggesting that the ribotypes identified within each morphological species might be considered as different phylogenetic species as for *Pisolithus* species (Martin et al. 2002). Hence, samples identified by morphological features as *S. verrucosum* included in fact three species (species 1, 2 and 3), two species for *S. dictyosporum* (species 1 and 2) and one species sequenced for *Scleroderma* sp2. In each clade, species were supported by high bootstrap value (100%).

Isolates of *Scleroderma* sp1, closely related to *S. verrucosum* with which they differed mainly by some deletions in ITS2, appear to belong to *S. verrucosum* morphotype (species 4). The two previously unidentified species (*Scleroderma* sp3, IR252 and *Scleroderma* sp4) presented a high degree of sequence homology (100% bootstrap) with the species 2 of *S. verrucosum* morphotype (IR252) and species 2 of *S. dictyosporum* morphotype (IR408; Fig. 6).

Discussion

The hypothesis in the present study was whether distinctive morphotypes of basidiocarps and mycelial cultures reflected variations in genetic and phylogenetic analyses of rDNA ITS and IGS1. We used a combination of three approaches, i.e. morphotyping, PCR/RFLP analyses and ITS sequencing, to investigate genetic and phylogenetic relationships between *Scleroderma* species collected from woodlands in Burkina Faso. We characterised them according to their morphological features, and then identified six species of *Scleroderma* from the 52 specimens collected from 20 sites (Table 1). PCR/RFLP variations in the ITS and IGS1 regions were used to discriminate these six *Scleroderma* morphotypes. After digestion of ITS and IGS1 with *Hinf* I and *Mbo*I, each of the six morphotypes produced one to

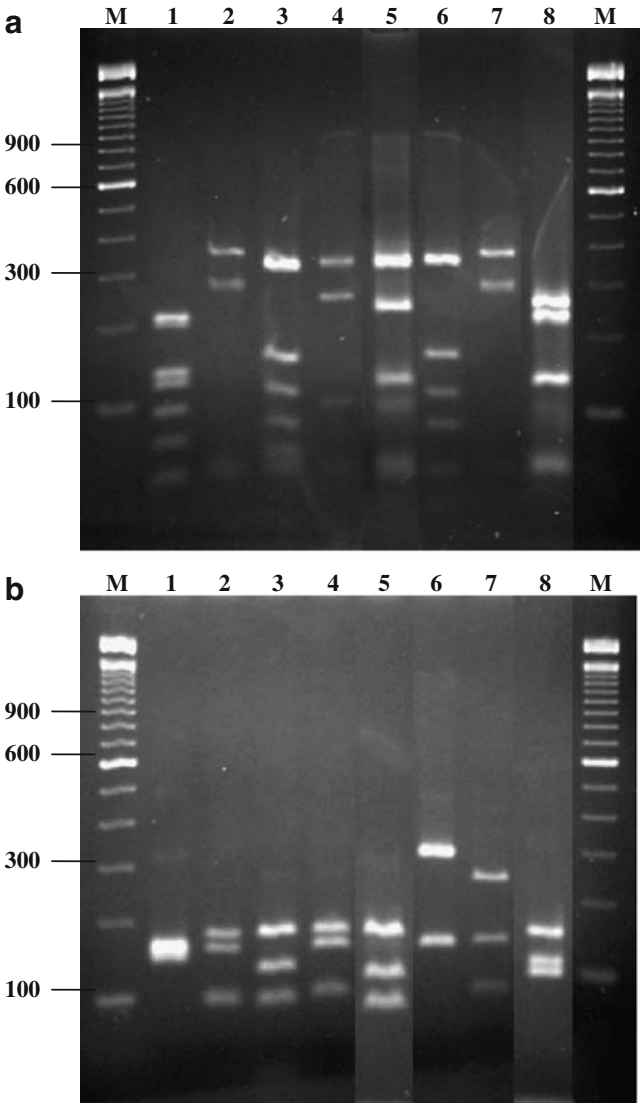

Fig. 4 Agarose gels of ITS (**a**) and IGS1 (**b**) of *Scleroderma* digested with *Hinf*I. (*M*) represents 100 bp ladder. The *bands* (*1*) and (*2*) are for *S. dictyosporum*; *bands* (*3*), (*4*) and (*5*) represent *S. verrucosum*; *band* (*6*) *Scleroderma* sp1 and *bands* (*7*) and (*8*) stand for *Scleroderma* sp2

three distinct patterns referred to as ribotypes with two to five restricted fragments per pattern. In this respect, the number of ribotypes was greater than the number of morphotypes. Eight patterns were identified for each rDNA region. Identical ribotypes were observed in samples assigned to different morphotypes. Two morphotypes, *Scleroderma* sp3 and *Scleroderma* sp4 showed the same patterns as ribotype D of *S. verrucosum* and ribotype A of *S. dictyosporum*. We therefore suggested that *Scleroderma* sp3 and *Scleroderma* sp4 are species of *S. verrucosum* and *S. dictyosporum*, respectively. We also found that sporocarps of *Scleroderma* sp3 were closely related to *S. verrucosum* while *Scleroderma* sp4 was morphologically very different from *S. dictyosporum*. However, one should note that the morphological characterisations of basidiocarps and spores were based on one single specimen in the case of *Scleroderma* sp3 (Table 1).

The IGS region has been reported to be a more intraspecific variable than ITS (James et al. 2001; Kim et al. 2006; Sha et al. 2007). The polymorphism of our samples of *Scleroderma* revealed by ITS restriction fragments was confirmed by IGS1 digestion with the two endonucleases, as also observed for other species of fungi (Henrion et al. 1992; Erland et al. 1994; Di Battista et al. 1996). However, when monitoring isolates, either introduced in nursery and/or in the field, it is important to identify specific markers. It would have been therefore interesting to test other markers, e.g. IGS2 RFLP and sequencing, in order to allow characterising isolates within ribotypes as suggested by Zhang et al. (2006).

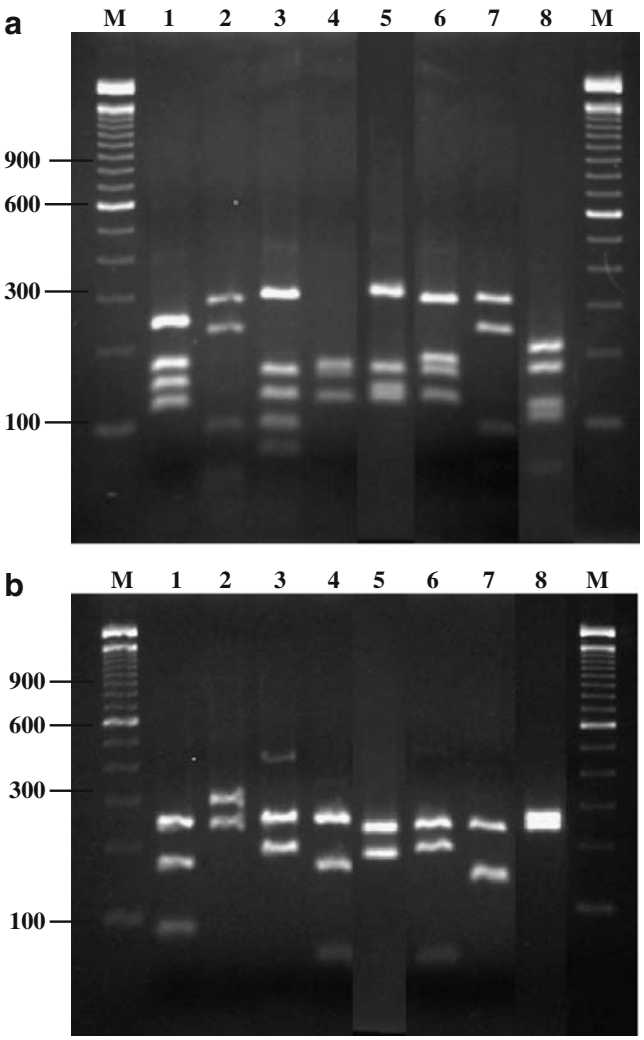

Fig. 5 Agarose gels of ITS (**a**) and IGS1 (**b**) of *Scleroderma* digested with *Mbo*I. (*M*) represents 100 bp ladder. The *bands* (*1*) and (*2*) are for *S. dictyosporum*; *bands* (*3*), (*4*) and (*5*) represent *S. verrucosum*; *band* (*6*) *Scleroderma* sp1 and *bands* (*7*) and (*8*) stand for *Scleroderma* sp2

Table 4 Sporocarps and mycelial cultures of *Scleroderma* species studied with their accession number and best aligned fungal sequence in GenBank

Species/ribotypes/N° of voucher material	GenBank accession no.	Best ITS match in GenBank (accession no.; size of compared base pairs and identity)
S. dictyosporum		
Ribotype A		
IR109	FJ840442	*S. areolatum* voucher JMP0080 (EU819438; 496/583, 85%)
IR250	FJ840444	*S. areolatum* voucher JMP0080 (EU819438; 475/563, 84%)
IR412	FJ84044	*S. areolatum* voucher RT00036 (EU819518; 439/513, 85%)
IR602	FJ840447	*S. areolatum* voucher JMP0080 (EU819438; 499/587, 85%)
IR408	FJ840445	*S. areolatum* voucher RT00036 (EU819518; 439/513, 85%)
ORS7731	FJ840448	*S. areolatum* voucher RT00036 (EU819518; 547/665, 82%)
Ribotype B		
IR215	FJ840443	*S. bovista* voucher RT00034 (EU81951; 423/503, 84%)
SD-4901	FJ840449	*S. areolatum* voucher JMP0080 (EU819438; 378/438, 86%)
S. verrucosum		
Ribotype C		
IR256	FJ840454	*S. cepa* voucher SOC541 (DQ453694; 545/615, 88%)
IR114	FJ840451	*S. cepa* voucher SOC541 (DQ453694; 579/654, 88%)
IR110	FJ840450	*S. cepa* voucher SOC541 (DQ453694; 584/661, 88%)
IR118	FJ840452	*S. cepa* voucher SOC541 (DQ453694; 583/661, 88%)
SV1803	FJ840458	*S. cepa* voucher SOC541 (DQ453694; 586/665, 88%)
SV2802	FJ840460	*S. cepa* voucher SOC541 (DQ453694; 589/665, 88%)
SV5602	FJ840461	*S. cepa* voucher SOC541 (DQ453694; 545/614, 88%)
Ribotype D		
IR500	FJ840455	Sclerodermataceae sp. TU103614 (AM412304; 544/613, 88%)
IR600	FJ840456	Sclerodermataceae sp. TU103614 (AM412304; 541/611, 88%)
IR252	FJ840453	Sclerodermataceae sp. TU103614 (AM412304; 570/642, 88%)
Ribotype E		
SV1801	FJ840457	*S. verrucosum* strain VERSCLE-4 (AJ629886; 488/546, 89%)
SV1804	FJ840459	*S. verrucosum* strain VERSCLE-4 (AJ629886; 489/546, 89%)
Scleroderma sp1		
Ribotype F		
IR406	FJ840462	*S. cepa* voucher SOC541 (DQ453694; 551/630, 87%)
IR410	FJ840463	*S. cepa* voucher SOC541 (DQ453694; 530/602, 88%)
Scleroderma sp2		
Ribotype H		
SSP2-1806	FJ840464	*S. bovista* strain: Nara_ScB84e3 (AB211267; 577/618, 83%)
SSP2-2803	FJ840465	*S. bovista* strain: Nara_ScB84e3 (AB211267; 498/592, 84%)
SSP2-3901	FJ840466	*S. bovista* strain: Nara_ScB84e3 (AB211267; 484/585, 82%)

Mycelial cultures were referred to as "IR" and sporocarps as SD, SV, SP1, SP2, SP3 or SP4

Morphotyping and grouping of morphotypes by RFLP analyses showed that these species could not be clustered per specimen from different host trees except *Scleroderma* sp1, which was specifically collected only in riverside forest and especially under *U. guineensis* (Phyllanthaceae) and *B. grandiflora* (Caesalpinioideae). This suggested that *Scleroderma* sp1 has its microhabitat preference where it propagates. Basidiocarps or mycelial culture of morphotypes harvested in different years but from the same sites showed identical ribotypes. There was a poor correlation between the occurrence of morphotypes and ribotypes. Morphotypes that occurred in different host trees were comparable in spite that they belonged to different ribotypes.

Our samples of *Scleroderma* did not present morphological or genetic features that correlated to their host trees.

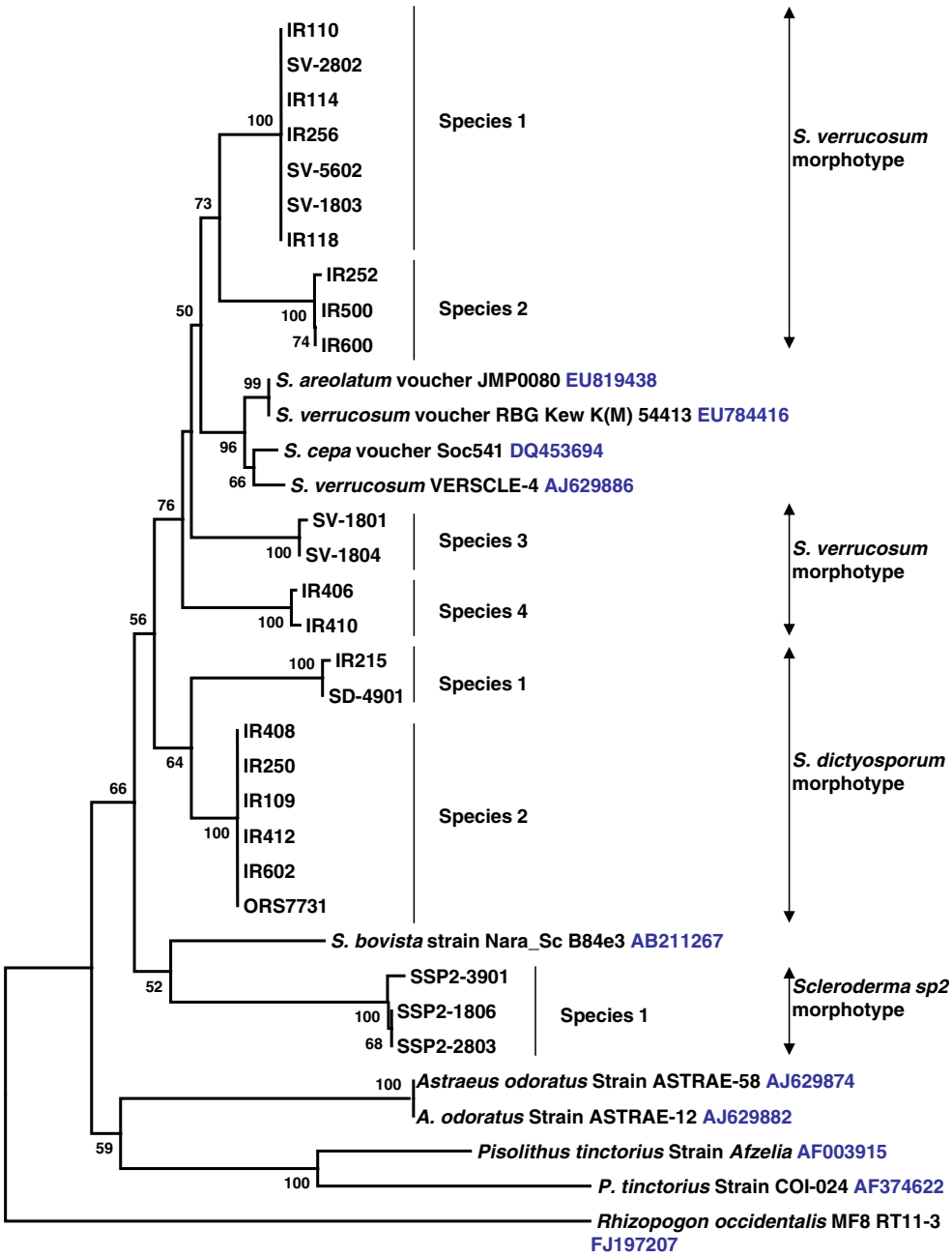

Fig. 6 Neighbor-joining phylogenetic tree of *Scleroderma* species based on ITS sequences. *R. occidentalis* is used as outgroup. Numerical values on the branches are the bootstrap values of 1,000 replications

Isolates from different host trees (Caesalpinioideae, Phyllanthaceae and Dipterocarpaceae) and from different collection sites as far distant as 2 to 1000 km from each other (Diapaga in East to Tenakourou in South-west), presented identical RFLP patterns. Although only two basidiocarps were analysed, ribotype E of *S. verrucosum* appeared to be specific to the tree species *Afzelia africana*. These results suggested that, even though *Scleroderma* species showed identical genetic pattern, they displayed different morphological basidiospores and basidiocarps. Thus, it is difficult to distinguish between *Scleroderma* species based only on fruiting organs and basidiospore morphology. We examined a small number of samples of only one culture of *Scleroderma* sp3 (IR252) and four samples of *Scleroderma* sp4. This was limited to allow us to assess whether the fruiting organ morphology was susceptible to environmental influences or not.

In the present investigations, PCR/RFLP analyses allowed a clear separation of isolates of *S. dictyosporum* and *S. verrucosum* morphotypes. The results confirmed those shown in the previous study carried out on samples, also originated from Burkina Faso (Sanon 1999). In addition, the main ribotype of *S. dictyosporum* (A) was also observed in another isolate (ORS7731) of the same

species that were collected under *A. africana* in Senegal (Bâ 1990; Sanon 1999). Four isolates of *S. verrucosum* harvested beneath *Afzelia africana* tree from Guinea were also identified as *S. verrucosum*, ribotype D (Sanon, unpublished data). This indicates that isolates from different basidiocarps could generate from the same vegetative clones.

Phylogenetic analyses of ITS sequences of 25 mycelial cultures and basidiocarps showed that *Scleroderma* species analysed were clustered in three distinct clades. The neighbor-joining tree clearly separated the different morphotypes with terminal clades, grouping samples referred to phylogenetic species that were supported each by high bootstrap value (100%). Within each morphotype, pairwise distance between species varies from 7% for *S. dictyosporum* to 9% for *S. verrucosum*. There was no correlation between the different morphotypes, the host-trees and the three clades. This contrasted with *Pisolithus* species, where sequenced polymorphism was correlated with basidiocarps morphology, host range and geographic origin (Martin et al. 1998, 2002). The results obtained from ITS sequence analyses allowed identifying seven phylogenetic species corresponding to the seven PCR/RFLP ribotypes. Hence, the morphological descriptions used were insufficient to differentiate these species.

Scleroderma sp3 (IR252) and *Scleroderma* sp4 (IR408) were supported by 100% bootstrap with species 2 of *S. verrucosum* and *S. dictyosporum* morphotypes, respectively. These data confirmed the results obtained with PCR/RFLP analyses and showed that *Scleroderma* sp3 and *Scleroderma* sp4 were morphotypes of *S. verrucosum* and *S. dictyosporum*, respectively. *Scleroderma* sp1 is genetically closer to species *S. verrucosum* than to any other species with 76% bootstrap value and therefore belongs to the *S. verrucosum* clade. By contrast, *Scleroderma* sp2 is genetically distant from other *Scleroderma* species analysed, but it was grouped with *Scleroderma* genus. The specific morphology of its spores suggests that it could be a new *Scleroderma* species which would be specific in tropical Africa. Nevertheless, it would be important to study in detail more samples of this potential new species. The *Scleroderma* sequences from GenBank, although grouped into *S. verrucosum* and *Scleroderma* sp2 clades, did not show any significant homology with our samples (73% and 52%).

The populations within *S. dictyosporum*, *S. verrucosum* and *Scleroderma* sp2 were remarkably homogeneous considering distances (of 2 to 1000 km) that separated sites in which the different mycelial cultures and basidiocarps were harvested. This molecular homogeneity of the populations of *Scleroderma* from Burkina Faso could partly be explained by a predominance of vegetative multiplication, as opposed to sexual reproduction. It would have been interesting to determine by what mechanisms, for example, sexual incompatibility, this homogeneity persists into these populations of Sclerodermatales. The morphological criteria which we used to identify the *Scleroderma* species were insufficient to highlight the phylogenetic species. These data show the limits of morphological criteria and the importance of genetic and phylogenetic characteristics for the identification of *Scleroderma* species as for *Pisolithus* species (Martin et al. 2002).

Nonetheless, the approach of using combined species morphology, molecular tools (PCR-RFLP, ITS sequencing) and the phylogenetic analyses paved the way for the identification of larger populations of *Scleroderma* species from Africa and other tropical zones.

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