Turkish Journal of Botany
http://journals.tubitak.gov.tr/botany/
Research Article

Turk J Bot
(2014) 38: 375-385
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doi:10.3906/bot-1301-36

Rechecking of the genus *Scleroderma* (Gasteromycetes) from Macedonia using barcoding approach

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Received: 16.01.2013 • **Accepted:** 04.12.2013 • **Published Online:** 17.01.2014 • **Printed:** 14.02.2014

Abstract: In order to verify some doubtful collections of *Scleroderma* Pers. species from Macedonia, 54 specimens were revised using morphological and molecular analyses. The 12 internal transcribed spacer of nuclear ribosomal DNA (ITS nrDNA) sequences obtained in this study were compared with previously published sequences included in public databases. According to the barcoding sequences, some Macedonian *Scleroderma* specimens were confirmed to belong to 4 species: *S. areolatum* Ehrenb., *S. bovista* Fr., *S. meridionale* Demoulin & Malençon, and *S. verrucosum* (Vaill.) Pers.

Key words: Sclerodermatales, taxonomy, molecular identification, ITS nrDNA

1. Introduction

Scleroderma Pers. species are ectomycorrhizal with a worldwide distribution. The genus *Scleroderma* was erected by Persoon (1801) with 11 species. Guzmán (1970) cited 21 species and the revision by Sims et al. (1995) included 25 species. Phosri et al. (2009), based on morphological characters compared with internal transcribed spacer of nuclear ribosomal DNA (ITS nrDNA) analyses of a number of *Scleroderma* spp. from different geographical origins, observed a good relationship among phylogenetic clades and basidiospore ornamentation. In Macedonia, 3 species have been cited frequently: *Scleroderma areolatum* Ehrenb. (Karadelev et al., 2003b; Karadelev and Rusevska, 2004), *S. citrinum* Pers. (Karadelev, 2000; Karadelev et al., 2002b, 2003a, 2004), and *S. verrucosum* (Vaill.) Pers. (Pilát and Lindtner, 1939; Tortić, 1988; Karadelev et al., 2002a; Rusevska and Karadelev, 2004). Karadelev et al. (2008) included a total of 8 species for Macedonia [including *Scleroderma bovista* Fr., *S. cepa* Pers., *S. meridionale* Demoulin & Malençon, *S. polyrhizum* (J.F.Gmel.) Pers., and *S. septentrionale* Jeppson]; this is a number quite similar to those of other Mediterranean areas, such as in Catalonia (6 species, Martín, 1988) and the rest of the Iberian Peninsula (7 species, Calonge, 1998), as well as in Asia Minor (7 species, Sesli and Denchev, 2008; Kaya, 2009; Demirel et al., 2010; Alli, 2011; Doğan et al. 2012; Solak et al., 2013), while only 1 species (*S. verrucosum*) is known from the European part of Turkey (Stojchev et al., 1998).

Being in the barcoding era, we aim to exploit this approach and clarify the diversity of the *Scleroderma* species mentioned by Karadelev et al. (2008), rechecking the 54 specimens mentioned in this paper under morphological and molecular analysis of ITS nrDNA and following the methodology described by Phosri et al. (2009).

2. Materials and methods

2.1. Taxon sampling and morphological studies

All collections are deposited in the Macedonian Collection of Fungi (MCF) at the Mycological Laboratory, Institute of Biology, Faculty of Natural Sciences and Mathematics, Ss. Cyril and Methodius University (Table 1). The morphological revision of the specimens has been done according to Guzmán (1970), Jülich (1984), Breitenbach and Kränzlin (1986), Pegler et al. (1995), Hansen and Knudsen (1997), Calonge (1998), and Krieglsteiner (2000). The most important morphological features of the basidiomata defining the genus *Scleroderma* are spore morphology (size, ornamentation), peridium (thickness, scaliness), and

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Table 1. Total Macedonian collection of *Scleroderma* included in this study. Complete data for each specimen including accession number in MCF, country name, precise locality, altitude (meters above sea level, m a.s.l.), habitats, date of collection, and results of molecular analyses are given. Asterisk indicates the new sequences obtained in this study.

Taxon name	Specimen ID	Country	Locality	Habitat description	Date collected	Molecular results
Scleroderma areolatum	MCF 99/2293	Macedonia	Jakupica Mountain: Orashche village, 800–900 m a.s.l.	*Quercetum frainetto-cerris*	15-Jul-1999	Contamination: *Candida* spp.
S. areolatum	MCF 02/4202	Macedonia	Osogovski Planini Mountain: Konopnica village, 1100 m a.s.l.	*Quercus* and *Fagus* forest	3-Sep-2002	HF933231*
S. areolatum	MCF 04/4954	Macedonia	Baba Mountain (Pelister): Rotino village, 1000 m a.s.l.	Pasture	26-Oct-2004	Not PCR product
S. areolatum	MCF 05/5300	Macedonia	Skopje (vicinity): Vodno, near Krushopek village, 800 m a.s.l.	Deciduous forest	16-Oct-2005	Not PCR product
S. areolatum	MCF 05/5455	Macedonia	Skopska Crna Gora Mountain: Ljubanci vill., around St. Nikola monastery, 800 m a.s.l.	*Querco-Carpinetum orientalis*	9-Oct-2009	Contamination: *Candida* spp.
S. areolatum	MCF 07/8552	Macedonia	Dobra Voda Mountain: Jagol village (above)	*Quercetum frainetto-cerris*	1-Nov-2007	Not PCR product
S. bovista	MCF 98/4525	Macedonia	Kozhuf Mountain: Oreovica village (above), 850 m a.s.l.		19-Jul-1998	Contamination: *Candida* spp.
S. bovista	MCF 01/168	Macedonia	Baba Mountain (Pelister): Gjavato, 1000 m a.s.l.	*Abieti-Fagetum*	9-Oct-2001	HF933234*
S. bovista	MCF 05/788	Macedonia	Kozhuf Mountain: Smrdliva Voda, 800 m a.s.l.	*Fagus* forest	23-Oct-2005	HF933235*
S. bovista	MCF 05/5304	Macedonia	Jablanica Mountain: Gorna Belica village (below), 1300 m a.s.l.	*Fagus* forest	16-Oct-2005	HF933236*
S. bovista	MCF 06/330	Macedonia	Bogdanci: Bolovan, 250 m a.s.l.	*Juglando-Platanetum orientalis*	11-Nov-2006	Not PCR product
S. bovista	MCF 09/11184	Serbia	Leskovac town (vicinity): Vuchje	Edge of deciduous forest	12-Sep-2009	HF933242*
S. cepa	MCF 01/5503	Macedonia	Baba Mountain (Pelister): Gjavato, 1000 m a.s.l.	*Abieti-Fagetum*	9-Oct-2011	Contamination: *Candida* spp.
S. cepa	MCF 05/800	Macedonia	Galichica Mountain: Trpejca village		22-Oct-2005	Contamination: *Candida* spp.
S. cepa	MCF 05/5161	Macedonia	Skopska Crna Gora Mountain: Ljubanci vill., around St. Nikola monastery, 800 m a.s.l.	At roadsides, grassy place; silicate soil	11-Sep-2005	Contamination: *Candida* spp.
S. cepa	MCF 06/6180	Macedonia	Skopje city: Gazi Baba, Botanical Garden (Faculty of Natural Science and Mathematics), 250 m a.s.l.	Park	20-Oct-2006	Contamination: *Candida* spp.
S. cepa	MCF 07/8290	Macedonia	Valandovo: Chalakli village, 200 m a.s.l.	*Juniperus excelsa* forest	26-Oct-2007	Contamination: *Candida* spp.
S. cepa	MCF 07/8355	Macedonia	Tetovo town (vicinity)		Nov-2007	Contamination: *Candida* spp.
S. cepa	MCF 08/4530	Macedonia	Dobra Voda Mountain: Gorica, west of Popovjane village, 800–850 m a.s.l.	*Quercetum frainetto-cerris*	8-Nov-2008	Contamination: *Candida* spp.
S. cepa	MCF 09/10600	Macedonia	Skopska Crna Gora Mountain: Kuchevishte village, St. Arhangel monastery, 700 m a.s.l.	Roof construction, old guest house	12-May-2005	Not PCR product

Table 1. (Continued).

Taxon name	Specimen ID	Country	Locality	Habitat description	Date collected	Molecular results
S. cf. septentrionale	MCF 06/5993	Macedonia	Skopska Crna Gora Mountain: Ljubanci vill., above St. Nikola monastery, 900 m a.s.l.	Oak forest with *Castanea* plantings	29-Aug-2006	HF933240*
S. citrinum	MCF 89/4709	Macedonia	Bogdanci (vicinity), 150 m a.s.l.	Meadow	5-Jul-1989	HF933237*
S. citrinum	MCF 02/5498	Macedonia	Nidze Mountain: r. Prava Reka, 1400 m a.s.l.	*Pinetum sylvestris macedonicum*	16-Jul-2002	Contamination: *Candida* spp.
S. citrinum	MCF 02/7981	Macedonia	Dobra Voda Mountain: between Tuin village and Popovjane village, 800–1100 m a.s.l.	Oak forest	10-Oct-2002	Contamination: *Candida* spp.
S. citrinum	MCF 06/6069	Macedonia	Tetovo town, 450 m a.s.l.	Yard	11-Oct-2006	Contamination: *Candida* spp.
S. citrinum	MCF 06/6268	Macedonia	Skopska Crna Gora Mountain: Ljubanci vill., above St. Nikola monastery, 800–900 m a.s.l.	Edge of *Quercus* and *Carpinus* forest	29-Oct-2006	Contamination: *Candida* spp.
S. citrinum	MCF 06/6291	Macedonia	Skopje city: Gazi Baba, above Faculty of Natural Science and Mathematics, 250 m a.s.l.	Oak-planted forest	18-Oct-2006	Contamination: *Candida* spp.
S. meridionale	MCF 05/5505	Macedonia	Bogdanci, 150 m a.s.l.	Meadow in *Coccifero-Carpinetum orientalis*	12-Dec-2005	HF933238* HF933239*
S. meridionale	MCF 08/10263	Macedonia	Bogdanci: Strelishte, 150 m a.s.l.	*Coccifero-Carpinetum orientalis*	14-Nov-2008	Contamination: *Candida* spp.
S. meridionale	MCF 01/10050 (TR 2010)	Turkey	Ağacaltı village: Ihlara Vadisi	Near stream or river	16-Apr-2001	Contamination: *Candida* spp.
S. polyrhizum	MCF 03/4749	Macedonia	Bogdanci: Kuchalat (vicinity), 200–300 m a.s.l.	*Coccifero-Carpinetum orientalis*	2-Jan-2003	Contamination: *Candida* spp.
S. polyrhizum	MCF 05/1199	Macedonia	Skopska Crna Gora Mountain: Ljubanci village, Zgurovci, 800 m a.s.l.	Meadow	9-Oct-2005	Contamination: *Candida* spp.
S. polyrhizum	MCF 05/5507	Macedonia	Skopska Crna Gora Mountain: Ljubanci village, between St. Nikola monastery and Zgurovci, 800 m a.s.l.	Roadsides	2-Oct-2005	Contamination: *Candida* spp.
S. polyrhizum	MCF 05/5508	Macedonia	Skopska Crna Gora Mountain: Ljubanci village, Zgurovci, 800 m a.s.l.	Forest edges (*Querco-Carpinetum orientalis*)	2-Oct-2005	Contamination: *Candida* spp.
S. polyrhizum	MCF 07/8229	Macedonia	Dobra Voda Mountain: Jagol village (above), 850 m a.s.l.	Meadow in *Quercetum frainetto-cerris*	24-Oct-2007	Contamination: *Candida* spp.
S. polyrhizum	MCF 07/8354	Macedonia	Kichevo town: Rashtanski Pat	Meadow	6-Nov-2007	Contamination: *Candida* spp.
S. verrucosum	MCF 07/7984	Macedonia	Skopska Crna Gora Mountain: Ljubanci vill., near st. Nikola monastery, 800 m a.s.l.	At roadsides, near deciduous forest	Jan-2007	HF933232*
S. verrucosum	MCF 08/10124	Macedonia	Prespa Lake: Ezerani strict nature reserve, 860 m a.s.l.	*Salicetum*	10-Nov-2008	HF933233*
S. verrucosum	MCF 02/2804	Macedonia	Skopje city (vicinity): Vodno, 750–1000 m a.s.l.	*Querco-Carpinetum orientalis*	16-Oct-2002	Contamination: *Candida* spp.
S. verrucosum	MCF 02/3032	Macedonia	Kumanovo town: Skachkovce village, 500–700 m a.s.l.	*Querco-Carpinetum orientalis*	18-Nov-2002	Contamination: *Candida* spp.
S. verrucosum	MCF 04/4776	Macedonia	Demir Hisar: Smilevo village	Meadow	6-Nov-2004	Contamination: *Candida* spp.

Table 1. (Continued).

S. verrucosum	MCF 05/4303	Macedonia	Ograzhden Mountain: Suvi Laki, 1200 m a.s.l.	mixed forest (*Pinus*, *Quercus*, *Fagus*)	23-Oct-2005	Contamination: *Candida* spp.
S. verrucosum	MCF 05/5336	Macedonia	Kichevo town (vicinity), 800 m a.s.l.	Oak forest (*Quercus frainetto*)	10-Oct-2005	Not PCR product
S. verrucosum	MCF 05/5448	Macedonia	Skopska Crna Gora Mountain: Ljubanci village, Zgurovci, 800 m a.s.l.	Oak forest with *Castanea* plantings	2-Oct-2005	Not PCR product
S. verrucosum	MCF 06/5504	Macedonia	Skopska Crna Gora Mountain: Ljubanci vill., around St. Nikola monastery, 800 m a.s.l.	Oak forest	8-Jan-2006	Not PCR product
S. verrucosum	MCF 06/7265	Macedonia	Galichica Mountain: Pljuska, 1000 m a.s.l.	*Quercetum frainetto-cerris*	15-Sep-2006	HF933241*
S. verrucosum	MCF 07/7650	Macedonia	Skopje city: Gazi Baba, Botanical Garden (Faculty of Natural Science and Mathematics), 250 m a.s.l.	Park	19-Jun-2007	Contamination: *Candida* spp.
S. verrucosum	MCF 07/7987	Macedonia	Skopska Crna Gora Mountain: Ljubanci vill., above St. Nikola monastery, 800–900 m a.s.l.	Deciduous forest (*Carpinus*, *Cornus mas*, *Corylus avellana*)	23-Sep-2007	Not PCR product
S. verrucosum	MCF 08/10068	Macedonia	Skopska Crna Gora Mountain: Ljubanci vill., above St. Nikola monastery, 773 m a.s.l.	Oak forest (*Quercus frainetto*, *Q. petraea*, *Castanea*, *Carpinus*)	11-Oct-2008	Not PCR product
S. verrucosum	MCF 08/10264	Macedonia	Skopje city: Gazi Baba, Botanical Garden (Faculty of Natural Science and Mathematics), 250 m a.s.l.	Park	26-Nov-2008	Contamination: *Candida* spp.
S. verrucosum	MCF 08/10813	Macedonia	Galichica Mountain: Prchno Brdo		5-Nov-2008	Contamination: *Candida* spp.
S. verrucosum	MCF 08/10054	Macedonia	Skopje city: Gazi Baba, Botanical Garden (Faculty of Natural Science and Mathematics), 250 m a.s.l.	Park	16-Oct-2008	Not PCR product
S. verrucosum	MCF 09/10474	Macedonia	Kichevo town (vicinity): Krushino, 1000–1100 m a.s.l.	*Quercetum frainetto-cerris*	6-Jul-2009	Not PCR product
S. verrucosum	MCF 09/11237	Macedonia	Shar Planina Mountain: Varvara village (above), 830–900 m a.s.l.	Deciduous forest	17-Oct-2009	Not PCR product

presence of a stalk. Dried specimens were used for light microscope (LM) and scanning electron microscope (SEM) studies. Measurements and photographs were made from microscopic sections mounted in a 5% aqueous solution of potassium hydroxide (KOH) or Melzer's reagent and examined with a Nikon Eclipse 80*i* with digital camera Nikon Coolpix 6000. SEM studies were carried out after coating dried glebal samples in gold with the Balzers SCD 004 sputter coater with a Hitachi S-3000N SEM. The species names follow Index Fungorum (Kirk, 2013) and MycoBank (Stalpers and Cock, 2013).

2.2. Molecular methods

DNA extraction, amplification, and sequencing of the ITS regions including the 5.8S of the ribosomal RNA gene cluster followed the protocols mentioned by Phosri et al. (2009), with the primer pair ITS1F/ITS4 (White et al., 1990; Gardes and Bruns, 1993), and the cycling protocol described by Martín and Winka (2000). Aliquots of the purified products were mixed separately with the direct and reverse primers before sending them to Macrogen (South Korea) for sequencing. Sequences obtained in this study are included in Table 2 and marked with an asterisk.

Consensus sequences were assembled using Navigator Sequence comparison software (PerkinElmer Applied Biosystems) or Sequencher (Gene Codes Corporation Inc., Ann Arbor, MI, USA). Previous to the alignment, sequences were compared with homologous sequences from the EMBL/GenBank/DDBJ (Cochrane et al., 2011) using the BLASTn algorithm (Altschul et al., 1997). Multiple sequence alignment of the consensus sequences obtained in this study and homologous sequences from the EMBL/GenBank/DDBJ, mainly described by Phosri et al. (2009) (Tables 2 and 3), were performed using SEQAPP software (PerkinElmer Applied Biosystems). The alignment was optimised visually. Alignment gaps were indicated with "-" and ambiguous nucleotides were

Table 2. List of *Scleroderma* collections used in this study compared with collections published in Phosri et al. (2009). The DNA isolation code appears as in Figure 1. Asterisk after GenBank accession number indicates the new sequences obtained in this study.

Morphological identification/ name in GenBank	Molecular identification	DNA isolation code	Herbarium number	Geographical origin	Collection date	Gen bank acc. num.
Scleroderma areolatum	*S. areolatum*	02MCF4202Sar	MCF 02/4202	Macedonia	03.09.2002	HF933231*
S. areolatum	*S. areolatum*	ARESCL_1	E00278288	USA	05.09.1998	FM213351
S. areolatum	*S. areolatum*	ARESCL_2	E00278290	USA	05.09.1998	FM213352
S. areolatum	*S. areolatum*	ARESCL_3	E00278286	USA	08.09.2001	FM213353
S. bovista	*S. bovista*	01MCF168Sbov	MCF 01/168	Macedonia	09.10.2001	HF933234*
S. bovista	*S. bovista*	05MCF5304Sbov	MCF 05/5304	Macedonia	16.10.2005	HF933236*
S. bovista	*S. bovista*	05MCF788Sbov	MCF 05/788	Macedonia	23.10.2005	HF933235*
S. bovista	*S. bovista*	09MCF11184Sbov	MCF 09/11184	Serbia	19.09.2009	HF933242*
S. bovista	*S. bovista*	BOVSCL_1	BCN-MPM1989	Spain	12.07.1995	FM213340
S. cepa	*S. cepa*	CEPSCL_2	BCN-MPM2525	Spain	26.11.1995	FM213354
S. cepa	*S. cepa*	CEPSCL_5	E00278296	USA	21.09.2001	FM213355
S. cf. *bovista*	*S. septentrionale*	BOVSCL_2	BOVSCL_2	USA	03.10.1993	FM213339
S. cf. *septentrionale*	*S. bovista*	06MCF5993Ssepcf	MCF 06/5993	Macedonia	29.08.2006	HF93324*
S. cf. *septentrionale*	*S. septentrionale*	SEPSCL_1	E00278318	USA	14.10.2000	FM213337
S. citrinum	*S. verrucosum*	89MCF4709Scitcf	MCF 89/4709	Macedonia	05.07.1989	HF933237*
S. citrinum	*S. citrinum*	CITSCL_1	E00278300	USA	20.09.1985	FM213344
S. citrinum	*S. citrinum*	CITSCL_2	-	USA	22.09.2001	FM213345
S. citrinum	*S. citrinum*	UNSCL_2	SCL3	UK	28.01.2002	FM213333
S. citrinum	*S. citrinum*	UNSCL_3	SCL5	UK	28.01.2002	FM213334
S. citrinum	*S. citrinum*	UNSCL_4	SCL7	UK	28.01.2002	FM213335
S. meridionale	*S. meridionale*	05MCF5505Smer	MCF 05/5505	Macedonia	12.12.2005	HF933238* HF933239*
S. michiganense	*S. michiganense*	MICSCL_1	E00278306	USA	14.09.1991	FM213346
S. michiganense	*S. michiganense*	MICSCL_2	E00278311	USA	05.09.1998	FM213347
S. michiganense	*S. michiganense*	MICSCL_3	E00278309	USA	30.08.1992	FM213348
S. polyrhizum	*S. polyrhizum*	POLSCL_1	E00278315	USA	23.09.2001	FM213349
S. polyrhizum	*S. polyrhizum*	POLSCL_2	E00278313	USA	10.10.2000	FM213350
S. septentrionale	*S. septentrionale*	SEPSCL_2	J Nitare (12.09.1986)	Sweden	12.09.1986	FM213336
S. septentrionale	*S. septentrionale*	SEPSCL_3	AD Parker (02.10.1997)	USA	02.10.1997	FM213338
S. sinnamariense	*S. sinnamariense*	SINSCL_1	SCLK4	Thailand	28.01.2002	FM213356
S. sinnamariense	*S. sinnamariense*	SINSCL_2	SCLP3	Thailand	28.01.2002	FM213357
S. sinnamariense	*S. sinnamariense*	SINSCL_3	SCLN	Thailand	28.01.2002	FM213358
S. sinnamariense	*S. sinnamariense*	SINSCL_4	SCLY5	Thailand	28.01.2002	FM213359
S. sinnamariense	*S. sinnamariense*	SINSCL_5	SC1	Thailand	28.01.2002	FM213360
S. sinnamariense	*S. sinnamariense*	SINSCL_6	SCLD1	Thailand	28.01.2002	FM213361
S. sinnamariense	*S. sinnamariense*	SINSCL_7	SINSCL_7	Thailand	12.08.2001	FM213362
S. sinnamariense	*S. sinnamariense*	SINSCL_8	SINSCL_8	Thailand	22.08.2001	FM213363
S. sinnamariense	*S. sinnamariense*	SINSCL_9	SINSCL_9	Thailand	24.08.2001	FM213364
Scleroderma sp.	*S. septentrionale*	UNSCL_5	E00278667	USA	25.09.1993	FM213342
Scleroderma sp.	*S. cepa*	UNSCL_7	-	Thailand	11.12.2001	FM213343
Scleroderma sp.	*S. bovista*	UNSCL_8	MJ6006	Hungary	01.11.2002	FM213341
S. verrucosum	*S. verrucosum*	06MCF7265Sver	MCF 06/7265	Macedonia	15.09.2006	HF933241*
S. verrucosum	*S. verrucosum*	07MCF7984Sver	MCF 07/7984	Macedonia	01.01.2007	HF933232*
S. verrucosum	*S. verrucosum*	08MCF10124Sver	MCF 08/10124	Macedonia	10.11.2008	HF933233*
S. verrucosum	*S. verrucosum*	VERSCL_4	BCN-MPM2605	Spain	29.09.1996	AJ629886

Table 3. The information for the sequences of *Scleroderma* included in Figure 1 obtained from GenBank DNA database not obtained by Phosri et al (2009).

Name in GenBank	Molecular identification	Geographical origin	Gen bank acc. num.
Scleroderma areolatum	*S. areolatum*	England	EU784408
S. bovista	*S. bovista*	Japan	AB211267
S. bovista	*S. bovista*	Japan	AB099901
S. cepa	*S. cepa*	USA	DQ453694
S. citrinum	*S. meridionale*	Spain	AY935514

marked as "N". A sequence of *Pisolithus arhizus* (Scop.) Rauschert (FM213365; *Pisolithus* sp. in Phosri et al., 2009) was used as an outgroup since species of this genus are closely related to *Scleroderma* (Watling, 2006).

2.3. Phylogenetic analyses

The alignment was analysed under a heuristic search, using the programme PAUP 4.0b10 (Swofford, 2003), and under a Bayesian approach (Larget and Simon, 1999; Huelsenbeck et al., 2000) assuming an HKY+G model using the programme MrBAYES 3.0 (Huelsenbeck and Ronquist, 2001), as described by Phosri et al. (2009). The phylogenetic tree was drawn with the programme TreeView (Page, 1996) and edited in Adobe Illustrator CS3; names of clades and subclades are according to Phosri et al. (2009).

3. Results

Genomic DNA concentration ranged from 1.5 to 15 ng/µL. A total of 62 polymerase chain reactions (PCRs) were performed, where 2 of them were weak (concentration of less than 10 ng/µL) and were not purified for sequencing. In general, after purification of the amplified product, if the DNA concentration was greater than 20 ng/µL, the sample was sequenced directly (Macrogen). From the 42 consensus sequences, 29 were suspected of being contaminated, such as sequences obtained from collections 08MCF10263 Smer (*Scleroderma meridionale*) and 08MCF10264Sver (*S. verrucosum*) (Table 1) that always gave a higher BLAST score (98%) associated with 1 sequence of *S. areolatum* [EU784408, collection K(M)88037 from Kew RBG], but the rest of the sequences with high BLAST scores were *Candida* species (such as FM178338). Since sequence EU784408 from GenBank was also suspected of contamination, sequences obtained in this study and with this kind of BLAST result were excluded from the molecular analyses.

The *Scleroderma* ITS region was successfully amplified from 12 dried basidiomes using the ITS1F/ITS4 primer pair (Table 2). The total length of the *Scleroderma* sequences, including the ITS1, 5.8S, and ITS2 genes and small flanking regions of the SSU and LSU genes, ranged from around 624 to 710 bp. The new *Scleroderma* sequences obtained in this study were submitted to the international database (EMBL) with accession numbers as provided in Table 2.

The ITS nrDNA dataset contains 49 *Scleroderma* sequences and a variable region of 66 bp (excluded in the phylogenetic analyses). Out of the total 884 positions, 625 were constant, 69 variable parsimony-uninformative, and 190 parsimony-informative. In the phylogenetic analysis under heuristic search, the 100 most parsimonious trees were obtained [tree length = 435 steps long, consistency index (CI) = 0.7195, retention index (RI) = 0.9316, rescaled consistency index (RC) = 0.6703]. The 50% majority-rule tree of the Bayesian analysis (Figure 1) has a similar topology to the parsimony strict consensus tree (data not shown) and fits with the results of Phosri et al. (2009). In this tree, Macedonian *Scleroderma* collections appeared mainly in 4 well-supported terminal subclades: *S. areolatum*, *S. bovista*, *S. meridionale*, and *S. verrucosum*. Figure 2 shows the basidioma morphology of selected specimens of each subclade, and the spore morphology under LM and SEM is shown in Figures 3–4.

In the *Scleroderma areolatum* subclade [bootstrap support (BS) = 95%, posterior probability (PP) = 1.0] appeared 1 sequence morphologically identified as *S. areolatum* (02MCF4202 Sare), with 3 sequences from US specimens, and in the *S. verrucosum* subclade (BS = 86%, PP = 0.89), 3 Macedonian sequences appeared previously identified as *S. verrucosum*, with 2 sequences from Spanish specimens. In Clade I, a Macedonian collection group was close to the *S. verrucosum* subclade, although morphologically it was identified as *S.* cf. *citrinum* (89MCF4709 Scitcf). As shown in Figure 3 and 4, specimens of these subclades had echinulate spores.

In the *Scleroderma meridionale* subclade (BS = 100%, PP = 1.00) appeared 2 sequences from 1 specimen morphologically identified as *S. meridionale* (05MCF5505_1_Smer and 05MCF5505_2_Smer); these sequences grouped together with a GenBank sequence

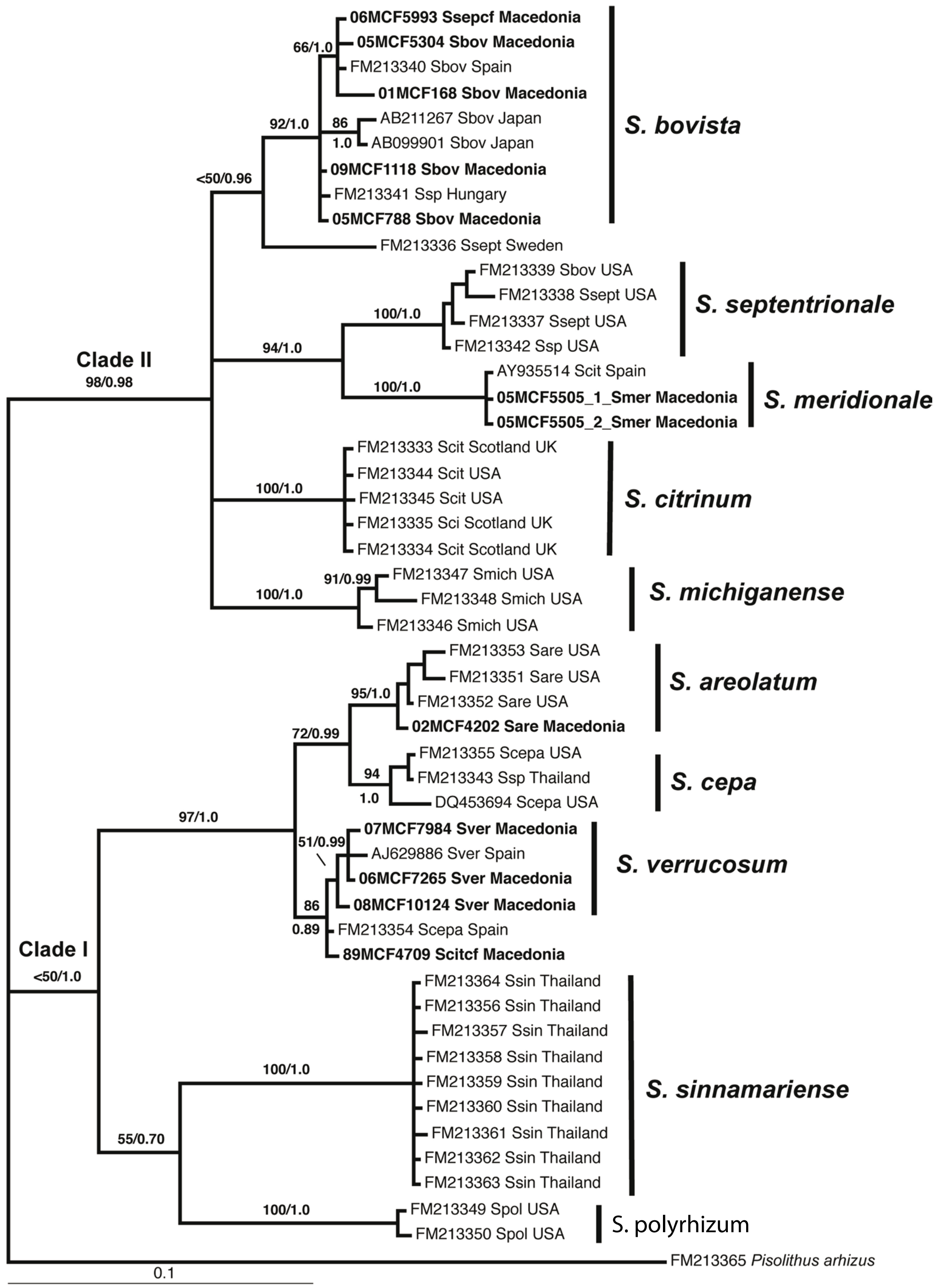

Figure 1. The 50% majority-rule consensus tree of Bayesian analysis inferred from ITS nrDNA sequences from *Scleroderma* specimens from Macedonia (indicated with the DNA isolation code), as well as sequences retrieved from the GenBank included in Tables 1 and 3. Bootstrap and posterior probabilities values are indicated on the branches. *Pisolithus arhizus* was included as outgroup. Names of main clades (I and II) are according to Phosri et al. (2009).

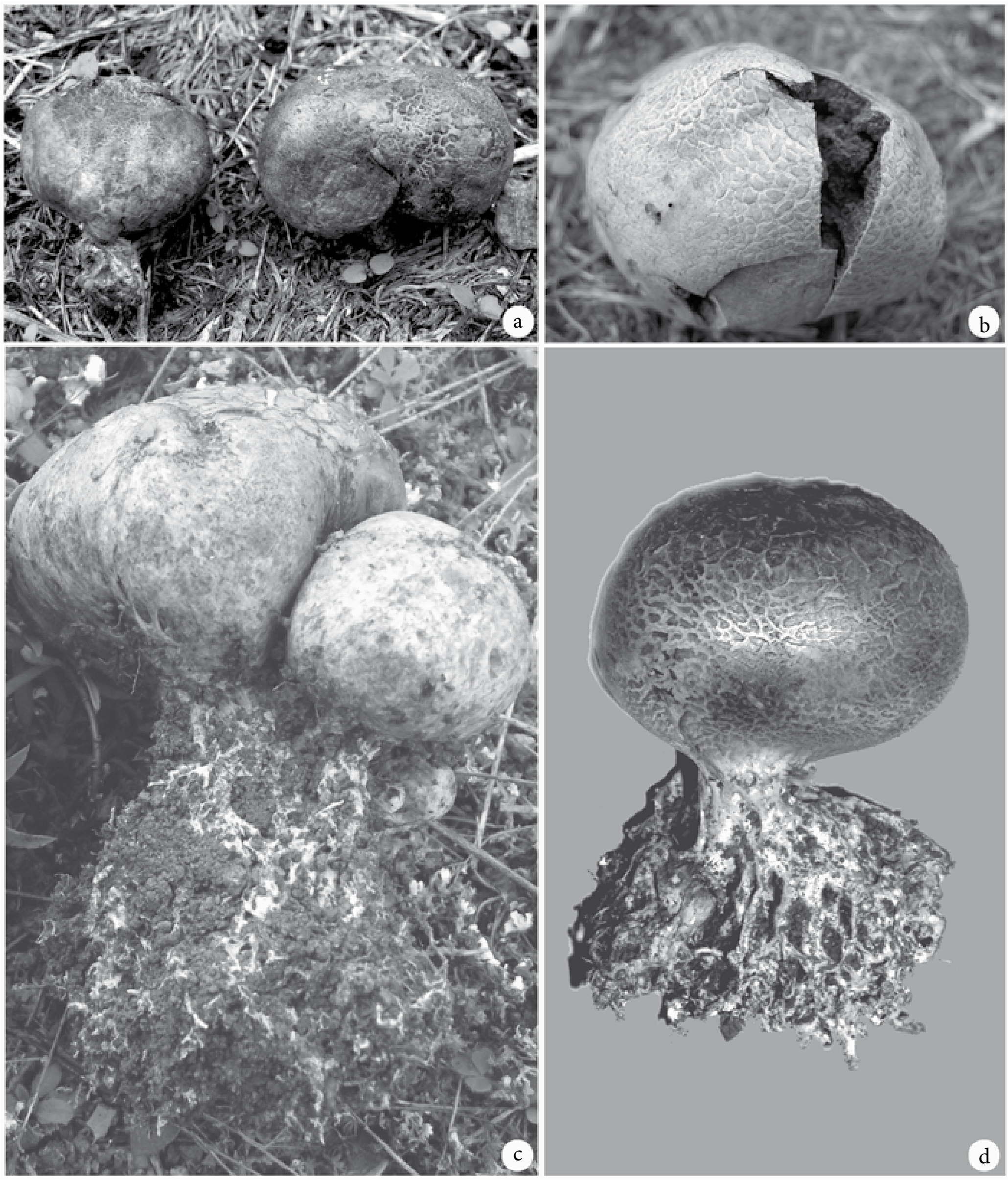

Figure 2. Basidiocarps. a- *Scleroderma areolatum* (MCF 07/8552), b- *S. bovista* (MCF 05/5304), c- *S. meridionale* (MCF 08/10263), d- *S. verrucosum* (MCF 02/3032).

from Spain (AY935514, under *S. citrinum*; a reexamination of the Spanish collection confirmed it as *S. meridionale*). In the *S. bovista* clade (BS = 92%, PP = 1.00), 5 sequences from Macedonia were grouped with 4 sequences from Spain, Japan, and Hungary; 4 Macedonian specimens were previously identified as *S. bovista* (05MCF5304 Sbov, 01MCF168 Sbov, 09MCF1118 Sbov, and 05MCF788 Sbov) and 1 as *S.* cf. *septentrionale* (06MCF5993 Ssepcf); reexamination of morphological characters confirmed this last specimen as belonging to *S. bovista*.

4. Discussion

In general, morphological studies fit with molecular analyses. The molecular analyses resulted in 4 well-defined subgroups (except collection 89MCF4709 Scitcf).

Morphologically *Scleroderma areolatum* and *S. verrucosum* are very similar. *Scleroderma verrucosum* has an almost always globose, sometimes subglobose fruit body with well-developed pseudostipe in contrast to *S. areolatum*, which has a sessile basidiocarp or short pseudostipe. In both species, the peridium is covered with numerous very small patches or scales, which in

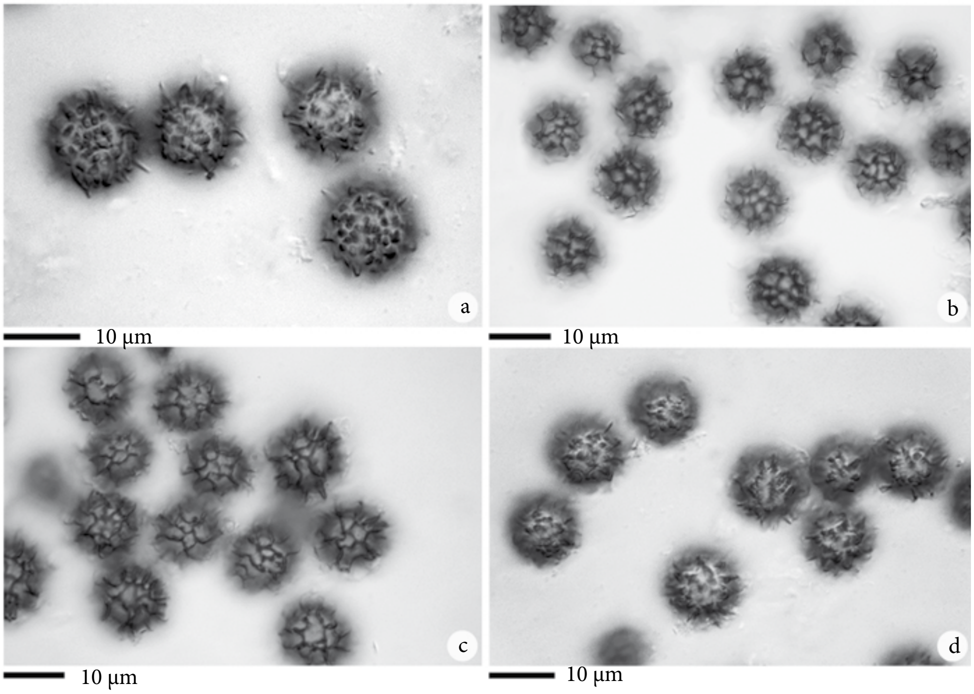

Figure 3. Spores of *Scleroderma* species photographed by LM. a- *Scleroderma areolatum* (MCF 02/4202), b- *S. bovista* (MCF 06/330), c- *S. meridionale* (MCF 05/5505), d- *S. verrucosum* (MCF 07/7984).

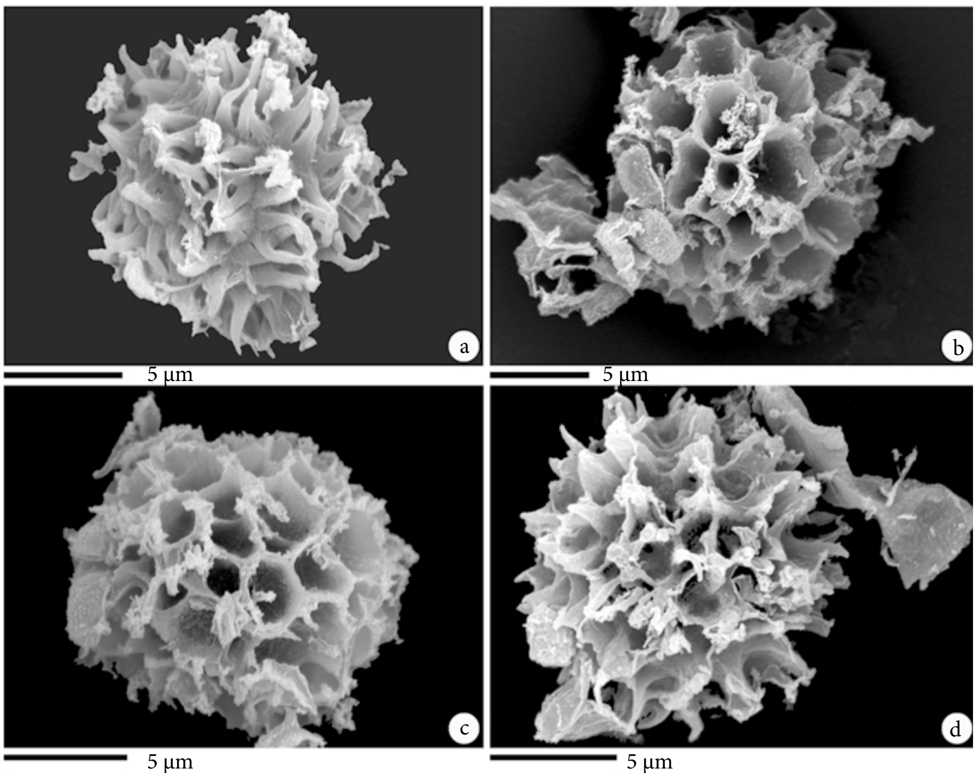

Figure 4. Spores of *Scleroderma* spp. photographed by SEM. a- *Scleroderma areolatum* (MCF 02/4202), b- *S. bovista* (MCF 09/11184), c- *S. meridionale* (MCF 05/5505), d- *S. verrucosum* (MCF 08/10124).

S. areolatum are surrounded by a ring zone, giving an areolate structure. The echinulate spores also differ in size (including ornamentation): *S. areolatum* 11–15(–16) µm and *S. verrucosum* (8–)9–14 µm.

Scleroderma meridionale and *S. bovista* both have reticulate spores. *Scleroderma meridionale* is characterised by almost globose fruit body with well-developed pseudostipe; the peridium is smooth and firm, with a star-like opening in maturity, showing dark olivaceous to grey-brownish gleba; and globose basidiospores (11–15 µm) with a reticulum formed by projecting spines connected only in their bases. *Scleroderma bovista* has ovoid to subovoid tuber-like fruit bodies, usually sessile or with short pseudostipe; the peridium is pale yellowish to ochre or brownish, almost smooth or with fine scales primary in the apical part; mature gleba is olivaceous, grey to dark brown; and the globose basidiospores are (9–)10–16 µm.

The ITS became the default marker for species-level studies for most fungi, with the notable exception of the yeasts, where the LSU became the standard for identification (Seifert, 2009). Schoch et al. (2012) proposed ITS as a standard barcode for fungi and data from their study indicated that ITS and LSU performed similarly to barcodes and that differences in their sequences correlated well with current species concepts.

As indicated by Telleria et al. (2010), in the era of DNA barcoding it is very important to sequence the barcode loci from well-annotated herbarium specimens. In our study, from a large number of herbarium collections located at the MCF it was not possible to obtain ITS nrDNA sequence due to poor conservation of these collections. New specimens should be collected in order to confirm, through morphological and molecular analyses, the presence of the *S. cepa*, *S. citrinum*, *S. polyrhizum*, and *S. septentrionale* species mentioned by Karadelev et al. (2008) in Macedonia.

Acknowledgements

This research received support from the SYNTHESYS Project (http://www.synthesys.info/), which is financed by the European Community Research Infrastructure Action under the FP7 "Capacities" Program.

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